Drug Safety Evaluation

Second Edition

Shayne Cox Gad
Gad Consulting

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To the memory of my mother, Norma Jean Cox Gad, who crossed over a year ago. I hope that all your beloved little friends are there with you.

Contents

Preface

The second edition of *Drug Safety Evaluation* is a complete revision of the initial volume which maintains the central objective of presenting an all-inclusive practical guide for those who are responsible for ensuring the safety of drugs and biologics to patients, health care providers, those involved in the manufacture of medicinal products, and all those who need to understand how the safety of these products is evaluated. The many changes in regulatory requirements, pharmaceutical development, and technology have required both extensive revision to every chapter and the addition of a number of new chapters.

This practical guide presents a road map for safety assessment as an integral part of the development of new drugs and therapeutics. Individual chapters also address specific approaches to evaluating hazards, including problems that are encountered and their solutions. Also covered are the scientific and philosophical bases for evaluation of specific concerns (e.g., carcinogenicity, development toxicity) to provide both understanding and guidance for approaching new problems. *Drug Safety Evaluation* is aimed specifically at the pharmaceutical and biotechnology industries. It not only addresses the general cases for safety evaluation of small and large molecules but also all of the significant major subcases: imaging agents, dermal and inhalation route drugs, vaccines, and gene therapy products. It is hoped that the approaches and methodologies presented here will show a utilitarian, yet scientifically valid path to the everyday challenges of safety evaluation and the problem solving that is required in drug discovery and development.

Cary, North Carolina SHAYNE C. GAD

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About the Author

Shayne C. Gad, B.S. (Whittier College, Chemistry and Biology, 1970) and Ph.D. in Pharmacology/Toxicology (Texas, 1977) DABT, ATS, is the principal of Gad Consulting Services, a fifteen-year-old consulting firm with six employees and more than 350 clients (including 120 pharmaceutical companies in the United States and 35 overseas). Prior to this, he served in director-level and above positions at Searle, Synergen, and Becton Dickinson. He has published 39 books and more than 350 chapters, articles, and abstracts in the fields of toxicology, statistics, pharmacology, drug development, and safety assessment. He has more than 32 years of broad-based experience in toxicology, drug and device development, statistics, and risk assessment. He has specific expertise in neurotoxicology, *in vitro* methods, cardiovascular toxicology, inhalation toxicology, immunotoxicology, and genotoxicology. Past president of the American College of Toxicology, the Roundtable of Toxicology Consultants and three of SOT's specialty sections. He has direct involvement in the preparation of INDs (88 successfully to date), NDA, PLA, ANDA, 510(k), IDE, CTD, clinical data bases for phase 1 and 2 studies, and PMAs. He has consulted for FDA, EPA and NIH, and has trained reviewers and been an expert witness for FDA. He has also conducted the triennial toxicology salary survey as a service to the profession for the last 19 years.

Dr. Gad is also a retired Navy officer.

1

Drug Development Process and Global Pharmaceutical Marketplace

Much more so then when the last edition of this book was written, pharmaceuticals[1] are global in impact, their regulation, and market.

This volume focuses on the assessment of the safety of new drugs. In the broadest sense, this means it must address not only the traditional "small molecules" that have dominated the field for the last century and the large therapeutic molecules derived from biotechnology sources but also vaccines, biologics such blood and blood products, and excipients. The globalization of the regulation of the safety, efficacy, and manufacture of their products comes from the success of the ICH (International Conference on Harmonisation) process. But, as will be seen, the same globalization of the industry and continuous our advance of science have also led to market diversification of the types and use of drugs and, with this, a fragmentation of regulatory drug safety evaluation requirements which has made things more complex rather than simpler.

[1]The term *pharmaceuticals* is here used in the broadest sense of man-made therapeutics: small molecules and large pills and vaccines, blood products, and, as must be, their attendant components (excipients, impurities, and all) to different degrees and in different products.

Drug Safety Evaluation, Second Edition, by Shayne Cox Gad

1.1 THE MARKETPLACE

The world marketplace for drugs is large, although the majority of sales are in three regions: about 51% of the pharmaceutical market resides in the United States, about 25% in Europe, and 15% in Japan. The balance of sales is spread across the globe. This does not mean, however, that the marketing applicant should ignore the requirements of other countries (e.g., Indonesia). Approval processes in these countries can, at times, be as rigorous as in any other regulatory authority domain.

Pharmaceuticals in all their forms compete today as part of a global market, though one which serves (and is available to) different parts of the world's population too.

According to the Intercontinental Marketing Service (IMS) 2008 global pharmaceutical market and therapy forecast, the global market for regulated drugs (as differentiated from dietary supplements, herbal products, and nutraceuticals) is estimated to be some $690 billion in 2007 (U.S. dollars). The same forecast projected growth to $735–745 billion in 2008 (Goozner, 2004). In 2006, there were 109 individual products with annual sales excess of $1 billion (Greider, 2003) which have tended to be the focus of pharmaceutical development until recently, but the impending demise of patents on which is changing the industry (Table 1.1).

This concentration of total sales in a limited number of products (e.g., there are currently more than 21,000 approved drugs in the United States) is widely held to have distorted the therapeutic aspects of new drug development but is now starting to undergo change (back to) a paradigm that looks at a decreased emphasis on the $1 billion "blockbuster" drugs.

Widely misunderstood is the extent of the pharmaceutical research and development (R&D) sector. While precise numbers are unavailable (and meaningless, as companies are continuously starting, merging, or going out of business though the overall trend is to increased numbers), best estimates place the number of companies directly involved in discovering and developing new drugs in the United States and Canada at 3700. There are an equal number in Europe and significant numbers in many other parts of the world (China, Australia, India, and Israel, to name a few). While most of the public focuses on the very large companies, such as those in Table 1.2, there are many more midsize and small companies.

One factor to consider in the regulatory requirements for early development of new therapeutic entities is the degree of barrier which costs may present to the smaller, innovative companies. This is commonly overlooked by many who also do not recognize that such small companies (most of which fail) are the primary initial source of new therapeutics.

A second complicating factor in considering the "pharmaceutical" market sector is the diversity of products involved. The most basic expression of this is the division of drugs into "small molecules" [which currently constitute about two-thirds of both investigational new drugs (INDs)—applications for clinical

TABLE 1.1 Top 20 Selling Pharmaceuticals (2006)

Rank (2005)	Medicine	2005 Sales ($M)	2004 Sales ($M)	Company	Primary Diseased Medical Use	First Approval Date(s)	Route(s)
1	Lipitor	12,986	11,587	Pfizer, Astellas Pharma	Cholesterol	Dec.17, 1996	Oral
2	Plavix/Iscover	6,345	5,434	Bristol-Myres Squibb, Sanofi-Aventis	Thrombotic events	Nov. 17, 1997	Oral
3	Advair/Seretide	5,465	4,503	GlaxoSmithKline	Asthma	Nov. 23, 1999/ Sept. 7, 1998	Inhalation
4	Norvasc	4,706	4,463	Pfizer	Hypertension	July 31, 1992	Oral
5	Nexium	4,633	3,883	AstraZeneca	Gastrointestinal disorders	Mar. 2000	Oral, parenteral
6	Zocor	4,382	5,197	Merck & Co.	Cholesterol	Dec. 23, 1991	Oral
7	Zyprexa	4,202	4,420	Eli Lilly	Schizophrenia	Sept. 27, 1996	Oral, injection
8	Prevacid/ Takepro	3,996	4,050	Tap Pharmaceutical, Takeda Pharmaceutical	Gastrointestinal disorders	May 10, 1995	Oral, injection
9	Diovan group	3,676	3,093	Novartis	Hypertension	Dec. 23, 1996	Oral
10	Enbrel	3,657	2,580	Amgen, Wyeth	Rheumatoid arthritis	Nov. 2, 1998	Injection
11	Risperdal	3,552	3,050	Johnson & Johnson	Schizophrenia	Dec. 29, 1993	Oral, injection
12	Remicade	3,547	2,920	Johnson & Johnson, Schering-Plough, Tanabe	Rheumatoid arthritis	Aug. 24, 1998	Injection
13	Effexor	3,459	3,347	Wyeth	Depression	Dec. 28, 1993	Oral
14	Protonix/ Pantozol	3,428	3,105	Wyeth, Atlana	Gastrointestinal disorders	Feb. 2, 2000/1994	Oral, injection
15	Rituxan/ MabThera	3,334	2,711	Roche, Genentech	Non-Hodgkin's lymphoma	Nov. 26, 1997	Injection
16	Procrit/Eprex	3,324	3,589	Johnson & Johnson	Anemia	Dec. 31, 1990/ May 4, 1995	Injection
17	Aranesp	3,273	2,473	Amgen	Anemia	June 11, 2001	Injection
18	Zoloft	3,256	3,361	Phizer	Depression	Dec. 30, 1991	Oral
19	Fosamax	3,191	3,160	Merck & Co.	Osteoporosis	Sept. 29, 1995	Oral
20	Cozaar, Hyzaar	3,037	2,824	Merck & Co.	Hypertension	Apr. 14, 1995, Apr. 28, 1995	Oral

TABLE 1.2 Top 25 Drug Companies by Sales (2006)

Company	Pharma Sales ($ million)	% Change	Pharma Sales as % of Total Sales
Pfizer	45,083	1.8	95.9
GlaxoSmithKline	40,156	25.1	86.5
Sanofi-Aventis	38,555	29	100
Roche	27,290	31.8	79.2
AstraZeneca	26,475	10.5	100
Johnson & Johnson	23,267	4.2	43.6
Novartis	22,576	11.4	62.7
Merck & Co	20,375	–6.6	90
Wyeth	16,884	10.2	83
Lilly	15,691	13.9	100
Bristol-Myers Squibb	13,861	–9.1	77.4
Boehringer Ingelheim	13,860	27.6	96.5
Amgen	13,858	15.3	100
Abbott Laboratories	12,395	–8.9	55.2
Bayer	10,162	–9	25.8
Takeda	8,716	–3.9	88.6
Schering-Plough	8,561	13.2	80.8
Teva	7,821	65.5	93.1
Genentech	7,640	39.2	100
Astellas	7,390	71.2	98.9
Novo Nordisk	7,087	32.3	100
Daiichi Sankyo	6,790	6.7	90.1
Baxter International	6,461	67.7	62.3
Merck KGaA	5,643	22.4	65.8
Eisai	4,703	8.3	96.4

evaluation of a new drug in humans–and current new drug approvals] and biotechnology products (which constitute the other third). The challenges in both developing and assessing the safety of these are very different. As will also be seen, if one considers further division into therapeutic claim areas [e.g., oncology, anti-infectives, cardiovascular, central nervous system (CNS)], the differences become even more marked. Most of what will be presented and discussed in this volume speaks to regulatory requirements for nonclinical safety assessment in the general case for either small molecules or protein therapeutics. It should be kept in mind that this general case development model never applies.

Additionally, there is now a significant hybrid area—combination products, which include both device and drug (small-molecule or biological) components. These will be addressed in a separate chapter of the book, though there is no single dedicated regulatory arm [such as a center within the U.S. Food and Drug Administration (FDA) dedicated to only their regulation] in any major market country. For that reason, more exploration of regulatory considerations will be provided in the chapter on these products.

The extent of regulations and practices for drug approval cause pharmaceutical companies to spend an enormous amount of resources on developing

applications, following different standards for preclinical and nonclinical programs for specific therapeutic areas as well as time and resources to satisfy the regulatory processes for clinical trials. Because of the regulatory diversity that existed, representatives from the regulatory authorities and trade associations came together in the late 1980s and early 1990s to attempt at harmonizing the process for drug approvals. Clearly this was a daunting task. With time, however, the International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceutical for Human Use has become increasingly more effective. Japan, Europe, and the United States represent the major pharmaceutical markets for the world, and these regions have the most influence on developments within the ICH and tend to follow the guidance documents that are prepared. However, other countries [rest of the world (ROW)] follow the developments within the ICH and tend to follow the guidance offered by the ICH. However, when seeking registration of pharmaceuticals, it remains important to be aware of local country regulations. For example, China is becoming a major economic force in many aspects. Placement of pharmaceutical manufacturing facilities and the marketing of drugs in China may potentially represent a significant marketing advantage to companies. With this new market area in Asia, regulatory processes are being developed sometimes—it seems at the whim of the government. With time it is hoped that China aligns itself more with the processes and guidance that have been developed by the ICH, FDA, and other developed countries.

1.2 HISTORY OF MODERN THERAPEUTICS

Although prior to the nineteenth century preventive medicine had made some spectacular advances, for example, through nutrition (scurvy), controlling infectious diseases (vaccination), public health through sanitation, and controlling childbirth fever and surgical infections using antiseptic techniques, truly therapeutic medicine was virtually nonexistent until the end of the nineteenth century (Mathieu, 2000; Rang, 2006).

Oliver Wendell Holmes (a physician and U.S. Supreme Court justice) wrote in 1860: "I firmly believe that if the whole material medica, as now used, could be sunk to the bottom of the sea, it would be all the better for mankind—and the worse for the fishes." While there were a few effective medicines—digitalis, extract of willow bark, and quinine—on balance Holmes was quite correct—medicines did more harm than good.

An idea of the state of therapeutics at the time comes from the first edition of the British Pharmacopoeia, in 1864, which listed 311 preparations. Of these, 187 were plant-derived materials, only 9 of which were purified substances. Most of the plant products—lemon juice, rose hips, yeasts, and so on—lacked any components we would now regard as therapeutically relevant, but some—digitalis, castor oil, ergot, colchicum—were pharmacologically active. Of the 311 preparations, 103 were truly synthetic inorganic chemicals, such as iodine,

ferrous sulfate, sodium bicarbonate, and toxic salts of bismuth, arsenic, lead, and mercury, with but a few synthetic chemicals (diethyl ether and chloroform). The remainder was miscellaneous materials and a few animal products, such as lard, cantharidin, and cochineal.

For the pharmaceutical industry, the transition from theoretical to actual an history occurred late in the nineteenth century, when three essential technologies came together—biomedicine (especially pharmacology), synthetic organic chemistry, and the development of a chemical industry in Europe—coupled with development of a medical supplies products-trade.

Science began to be applied wholeheartedly to medicine—as to almost every other aspect of life—only late in the nineteenth century. Among the most important milestones from the point of view of drug discovery was the elaboration in 1858 of cell theory. This tremendous reductionist leap of cell theory gave biology—and the pharmaceutical industry—the fundamental scientific underpenning it required. Only by thinking of living systems in terms of the function of their cells can one begin to understand how molecules affect them.

A second milestone was the birth of pharmacology as a scientific discipline when the world's first Pharmacological Institute was set up in 1874 at Dorpat by Rudolf Buchheim—literally by Buchheim himself, as the institute was in his own house and funded by him personally. This was advanced by pioneers such as Magendie and Claude Bernard, who linked pharmacology to therapeutics.

Another vital spark on this road came with Louis Pasteur's germ theory of disease, proposed in Paris in 1878. A chemist by training, Pasteur's initial interest was in the process of fermentation of wine and beer and the souring of milk. He showed, famously, that airborne infection was the underlying cause and concluded that the air was alive with microorganisms. Particular types, he argued, were pathogenic to humans and accounted for many forms of disease, including anthrax, cholera, and rabies. Pasteur successfully introduced several specific immunization procedures to give protection against infectious diseases. Robert Koch, Pasteur's rival and near contemporary, clinched the infection theory by observing anthrax and other bacilli in the blood of infected animals.

The founder of chemotherapy—some would says the father of molecular pharmacology—was Paul Ehrlich. He invented "vital staining"—staining by dyes injected into living animals—and described how the chemical properties of the dyes, particularly their acidity and lipid solubility, influenced the distribution of dye to particular tissues and cellular structures. Thence came the idea of specific binding of molecules to particular specific binding of molecules to particular cellular components. This not only led to Ehrlich's study of chemotherapeutic agents but also became the basis of pharmacological thinking to the present day. "Receptors" and "magic bullets" were Ehrlich's terms, though he envisaged receptors as targets for toxins, rather than physiological mediators. Working at Koch's Institute, Ehrlich developed diphtheria antitoxin

for clinical use and put forward a theory of antibody action based on specific chemical recognition of microbial molecules, for which he won the 1908 Nobel Prize.

The first synthetic organic chemicals to be used for medical purposes were not therapeutic agents at all, but rather anesthetics. Diethyl ether ("sweet oil of vitriol") was first made and described in 1540. It and nitrous oxide (prepared by Humphrey Davy in 1799 and found, by experiments on himself, to have stupor-inducing properties) had their usefulness as surgical anesthetics demonstrated only in the 1840s, by which time chloroform had also made its appearance. Synthetic chemistry at the time could deal only with very simple molecules, made by recipe rather than reason, as our understanding of chemical processes and molecular structure was still in its infancy. The first therapeutic drug to truly come from synthetic chemistry was amyl nitrite, prepared in 1859 by Guthrie and used to treat angina by Brunton in 1864. This was the first example of a drug born in a recognizably "modern" way, through the application of synthetic chemistry, physiology, and clinical medicine. This was a landmark indeed, for it was nearly 40 years before synthetic chemistry made any further significant contribution to therapeutics, and it was not until well into the twentieth century that physiological and pharmacological knowledge began to be applied to the invention of new drugs.

During the latter half of the nineteenth century the foundations of synthetic organic chemistry were laid, the impetus coming from work on aniline, a copious byproduct of the coal-tar industry, with the discovery of how to produce a purple dye. This discovery gave birth to the synthetic dyestuff industry, which played a major part in establishing the commercial potential of synthetic organic chemistry—a technology which became the underpinning of the evolving pharmaceutical industry for the next century. A systematic approach to organic synthesis went hand in hand with improved understanding of chemical structure.

Despite the limited efficacy of the pharmaceutical preparations available in the nineteenth century ("patent medicines") the pharmacists' trade flourished; then, as now, physicians felt obligated to issue prescriptions to satisfy the expectations of their patients for some therapeutic action—or at least cause for hope. Early in the nineteenth century, a few enterprising chemists undertook the task of isolating the active substances from the plants extracts. The trend began with Friedrich Serturner, a junior apothecary in Westphalia, who in 1805 isolated and purified morphine, barely surviving a test of its potency on himself. This was the first "alkaloid," so named because of its ability to neutralize acids and form salts. This discovery in turn led to the isolation of other plant alkaloids, including strychnine, caffeine, and quinine. The recognition that medicinal plants owed their properties to their individual chemical constituents, rather than to some intangible property associated with their living nature, marks a critical point in the history of the pharmaceutical industry which can be recognized as the point of origin of two of the three roads from which the industry grew—the beginnings of the "industrialization" of the

pharmaceutical trade. It hinted at the future possibility of making drugs artificially.

The first local apothecary business to move into large-scale production and marketing of pharmaceuticals was the old-established Darmstadt firm Merck, founded in 1668. This development, in 1827, was stimulated by the advances in purification of natural products. Merck was closely followed in this astute business move by other German- and Swiss-based apothecary businesses, giving rise to some which later also became giant pharmaceutical companies, such as Schering and Boehringer. The American pharmaceutical industry emerged in the middle of the nineteenth century. Squibb began in 1858, with ether as its main product. The move to pharmaceuticals was also followed by several chemical companies, such as Bayer, Hoechst, Agfa, Sandoz, Geigy, and others which began as dyestuff manufacturers. The dyestuff industry at that time was also based largely on plant products, which had to be refined and were sold in relatively small quantities, so the commercial parallels with the pharmaceutical industry were plain.

After 1870, with the crucial discovery by Kekule of the structure of benzene, the dyestuff industry turned increasingly to synthetic chemistry as a source of new compounds, starting with aniline-based dyes. A glance through any modern pharmacopeia will show the overwhelming preponderance of synthetic aromatic compounds, based on the benzene ring structure, among the list of useful drugs. Understanding the nature of aromaticity was critical.

Thus the beginning of the pharmaceutical industry as we now know it dates from the 1800s, with origins in the apothecary and patent medicine trades on the one hand and the dyestuff industry on the other. Unfortunately, these firms had few effective products to sell (mainly inorganic compounds of varying degrees of toxicity and others most charitably described as concoctions).

Entering the 1900s, synthetic drugs were made and tested, including the "antipyretics" and various CNS depressants. Chemical developments based on chloroform produced chloral hydrate, the first nonvolatile CNS depressant, which was in clinical use for many years as a hypnotic drug. Independently, various compounds based on urea were found to act similarly, and von Mering followed this lead to produce the first barbiturate, barbitone (since renamed barbital), which was introduced in 1903 by Bayer and gained widespread clinical use as a hypnotic, tranquillizer, and antiepileptic drug—the first blockbuster. Barbitone and procaine were triumphs for chemical ingenuity but owed little or nothing to physiology or indeed pharmacology. The physiological sites of action of barbiturates remain unclear to this day, and their mechanism of action at the molecular level was unknown until the 1980s.

The pattern of drug discovery driven by synthetic chemistry—with biology often struggling to keep up—became the established model in the early part of the twentieth century and prevailed for at least 50 years. The balance of research in the pharmaceutical industry up to the 1970s clearly placed chemistry as the key discipline in drug discovery, the task of biologists being mainly

to devise and perform assays capable of revealing possible useful therapeutic activity among the many anonymous white powders that arrived for testing. Research management in the industry was largely in the hands of chemists. This strategy produced many successes, including benzodiazepine tranquillizers, several antiepileptic drugs, antihypertensive drugs, antidepressants, and antipsychotic drugs. The surviving practice of classifying many drugs on the basis of their chemical structure rather than on the more logical basis of their site or mode of action (therapeutic class) stems from this era. We have mentioned the early days of pharmacology, with its focus on plant-derived materials, such as atropine, tubocurafine, strychnine, digitalis, and ergot alkaloids, which were almost the only drugs that existed until well into the twentieth century. Despite the rise of synthetic chemistry natural products remain a significant source of new drugs, particularly in the field of chemotherapy, but also in other applications. Following the discovery of penicillin by Fleming in 1929—and its development as an antibiotic for clinical use by Chain and Florey in 1938—an intense search was undertaken for antibacterial compounds produced by fungi and other microorganisms which yielded many useful antibiotics, including chloramphenicol (1947), tetracyclines (1948), and streptomycin (1949). The same fungal source that yielded streptomycin also produced actinomycin D, used in cancer chemotherapy Higher plants have continued to yield useful drugs, including vincristine and vinblastine (1958), and paclitaxel (or taxol, 1971).

Outside the field of chemotherapy, successful drugs derived from natural products include cyclosporin (1972) and tacrolimus (1993), both of which come from fungi and are used to prevent transplant rejection. Soon after came mevastatin (1976), another fungal metabolite, which was the first of the "statin" series of cholesterol-lowering drugs which act by inhibiting the enzyme human menopausal gonadotrophin (HMG) coenzyme A (CoA) reductase.

Overall, the pharmaceutical industry continues to have something of a on-again, off-again relationship with natural products. They often have weird and wonderful structures that cause hardened chemists to turn pale, they are often near-impossible to synthesize and troublesome to produce from natural sources, and "optimizing" such molecules to make them suitable for therapeutic use is prone to frequent failure. But nature continues to unexpectedly provide some of our most useful drugs, and most of its potential remains untapped.

Although chemistry was the preeminent discipline in drug discovery until at least the 1970s, the seeds of the biological revolution were sown long before. It started foremost in the field of chemotherapy, where Ehrlich defined the principles of drug specificity in terms of a specific interaction between the drug molecule and a target molecule—the "receptor site"—in the organism. Although we now take it for granted that in almost all cases a highly specific chemical target molecule, as well as the "pharmacophore" or an outline portion of the drug molecule, determines what effects a therapeutic will yield, before Ehrlich no one had envisaged drug action in this way. By

TABLE 1.3 Examples of Drugs from Different Sources

Natural Products	Synthetic Chemistry	Biopharmaceuticals Produced by Recombinant DNA Technology
Antibiotics (penicillin, streptomycin, tetracyclines, cephalosporins, etc.)	Early successes include:	Human insulin (the first biotech product, registered 1982)
Anticancer drugs (doxorubicin, bleomycin, actinomycin, vincristine, vinblastine, taxol etc.)	Antiepileptic drugs	Human growth hormone
Atopine, hyoscine	Antihypertensive drugs	α-Interferon, γ-interferon
Ciclosporin	Antimetabolites	Hepatitis B vaccine
Cocaine	Barbiturates	Tissue plasminogen activator (t-PA)
Colchicine	Bronchodilators	Hirudin
Digitalis (digoxin)	Diuratics	Blood-clotting factors
Ephedrine	Local anaesthetics	Erythropoietin
Heparin	Sulfonamides	Granulocyte and granulocyte–monocyte colony-stimulating factor (G-CSF, GM-CSF)
Human growth hormone[a]	(Since ca.1950, synthetic chemistry has accounted for the great majority of new drugs)	
Insulin (porcine, bovine)[a]		
Opium alkaloids (morphine, papaverine)		
Physostigmine		
Rauwolfia alkaloids (reserpine)		
Statins		
Streptokinase		
Tubocurarine		
Vaccines		

[a]Now largely or entirely replaced by material prepared by recombinant DNA technology.

linking chemistry and biology, Ehrlich defined the parameters of modern drug discovery.

Despite these discoveries in Ehrlich's field, chemotherapy remained empirical rather than target directed. For many years, Ehrlich's preoccupation with the binding of chemical dyes, as exemplified by biologicals in the 1950s onward, steadily shifted the industry's focus from chemistry to biology (Lednicer, 1993). The history of successes in the field of chemotherapy prior to the antibiotic era (Table 1.3) demonstrates the diversity of sources of new therapeutic entities. The popular image of magic bullets (a phrase invented by Ehrlich) is the essence of today's target-directed approaches to drug discovery.

1.3 DRUG DEVELOPMENT PROCESS

While processes for the discovery of new potential therapeutic drugs are very diverse (Gad, 2007), once the decision is made to move a candidate com-

pound forward to (hopefully) market approval, the process is well defined in the components of its regulatory requirements. It has many components which are beyond the scope of safety assessment and therefore of this volume (including chemical development, clinical evaluation, and a host of regulatory actions.)

The process generally proceeds by way of getting regulatory concurrences for entering clinical trials, then proceeding through three (not strictly defined) stages of clinical trials (phases I–III), followed by submission of a full set of documents, data, and a proposed label seeking regulatory approval for a marketing application.

The metrics of this process as it now operates makes cancer the most prevalent therapeutic target for new drugs, with perhaps as many as one-third of all new drug candidates being in this claim area. Heart diseases, CNS diseases, nervous system diseases, and immune system disorders follow in order of current popularity (Table 1.4).

According to Pharma & BioIngredients (www.pharmabioingredients.com), the more than 16,000 different drugs in development in 2006 were spread across the entire course of the development process (Table 1.5).

At the same time, the metrics of regulatory applications for the development of new drugs in the United States (where the best data are available) show a continued increase in the number of candidates entering the development process, as indicated by the number of new (or original) INDs filed, with the proportion of these that are commercial (or traditional INDs) continuing to increase (see Table 1.6).

TABLE 1.4 Potential New Drugs in U.S. Clinical Trials, 2005–2006

Cancer	5468
Heart disease	2342
Mental and behavioral disorders	2397
Bacterial and fungal diseases	1591
Blood and lymph conditions	1654
Digestive system diseases	1527
Nervous system diseases	2928
Rare diseases	5765
Respiratory tract diseases	1548
Viral diseases	1168
Injuries, poisonings, and occupational diseases	832
Immune system disorders (not including HIV/AIDS)	2578
Disease abnormalities at or before birth	1090
Gland- and hormone-related diseases	1216
Muscle, bone, and cartilage diseases	699
Nutritional and metabolic diseases	1296
Skin and connective tissue diseases	1727
Symptoms and general pathology	4227
Urinary tract and sexual organs and pregnancy	1756

TABLE 1.5 2006 Status of Drug in Development

Stage	Drugs
New drug application (NDA)/Biological license application (BLA) filed	482
Phase III	1,179
Phase II	2,622
Phase I/IND filed	2,415
Preclinical/discovery	7,569
Recent product launches	2,002
Total	16,269

TABLE 1.6 INDs Received and Active at Center for Drug Evaluation and Research

Calendar Year Received	Original INDs Received	Number of Active INDs at Year End	NDAs
1998	2,419	12,723	121
1999	1,763	12,584	139
2000	1,812	11,838	115
2001	1,872	10,873	98
2002	2,374	11,544	105
2003	2,120 (426 commercial)	12,661 (4544 commercial)	109
2004	1,837 (621 commercial)	12,778 (4827 commercial)	115
2005	1,936 (637 commercial)	13,360 (5029 commercial)	116
2006	1,863 (713 commercial)	14,117 (5445 commercial)	123

At the same time the rate of approval of new molecular entities has decreased (and stayed stable at) 17 or 18 a year for the last three years. This has finally caused recognition that the traditional/existing system of development that focused on blockbusters is irretrievably broken.

1.4 STRATEGIES FOR DEVELOPMENT: LARGE VERSUS SMALL COMPANY OR SHORT VERSUS LONG GAME

While harmonization and societal concern for safety are driving the changes in regulatory processes for device and drug development to become less confused, strategies for product development and the associated nonclinical safety assessment can still be viewed in broad trends.

The truths driving strategies in developing new drugs are as follows:

1. Most molecules will fail. While the true success rate is certainly greater than the often-quoted 1 in 10,000, it is clear that only 3–5% of those that enter initial clinical evaluation (that is, for which an IND "opens") become marketed drugs. This rate varies depending on the therapeutic class (oncology drugs dosing at a rate as low as 1–2% and CNS therapeutics being only somewhat higher) (Czerepak and Rysef, 2008; Choerghade, 2006).

2. The cost of developing drugs is high. While not the currently quoted "average" of $1.4 billion, just getting to the point of an IND opening will cost a minimum of $2 million. One can spread out the rate of expenditure over time or shorten the required time by spending money more rapidly, but there are fixed minimums for cost and time.

And costs of development go up sharply with time/progress. Subsequent to a plain vanilla first-in-man (FIM) trial, outlays come to be spoken of first in tens of millions and (frequently), before a marketing approval filing, in the hundreds of millions. Once the decision is made to develop a molecule into a drug, the process takes years. One can dispute how many (from 5 to 16 years covers the extreme range), and at no point up to the end is success (achieving marketing approval and economically successful therapeutic use) assured. These truths conspire to produce the principal general goals behind a drug development strategy:

1. Kill the losers as early as possible, before too much money is spent on them.
2. Do all you can to minimize the time spent in developing a drug.

These principles produce a spectrum of strategies in the nonclinical safety assessment of drugs, best illustrated by looking at the two extreme cases.

Do only what you must. Driven by financial limitations and the plan that, at an optimal point in development (most commonly after either FIM/phase I trials or a "proof-of-concept" phase II trial), the candidate therapeutic will be licensed to or partnered with a large company, only the technical and regulatory steps necessary to get a molecule to this point are to be performed. For those pursuing this case, the guidance provided by this book should prove essential (though not generally completely sufficient). This approach in summarized in Figure 1.1.

Minimize the risk of subsequent failure. This is considered the traditional big company model. Studies and technical tasks are not limited to the minimum but rather are augmented by additional components. Development proceeds through a series of well-defined and carefully considered "go/no-go" decision points. This approach is summarized in Figure 1.2. Many of the additional components are either limited, non-GLP (good laboratory practice) forms of studies which will be required later [such as Ames, acute toxicity, human ether-a-gogo related gene (hERG) at only one concentration and 7 days to 4 weeks repeat-dose studies] or studies which are inexpensive and could be done later [cytochrome p. 450 (CYP) inhibitors and induction, metabolic stability, and longer than required repeat-dose toxicity studies before proceeding into phase II]. Exactly which "extra" components are included vary from company to company and frequently reflect past experiences of the organization or individuals involved.

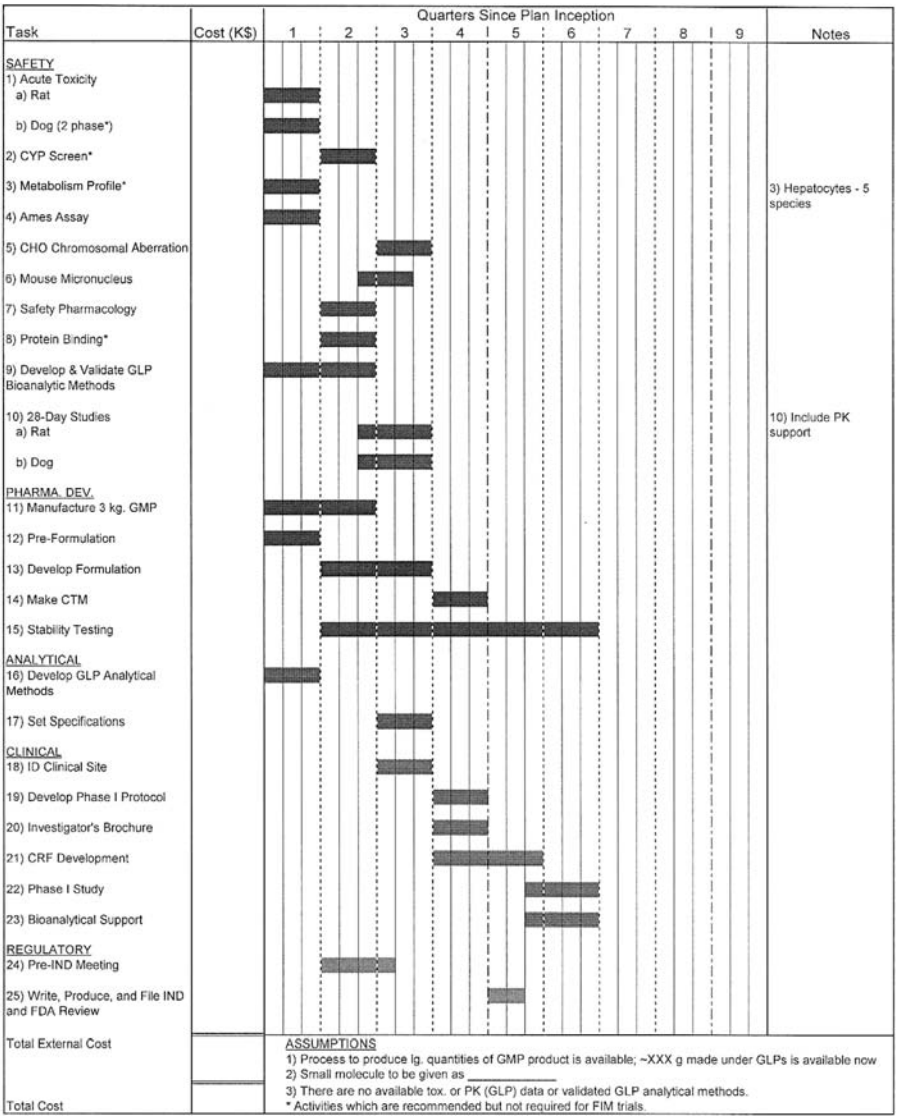

Figure 1.1 *General case oral drug: lead through phase 1 (do only what you must).*

The studies performed to meet regulatory nonclinical safety assessment requirements (which must be considered to include all of the supportive toxicokinetic and metabolism activities and studies) can be thought of as belonging to three major categories.

(a) Those necessary to support the successful filing/opening of an IND, clinical trial application (CTA), or equivalent application and of the subsequent FIM clinical studies.

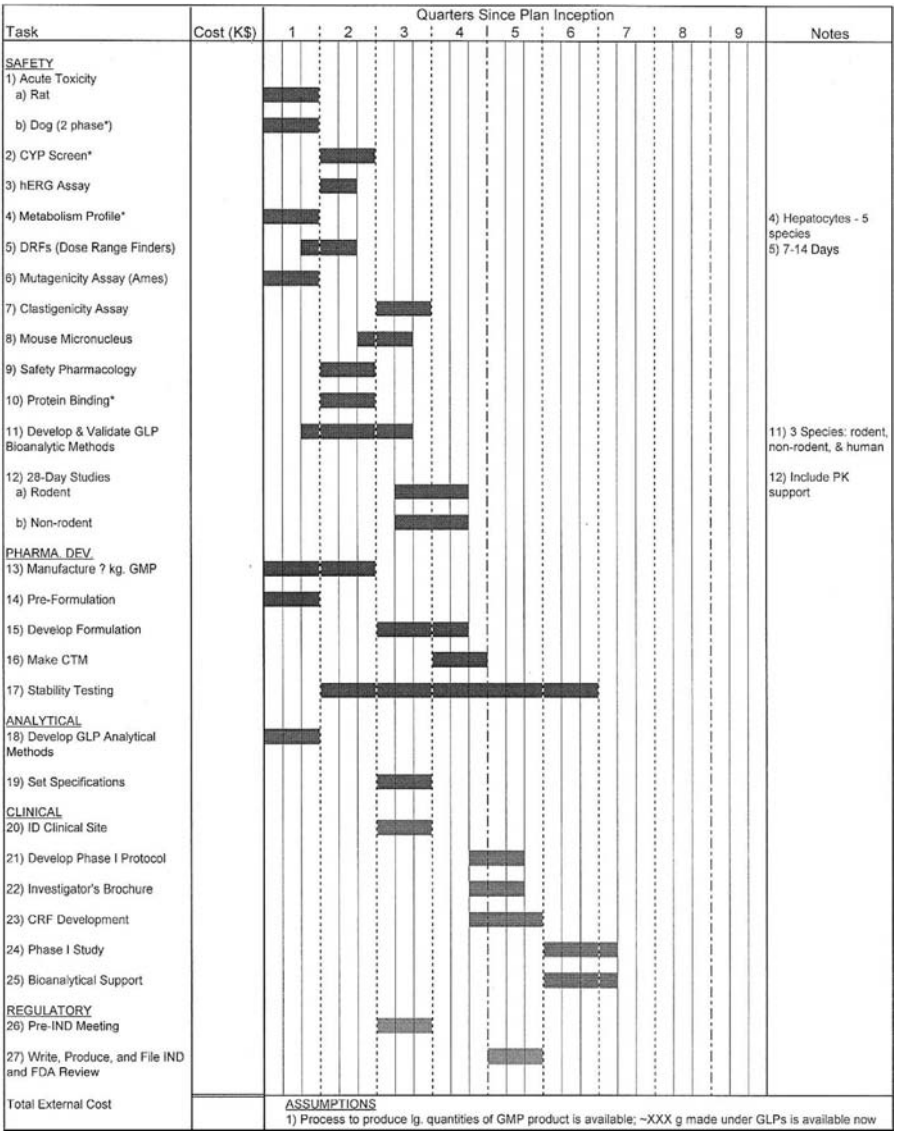

Figure 1.2 *General case oral drug: lead through phase 1 (minimize risk).*

(b) Those required to support continuation of clinical evaluation and development of up to and through successful phase III studies.

(c) Those studies required to support a successful marketing, approval application (NDA, BLA, or equivalent), but only required as such. This group is typically exemplified for carcinogenicity studies and the formal reproductive (as opposed to developmental) toxicity studies.

Which studies fit into what category is somewhat fluid and influenced by what patient population will be served (therapeutic claim) and the mechanism of action of the drug.

1.5 SAFETY ASSESSMENT AND EVOLUTION

In the mid-nineteenth century restrictions on the sale of poisonous substances were imposed in the United States and United Kingdom, but it was not until the early 1900s that system of "prescription-only" medicines was introduced, requiring approval of purchase by a licensed medical practitioner. Soon afterward, restrictions began to be imposed on what "cures" could be claimed in advertisements for pharmaceutical products and what information had to be given on the label; legislation evolved at a leisurely pace. Most of the concern was with controlling poisonous or addictive substances or contaminants, not with the efficacy and possible harmful effects of new drugs.

In 1937, the use of diethylene glycol as a solvent for a sulfonamide preparation caused the deaths of 107 children in the United States, and a year later the 1906 Food and Drugs Act was revised, requiring safety to be demonstrated before new products could be marketed as well as federal inspection of manufacturing facilities. The requirement for proven efficacy as well as safety was added in the Kefauver–Harris amendment in 1962 (said amendment being brought about largely by a safety issue—the thalidomide disaster in Europe).

In Europe, preoccupied with the political events in the first half of the century, matters of drug safety and efficacy were a minor concern, and it was not until the mid-1960s, in the wake of the thalidomide disaster—a disaster averted in the United States by an officer who used the provisions of the 1938 Food and Drugs Act to delay licensing approval—that the United Kingdom began to follow the U.S. lead in regulatory laws. Until then, the ability of drugs to do harm—short of being poisonous or addictive—was not really appreciated, most of the concern having been about contaminants. In 1959, when thalidomide was first put on the market by the German company Chemie Grumenthal, it was up to the company to decide how much research was needed to satisfy itself that the drug was safe and effective. Grumenthal made a disastrously wrong judgment [see Sjostrom and Nilsson (1972) for a full account], which resulted in an estimated 10,000 cases of severe congenital malformation following the company's specific recommendation that the drug was suitable for use by pregnant women. This single event caused an urgent reappraisal on a global scale, leading to the introduction of much tighter government controls.

By the end of the 1960s, the primary planks in the regulatory platform—evidence of safety, efficacy, and chemical purity—were in place in most developed countries. Subsequently, the regulations were adjusted in various minor ways and were adopted with local variations in most countries.

In 1988, Alder and Zbinden published national and international drug safety guidelines which set forth the wide differences in safety assessment requirements between the different nations of the world, at the time global development of a drug required multiple safety assessment programs, with a great number of repetition of studies and attendant extra costs and increased usage of test animals.

The solution to this was the ICH paradigm, which starting in the late 1980s sought to have a harmonized set of global requirements for all aspects of drug development (not just assessment). The safety assessment aspects were embodied primarily in the S series ICH guidelines (M4, which sets forth the overall structure of nonclinical requirements, being an exception). This did serve to largely regularize global requirements with minor differences.

As the rest of this book will make clear, this system is now fraying a bit of the edges. Recent additions of new guideline topic areas (e.g., immunotoxicology) and revisions to existing guideline (on genotoxicity and biotechnology) as well as recent regional guideline responses to recent occurrences [the case in point being the failed TGN-412 FIM trial and the resulting two European Medicines Agency (EMEA) special guidances issued in response to it] and differences in requirements for different therapeutic classes have reversed the harmonization trend.

1.6 THREE STAGES OF DRUG SAFETY EVALUATION ON GENERAL CARE

Nonclinical safety assessment studies fall into three categories, as will be examined in detail in the remainder of this book:

1. IND enabling ("FIM"): The studies necessary to support the initiation of clinical trials in human beings. These are generally as specified in ICH M3, and this is most commonly performed of the three sets.
2. To support continued clinical development: As clinical development proceeds, longer repeat drug studies must be performed, reproductive and developmental toxicology studies must be done, and other ancillary studies are required.
3. To support filing for marketing approval: The final studies generally required to support marketing of drugs—such as carcinogenicity.

Which studies fall into each of these categories and exactly what studies must be done to support the development of a drug for a specific therapeutic claim are extremely variable. The general case—much as specified in M3—gives us a starting place for understanding what must be done.

But the general case really applies to the simplest oral drug intended for chronic use and more often than not does not apply. In fact, it may never fully apply.

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2

Regulation of Human Pharmaceutical Safety

2.1 INTRODUCTION

The safety of pharmaceutical agents, medical devices, and food additives are the toxicology issues of the most obvious and longest-standing concern to the public. A common factor among the three is that any risk associated with a lack of safety of these agents is likely to affect a very broad part of the population, with those at risk having little or no option as to undertaking this risk. Modern drugs are essential for life in our modern society, yet there is a consistent high level of concern about their safety.

This chapter examines the regulations which establish how the safety of human pharmaceutical products are evaluated and established in the United States and the other major international markets. As a starting place, the history of this regulation will be reviewed and the current organizational structure of the U.S. Food and Drug Administration (FDA) will be briefly reviewed along with the other quasi-governmental bodies that also influence the regulatory processes. The current structure and context of the regulations in the United States and overseas will also be presented. From this point the general case of regulatory product development and approval will be presented. Toxicity assessment study designs will be presented. The broad special case of biotechnology-derived therapeutic products and environmental concerns associated with the production of pharmaceuticals will be briefly addressed. The significant changes in regulation brought about by harmonization are also reflected.

Drug Safety Evaluation, Second Edition, by Shayne Cox Gad

As an aid to the reader, appendices are provided at the end of this book: a codex of acronyms that are used in this field followed by a glossary which defines some key terms.

2.2 BRIEF HISTORY OF U.S. PHARMACEUTICAL LAW

A synopsis of the history of U.S. drug legislation is presented in Table 2.1. Here we will review the history of the three major legislative acts covering pharmaceuticals.

2.2.1 1906: Pure Food and Drug Act

As so eloquently discussed by Temin (1991), the history of health product legislation in the United States largely involves the passage of bills in Congress which were primarily in response to public demand. In 1902, for example, Congress passed the Biologics Act in response to a tragedy in St. Louis where 10 children died after being given contaminated diphtheria toxins. Interestingly, the background that led to the passage of the first Pure Food and Drug Act in 1906 had more to do with food processing than with drugs. The conversion from an agrarian to an urban society fostered the growth of a food-processing industry that was rife with poor practice. Tainted and adulterated food was commonly sold. Such practices were sensationalized by the muckraking press, including books such as *The Jungle* by Sinclair Lewis.

In the early debates in the U.S. Congress on the Pure Food and Drug Act (passed in 1906), there was little mention of toxicity testing. When Harvey Wiley, chief of the Bureau of Chemistry. Department of Agriculture, and driving force in the enactment of this early law, did his pioneering work (beginning in 1904) on the effects of various food preservatives on health, he did so using only human subjects and with no prior experiments with animals (Anderson, 1988). Ironically, work that led to the establishment of the FDA would probably not have been permitted under the current guidelines of the agency. Wiley's studies were not double blinded, so it is also doubtful that his conclusions would have been accepted by the present agency or the modern scientific community. Legislation in place in 1906 consisted strictly of a labeling law prohibiting the sale of processed food or drugs that were misbranded. No approval process was involved and enforcement relied on postmarketing criminal charges. Efficacy was not a consideration until 1911, when the Sherley amendment outlawed fraudulent therapeutic claims.

2.2.2 1938: Food, Drug and Cosmetic Act

The present regulations are largely shaped by the law passed in 1938. It will therefore be discussed in some detail. The story of the 1938 Food, Drug and Cosmetic Act (FDCA) actually started in 1933. Franklin D. Roosevelt had

TABLE 2.1 Important Dates in U.S. Federal Drug Law

Year	Event
1902	Passage of the Virus Act, regulating therapeutic serums and antitoxins. Enforcement by the Hygienic Laboratory (later to become the National Institute of Health), Treasury Department.
1906	Passage of Pure Food Act, including provisions for the regulations of drugs to prevent the sale of misbranded and adulterated products. Enforcement by the Chemistry Laboratory, Agriculture.
1912	Passage of the Sherley Amendment. Specifically outlawed any false label claims as to curative effect.
1927	Bureau of Chemistry renamed the Food, Drug and Insecticide Administration.
1931	Renamed again to Food and Drug Administration.
1938	Passage of the Food, Drug and Cosmetic Act. Superseded the law of 1906. Required evidence of safety, e.g., studies in animals. Included coverage of cosmetics and medical devices. Specifically excluded biologics.
1944	Administrative Procedures Act, codifying public health laws: included provision that for a biological license to be granted, a product must meet standards for safety, purity, and potency. The National Institutes of Health (NIH) also given the responsibility for developing biologics not developed by the private sector.
1945	Amendment to the 1936 act requiring that the FDA examine and certify for release each batch of penicillin. Subsequently amended to include other antibiotics.
1949	Publication of the first set of criteria for animal safety studies. Following several revisions, guidelines published in 1959 as *Appraisals Handbook*.
1951	Passage of Durham–Humphrey Amendment. Provided the means for manufacturers to classify drugs as over the counter (not requiring prescription).
1953	Transfer of FDA to the Department of Health, Education and Welfare from Agriculture (now the Department of Health and Human Services).
1962	Passage of major amendments (the Kefauver Bill) to the 1938 FDCA, which required proof of safety and effectiveness (efficacy) before granting approval of NDAS. Required affirmative FDA approval.
1968	FDA placed under the Public Health Service of Health, Education and Welfare (HEW).
1970	Controlled Substances Act and Controlled Substances Import and Export Act. Removed regulation of drug abuse from FDA (transferred to the Drug Enforcement Agency) and provided for stringent regulation of pharmaceuticals with abuse potential.
1972	Transfer of authority to regulate biologics transferred from NIH to FDA. The NIH retained the responsibility of developing biologics.
1973	Consumer Product Safety Act, leading to the formation of separate Consumer Product Safety Commission, which assumes responsibilities once handled by the FDA's Bureau of Product Safety.
1976	Medical Device Amendment to the FDCA requiring for devices that not only effectiveness be proven but also safety.
1979	Passage of the Good Laboratory Practices Act.
1983	Passage of the first Orphan Drug Amendment to encourage development of drugs for small markets.
1984	Drug Price Competition and Patent Term Restoration Act intended to allow companies to recover some of the useful patent life of a novel drug lost due to the time it takes the FDA to review and approve. Also permits the marketing of generic copies of approved drugs.

TABLE 2.1 ***Continued***

Year	Event
1985	The "NDA rewrite" final rule. An administrative action streamlining and clarifying the NDA process. Now embodied in 21 CFR 314.
1986	The United States Drug Export Amendment Act of 1986. Permitted the export of drugs outside the United States prior to approval for the U.S. market.
1987	The "IND rewrite" final rule: "to encourage innovation and drug development while continuing to assure the safety of (clinical) test subjects." *Federal Register* 52:8798, 1987. Now embodied in 21 CFR 312.
1990	Safe Medical Device Act, providing additional authority to the FDA for regulation of medical devices.
1992	Safe Medical Device Amendments requiring more extensive testing of devices.
1992	Prescription Drug User Fee Act. Established the payment of fees for the filing of applications (e.g., IND, NDA, PLA).
1994	Orphan Drug Amendment.
1997	The Food and Drug Administration Modernization Act: to streamline the drug and device review and approval process.
2002, 2007	Food and Drug Administration Modernization Act Amendments.

Note: Laws and amendments that have covered other aspects of FDA law, such as those governing food additives [e.g., Food Quality Production Act (FQPA)], are not included in this table.

just won his first election and installed his first cabinet. Walter Campbell was the chief of the FDA, reporting to Rexford Tugwell, the undersecretary of agriculture. The country was in the depths of its greatest economic depression. This was before the therapeutic revolution wrought by antibiotics in the 1940s, and medicine and pharmacy as we know it in the 1990s were not practiced. Most medicines were, in fact, self-prescribed. Only a relatively small number of drugs were sold via physicians' prescription. The use of so-called patent (because the ingredients were kept secret) preparations was rife, as was fraudulent advertising. Today, for example, it is difficult to believe that in the early 1930s a preparation such as Radithor (nothing more than a solution of radium) was advertised for treatment of 160 diseases. It is in this environment that one day in the winter of 1933 Campbell delivered a memo to Tugwell on an action level of an insecticide (lead arsenite) used on fruits. Tugwell briskly asked why, if the chemical was so toxic, was it not banned outright. He was amazed to find out from Campbell that the agency had no power to do so.

The 1906 law was designed to control blatantly misbranded and/or adulterated foods and drugs that relied on post facto criminal charges for enforcement. Safety and efficacy were not an issue so long as the product was not misbranded with regard to content. Premarketing review of a drug was an unknown practice. Thus, attempts at rewriting the old 1906 law to include control of bogus therapeutic claims and dangerous preparations proved to be unsatisfactory. Paul Dunbar of the FDA suggested to Campbell that an entirely new law was needed. A committee of FDA professionals and outside academic consultants drafted a new bill which immediately ran into trouble because no one in Congress was willing to sponsor it. After peddling the bill

up and down the halls of Congress, Campbell and Tugwell convinced Senator Royal Copeland of New York to sponsor the bill. Unknowingly at the time, Copeland put himself in the eye of a hurricane that would last for five years.

The forces that swirled around Copeland and the Tugwell Bill (Senate bill S.1944) were many. First was the immediate and fierce opposition from the patent medicine lobby. Flyers decried S.1944 as everything from a communist plot to un-American, stating it "would deny the sacred right of self-medication." In opposition to the patent trade organizations were two separate but unlikely allies: a variety of consumer advocacy and women's groups (such as the American Association of University Women, whose unfaltering support for the bill eventually proved critical to passage) and the mainline professional organizations. Interestingly, many of these organizations at first opposed the bill because it was not stringent enough. There were also the mainline professional pharmacy and medical organizations [such as the American Medical Association (AMA) and the American Association of Colleges of Pharmacy] whose support for the bill ranged from neutral to tepid but did grow over the years from 1933 to 1938.

Second, there was the basic mistrust on the part of Congress toward Tugwell and other "New Dealers." At the same time, Roosevelt gave the measure only lukewarm support at best (tradition has it that if it had not been for the First Lady, Eleanor, he would have given it no support at all) because of his political differences with Royal Copeland.

Third, there was a considerable bureaucratic turf war over the control of pharmaceutical advertising. Finally, despite the efforts of the various lobbying groups, there was no popular interest or support for the bill. At the end of the congressional period, S.1944 died for lack of passage.

The next five years would see the introduction of new bills, amendments, competing measures, committee meetings and hearings, lobbying, and House/Senate conferences. The details of this parliamentary in-fighting make for fascinating history but are outside the scope of this book. The reader is referred to the excellent history of this period by Jackson (1970).

The FDA was surprised by the force and depth of the opposition to the bill. The proposed law contained a then-novel idea that a drug was misbranded if its labeling made any therapeutic claim which was contrary to general medical practice and opinion. The definition of a drug was broadened to include devices used for medical purposes.[1] *Adulteration* was defined as any drug product dangerous to health when used according to label directions. The patent manufacturers charged that no bill granted too much discretionary power to a federal agency—that no manufacturer could stay in business except by the grace of the Department of Agriculture, a charge that may have been

[1]The use of a broad definition of what constitutes a drug for regulatory purposes is a precedent that remains in place today. For example, the computer software used in diagnostic systems is considered to be a pharmaceutical for purposes of regulation.

correct. In response to the patent trade lobbying effort, the FDA launched its own educational drive of radio spots, displays (such as the sensationalized chamber-of-horrors exhibition, in which the toxicity of a variety of useless medicines was clearly displayed), mimeographed circulars, speaking engagements, posters, and so on.

Ruth Lamb, FDA information officer at the time, was perhaps one of the hardest working and most quotable of the FDA staffers working the street at the time. For example, in reference to one of the counterbills that had language similar to the original Copeland Bill, but with extremely complicated enforcement provisions, Ruth Lamb called it "an opus for the relief of indigent and unemployed lawyers" (Jackson, 1970). She once described the Bailey amendment, which would have made proprietary drugs virtually immune to multiple seizures, as permitting the "sale of colored tap water as a cure for cancer … unless arsenic was added to each dose making [it] immediately dangerous." After 1934, however, the educational efforts of the FDA were greatly attenuated by federal laws prohibiting lobbying by federal agencies.

The fall of 1937 witnessed the beginning of the often-told elixir-of-sulfanilamide incident, which remains one of the nation's worst drug tragedies. The Massengil Company was not one of the industry giants, but neither was it a "snake oil peddler." The company's chief chemist, Harold Watkins, was simply trying to develop a product and, in fact, did so in a manner consistent with the norms of the time. There was a perceived need for a liquid form of sulfanilamide, but it was difficult to dissolve. Watkins hit upon diethylene glycol. No toxicity tests were performed on the finished product, although the product did pass through the "control lab" where it was checked for appearance, fragrance, and consistency.

The first reports of human toxicity occurred in October 1937 when Dr. James Stevenson of Tulsa requested some information from the AMA because of six deaths in his area that were attributable to the elixir. At the time, no product of Massengil stood accepted by the Council on Pharmacy and Chemistry, and the council recognized no solution of sulfanilamide. The AMA telegraphed Massengil, requesting samples of the preparation for testing. Massengil complied. The test revealed the diethylene glycol to be the toxic agent and the AMA issued a general warning to the public on October 18, 1937. In the meantime, the FDA had become aware of the deaths and launched an investigation through its Kansas City station. By October 20, when at least 14 people had died, Massengil wired the AMA to request an antidote for their own product. By the end of October, at least 73 people had died and another 20 suspicious deaths were linked to the drug. Had it not been for the response of the FDA, more deaths may have occurred. The agency put its full force of field investigators (239 members) on the problem and eventually recovered and accounted for 99.2% of the elixir produced. Massengil fully cooperated with the investigation and in November published a public letter expressing regret over the matter but further stating that no law had been broken. In fact, the company was eventually convicted on a

long list of misbranding charges and fined a total of $26,000 (the largest fine ever levied under the 1906 law).

The Massengil incident made the limits of the 1906 law quite clear. Because there were no provisions against dangerous drugs, the FDA could move only on the technicality of misbranding. The term *elixir* was defined by the U.S. Pharmacopeia (USP) as "a preparation containing alcohol," which elixir of sulfanilamide was not. It was only this technicality that permitted the FDA to declare the "elixir" misbranded, to seize the inventory, and to stop the sale of this preparation. If it had been called *solution of sulfanilamide*, no charges could have been brought.

The extensive press coverage of the disaster became part of the national dialogue. Letters poured in to congressmen demanding action to prevent another such tragedy. Medical and pharmacy groups and journals insisted that a new law was required. Congress was in special session in November 1937 and did not need to be told about the tragedy. Copeland and Representative Chapman (of Kentucky) pressed resolutions calling for a report from the FDA on the tragedy. When issued, the FDA report stunned Congress, not only because of the human disaster but also because it made apparent that, even had the bill then before Congress been law, the entire tragedy would still have occurred because there were no provisions for toxicity testing before new drugs entered the market. By December 1937 a new bill, S.3037, was introduced which stated that manufacturers seeking to place new drugs on the market would be required to supply records of testing, lists of components, descriptions of each manufacturing process, and sample labels. Drugs would require certification by the FDA before sale was permitted. A similar bill was introduced in the House by Chapman, although the issues of which agency was to control advertising of drugs was still festering in the House. In January 1938, debate started on the Wheeler–Lea Bill, which would ensure that all controls over drug advertising would remain with the Federal Trade Commission (FTC). Despite strong opposition by the FDA, the Wheeler–Lea Bill was signed into law March 1938. While the loss of advertising control was a blow to the FDA, the Wheeler–Lea Bill did facilitate the passage of the new Food and Drug Law.

With the issue of advertising controls settled, the Copeland–Chapman Bill faced one last hurdle. Section 701, which had been added in committee, provided for appeal suits that could be entered in any federal district court to enjoin the agency from enforcing new regulations promulgated as a result of the act. Interestingly, this issue had more to do with foods than with drugs, as its major focus was on acceptable tolerance limits for insecticides in food. The new bill defined an *adulterated food* as one containing any poison. However, because efforts to remove insecticides from fresh fruits and vegetables had never been completely successful, the secretary of agriculture needed this power to set tolerance levels. Allies of food producers tried to introduce provisions in the new bill that provided methods for stalling a tolerance regulation with rounds of appeals. The bill passed the House despite such provisions

(Section 701) and the resistance of consumer groups and the FDA and went into joint committee. Roosevelt, in one of his rare efforts to support the FDA, made it clear that he would not accept the bill with such a cumbersome appeals process. The resulting compromise was an appeals process which limited the new evidence that could be introduced into one of the 10 circuit courts. Other provisions regarding labeling were also rectified in joint committee. In May 1938, S.3073 passed by unanimous vote. Both chambers ratified the joint committee report, and Roosevelt signed the new law in June of 1938.

A historical note to this story was that Royal Copeland did not live to see his measure passed. In May 1938, he collapsed on the Senate floor. His death occurred one month before President Roosevelt signed his bill into law.

2.2.3 1962: Major Amendment

The 1938 law very much changed the manner in which Americans purchased pharmaceutical agents. In effect, it changed the pharmaceutical industry from a traditional consumer product industry to one in which purchases were made as directed by a third party (the physician). In 1929, ethical pharmaceuticals (prescription drugs) comprised only 32% of all medicines, while by 1969 this was up to 83% (Termini, 1980). This led to a peculiar lack of competition in the ethical market. In 1959, Senator Estes Kefauver initiated his now-famous hearings on the drug industry. Interestingly, almost 30 years later, Senator Edward Kennedy had hearings on exactly the same matter. In 1961, Kefauver submitted a proposed legislation to amend the 1938 act in such a way as to increase FDA oversight of the drug industry. The proposed amendment contained two novel propositions. The first was compulsory licensing, which would have required, for example, company A to license (with a royalty of no greater than 8% of sales) company B to market a drug patented by company A. Company A would have only three years' exclusivity with its patent. The second novel provision was that new drugs had to be not only "safe" but also "efficacious." There was not a groundswell of support for this legislation. When it was reported out of committee, it had been rewritten (including the removal of the licensing requirement) to the point that even Kefauver refused to support it. The Kennedy administration wanted new legislation but did not specifically support the Kefauver Bill; rather it introduced its own legislation, sponsored by Representative Orren Harris of Arkansas. It also had little support.

As in 1938, a tragic incident would intercede in the legislative process: 1961 would see the development of the thalidomide tragedy. An antianxiety agent marketed in Europe, thalidomide was prescribed for pregnancy-related depression and taken by countless numbers of women. At about the same time, phocomelia, a birth defect marked by the imperfect development of arms and legs, appeared in Europe. Thalidomide was eventually determined to be the causative teratogen in 1961 and subsequently taken off the market in

Europe. The William S. Merrill Company had applied for a new drug application (NDA) for thalidomide in the United States in 1960. It was never approved because the FDA examiner, Dr. Frances Kelsey, had returned the application for lack of sufficient information. Eventually, the company withdrew the application. Senator Kefauver's staff had uncovered the thalidomide story as it was unfolding and had turned its findings over to the *Washington Post*. The *Post* reported the episode under the headline "Heroine of the FDA Keeps Bad Drug Off the Market" in July 1962, three days after the Kefauver Bill was reported out of committee. Needless to say, the news created public support for the bill, which was sent back to committee and reported out again with new language in August 1962. The Kefauver–Harris Bill was signed into law in October 1962. It was demonstrated after the fact that thalidomide was teratogenic in the rabbit; out of the episode grew the current practice that new human pharmaceuticals are tested for teratogenicity in two species, one generally being the rabbit.

The 1962 Drug Amendment made three major changes in the manner in which new drugs could be approved (Merrill, 1994). First, and perhaps the most important, was that it introduced the concept of effectiveness into the approval process. An NDA had to contain evidence that the drug was not only safe but also effective. The 1938 law contained no such specification. The effectiveness requirement necessitated that a drug company had to do more extensive clinical trials. The new law required that companies apply to the FDA for approval of its clinical testing plan under an investigational new drug application (INDA). No response from the FDA was deemed to be acceptance. As each level of clinical testing came to require FDA review and approval, the new law made the FDA an active partner in the development of all drugs.

The second major change enacted under the 1962 amendment was the change in the approval process from premarket notification to a premarket approval system. Under the terms of the 1938 law, an NDA would take effect automatically if the FDA did not respond. For example, the only reason thalidomide was not approved was because Dr. Kelsey returned the application to the sponsor with a request for more information. In contrast, the 1962 law required affirmative FDA action before a drug could be put on the market. Under the terms of the 1962 amendments, the FDA was also empowered to withdraw NDA approval and remove the drug from the market for a variety of reasons, including new evidence that the product was unsafe or that the sponsor had misrepresented or underreported data.

The third major change enlarged the FDA's authority over clinical testing of new drugs. Thus, not only was evidence of effectiveness required, but Section 505(d) of the act specified the types of studies required: "Substantial evidence consisting of adequate and well-controlled investigations, including clinical investigations by qualified expert." In meeting the statutory requirement for setting standards of clinical evidence, the FDA has become highly influential in the design of drug-testing regimens (Merrill, 1994). Interestingly,

discussed in detail by Hutt (1987), the FDA was initially quite unprepared for this new level of responsibility. It was not until 1973 that audited regulations on the determination of safety and effectiveness were put into place (these were, in fact, approved by the Supreme Court). While there have been several procedural changes [e.g., the 1985 investigational new drug (IND) rewrite] and additions (e.g., the 1988 IND procedures for life-threatening disease treatment), there have actually been no major changes in the law through 1992 with the Prescription Drug User Fee Act (PDUFA) and 1997 with the Food and Drug Administration Modernization Act (FDAMA).

We must interject an interesting historical sidelight at this point. Despite its reputation, thalidomide made a bit of a comeback in the 1990s (Blakeslee, 1994). Among other properties, thalidomide has been shown to have good anti-inflammatory properties due to the fact that it apparently decreases the synthesis and/or release of tissue necrosis factor.

2.2.4 1992, 1997, 2002, and 2007: PDUFA and FDAMA

The history of pharmaceutical regulations has been dominated by two often-opposing schools of thought: the need to provide the citizenry with effective medicaments and the need to protect the consumer from unsafe and misbranded products. The reader is referred to Peter B. Hutt's in-depth reviews (1983a,b) on the subject. For example, the first federal drug legislation in the United States was the Vaccine Act of 1813, which mandated the provision of the smallpox vaccine to the general public. In the modern era, legislative debate could be further defined as the constant swing back and forth on these two issues (see Hutt, 1983a,b), that is, safety versus development costs. In 1963, for example, Senator Hubert Humphrey presided over hearings on the FDA's implementation of the Drug Amendment of 1962. The FDA came under substantial criticism for failure to take strong action to protect the public from dangerous drugs. Eleven years later (1974), Senator Edward Kennedy conducted hearings addressing exactly the same issue. Commissioner Schmidt pressed the point that the FDA is under constant scrutiny regarding the approval of "dangerous" drugs, but no hearing had ever been conducted (up to that time) on the failure of the FDA to approve an important new therapy.

The next decade and a half saw a proliferation of work that analyzed the impact of regulation on competitiveness and the introduction of new therapies [see Hutt (1983b) for a complete review]. This included, for example, Grabowski and Vernon's work (1983), which concluded that regulation had significant adverse effect on pharmaceutical innovation. This examination of the cost of regulation continued into the 1990s. In a meticulous and well-research study DiMasi et al. (1994) reported that throughout the 1980s the number of INDAS were decreasing while the NDA success rate was also dropping, and the length of time between discovery and approval was

increasing. Clearly this is a situation that could not go on forever. The cost of new drug development rose from $54 million (U.S.) in 1976 to $359 million (U.S.) in 1990 (Anonymous, 1998a). Members of the pharmaceutical industry and the biotechnology industry were becoming increasingly alarmed by the negative synergy caused by increased costs and increased time to market. In 1991, Dranove published an editorial examining the increased costs and decreased product flow that resulted from the 1962 amendment. He made the observation that European requirements are less stringent than those of the United States yet the Europeans did not seem to be afflicted by a greater number of dangerous drugs (see Table 1.2). Yet, if one looks at an analysis of worldwide withdrawals for safety from 1960 to 1999 (Fung et al., 2001), one sees that of 121 products identified 42.1% were withdrawn from European markets alone, 5% from North America, 3.3% from Asia Pacific, and 49.6% from multiple markets. The top five safety reasons for withdrawal were hepatic (26.2%), hematological (10.5%), cardiovascular (8.7%), dermatological (6.3%), and carcinogenic (6.3%) issue.

In an age of decreasing regulatory recourses, the FDA (as well as Congress) was under increasing pressure to review and release drugs more quickly. In response, Congress passed the 1992 PDUFA. Under the terms of this act, companies would pay a fee to the agency to defray costs associated with application review. They would supposedly provide the FDA with the resources available to decrease application review time. In return, companies were guaranteed a more rapid review time. By all accounts, PDUFA has been successful. In 1992 (the year PDUFA was passed), 26 NDAs were approved, requiring on average 29.9 months for data review, while in 1996, 53 new drug (or biological) products were approved, each requiring an average of 17.8 months of review time. PDUFA was successful in decreasing review times but has not really streamlined the procedures.

The AIDS activist community was particularly vocal and effective in demanding more rapid approvals and increased access to therapies. There was also demand for FDA reform on a number of other fronts (e.g., medical devices, pediatric claims, women and minority considerations, manufacturing changes, etc.). In 1993 the House Commerce Committee on Oversight and Investigations, chaired by John Dingel (D-MI), released a comprehensive investigation and evaluation of the FDA entitled *Less Than the Sum of Its Parts*. The report was highly critical of the FDA and made a number of recommendations (Pilot and Waldemann, 1998). The mid-1990s also saw the reinventing government initiatives (RIGO) chaired by Vice President AL Gore. Under RIGO, the FDA sought to identify and implement administrative reform. The RIGO report issued was entitled *Reinventing Regulation of Drugs and Medical Devices*. The 104th Congress started hearings on FDA reform again in the winter of 1995. Two bills were introduced that provided the essential outline of what would become FDAMA. Senator Nancy Kassebaum (R-KS), chair of the Senate Committee on Labor and Human Resources, introduced S-1477. The second was H.R. 3201, introduced by Rep. Joe Barton

(R-TX). Other bills were introduced by Senator Paul Wellstone (D-MN) and Rep. Ron Weyden (D-OR), which focused more on medical devices but still paved the way for bipartisan support of FDA reform (Pilot and Wladerman, 1998@@). Eventually, the 105th Congress passed FDAMA, which was signed into law by President Clinton in November 1997. The various sections of FDAMA are listed in Table 2.2. By any measure, it was a very broad and complex, if not overdeep, piece of legislation. In 1998, Marwick (1998, p. 815) observed, "a measure of the extent of the task is that implementation of the act will require 42 new regulations, … 23 new guidance notices, and 45 reports and other tasks." The FDA has identified these various tasks, regulations, and guidances necessary for the implementation of FDAMA. (The FDAMA implementation chart is available at http://www.fda.gov/po/modact97.html, and the reader is urged to explore this site. There is an FDAMA icon on the FDA home page, and both the Center for Biologics Evaluation and Research (CBER) and the Center for Drug Evaluation and Research (CDER) have issued various guidance documents. Some of the more interesting sections of the act that may be of interest to toxicologists included:

- Two successive renewals of PDUFA for another five years.
- Fast track for break-through products.
- Change in the fashion biologicals are regulated [elimination of the establishment and product licenses, both replaced with a biological license application or (BLA)].
- Change in the fashion antibiotics are developed and regulated.
- Incentives for the development of pediatric claims.
- Companies will be permitted to disseminate information about approved uses for their products.
- FDAMA requires that the FDA establishes a clinical trials database for drugs used to treat serious and life-threatening diseases, other than AIDS and cancers (databases for these diseases have already been established).

The full impact of FDAMA in the pharmaceutical industry in general and on toxicology within this industry in particular remains to be determined.

This is a debate that has continued to the present and has been highlighted by the demands for anti-HIV chromotherapeutic agents.

While it is not possible to review the history of regulations worldwide, it is possible to point out some differences. We will highlight specific differences where appropriate throughout the remainder of the text.

The strength of the United States regulatory system was highlighted at the BioEurope 1993 Conference. David Holtzman stated: "the main subject of the conference was regulation, and the U.S. was perceived to have the superior regulatory agency. It may be more difficult to satisfy but it is more predictable and scientifically based" (Holtzman, 1993, p. 87). This predictability has not

TABLE 2.2 Summary of Contents of 1997 Food and Drug Administration Modernization Act

Title/Subtitle	Section
I. Improving Regulatory Drugs	
A. Fees Relating to Drugs	101. Findings
	102. Definitions
	103. Authority to assess and use drug fees
	104. Annual reports
	105. Savings
	106. Effective date
	107. Termination of effectiveness
B. Other Improvements	111. Pediatric studies of drugs
	112. Expanding study and approval of fast-track drugs
	113. Information program on trials for serious disease
	114. Health care economic information
	115. Manufacturing changes for drugs
	116. Streamlining clinical research for drugs
	118. Data requirements for drugs and biologics
	119. Content and review of applications
	120. Scientific advisory panels
	121. Positron emission tomography
	122. Requirements for radiopharmaceuticals
	123. Modernization of regulation
	124. Pilot- and small-scale manufacture
	125. Insulin and antibiotics
	126. Elimination of certain labeling requirements
	127. Application of federal law to pharmacy compounding
	128. Reauthorization of clinical pharmacology program
	129. Regulation of sunscreen products
	130. Report of postmarketing approval studies
	131. Notification of discontinuance of a life-saving product
II. Improving Regulation of Devices	201. Investigational device exemptions
	202. Special review for certain devices
	203. Expanding humanitarian use of devices
	204. Device standards
	205. Collaborative determinations of device data requirements
	206. Premarket notification
	207. Evaluation of automatic class III designation
	208. Classification panels
	209. Certainty of review time frames
	210. Accreditation of person for review of premarket notification reports

TABLE 2.2 ***Continued***

Title/Subtitle	Section
	211. Device tracking
	212. Postmarket notification
	213. Reports
	214. Practice of medicine
	215. Noninvasive blood glucose meter
	216. Data relating to premarket approval: product development protocol
	217. Number of required clinical investigations for approval
III. Improving Regulation of Food	301. Flexibility for regarding claims
	302. Petitions for claims
	303. Health claims for food products
	304. Nutrient content claims
	305. Referral statements
	306. Disclosure of radiation
	307. Irradiation petition
	308. Glass and ceramic ware
	309. Food contact substance
IV. General Provisions	401. Dissemination of information new uses
	402. Expanded access of investigational therapies and diagnostics
	403. Approval of supplemental applications for approved products
	404. Dispute resolution
	405. Informal agency statements
	406. FDA mission and annual report
	407. Information system
	408. Education and training
	409. Centers for education and research on therapeutics
	410. Mutual recognition of agreements and global harmonization
	411. Environmental impact review
	412. National uniformity for nonprescription drugs and cosmetics
	413. FDA study of mercury in drugs and foods
	414. Interagency collaboration
	415. Contracts for expert review
	416. Product classification
	417. Registration of foreign establishments
	418. Clarification of seizure authority
	419. Interstate commerce
	420. Safety report disclaimers
	421. Labeling and advertising compliance with statutory requirements
	422. Rule of construction
V. Effective Date	501. Effective date

stultified the growth and biotechnology industry in the United States. In fact, it has made the United States a more inciting target for investment than Europe. It is also a system that, while not perfect, has permitted very few unsafe products on the market.

2.2.5 FDAMA: Summary—Consequences and Other Regulations

In summary, federal regulation of the safety of drugs has had three major objectives:

- Requiring testing to establish safety and efficacy
- Establishing guidelines as to which tests are required and how they are designed
- Promulgating requirements of data recording and reporting

The first of these objectives was served by the 1906 act, which required that agents be labeled appropriately. This was amended in 1938, in response to the tragedies associated with elixir of sulfanilamide and Lash Lure, to require that drugs and marketed formulations of drugs be shown to be safe when used as intended. In the aftermath of the thalidomide tragedy, the 1962 Kefauver–Harris Amendment significantly tightened requirements for preclinical testing (the INDA) and premarket approval (the NDA) of new drugs. The regulations pertaining to INDAs and NDAs have been modified (most recently in 1988) but essentially remain as the backbone of regulations of the toxicity evaluation of new human pharmaceutical agents.

The Good Laboratories Practice (GLP) Act, which specifies standards for study planning, personnel training, data recording, and reporting, came out in 1978 in response to perceived shoddy practices of the operations of laboratories involved in the conduct of preclinical safety studies. It was revised in 1985 and is discussed elsewhere in this book.

The final major regulatory initiative on how drugs will be preclinically evaluated for safety arose out of the AIDS crisis. To that point, the process of drug review and approval had very generally been perceived as slowing down, the FDA pursuing a conservative approach to requiring proof of safety and efficacy before allowing new drugs to become generally available. In response to AIDS, in 1988 the Expedited Delivery of Drugs for Life-Threatening Diseases Act established a basis for less rigorous standards (and more rapid drug development) in some limited cases.

In the United Kingdom, the Committee on Safety of Medicines (reporting to the minister of health) regulates drug safety and development under the Medicines Act of 1968 (which has replaced the Therapeutic Substances Act of 1925). Details on differences in drug safety regulations in the international marketplace can be found in Alder and Zbinden (1988), but key points are presented in this chapter.

2.3 OVERVIEW OF U.S. REGULATIONS

2.3.1 Regulations: General Considerations

The U.S. federal regulations that govern the testing, manufacture, and sale of pharmaceutical agents and medical devices are covered in Chapter 1, Title 21, of the Code of Federal Regulations (21 CFR). These comprise nine 6 × 8-in. (printing on both sides of the pages) volumes which stack 8 in. high. This title also covers foods, veterinary products, and cosmetics. As these topics will be discussed elsewhere in this book, here we will briefly review those parts of 21 CFR that are applicable to human health products and medicinal devices.

Of most interest to a toxicologist working in this arena would be Chapter 1, Subchapter A (Parts 1–78), which cover general provisions, organization, and so on. The GLPs are codified in 21 CFR 58.

General regulations that apply to drugs are in Subchapter C (Parts 200–299). This covers topics such as labeling, advertising, commercial registration, manufacture, and distribution. Of most interest to a toxicologist would be a section on labeling (Part 201, Subparts A–G, which covers Sections 201.1–201.317 of the regulations) as much of the toxicological research on a human prescription drug goes toward supporting a label claim. For example, specific requirements on content and format of labeling for human prescription drugs are covered in Section 201.57. Directions for what should be included under the "Precautions" section of a label are listed in 201.57(f). This included 201.57(f)(6), which covers categorization of pregnancy risk, and the reliance upon animal reproduction studies in making these categorizations is made quite clear. For example, a drug is given a pregnancy category B if "animal reproduction studies have failed to demonstrate a risk to the fetus." The point here is not to give the impression that the law is most concerned with pregnancy risk. Rather, we wish to emphasize that much basic toxicological information must be summarized on the drug label (or package insert). This section of the law is quite detailed as to what information is to be presented and the format of the presentation. Toxicologists working in the pharmaceutical arena should be familiar with this section of the CFR.

2.3.2 Regulations: Human Pharmaceuticals

The regulations specifically applicable to human drugs are covered in Subchapter D, Parts 300–399. The definition of a new drug is covered in Part 310(g):

> A new drug substance means any substance that when used in the manufacture, processing or packaging of a drug causes that drug to be a new drug but does not include intermediates used in the synthesis of such substances.

The regulation then goes on to discuss "newness with regard to new formulations, indications, or in combinations." For toxicologists, the meat of the

regulations can be found in Section 312 (INDA) and Section 314 (applications for approval to market a new drug or antibiotic drug or NDA). The major focus for a toxicologist working in the pharmaceutical industry is on preparing the correct toxicology "packages" to be included to "support" these two types of applications. (The exact nature of these packages will be covered below.)

In a nutshell, the law requires solid scientific evidence of safety and efficacy before a new drug will be permitted in clinical trials or (later) on the market. The INDA (covered in 21 CFR 310) is for permission to proceed with clinical trials on human subjects. Once clinical trials have been completed, the manufacturer or "sponsor" can then proceed to file an NDA (covered in 21 CFR 314) for permission to market the new drug.

As stated in 321.21, "A sponsor shall submit an IND if the sponsor intends to conduct a clinical investigation with a new drug … [and] shall not begin a clinical investigation until … an IND … is in effect." Similar procedures are in place in other major countries. In the United Kingdom, for example, a clinical trials certificate (CTC) must be filed or a CTX (clinical trial exemption) obtained before clinical trials may proceed. Clinical trials are divided into three phases, as described in 312.21. Phase I trials are initial introductions into healthy volunteers primarily for the purposes of establishing tolerance (side effects), bioavailability, and metabolism. Phase II clinical trials are "controlled studies … to evaluate effectiveness of the drug for a particular indication or disease." The secondary objective is to determine common short-term side effects; hence the subjects are closely monitored. Phase III studies are expanded clinical trials. It is during this phase that the definitive, large-scale, double-blind studies are performed.

The toxicologist's main responsibilities in the IND process are to design, conduct, and interpret appropriate toxicology studies (or "packages") to support the initial IND and then design the appropriate studies necessary to support each additional phase of investigation. Exactly what may constitute appropriate studies are covered elsewhere in this chapter. The toxicologist's second responsibility is to prepare the toxicology summaries for the (clinical) investigator's brochure [described in 312.23(a)(8)(ii)]. This is an integrated summary of the toxicological effects of the drug in animals and in vitro. The FDA has prepared numerous guidance documents covering the content and format of INDs. It is of interest that in the guidance for industry (Lumpkin, 1995) an in-depth description of the expected contents of the pharmacology and toxicology sections was presented. The document contains the following self-explanatory passage:

> Therefore, if final, fully quality-assured individual study reports are not available at the time of IND submission, an integrated summary report of toxicological findings based on the unaudited draft toxicologic reports of the completed animal studies may be submitted.

If unfinalized reports are used in an initial IND, the finalized report must be submitted within 120 days of the start of the clinical trial. The sponsor must

also prepare a document identifying any differences between the preliminary and final reports and the impact (if any) on interpretation.

Thus, while the submission of fully audited reports is preferable, the agency does allow for the use of incomplete reports.

Once an IND or CTC/X is opened, the toxicologists may have several additional responsibilities. First is to design, conduct, and report the additional tests necessary to support a new clinical protocol or an amendment to the current clinical protocol (Section 312.20). Second is to bring to the sponsor's attention any finding in an ongoing toxicology study in animals "suggesting a significant risk to human subjects, including any finding of mutagenicity, teratogenicity or carcinogenicity," as described in 21 CFR 312.32. The sponsor has a legal obligation to report such findings within 10 working days. Third is to prepare a "list of the preclinical studies … completed or in progress during the past year" and a summary of the major preclinical findings. The sponsor is required (under Section 312.23) to file an annual report (within 60 days of the IND anniversary date) describing the progress of the investigation. INDs are never "approved" in the strict sense of the word. Once filed, an IND can be opened 30 days after submission unless the FDA informs the sponsor otherwise. The structure of an IND is outlined in Table 2.3. Complete and thorough reports on all pivotal toxicological studies must be provided with the application.

If the clinical trials conducted under an IND are successful in demonstrating safety and effectiveness [often established at a pre-NDA meeting, described in 21 CFR 312.47(b)(2)], the sponsor can then submit an NDA. Unlike an IND, the NDA must be specifically approved by the agency. The toxicologist's responsibility in the NDA/marketing authorization application (MAA) process is to prepare an integrated summary of all the toxicology and/or safety studies performed and be in a position to present and review the toxicology findings to the FDA or its advisory bodies. The approval process can be exhausting, including many meetings, hearings, appeals, and so on. The ground

TABLE 2.3 Composition of Standard INDA

1.	IND cover sheets (form FDA-1571)
2.	Table of contents
3.	Introductory statement
4.	General (clinical) investigation plan
5.	(Clinical) investigators brochure
6.	(Proposed) clinical protocol(s)
7.	Chemistry, manufacturing, and control information
8.	Pharmacology and toxicology information (includes metabolism and pharmacokinetic assessments done in animals)
9.	Previous human experience with the investigational drug
10.	Additional information
11.	Other relevant information

rules for all of these are described in Part A of the law. For example, all NDAs are reviewed by an "independent" (persons not connected with either the sponsor or the agency) scientific advisory panel which will review the findings and make recommendations as to approval. MAAs must be reviewed by and reported on by an expert recognized by the cognizant regulatory authority. Final statutory approval in the United States lies with the commissioner of the FDA. It is hoped that few additional studies will be requested during the NDA review and approval process. When an NDA is approved, the agency will send the sponsor an approval letter and will issue a summary basis of approval (SBA) (312.30), which is designed and intended to provide a public record of the agency's reasoning for approving the NDA while not revealing any proprietary information. The SBA can be obtained through Freedom of Information and can provide insights into the precedents for which types of toxicology studies are used to support specific types of claims.

2.3.3 Regulations: Environmental Impact

Environmental impact statements, while once important only for animal drugs, must now accompany all MDAs. This assessment must also be included in the drug master file (DMF). The procedures, formats, and requirements are described in 21 CFR 2531. This requirement has grown in response to the National Environmental Policy Act, the heart of which required that federal agencies evaluate every major action that could effect the quality of the environment. In the INDs, this statement can be a relatively short section claiming that relatively small amounts will post little risk to the environment. The European Economic Community (EEC) has similar requirements for drug entities in Europe, though data requirements are more strenuous. With NDAs, this statement must be more substantial, detailing any manufacturing and/or distribution process that may result in release into the environment. Environmental fate (e.g., photohydrolysis) and toxicity (e.g., fish, daphnia, and algae) studies will be required. While not mammalian toxicology in the tradition of pharmaceutical testing, preparing an environmental impact statement will clearly require toxicological input. The FDA has published a technical bulletin covering the tests it may require (FDA, 1987).

2.3.4 Regulations: Antibiotics

The NDA law (safety and effectiveness) applies to all drugs, but antibiotic drugs were treated differently until the passage of FDAMA in 1997. Antibiotic drugs had been treated differently by the FDA since the development of penicillin revolutionized medicine during World War II. The laws applicable to antibiotic drugs were covered in 21 CFR 430 and 431. Antibiotics such as penicillin or doxorubicin are drugs derived (in whole or in part) from natural sources (such as molds or plants) which have cytotoxic or cytostatic properties. They were treated differently from other drugs as the applicable laws required

a batch-to-batch certification process. Originally passed into law in 1945 specifically for penicillin, this certification process was expanded by the 1962 amendment (under Section 507 of the FDCA) to require certification of all antibiotic drugs, meaning that the FDA would assay each lot of antibiotic for purity, potency, and safety. The actual regulations were covered in 21 CFR Subchapter D, Parts 430–460 (over 600 pages), which describes the standards and methods used for certification for all approved antibiotics. Section 507 was repealed by FDAMA (Section 125). As a result of the repeal of Sections 507, the FDA is no longer required to publish antibiotic monographs. In addition, the testing, filing and reviewing of antibiotic applications are now handled under Section 505 of the act like any other new therapeutic agent. The FDA has published a guidance document to which the reader is referred for more details (Anonymous, 1998a,b).

2.3.5 Regulations: Biologics

Biological products are covered in Subchapter F, Parts 600–680. As described in 21 CFR 600.3(h), "biological product means any virus, therapeutic serum, toxin, antitoxin or analogous product applicable to the prevention, treatment or cure of diseases or injuries of man." In other words, these are vaccines and other protein products derived from animal sources. Clearly the toxicological concerns with such products are vastly different than those involved with low-molecular-weight synthetic molecules. There is little rational basis, for example, for conducting a one-year, repeated dose toxicity study with a vaccine or a human blood product. The FDA definition for safety with regard to these products is found in 21 CFR 603.1(p): "Relative freedom from harmful effect to persons affected, directly or indirectly, by a product when prudently administered." Such safety consideration has more to do with purity, sterility, and adherence to good manufacturing standards than with the toxicity of the therapeutic molecule itself. The testing required to show safety is stated in the licensing procedures 21 CFR 601.25(d)(1): "Proof of safety shall consist of adequate test methods reasonably applicable to show the biological product is safe under the prescribed conditions." Once a license is granted, each batch or lot of biological product must be tested for safety, and the methods of doing so are written into the law. A general test for safety (i.e., required in addition to other safety tests) is prescribed using guinea pigs, as described in 610.11. Additional tests are often applied to specific products. For example, 21 CFR 630.35 describes the safety tests required for measles vaccines, which include tests in mice and in vitro assays with tissue culture. Many new therapeutic entities produced by biotechnology are seeking approval as biologics with the results being FDA approval of a product license application (PLA). Table 2.4 presents general guidance for the basis of deciding if an individual entity falls under CDER or the CBER authority for review.

The International Conferences on Harmonisation (ICH) has published its document S6, *Preclincial Safety Evaluation of Biotechnology-Derived*

TABLE 2.4 Product Class Review Responsibilities

Center for Drug Evaluation and Review
Natural products purified from plant or mineral sources
Products produced from solid tissue sources (excluding procoagulants, venoms, blood products, etc.)
Antibiotics, regardless of method of manufacture
Certain substances produced by fermentation
Disaccharidase inhibitors
HMG–CoA inhibitors
Synthetic chemicals
Traditional chemical synthesis
Synthesized mononuclear or polynuclear products including antisense chemicals
Hormone products
Center for Biologics Evaluation and Review
Vaccines, regardless of manufacturing method
In vivo diagnostic allergenic products
Human blood products
Protein, peptide, and/or carbohydrate products produced by cell culture (other than antibiotics and hormones)
Immunoglobulin products
Products containing intact cells or microorganisms
Proteins secreted into fluids by transgenic animals
Animal venoms
Synthetic allergens
Blood banking and infusion adjuncts

Pharmaceuticals. The FDA (the CDER and CBER jointly) has published the document as a guidance for industry (Anonymous, 1997a,b).

A current list of regulatory documents available by email (including the most recent PTCs, or points to consider) can be found at doc_list@a1.fda.gov.

2.3.6 Regulations versus Law

A note of caution must be inserted here. The law (the document passed by Congress) and the regulations (the documents written by the regulatory authorities to enforce the laws) are separate documents. The sections in the law do not necessarily have numerical correspondence. For example, the regulations on the NDA process is described in 21 CFR 312, but the law describing the requirement for an NDA process is in Section 505 of the FDCA. Because the regulations rather than the laws themselves have a greater impact on toxicological practice, greater emphasis is placed on regulation in this chapter. For a complete review of FDA law, the reader is referred to the monograph by the Food and Drug Law Institute in 1999.

Laws authorize the activities and responsibilities of the various federal agencies. All proposed laws before the U.S. Congress are referred to committees for review and approval. The committees responsible for FDA

TABLE 2.5 Congressional Committees Responsible for FDA Oversight

Authorization	
Senate	All public health service agencies are under the jurisdiction of the Labor and Human Resources Committee.
House	Most public health agencies are under the jurisdiction of Health and the Environmental Subcommittee of the House Energy and Commerce Committee.
Appropriation	
Senate	Unlike most other public health agencies, the FDA is under the jurisdiction of the Agriculture, Rural Development, and Related Agencies Subcommittee of the Senate Appropriations Committee.
House	Under the jurisdiction of the Agriculture, Rural Development, and Related Agencies Subcommittee of the House Appropriations Committee.

oversight are summarized in Table 2.5. This table also highlights the fact that authorizations and appropriations (the funding necessary to execute authorizations) are handled by different committees.

2.4 ORGANIZATIONS REGULATING DRUG AND DEVICE SAFETY IN THE UNITED STATES

The agency formally charged with overseeing the safety of drugs in the United States is the FDA. It is headed by a commissioner who reports to the secretary of the Department of Health and Human Services (DHHS) and has a tremendous range of responsibilities. Drugs are overseen primarily by the CDER (though some therapeutic or health care entities are considered biologics and are overseen by the corresponding CBER). Figure 2.1 presents the organization of CDER. The organization of CBER is shown in Figure 2.2.

Most of the regulatory interactions of toxicologists are with the two offices of Drug Evaluation, which have under them a set of groups focused on areas of therapeutic claim (cardiorenal, neuropharmacological, gastrointestinal and coagulation, oncology and pulmonary, metabolism and endocrine, anti-infective and antiviral). Within each of these are chemists, pharmacologists/toxicologists, statisticians, and clinicians. When an INDA is submitted to the offices of Drug Evaluation, it is assigned to one of the therapeutic groups based on its area of therapeutic claim. Generally, it will remain with that group throughout its regulatory approval "life." When allowed, INDs grant investigators the ability to go forward into clinical (human) trials with their drug candidate in a predefined manner, advancing through various steps of evaluation in human (and in additional preclinical or animal studies) until an NDA can be supported, developed, and submitted. Likewise for biological products, the PLA or other applications (INDA, IND) are handled by the offices of Biological Products Review of the CBER.

For drugs, there is at least one nongovernmental body which must review and approve various aspects—the USP (established in 1820), which maintains

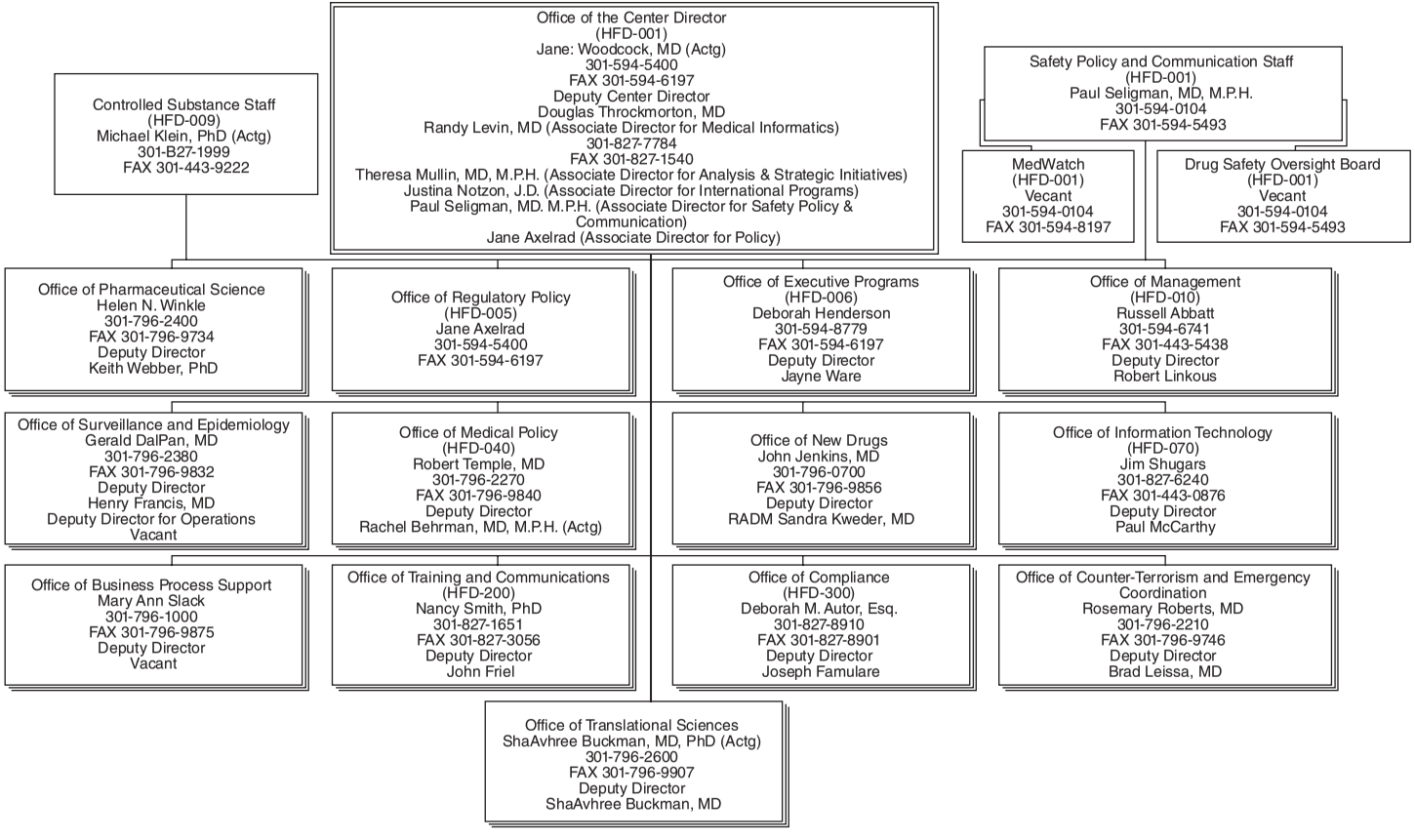

Figure 2.1 *Organizational Chart: Center for Drug Evaluation and Research.*

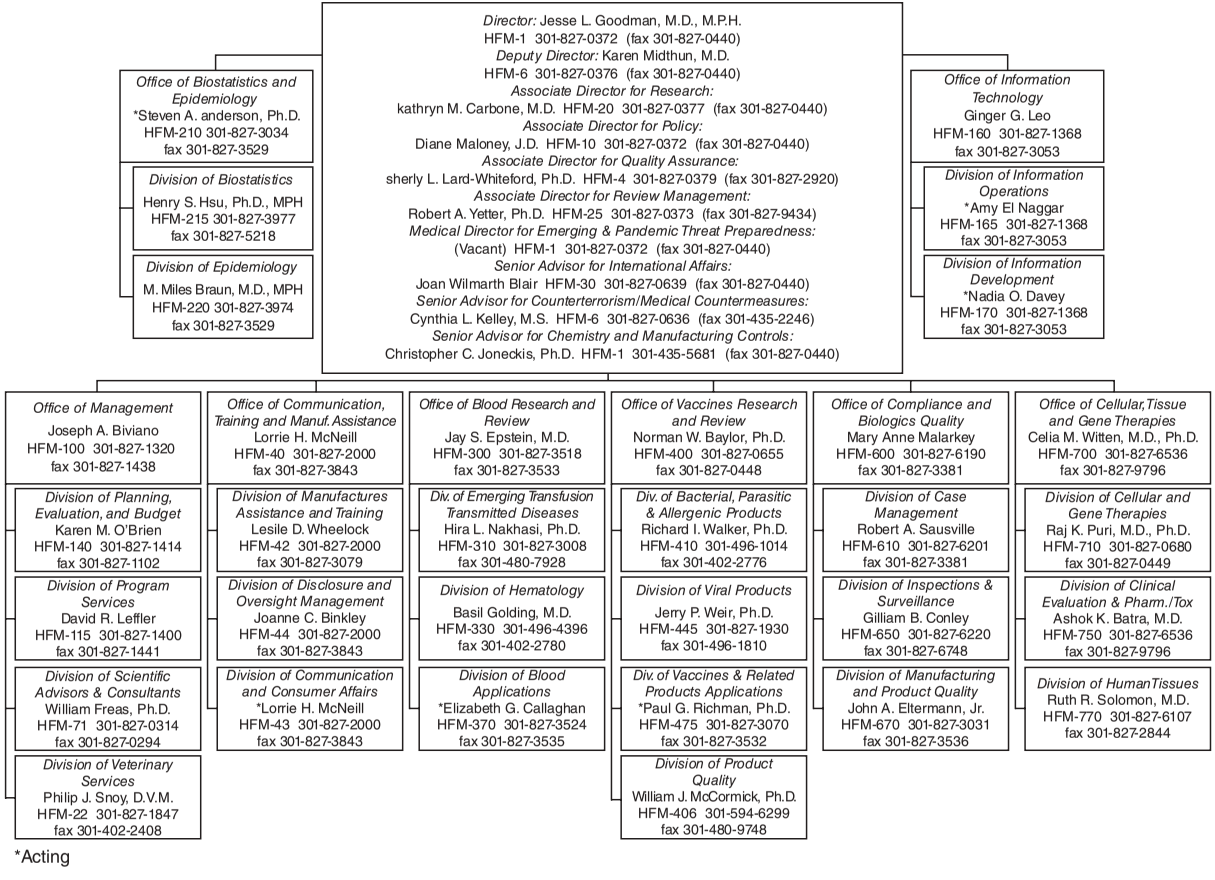

Figure 2.2 *Organizational Chart: Center for Biologics Evaluation and Research.*

(and revises) the compendia of the same name, and the National Formulary, which sets drug composition standards (Ember, 2001). This volume sets forth standards for purity of products in which residues may be present and tests for determining various characteristics of drugs, devices, and biologics. The USP also contains significant "guidance" for the evaluation.

2.5 PROCESS OF PHARMACEUTICAL PRODUCT DEVELOPMENT AND APPROVAL

Except for a very few special cases (treatments for life-threatening diseases such as cancer or AIDS), the safety assessment of new drugs as mandated by regulations are seemingly determined in a rather fixed manner. The IND is filed to support this clinical testing. An initial set of studies [typically, studies of appropriate length by the route intended for humans are performed in both a rodent (typically rat) and a nonrodent (usually a dog or a primate)] is required to support phase I clinical testing. Such phase I testing is intended to evaluate the safety ("tolerance" in clinical subjects), pharmacokinetics, and general biological effects of a new drug and is conducted in normal volunteers (almost always males).

Successful completion of phase I testing allows, with the approval of the FDA, progression into phase II clinical testing. Here, selected patients are enrolled to evaluate therapeutic efficacy, dose ranging, and more details about the pharmacokinetics and metabolism. Longer term systemic toxicity studies must be in conformity with the guidelines that are presented in the next section. Once a sufficient understanding of the actions, therapeutic dose response, and potential risk-to-benefit ratio of the drug is in hand (once again, with FDA approval), trials move into phase III testing.

Phase III tests are large, long, and expensive. They are conducted using large samples of selected patients and are intended to produce proof of safety and efficacy of the drug. Two studies providing statistically significant proof of the claimed therapeutic benefit must be provided. All the resulting data from preclinical and clinical animal studies are organized in a specified format in the form of a NDA, which is submitted to the FDA.

By the time that phase III testing is completed, some additional preclinical safety tests must also generally be in hand. These include the three separate reproductive and developmental toxicity studies (segments I and III in the rat and segment II in the rat and rabbit) and carcinogenicity studies in both rats and mice (unless the period of therapeutic usage is intended to be very short). Some assessment of genetic toxicity will also be expected.

The ultimate product of the pharmaceutical toxicologist will thus generally be the toxicology summaries of the IND and NDA (or PLA). For medical devices, the equivalents are the investigational device exemption (IDE) and product development notification (PDN). Data required to support each of these documents is specified in a series of guidelines, as will be discussed below.

Acceptance of these applications is contingent not only upon adherence to guidelines and good science but also adherence to GLPs.

2.6 TESTING GUIDELINES

2.6.1 Toxicity Testing: Traditional Pharmaceuticals

Although the 1938 act required safety assessment studies, no consistent guidelines were available. Guidelines were first proposed in 1949 and published in the *Food, Drug and Cosmetic Law Journal* that year (Burns, 1983). Following several revisions, these guidelines were issued as *The Appraisal Handbook* in 1959. While never formally called a guideline, it set the standard for preclinical toxicity test design for several years. The current basic guidelines for testing required for safety assessment in support of the phases of clinical development of drugs were first outlined by Goldenthal (1968) and later incorporated into a 1971 FDA publication entitled *FDA Introduction to Total Drug Quality*.

2.6.2 General or Systematic Toxicity Assessment

Table 2.6 presents an overview of the current FDA toxicity testing guidelines for human drugs. Table 2.7 presents the parallel ICH guidances (ICH, 2000), which are now largely supplanting the FDA guidelines. They are misleading in their apparent simplicity, however. First, each of the systemic toxicity studies in these guidelines must be designed and executed in a satisfactory manner. Sufficient animals must be used to have confidence in finding and characterizing any adverse drug actions that may be present. In practice, as the duration of the study increases, small doses are administered and larger numbers of animals must be employed per group. These two features—dosage level and group size—are critical to study designs. Table 2.8 presents general guidance on the number of animals to be used in systemic studies. These and other technical considerations for the safety assessment of pharmaceuticals are presented in detail in Gad (1994).

The protocols discussed thus far have focused on general or systemic toxicity assessment. The agency and, indeed, the lay public have a special set of concerns with reproductive toxicity, fetal/embryo toxicity, and developmental toxicity (also called *teratogenicity*). Collectively, these concerns often go by the acronym DART (developmental and reproductive toxicity) or RTF (reproduction, teratogenicity, fertility). Segment II studies are more designed to detect developmental toxicity. Only pregnant females are dosed during critical period of organogenesis. Generally, the first protocol DART test (exclusive of range-finding studies) is a segment I study of rats in fertility and general reproductive performance. This is generally done while the drug is in phase II clinical trials. Alternatively, many companies are now performing the segment II teratology study in rats before the segment I study because the

TABLE 2.6 Synopsis of General Guidelines for Animal Toxicity Studies for Drugs

Category	Duration of Human Administration	Clinical Phase	Subacute or Chronic Toxicity	Special Studies
Oral or parenteral	Several days	I, II, III, NDA	2 Species; 2 weeks	For parentally administered drugs
	Up to 2 weeks	I	2 Species; 4 weeks	
		II	2 Species; up to 4 weeks	
		III, NDA	2 Species; up to 3 months	Compatibility with blood where applicable
	Up to 3 months	I, II	2 Species; 4 weeks	
		III	2 Species; 3 months	
		NDA	2 Species; up to 6 months	
	6 Months to unlimited	I, II	2 Species; 3 months	
		III	2 Species; 6 months or longer	
		NDA	2 Species; 9 months (nonrodent) and 6 months (rodent) +2 rodent species for CA; □18 months (mouse), 24 months (rat). Mouse may be replaced with an allowable transgenic mouse study.	
Inhalation□(General □anesthetics), dermal		I, II, III, NDA	4 Species; 5 days (3 hours/day)	
	Single application	I	1 Species; single 24-h exposure followed by 2-week observation	Sensitization
	Single or short-term application	II	1 Species; 20-day repeated exposure (intact and abraded skin)	
	Short-term application	III	As above	
	Unlimited application	NDA	As above, but intact skin study extended up to 6 months	
Ophthalmic	Single application	I		Eye irritation tests with graded doses
	Multiple application	I, II, III	1 Species; 3 weeks daily applications, as in clinical use	

TABLE 2.6 ***Continued***

Category	Duration of Human Administration	Clinical Phase	Subacute or Chronic Toxicity	Special Studies
		NDA	1 Species; duration commensurate with period of drug administration	
Vaginal or rectal	Single application	I		Local and systematic toxicity after vaginal or rectal application in 2 species
	Multiple application	I, II, III, NDA	2 Species; duration and number of applications determined by proposed use	
Drug combinations		I, II, III, NDA	2 Species; up to 3 months	Lethality by appropriate route, compared to components run concurrently in 1 species

TABLE 2.7 Duration of Repeated-Dose Toxicity Studies to Support Clinical Trials and Marketing[a]

Duration of Clinical Trials	Minimum Duration of Repeated-Dose Toxicity Studies[b]		Duration of Clinical Trials	Minimum Duration of Repeated-Dose Toxicity Studies[c]	
	Rodents	Nonrodents		Rodents	Nonrodents
Single dose	2 Weeks[d]	2 Weeks	Up to 2 Weeks	1 Month	1 Month
Up to 2 weeks	2 Weeks[d]	2 Weeks	Up to 1 Month	3 Months	3 Months
Up to 1 month	1 Month	1 Month	Up to 3 Months	6 Months	3 Months
Up to 6 months	6 Months	6 Months[e]	>3 Months	6 Months	Chronic[d]
>6 Months	6 Months	Chronic[e]			

[a]In Japan, if there are no phase II clinical trials of equivalent duration to the planned phase III trials, conduct of longer duration toxicity studies is recommended as given above.
[b]Data from 6 months of administration in nonrodents should be available before the initiation of clinical trials longer than 3 months. Alternatively, if applicable, data from a 9-month nonrodent study should be available before the treatment duration exceeds that which is supported by the available toxicity studies.
[c]The table also reflects the marketing recommendations in the three regions except that a chronic nonrodent study is recommended for clinical use >1 month.
[d]In the United States, as an alternative to 2-week studies, single-dose toxicity studies with extended examinations can support single-dose human trials (4).
[e]To support phase I and II trials in the EU and phase I, II, and III trials in the United States and Japan.

TABLE 2.8 Numbers of Animals per Dosage Group in Systemic Toxicity Studies

Study Duration (per Sex)	Rodents (per Sex)	Nonrodents
2–4 Weeks	5	3
13 Weeks	20[a]	6
26 Weeks	30	8
Chronic	50	10
Carcinogenicity	60[b]	Applies only to contraceptives
Bioassays		Applies only to contraceptives

[a]Starting with 13-week studies, one should consider adding animals (particularly to the high dose) to allow evaluation of reversal of effects.

[b]In recent years there have been decreasing levels of survival in rats on 2-year studies. What is required is that at least 20–25 animals/sex/group survive at the end of the study. Accordingly, practice is beginning to use 70 or 75 animals per sex per group.

former is less time and resource intensive. One or both should be completed before including women of child-bearing potential in clinical trials. The FDA requires teratogenicity testing in two species—a rodent (rat or mouse) and the rabbit. The use of the rabbit was instituted as a result of the finding that thalidomide was a positive teratogen in the rabbit but not in the rat. On occasion, when a test article is not compatible with the rabbit, teratogenicity data in the mouse may be substituted. There are also some specific classes of therapeutics (e.g., the quinalone antibiotics) where segment II studies in primates are effectively required prior to product approval. Both should be completed before entering phase III clinical trials. The most complicated of the DART protocols—segment III—is generally commenced during phase III trials and should be part of the NDA. There are differences in the different national guidelines (as discussed later with international considerations) regarding the conduct of these studies. The large multinational drug companies try to design their protocols to be in compliance with as many of the guidelines as possible to avoid duplication of testing while allowing the broadest possible approval and marketing of therapeutics.

2.6.3 Genetic Toxicity Assessment

Genetic toxicity testing generally focuses on the potential of a new drug to cause mutations (in single-cell systems) or other forms of genetic damage. The tests, generally short in duration, often rely on in vitro systems and generally have a single endpoint of effect (point mutations, chromosomal damage, etc.). For a complete review of protocols, technology, and so on, the reader is referred to Brusick (1989). It is of interest that the FDA has no standard or statutory requirement for genetic toxicity testing but generally expects to see at least some such tests performed and will ask for them if the issue is not addressed. If one performs such a study, any data collected, of course, must be sent to the agency as part of any INDA, PLA, or NDA. These studies have

yet to gain favor with the FDA (or other national regulatory agencies) as substitutes for in vivo carcinogenicity testing. However, even with completed negative carcinogenicity tests, at least some genetic toxicity assays are generally required. Generally, pharmaceuticals in the United States are evaluated for mutagenic potential (e.g., the Ames assay) or for chromosomal damage (e.g., the in vivo mouse micronucleus test). In general, in the United States, pharmaceutical companies apply genetic toxicity testing in the following fashion:

- *As Screen* An agent that is positive in one or more genetic toxicity tests may be more likely than one that is negative to be carcinogenic and, therefore, may not warrant further development.
- *As Adjunct* An agent that is negative in carcinogenicity testing in two species and also negative in a genetic toxicity battery is more likely than not to be noncarcinogenic in human beings.
- *To Provide Mechanistic Insight* For example, if an agent is negative in a wide range of genetic toxicity screens but still produces tumors in animals, then one could hypothesize that an epigenetic mechanism was involved.

While not officially required, the FDA does have the authority to request, on a case-by-case basis, as specific tests it feels may be necessary to address a point of concern. A genetic toxicity test could be part of such a request. In general, therefore, companies deal with genetic toxicity (after "screening") on a case-by-case basis, as dictated by good science. If more than a single administration is intended, common practice is to perform the tests prior to submitting an IND.

2.6.4 Toxicity Testing: Biotechnology Products

As mentioned, the regulation of traditional pharmaceuticals (small molecules such as aspirin or digitalis) and the regulation of biologicals (proteins such as vaccines and antitoxins derived from animal sources) have very different histories. See the discussion on biologics in Section 2.3.5. Until 1972, the National Institutes of Heath (NIH; or its forerunning agency, the Hygienic Laboratory of the Department of the Treasury) was charged with the responsibilities of administering the Virus Act of 1902. With the passage of the Food and Drug Laws of 1906, 1938, and 1962, there was reoccurring debate about whether these laws applied or should apply to biologics (Pendergast, 1983). This debate was resolved when the authority for the regulation of biologics was transferred to the FDA's new Bureau of Biologics (now the CBER) in 1972. Since then, there appears to have been little difference in the matter of regulation for biologics and pharmaceuticals. The FDA essentially regulates biologics as described under the 1902 act but then uses the rule-making authority granted under the Food and Drug Act to "fill in the gaps."

The Bureau of Biologics was once a relatively "sleepy" agency, primarily concerned with the regulation of human blood products and vaccines used for mass immunization programs. The authors of the 1902 law could hardly have foreseen the explosion in biotechnology that occurred in the 1980s. New technology created a welter of new biological products, such as recombinant-DNA-produced proteins (e.g., tissue plasminogen activator), biological response modifiers (cytokinins and colony-stimulating factors), monoclonal antibodies, antisense oligonucleotides, and self-directed vaccines (raising an immune response to self-proteins such as gastrin for therapeutic reasons). The new products raised a variety of new questions on the appropriateness of traditional methods of evaluating drug toxicity that generated several PTC documents. For the sake of brevity, this discussion will focus on the recombinant DNA (rDNA) proteins. Some of the safety issues that have been raised over the years are as follows:

- The appropriateness of testing a human-specific peptide hormone in non-human species
- The potential that the peptide could break down due to nonspecific metabolism, resulting in products that had no therapeutic value or even a toxic fragment
- The potential sequelae to an immune response (formation of neutralizing antibodies, provoking an autoimmune or a hypersensitivity response), pathology due to immune precipitation, and so on
- The presence of contamination with oncogenic virus DNA (depending on whether a bacterial or mammalian system was used on the synthesizing agent) or endotoxins
- The difficulty interpreting the scientific relevance of response to supra-physiological systemic doses of potent biological response modifiers.

The intervening last few years have shown that some of these concerns were more relevant than others. The "toxic peptide fragment" concern, for example, has been shown to be without merit. The presence of potentially oncogenic virus DNA and endotoxins is a quality assurance concern and is not truly a toxicological problem. Regardless of the type of synthetic pathway, all proteins must be synthesized in compliance with good manufacturing practices (GMPs). Products must be as pure as possible, free of not only rDNA but also other types of cell debris (endotoxin). Batch-to-batch consistency with regard to molecular structure must also be demonstrated using appropriate methods (e.g., amino acid). The regulatory thinking and experience over the last 15 years has come together in the document *S6 Preclincial Safety Evaluation of Biotechnology-Derived Pharmaceuticals*, prepared by the ICH. The FDA (the CDER and CBER jointly) has published the document as a guidance for industry (Anonymous, 1997a,b). The document intended to provide basic guidance for the preclinical evaluation of biotechnology-derived products,

including proteins and peptides, either produced by cell culture using rDNA technology (but did not cover antibiotics, allergenic extracts, heparin, vitamins, cellular drug products vaccines) or as other products regulated as biologics. Items covered are summarized as follows:

- *Test Article Specifications* In general, the product that is used in the definitive pharmacology and toxicology studies should be comparable to the product proposed for the initial clinical studies.
- *Animal Species/Model Selection* Safety evaluation should include the use of relevant species, in which the test article is pharmacologically active due, for example, to the expression of the appropriate receptor molecule. These can be screened with in vitro receptor-binding assays. Safety evaluation should normally include two appropriate species if possible and/or feasible. The potential utility of gene knockout and/or transgenic animals in safety assessment is discussed.
- *Group Size* No specific numbers are given, but it does state that a small sample size may lead to failure to observe toxic events.
- *Administration* The route and frequency should be as close as possible to that proposed for clinical use. Other routes can be used when scientifically warranted.
- *Immunogenicity* It has also been clearly demonstrated in the testing of rDNA protein products that animals will develop antibodies to foreign proteins. This response has been shown to neutralize (rapidly remove from circulation) the protein, but no pathological conditions have been shown to occur as a sequelae to the immune response. Bear in mind, however, that interleukins have powerful effects on immune response, but these are due to their physiological activity and not to an antigen–antibody response. The first has to do with "neutralizing antibodies"; that is, is the immune response so great that the test article is being removed from circulation as fast as it is being added? If this is the case, does long-term testing of such a chemical make sense? In many cases, it does not. The safety testing of any large molecule should include the appropriate assays for determining whether the test system has developed a neutralizing antibody response. Depending on the species, route of administration, intended therapeutic use, and development of neutralizing antibodies (which generally takes about two weeks), it is rare for a toxicity test on an rDNA protein to be longer than four weeks duration. However, if the course of therapy in humans is to be longer than two weeks, formation of neutralizing antibodies must be demonstrated or longer term testing performed. The second antigen–antibody formation concern is that a hypersensitivity response will be elicited. Traditional preclinical safety assays are generally adequate to guard against this if they are two weeks or longer in duration and the relevant endpoints are evaluated.

- *Safety Pharmacology* It is important to investigate the potential for unwanted pharmacological activity in appropriate animal models and to incorporate monitoring for these activities in the toxicity studies.
- *Exposure Assessment* Single- and multiple-dose pharmacokinetics, toxicokinetics, and tissue distribution studies in relevant species are useful. Proteins are not given orally, demonstrating absorption and mass balance are not typically primary considerations. Rather, this segment of the test should be designed to determine the half-life (and other appropriate pharmacokinetic descriptor parameters), the plasma concentration associated with biological effects, and potential changes due to the development of neutralizing antibodies.
- *Reproductive Performance and Developmental Toxicity Studies* These will be dictated by the product, clinical indication, and intended patient population.
- *Genotoxicity Studies* The S6 document states that the battery of genotoxicity studies routinely conducted for traditional pharmaceuticals are not appropriate for biotechnology-derived pharmaceuticals. In contrast to small molecules, genotoxicity testing with a battery of in vitro and in vivo techniques of protein molecules has not become common U.S. industry practice. Such tests are not formally required by the FDA but, if performed, have to be reported. They are required by European and Japanese regulatory authorities. This has sparked a debate as to whether or not genotoxicity testing is necessary or appropriate for rDNA protein molecules. It is the author's opinion that such testing is scientifically of little value. First, large protein molecules will not easily penetrate the cell wall of bacteria or yeast, and (depending on size, charge, lipophilicity, etc.) penetration across the plasma lemma of mammalian cells will be highly variable. Second, if one considers the well-established mechanism(s) of genotoxicity of small molecules, it is difficult to conceive how a protein can act in the same fashion. For example, proteins will not be metabolized to be electrophilic active intermediates that will crosslink guanine residues. In general, therefore, genotoxicity testing with rDNA proteins is a waste of resources. It is conceivable, however, that some proteins, because of their biological mechanism of action, may stimulate the proliferation of transformed cells. For example, it is a feasible hypothesis that a colony-stimulating factor could stimulate the proliferation of leukemic cells (it should be emphasized that this is a hypothetical situation, presented here for illustrative purposes). Again, this is a question of a specific pharmacological property, and such considerations should be tested on a case-by-case basis.
- *Carcinogenicity Studies* These are generally inappropriate for biotechnology-derived pharmaceuticals; however, some products may have the potential to support or induce proliferation of transformed cells, possibly leading to neoplasia. When this concern is present, further studies in relevant animal models may be needed.

These items are covered in greater detail in the S6 guidance document.

So, given the above discussion, what should the toxicology testing package of a typical rDNA protein resemble? Based on the products that have successfully wound their way through the regulatory process, the following generalizations can be drawn:

- The safety tests look remarkably similar to those for traditional tests. Most have been done on three species: the rat, the dog, or the monkey. The big difference has to do with the length of the test. It is rare for a safety test on a protein to be more than 13 weeks long.
- The dosing regimens can be quite variable and at times very technique intensive. These chemicals are almost always administered by a parenteral route of administration—normally intravenously or subcutaneously. Dosing regimens have run the range from once every 2 weeks for an antihormone "vaccine" to continuous infusion for a short-lived protein.
- As reviewed by Ryffel (1996) most side effects in man of a therapy with rDNA therapy may be predicted by data from experimental toxicology studies, but there are exceptions. Interleukin 6 (IL-6), for example, induced a sustained increase in blood platelets and acute phase proteins, with no increase in body temperature. In human trials, however, there were increases in temperature.
- The S6 document also mentions monoclonal antibody products. Indeed, many of the considerations for rDNA products are also applicable to monoclonal antibodies (including hybridized antibodies). With monoclonal antibodies, there is the additional concern of cross-reactivity with nontarget molecules.

As mentioned, the rapid development in the biotechnology industry has created some confusion as to what arm of the FDA is responsible for such products. In October 1992, the two major reviewing groups, CBER and CDER, reached a series of agreements to explain and organize the FDA's position on products that did not easily fall into its traditional classification schemes. CDER will continue to have responsibility for traditional chemically synthesized molecules as well as those purified from mineral or plant sources (except allergenics), antibiotics, hormones (including insulin, growth hormone, etc.), most fungal or bacterial products (disaccharidase inhibitors), and most products from animal or solid human tissue sources. CBER will have responsibility for products subject to licensure (BLA), including all vaccines, human blood or blood-derived products (as well as drugs used for blood banking and transfusion), immunoglobulin products, products containing intact cells, fungi, viruses, proteins produced by cell culture or transgenic animals, and synthetic allergenic products. This situation was further simplified by the introduction of the concept of "well-characterized biologics." When introduced during the

debate on FDA reform in 1996, the proposed section of S-1447 stated: "Biological products that the secretary determines to be well-characterized shall be regulated solely under the Federal Food, Drug and Cosmetic Act." Under this concept, highly purified, well-characterized therapeutic rDNA proteins would be regulated by CDER, regardless of therapeutic target (Anonymous, 1996).

2.6.5 Toxicity/Safety Testing: Cellular and Gene Therapy Products

Human clinical trials of cellular and gene therapies involve administration to patients of materials considered investigational biological, drug, or device products. Somatic cell therapy refers to the administration to humans of autologous, allogenic, or xenogenic cells which have been manipulated or processed ex vivo. Gene therapy refers to the introduction into the human body of genes or cells containing genes foreign to the body for the purposes of prevention, treatment, diagnosing, or curing disease.

Sponsors of cellular or gene therapy clinical trials must file an INDA or in certain cases an IDE with the FDA before initiation of studies in humans. It is the responsibility of the CBER to review the application and determine if the submitted data and the investigational product meet applicable standards. The critical parameters of identity, purity, potency, stability, consistency, safety, and efficacy relevant to biological products are also relevant to cellular and gene therapy products.

In 1991, the FDA first published "Points to Consider on Human Somatic Cell and Gene Therapy." At this time virtually all gene therapies were retroviral and were prepared as ex vivo somatic cell therapies. This was subsequently reviewed by Kessler et al. (1993). While the data for certain categories of information such as the data regarding the molecular biology were defined in previous guidance documents relating to recombinant DNA products, the standards for preclinical and clinical development were less well defined. Over the past five years, the field has advanced to include not only new vectors but also novel routs of administration. "Points to Consider on Human Somatic Cell and Gene Therapy" has thus been recently amended (1996) to reflect both the advancements in product development and more importantly the accumulation of safety information over the past five years.

FDA regulations state that the sponsor must submit, in the IND, adequate information about pharmacological and toxicological studies of the drug, including laboratory animals or in vitro studies on the basis of which the sponsor has considered that it is reasonably safe to conduct the proposed clinical investigation. For cellular and gene therapies, designing and conducting relevant preclinical safety testing have been a challenge to both the FDA and the sponsor. For genes delivered using viral vectors, the safety of the vector system per se must be considered and evaluated.

The preclinical knowledge base is initially developed by designing studies to answer fundamental questions. The development of this knowledge base is

generally applicable to most pharmaceuticals as well as biopharmaceuticals and includes data to support (1) the relationship of the dose to the biological activity, (2) the relationship of the dose to the toxicity, (3) the effect of route and/or schedule on activity or toxicity, and (4) identification of the potential risks for subsequent clinical studies. These questions are considered in the context of indication and/or disease state. In addition there are often unique concerns relating to the specific category or product class.

For cellular therapies safety concerns may include development of a database from studies specifically designed to answer questions relating to growth factor dependence, tumorigenicity, local and systemic toxicity, and effects on host immune responses, including immune activation and altered susceptibility to disease. For viral-mediated gene therapies, specific questions may relate to the potential for overexpression of the transduced gene, transduction of normal cells/tissues, genetic transfer to germ cells and subsequent alterations to the genome, recombination/rescue with endogenous virus, reconstitutions of replication competence, potential for insertional mutagenesis/malignant transformation, altered susceptibility to disease, and/or potential risk(s) to the environment.

To date cellular and gene therapy products submitted to the FDA have included clinical studies indicated for bone marrow marking, cancer, cystic fibrosis, AIDS, and inborn errors of metabolism and infectious diseases. Of the current active INDs approximately 78% have been sponsored by individual investigators or academic institutions and 22% have also been industry sponsored. In addition to the variety of clinical indications the cell types have also been varied. Examples include tumor-infiltrating lymphocytes (TILs) and lymphocyte-activated killer (LAK) cells, selected cells from bone marrow and peripheral blood lymphocytes (e.g., stem cells), myoblasts, tumor cells, and encapsulated cells (e.g., islet cells and adrenal chromaffin cells).

Cellular Therapies Since 1984 CBER has reviewed close to 300 somatic cell therapy protocols. Examples of the specific categories include manipulation, selection, mobilization, tumor vaccines, and others:

Manipulation Autologous, allogenic, or xenogenic cells which have been expanded, propagated, manipulated, or had their biological characteristics altered ex vivo (e.g., TIL or LAK cells; islet cells housed in a membrane).

Selection Products designed for positive or negative selection if autologous or allogenic cells are intended for therapy (e.g., purging of tumor from bone marrow, selection of $CD34^+$ cells).

Mobilization In vivo mobilization of autologous stem cells intended for transplantation.

Tumor Vaccines Autologous or allogenic tumor cells which are administered as vaccine (e.g., tumor cell lines, tumor cell lysates, primary

explant). This group also includes autologous antigen-presenting cells pulsed with tumor-specific peptides or tumor cell lysates.

Other Autologous, allogenic, and xenogenic cells which do not specifically fit above. This group includes cellular therapies such as extracorporeal liver assist devices.

Gene Therapies The types of vectors that have been used, or proposed, for gene transduction include retrovirus, adenovirus, adeno-associated viruses, other viruses (e.g., herpes, vaccinia), and plasmid DNA. Methods for gene introduction include ex vivo replacement, drug delivery, marker studies, and others and in vivo, viral vectors, plasmid vectors, and vector producer cells.

Ex Vivo

Replacement Cells transduced with a vector expressing a normal gene in order to correct or replace the function of a defective gene.

Drug Delivery Cells transduced with a vector expressing a gene encoding a therapeutic molecule which can be novel or native to the host.

Marker Studies Cells (e.g., bone marrow, stem cells) transduced with a vector expressing a marker or reporter gene used to distinguish it from other similar host tissues.

Other Products which do not specifically fit above (e.g., tumor vaccines in which cells are cultured or transduced ex vivo with a vector).

In Vivo

Viral Vectors The direct administration of a viral vector (e.g., retrovirus, adenovirus, adeno-associated virus, herpes, vaccinia) to patients.

Plasmid Vectors The direct administration of plasmid vectors with or without other vehicles (e.g., lipids) to patients.

Vector Producer Cells The direct administration of retroviral vector producer cells [e.g., murine cells producing the Hidden Markov Model ToolKit (HTK) vector] to patients.

Preclinical Safety Evaluation The goal of preclinical safety evaluation includes recommendation of an initial safe starting dose and safe dose escalation scheme in humans, identification of potential target organ(s) of toxicity, identification of appropriate parameters for clinical monitoring, and identification of "at-risk" patient population(s). Therefore, when feasible, toxicity studies should be performed in relevant species to assess a dose-limiting toxicity. General considerations in study design include selection of the model (e.g., species, alternative model, animal model or disease), dose (e.g., route, frequency, and duration), and study endpoint (e.g., activity and/or toxicity).

The approach to preclinical safety evaluation of biotechnology-derived products, including novel cellular and gene therapies, has been referred to as

the "case-by case" approach. This approach is science based, data driven, and flexible. The major distinction from past practices from traditional pharmaceuticals is that the focus is directed at asking specific questions across various product categories. Additionally, there is a consistent reevaluation of the knowledge base to reassess real or theoretical safety concerns and hence reevaluation of the need to answer the same questions across all product categories. In some cases there may even be conditions which may not need specific toxicity studies, for example, when there is a strong efficacy model which is rationally designed to answer specific questions and/or there is previous human experience with a similar product with respect to dose and regimen.

Basic Principles for Preclinical Safety Evaluation of Cellular and Gene Therapies

Biotechnology-Derived Products in General

- Use of product in animal studies that is comparable or the same as the product proposed for clinical trial(s)
- Adherence to basic principles of GLP to ensure quality of the study, including a detailed protocol prepared prospectively
- Use of the same or similar route and method of administration as proposed for clinical trials (whenever possible)
- Determination of appropriate doses delivered based upon preliminary activity obtained from both in vitro and in vivo studies (i.e., finding a dose likely to be effective and not dangerous, at no observed adverse effect level, and a dose causing dose-limiting toxicity)
- Selection of one or more species sensitive to the endpoint being measured, for example, infections or pathological sequelae and/or biological activity or receptor binding
- Consideration of animal model(s) of disease, which may be better to assess the contribution of changes in physiological or underlying physiology to safety and efficacy
- Determination of affect on host immune response
- Localization/distribution studies—evaluation of target tissue, normal surrounding tissue, and distal tissue sites and any alteration in normal or expected distribution
- Local reactogenicity at target tissue

Additional Considerations for Cellular Therapies

- Evaluation of cytopathogenicity
- Evaluation of signs of cell transformation/growth factor dependence effect on animal cells, normal human cells, and cells prone to transform easily

- Determination of alteration in cell phenotype, altered cell products, and/or function
- Tumorigenicity

Additional Considerations for Gene Therapies

- Determination of phenotype/activation state of effector cells
- Determination of vector/transgene toxicity
- Determination of potential transfer to germ line
- In vitro challenge studies—evaluation of recombination or complementation, potential for "rescue" for subsequent infection with wild-type virus
- Determination of persistence of cells/vector
- Determination of potential for insertional mutagenesis (malignant transformation)
- Determination of environmental spread (e.g., viral shedding)

2.7 TOXICITY TESTING: SPECIAL CASES

On paper, the general-case guidelines for the evaluation of the safety of drugs are relatively straightforward and well understood. However, there are also a number of special-case situations under which either special rules apply or some additional requirements are relevant. The more common of these are summarized below.

2.7.1 Oral Contraceptives

Oral contraceptives are subject to special testing requirements. These have recently been modified so that, in addition to those preclinical safety tests generally required, the following are also required (Berliner, 1974):

- A three-year carcinogenicity study in beagles (this is a 1987 modification in practice from earlier FDA requirements and the 1974 publication)
- A rat reproductive (segment I) study, including a demonstration of return to fertility

2.7.2 Life-Threatening Diseases (Compassionate Use)

Drugs to treat life-threatening diseases are not strictly held to the sequence of testing requirements as put forth in Table 2.3 because the potential benefit on any effective therapy in these situations is so high. In the early 1990s, this situation applied to AIDS-associated diseases and cancer. The development of more effective HIV therapies (protease inhibitors) has now made cancer therapy more the focus of these considerations. Though the requirements

for safety testing prior to initial human trials are unchanged, subsequent requirements are flexible and subject to negotiation and close consultation with the FDA's Division of Oncology (within CDER) (FDA, 1988c). The more recent thinking on anticancer agents has been reviewed by DeGeorge et al. (1998). The preclinical studies that will be required to support clinical trials and marketing of new anticancer agents will depend on the mechanism of action and the target clinical population. Toxicity studies in animals will be required to support initial clinical trials. These studies have multiple goals:

- To determine a starting dose for clinical trials
- To identify target organ toxicity and assess recovery
- To assist in the design of clinical dosing regimens

The studies should generally conform to the protocols recommended by the National Cancer Institute. as discussed by Grieshaber (1991). In general, it can be assumed that most antineoplastic cytotoxic agents will be highly toxic. Two studies are essential to support initial clinical trials (IND phase) in patients with advanced disease. These studies are 5–14 days long with longer recovery periods. A study in rodents is required that identifies those doses that produce either life-threatening or non-life-threatening toxicity. Using the information from this first study, a second study in nonrodents (generally the dog) is conducted to determine if the tolerable dose in rodents is life threatening. Doses are compared on a milligram-per-square-meter basis. The staring dose in initial clinical trials is generally one-tenth of that required to produce severe toxicity in rodents (STD10) or one-tenth the highest dose in nonrodents that does not cause severe irreversible toxicity. While not required, information on pharmacokinetic parameters, especially data comparing the plasma concentration associated with toxicity in both species, is very highly regarded. Special attention is paid to organs with high cell division rates, bone marrow, testes, lymphoid tissue testing, and gastrointestinal tract. As these agents are almost always given intravenously, special attention needs to be given relatively early in development to intravenous (IV) irritation and blood compatibility study. Subsequent studies to support the NDA will be highly tailored, depending on the following:

- Therapeutic indication and mechanism of action
- Results of the initial clinical trials
- Nature of the toxicity
- Proposed clinical regimen

Even at the NDA stage, toxicity studies with more than 28 days of dosing are rarely required. While not required for the IND, assessment of genotoxicity and developmental toxicity will need to be addressed. For genotoxicity, it will

be important to establish the ratio between cytotoxicity and mutagenicity. In vivo models (e.g., the mouse micronucleus test) can be particularly important in demonstrating the lack of genotoxicity at otherwise subtoxic doses. For developmental toxicity, ICH stage C–D studies (traditionally known as segment II studies for teratogenicity in rat and rabbits) will also be necessary.

The emphasis of this discussion has been on purely cytotoxic neoplastic agents. Additional considerations must be given to cytotoxic agents that are administered under special circumstances: those that are photoactivated, delivered as liposomal emulsions, or delivered as antibody conjugates. These types of agents will require additional studies. For example, a liposomal agent will need to be compared to the free agent and a blank liposomal preparation. There are also studies that may be required for a particular class of agents. For example, anthracyclines are known to be cardiotoxic, so comparison of a new anthracycline agent to previously marketed anthracyclines will be expected.

In addition to antineoplastic, cytotoxic agents, there are cancer therapeutic or preventative drugs that are intended to be given on a chronic basis. This includes chemopreventatives, hormonal agents, and immunomodulators. The toxicity assessment studies on these will more closely resemble those of more traditional pharmaceutical agents. Chronic toxicity, carcinogenicity, and full developmental toxicity (ICH A–B, C–D, E–F) assessments will be required. For a more complete review, the reader is referred to DeGeorge et al. (1998).

2.7.3 Optical Isomers

The FDA [and like regulatory agencies, as reviewed by Daniels et al. (1997)] has become increasingly concerned with the safety of stereoisomeric or chiral drugs. Stereoisomers are molecules that are identical to one another in terms of atomic formula and covalent bonding but differ in the three-dimensional projections of the atoms. Within this class are those molecules that are non-superimposable mirror images of one another. These are called enantiomers (normally designated as R or S). Enantiomeric pairs of a molecule have identical physical and chemical characteristics except for the rotation of polarized light. Drugs have generally been mixtures of optical isomers (enantiomers) because of the difficulties in separating the isomers. It has become apparent in recent years, however, that these different isomers may have different degrees of both desirable therapeutic and undesirable toxicological effects. Technology has also improved to the extent that it is now possible to perform chiral specific syntheses, separations, and/or analyses. It is now highly desirable from a regulatory (FDA, 1988a–c; Anonymous, 1992a) basis to develop a single isomer unless all isomers have equivalent pharmacological and toxicological activity. The FDA has divided enantiomeric mixtures in the following categories:

- Both isomers have similar pharmacologic activity, which could be identical, or they could differ in the degrees of efficacy.
- One isomer is pharmacologically active, while the other is inactive.
- Each isomer has completely different activity.

During preclinical assessment of an enantiomeric mixture, it may be important to determine to which of these three classes it belongs. The pharmacological and toxicological properties of the individual isomers should be characterized. The pharmacokinetic profile of each isomer should be characterized in animal models with regard to disposition and interconversion. It is not at all unusual for each enantiomer to have a completely different pharmacokinetic behavior.

If the test article is an enantiomer isolated from a mixture that is already well characterized (e.g., already on the market), then appropriate bridging guides need to be performed which compare the toxicity of the isomer to that of the racemic mixture. The most common approach would be to conduct a subchronic (three-month) and a segment-II-type teratology study with an appropriate "positive" control group which received the racemate. In most instances no additional studies would be required if the enantiomer and the racemate did not differ in toxicity profile. If, on the other hand, differences are identified, then the reasons for this difference need to be investigated and the potential implications for human subjects need to be considered.

2.7.4 Special Populations: Pediatric and Geriatric Claims

Relatively few drugs marketed in the United States (approximately 20%) have pediatric dosing information available. Clinical trials had rarely been done specifically on pediatric patients. Traditionally, dosing regimens for children have been derived empirically by extrapolating on the basis of body weight or surface area. This approach assumes that the pediatric patient is a young adult, which simply may not be the case. There are many examples of how adults and children differ qualitatively in metabolic and/or pharmacodynamic responses to pharmaceutical agents. In their review, Schacter and DeSantis (1998, p. 300) state:

> The benefit of having appropriate usage information in the product label is that health care practitioners are given the information necessary to administer drugs and biologics in a manner that maximizes safety, minimizes unexpected adverse events, and optimizes treatment efficacy. Without specific knowledge of potential drug effects, children may be placed at risk. In addition, the absence of appropriate proscribing information, drugs and biologics that represent new therapeutic advances may not be administered to the pediatric population in a timely manner.

In response to the need for pediatric information, the FDA had developed a pediatric plan. This two-phase plan called first for the development of

pediatric information on marketed drugs. The second phase focused on new drugs. The implementation of the plan was to be coordinated by the Pediatric Subcommittee of the Medical Policy Coordinating Committee of CDER. The Pediatric Use Labeling Rule was a direct result of phase 1 in 1994 (Anonymous, 1998b). Phase 2 resulted in 1997 from a proposed rule entitled "Pediatric Patients; Regulations Requiring Manufacturers to Assess the Safety and Effectiveness of New Drugs and Biologics." Soon after this rule was proposed, the FDA Modernization Act of 1997 was passed. FDAMA contained provisions that specifically addressed the needs and requirements for the development of drugs for the pediatric population.

The FDAMA bill essentially codified and expanded several regulatory actions initiated by the FDA during the 1990s. Among the incentives offered by the bill, companies will be offered an additional six months of patent protection for performing pediatric studies (clinical trials) on already approved products. In fact, the FDA was mandated by FDAMA to develop a list of over 500 drugs for which additional information would produce benefits for pediatric patients. The FDA is supposed to provide a written request for pediatric studies to the manufacturers (Hart, 1999).

In response to the pediatric initiatives, the FDA has published policies and guidelines and conducted a variety of meetings. CDER has established a website (http//www.fda.gov/cder/pediatric) which lists three pages of such information. Interestingly, the focus has been on clinical trials, and almost no attention has been given to the preclinical toxicology studies that may be necessary to support such trials. There are three pages of documents on the pediatric website. None appear to address the issue of appropriate testing. This situation is just now being addressed and is in a great deal of flux.

In the absence of any guidelines from the agency for testing drugs in young or "pediatric" animals, one must fall back on the maxim of designing a program that makes the most scientific sense. As a guide, the FDA-designated levels of postnatal human development and the approximate equivalent ages (in the author's considered opinion) in various animal models are given in Table 2.9. The table is somewhat inaccurate, however, because of differences in the stages of development at birth. A rat is born quite underdeveloped when compared to a human being. A one-day-old rat is not equivalent to a one-day-old full-term human infant. A four-day-old rat would be more appropriate. In terms of development, the pig may be the best model of those listed; however, one should bear in mind that different organs have different developmental schedules in different species.

Table 2.9 can be used as a rough guide in designing toxicity assessment experiments in developing animals. In designing the treatment period, one needs to consider not only the dose and proposed course of clinical treatment but also the proposed age of the patient and whether or not an equivalent dosing period in the selected animal model covers more than one developmental stage. For example, if the proposed patient population is human infants, initiating a toxicity study of the new pharmaceutical agent in

TABLE 2.9 Comparison of Postnatal Development Stages

Stage	Human	Rat	Dog	Pig
Neonate	Birth to 1 month	Birth to 1 week	Birth to 3 weeks	Birth to 2 weeks
Infant	1 Month to 2 years	1–3 Weeks	3–6 Weeks	2–4 Weeks
Child	2 Years to 12 years	3–9 Weeks	6 Weeks–5 months	4 Weeks–4 months
Adolescent	12 Years to 16 years	9–13 Weeks	5 Moths–9 months	4–7 Months
Adult	Over 16 years	Over 13 weeks	Over 9 months	Over 7 months

three-day-old rats is not appropriate. Furthermore, if the proposed course of treatment in adult children is two weeks, it is unlikely that this would cross over into a different developmental stage. A two-week treatment initiated in puppies, however, might easily span two developmental stages. Thus, in designing an experiment in young animals one must carefully consider the length of the treatment period balancing the developmental age of the animal model and the proposed length of clinical treatment. Where appropriate (infant animals), one needs to also assess changes in standard developmental landmarks (e.g., eye opening, pinnae eruption, external genitalia development) as well as the more standard indicators of target organ toxicity. The need for maintaining the experimental animals past the dosing period, perhaps into sexual maturity, to assess recovery or delayed effects needs also to be carefully considered.

To summarize, the current status of assessment of toxicity in postnatal mammals, in response to the pediatric initiatives covered in FDAMA, is an extremely fluid situation. One needs to carefully consider a variety of factors in designing the study and should discuss proposed testing programs with the appropriate office at CDER.

Drugs intended for use in the elderly, like those intended for the very young, may also have special requirements for safety evaluation, but geriatric issues were not addressed in the FDAMA of 1997. The FDA has published a separate guidance document for geriatric labeling. As was the case with pediatric guidance, this document does not address preclinical testing. With the elderly, the toxicological concerns are quite different than the developmental concerns associated with pediatric patients. With the elderly, one must be concerned with the possible interactions between the test article and compromised organ function. The FDA had previously issued a guidance for clinically examining the clinical safety of new pharmaceutical agents in patients with compromised renal and/or hepatic function (CDER, 1989). The equivalent ICH guideline (S5A) was issued in 1994. Whether this type of emphasis will require toxicity testing in animal models with specifically induced organ insufficiency remains to be seen. In the interim, we must realize that there is

tacit evaluation of test article–related toxicity in geriatric rodents for those agents that undergo two-year carcinogenicity testing. As the graying of America continues, labeling for geriatric use may become more of an issue in the future.

2.7.5 Orphan Drugs

The development of sophisticated technologies coupled with the rigors and time required for clinical and preclinical testing has made pharmaceutical development very expensive. In order to recoup such expenses, pharmaceutical companies have tended to focus on therapeutic agents with large potential markets. Treatment for rare but life-threatening diseases have been "orphaned" as a result. An orphan product is defined as one targeted at a disease which affects 200,000 or fewer individuals in the United States. Alternatively, the therapy may be targeted for more than 200,000 but the developer would have no hope of recovering the initial investment without exclusivity. The Orphan Drug Act (ODA) of 1983 was passed in an attempt to address this state of affairs. Currently applicable regulations were put in place in 1992 (Anonymous, 1992b). In 1994, there was an attempt in Congress to amend the act, but it failed to be passed into law. The current regulations are administered by the office of Orphan Product Development (OPD). The act offers the following incentives to encourage the development of products to treat rare diseases:

- Seven years exclusive market following the approval of a product for an orphan disease
- Written protocol assistance from the FDA
- Tax credits for up 50% of qualified clinical research expenses
- Available grant to support pivotal clinical trials

As reviewed by Haffner (1998), other developed countries have similar regulations.

The ODA did not change the requirements of testing drug products. The nonclinical testing programs are similar to those used for more conventional products. They will undergo the same FDA review process. A major difference, however, is the involvement of the OPD. A sponsor must request OPD review. Once the OPD determines that a drug meets the criteria for orphan drug status, it will work with the sponsor to provide the assistance required under the act. The ODA does not review a product for approval. The IND/NDA process is still handled by the appropriate reviewing division (e.g., cardiovascular) for formal review. The act does not waive the necessity for submission of an IND, not for the responsibility of toxicological assessment. As always, in cases where there is ambiguity, a sponsor may be well served to request a pre-IND meeting at the appropriate division to discuss the acceptability of a toxicology assessment plan.

2.7.6 Botanical Drug Products

There is an old saying, "What goes around, comes around": and so it is with botanicals. At the beginning of the twentieth century, most marketed pharmaceutical agents were botanical in origin. For example, aspirin was first isolated from willow bark. These led the way to modern drug development in the middle part of the century, for reasons having to do with patentability, manufacturing costs, standardization, selectivity, and potency. The dawning of the twenty-first century has seen a grass-roots return to botanical preparations (also sold as herbals or dietary supplements). These preparations are being marketed to the lay public as "natural" supplements to the nasty synthetic chemicals now proscribed as pharmaceutical products. In 1994, the Dietary Supplement Health and Education Act was passed which permitted the marketing of dietary supplements (including botanicals) with limited submissions to the FDA (Wu et al., 2000). If a producer makes a claim that an herbal preparation is beneficial to a specific part of the body (e.g., enhanced memory), then it may be marketed after a 75-day period of FDA review but without formal approval. On the other hand, if any curative properties are claimed, then the botanical will be regulated as a drug and producers will be required to follow the IND/NDA process. In 1997 and 1998 combined, some 26 INDs were filed for botanical products (Wu et al., 2000).

The weakness in the current regulation has to do with its ambiguity. The line between a beneficial claim and a curative claim is sometimes difficult to draw. What is the difference, for example, between an agent that enhances memory and one that prevents memory loss? Given the number of products and claims hitting the shelves every day, this situation will probably demand increased regulatory scrutiny in the future.

2.8 INTERNATIONAL PHARMACEUTICAL REGULATION AND REGISTRATION

2.8.1 International Conference on Harmonisation

The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use was established to make the drug regulatory process more efficient in the United States, Europe, and Japan. The U.S. involvement grew out of the fact that the United States is party to the General Agreement on Tariffs and Trade, which included the Agreement on Technical Barriers to Trade, negotiated in the 1970s, to encourage reduction of nontariff barriers to trade (Barton, 1998). The main purpose of ICH is, through harmonization, to make new medicines available to patients with a minimum of delay. More recently, the need to harmonize regulation has been driven, according to ICH, by the escalation of the cost of R&D. The regulatory systems in all countries have the same fundamental concerns about safety, efficacy, and quality, yet sponsors had to repeat many time-consuming

TABLE 2.10 ICH Representation

Country/Region	Regulatory	Industry
European Union	European Commission (2)	European Federation of Pharmaceutical Industries Associations (2)
Japan	Ministry of Health and Welfare (2)	Japanese Pharmaceutical Manufactures Association (2)
United States	Food and Drug Administration (2)	Pharmaceutical Research and Manufacturers of America (2)
Observing organizations	World Health Organization, European Free Trade Area, Canadian Health Protection Branch	International Federation of Pharmaceutical Manufactures Associations (2): also provides the secretariat

Note: Numbers in parentheses are number of representatives on the ICH steering committee.

and expensive technical tests to meet country-specific requirements. In addition, there was a legitimate concern over the unnecessary use of animals. Conference participants include representatives from the drug regulatory bodies and research-based pharmaceutical industrial organizations of three regions; the European Union, the United States, and Japan comprised over 90% of the world's pharmaceutical industry. Representation is summarized in Table 2.10. The biannual conference has met four times, beginning in 1991, rotating between sites in the United States, Europe, and Japan. The next meeting is scheduled for the year 2001 and will be held on the West Coast of the United States. The precise venue has yet to be named.

The ICH meets its objectives by issuing guidelines for the manufacturing, development, and testing of new pharmaceutical agents that are acceptable to all three major parties. For each new guideline, the ICH steering committee establishes an expert working group with representation from each of the six major participatory ICH bodies. Each new draft guideline goes through the five various steps of review and revision, summarized in Table 2.11. So far, the ICH has proposed or adopted over 40 safety, efficacy, and quality guidelines (listed in Table 2.12) for use by drug regulatory agencies in the United States, Europe, and Japan. The guidelines are organized under broad categories: the E series having to do with clinical trials, the Q series having to do with quality (including chemical manufacturing and control as wells as traditional GLP issues), and the S series having to do with safety. Guidelines may be obtained from the ICH secretariat, IFPMA, 30 rue de St.-Jean, PO Box 9, 1211 Geneva 18, Switzerland, or may be downloaded from a website set up by Nancy McClure (http://www.mcclurenet.com/index.html). They are also published in the *Federal Register*. It is the guidelines of the S series that will have the most impact on toxicologists. The biggest changes, having to do with toxicological assessment, are summarized as follows:

TABLE 2.11 Steps in ICH Guideline Development and Implementation

1. Building scientific consensus in joint regulatory/industry expert working groups
2. Agreement by the steering committee to release the draft consensus text for wider consultation
3. Regulatory consultation in the three regions; consolidation of the comments
4. Agreement on a harmonized ICH guideline; adopted by the regulators
5. Implementation in the three ICH regions

1. *Carcinogenicity Studies* Carcinogenicity studies are covered in guidelines S1A, S1B, and S1C. The guidelines are almost more philosophical than they are technical. In comparison to the EPA guidelines, for example, the ICH guidelines contain little in the way of concrete study criteria (e.g., the number of animals, the necessity for clinical chemistry). There is discussion on when carcinogenicity studies should be done, whether two species are more appropriate than one, and how to set dosages on the basis of human clinical pharmacokinetic (PK) data. The major changes wrought by these guidelines are:

- Only one two-year carcinogenicity study should be generally required. Ideally, the species chosen should be the one most like humans in terms of metabolic transformations of the test article.
- The traditional second long-term carcinogenicity study can be replaced by a shorter term alternative model. In practical terms, this guideline is beginning to result in sponsors conducting a two-year study in the rat and a six-month study in an alternative mouse model, such as the P53 or the TG.AC genetically manipulated mouse strains.
- In the absence of target organ toxicity with which to set the high dose at the maximally tolerated dose, the high dose can be set at the dose that produces an area under the curve (AUC). This is 25-fold higher than that obtained in human subjects.

2. *Chronic Toxicity* Traditionally, chronic toxicity of new pharmaceuticals in the United States was assessed in studies of one year duration in both rodent and nonrodent species of choice. The European view was that studies of six months are generally sufficient. The resulting guideline (S4A) was a compromise. Studies of six months duration were recommended for the rodent, as rodents would also be examined in two-year studies. For the nonrodent (dog, nonhuman primate, and pig) studies of nine months duration were recommended.

3. *Developmental and Reproductive Toxicity* This was an area in which there was considerable international disagreement and the area in which ICH has promulgated the most technically detailed guidelines (S5A and S5B). Some of the major changes include:

- The traditional segment I, II, and III nomenclature has been replaced with different nomenclature, as summarized in Table 2.13.

TABLE 2.12 International Conference on Harmonisation Guidelines

References	Guideline	Date
E1	The Extent of Population Exposure to Assess Clinical Safety	Oct. 94
E2A	Clinical Safety Data Management: Definitions and Standards for Expedited Reporting	Oct. 94
E2B	Clinical Safety Data Management: Data Elements for Transmission of Individual Case Safety Reports	May 05
E2C	Clinical Safety Data Management: Periodic Safety Update Reports for Marketed Drugs	May 97
E2D	Definitions and Standards for Expedited Reporting	Nov. 03
E2E	Pharmacovigilance Planning	Nov. 04
E3	Structure and Content of Clinical Study Reports	Nov. 95
E4	Dose Response Information to Support Drug Registration	Mar. 94
E5	Ethnic Factors in the Acceptability of Foreign Clinical Data	Feb. 98
E6	Good Clinical Practice: Consolidated Guideline; Notice of Availability	May 96
E6A	GCP Addendum on Investigator's Brochure	Mar. 95
E7	Studies in Support of Special Populations: Geriatrics	June 93
E8	Guidance on General Considerations for Clinical Trials; Notice	July 97
E9	Draft Guideline on Statistical Principles for Clinical Trials; Notice of Availability	Feb. 98
E10	Choice of Control Group and Related Issues in Clinical Trials	July 00
E11	Clinical Investigation of Medicinal Products in the Pediatric Population	July 00
E12	Principles for Clinical Evaluation of New Antihypertensive Drugs	
E14	The Clinical Evaluation of QT/QTc Interval Prolongation and Proarrhythmic Potenial for Non-Antiarrhythmic Drugs	May 05
E15	Definitions for Genomic Biomakers, Pharmacogenomics, Pharmacogenetics, Genomic Data and Sample Coding Categories	Nov. 07
M3	Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals; Notice	Nov. 97
Q1A	Stability Testing of New Drug Substances and Products	Feb. 03
Q1B	Stability Testing of New Drug Substances and Products	Nov. 96
Q1C	Stability Testing for New Dosage Forms	Nov. 96
Q1D	Bracketing and Matrixing Designs for Stability Testing of Drug Substances and Drug Products	Feb. 02
Q1E	Evaluation of Stability Data	Feb. 03
Q1F	Stability Data Package for Registration Applications in Climatic Zones III and IV	June 06
Q2	Validation of Analytical Procedures: Text and Methodology	Oct. 94
Q3A	Guideline on Impurities in New Drug Substances	Oct. 06
Q3B	Guideline on Impurities in New Drug Products	June 06
Q3C	Guideline on Impurities: Guideline for Residual Solvents	July 97
Q4	Pharmacopeias	Nov. 07
Q4A	Pharmacopeias Harmonisation	Nov. 07
Q4B	Evaluation and Recommendation of Pharmacopoeial Texts	Nov. 07
Q4B Annex 1	Evaluation and Recommendation of Pharmacopoeial Texts: Residue on Ignition/Sulphated Ash General Chapter	Nov. 07
Q4B Annex 2	*Evaluation and Recommendation of Pharmacopoeial Texts: Test for Extractable Volume of Parenteral Preparations General Chapter*	Nov. 07

TABLE 2.12 ***Continued***

References	Guideline	Date
Q4B Annex3	*Evaluation and Recommendation of Pharmacopoeial Texts: Test for Particulate Contamination: Sub-Visible Particles General Chapter*	Nov. 07
Q5A	Quality of Biotechnological Products Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin	Mar. 97
Q5B	Quality of Biotechnology Products Analysis of the Expression Construct in Cells Used for Production of r-DNA Derived Protein Product	Nov. 95
Q5C	Quality of Biotechnological Products: Stability Testing of Biotechnological/Biology Products	Nov. 95
Q5D	Availability of Draft Guideline on Quality of Biotechnological/ Biological Products: Derivation and Characterization of Cell Substrates Used for Production of Biotechnological/Biological Products	July 97
Q5E	*Comparability of Biotechnological/Biological Products Subject to Changes in Their Manufacturing Process*	Nov. 04
Q6A	*Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances (including Decision Trees)*	Oct. 99
Q6B	*Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products*	Mar. 99
Q7	*Good Manufacturing Practice Guide for Active Pharmaceutical Ingredients*	Nov. 00
Q8	*Pharmaceutical Development*	Nov. 05
Annex to Q8	*Pharmaceutical Development Annex*	Nov. 07
Q9	*Quality Risk Management*	Nov. 05
Q10	*Pharmaceutical Quality System*	May 07
Q6A	Draft Guidance on Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances; Notice	Nov. 97
Q6B	Specifications: Test Procedures and Acceptance Criteria for Biotechnology Products	Feb. 98
S1A	Guidance on the Need for Carcinogenicity Studies of Pharmaceuticals	Nov. 95
S1B	Draft Guideline on Testing for Carcinogenicity of Pharmaceuticals; Notice	July 97
S1C	Dose Selection for Carcinogenicity Studies of Pharmaceuticals	Oct. 94
S1Ca	Guidance on Dose Selection for Carcinogenicity Studies of Pharmaceuticals: Addendum on a Limit Dose and Related Notes; Availability; Notice	Dec. 97
S2A	Genotoxicity: Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals	July 95
S2B	Guidance on Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals; Availability; Notice	July 97
S3A	Toxicokinetics: Guidance on the Assessment of Systemic Exposure in Toxicity Studies	Oct. 94
S3B	Pharmacokinetics: Guidance for Repeated Dose Tissue Distribution Studies	Oct. 94
S4	Single Dose Acute Toxicity Testing for Pharmaceuticals; Revised Guidance; Availability; Notice	Sept. 98

TABLE 2.12 ***Continued***

References	Guideline	Date
S4A	Draft Guidance on the Duration of Chronic Toxicity Testing in Animals (Rodent and Nonrodent Toxicity Testing); Availability; Notice	Nov. 97
S5A	Detection of Toxicity to Reproduction for Medicinal Products	Nov. 95
S5B	Reproductive Toxicity to Male Fertility	Nov. 95
S6A	Guidance on Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals; Availability	July 97
S7A	*Safety Pharmacology Studies for Human Pharmaceuticals*	Nov. 00
S7B	*The Non-Clinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals*	May 05
S8	*Immunotoxicity Studies for Human Pharmaceuticals*	Sept. 05

- The dosing period of the pregnant animals during studies on embryonic development (traditional segment II studies) has been standardized.
- New guidelines for fertility assessment (traditional segment I) studies have shortened the premating dosing schedule (e.g., in male rats from 10 to 4 weeks). There has been an increased interest in assessment of spermatogenesis and sperm function.
- The new guidelines allow for a combination of studies in which the endpoints typically assessed in the traditional segment II and III studies are now examined under a single protocol.

For a more complete review of the various study designs, the reader is referred to Manson (1994).

While not quite as sweeping in approach as the aforementioned guidelines, a toxicologist working in pharmaceutical safety assessment should become familiar with all the other ICH guidelines in the S series.

In an interesting recent article, Ohno (1998) discussed not just the harmonization of nonclinical guidelines but also the need to harmonize the timing of nonclinical tests in relation to the conduct of clinical trials. For example, there are regional differences in the inclusion of women of childbearing potential in clinical trials. In the United States, including women in such trials is becoming more important, and therefore evaluation of embryo–fetal development will occur earlier in the drug development process than in Japan. Whether or not such timing or staging of nonclinical tests becomes part of an ICH guideline in the near future remains to be established.

2.8.2 Other International Considerations

The United States is the single largest pharmaceutical market in the world. But the rest of the world [particularly but not limited to the second and third

TABLE 2.13 Comparison of Traditional and ICH Guidelines for Reproductive and Developmental Toxicology

Traditional Protocol	Stages Covered	ICH Protocol	Dosing Regimen
Segment I (rats)	A. Premating to conception B. Conception to implantation	Fertility and early embryonic development, including implantation	Males: 4 weeks premating, mating (1–3 weeks) plus 3 weeks postmating Females: 2 weeks premating, mating through day 7 of gestation
Segment II (rabbits)	C. Implantation to closure of hard palate D. Closure of hard palate to end of pregnancy	Embryo–fetal development	Female rabbits: day 6 to day 20 of pregnancy
Study Title	**Termination**	**Endpoints: In Life**	**Endpoints: Postmortem**
Fertility and early embryonic development, including implantation	Females: Days 13–15 of pregnancy Males: Day after completion of dosing	Clinical signs and mortality Body weights and feed intake Vaginal cytology	Macroscopic examination plus histology on gross lesions Collection of reproductive organs for possible histology Quantitation of corpora lutea and implantation sites Seminology (count, motility, and morphology)
Embryo–fetal development		Clinical signs and mortality Body weights and changes Feed intake	Macroscopic examination plus histology on gross lesions Quantitation of corpora lutea and implantation sites Fetal body weights Fetal abnormalities
Pre- and postnatal development, including maternal function		Clinical signs and mortality Body weights and changes Feed intake Duration of pregnancy Parturition	Macroscopic examination plus histology on gross lesions Implantation Abnormalities (including terata) Live/dead offspring at birth Pre- and postweaning survival and growth (F_1) Physical development (F_1) Sensory functions and reflexes (F_1) Behavior (F_1)

largest markets, Japan and the European Union (EU)] represents in aggregate a much larger market, so no one develops a new pharmaceutical for marketing in just the United States. The effort at harmonization (exemplified by the ICH) has significantly reduced differences in requirements for these other countries but certainly not obliterated them. Though a detailed understanding of their regulatory schemes is beyond this volume, the bare bones and differences in toxicology requirements are not.

European Union The standard EU toxicology and pharmacologic data requirements for a pharmaceutical include:

- Single-dose toxicity
- Repeat-dose toxicity (subacute and chronic trials)
- Reproduction studies (fertility and general reproductive performance, embryotoxicity, and peri/postnatal toxicity)
- Mutagenic potential (in vitro and in vivo)
- Carcinogenicity
- Pharmacodynamics
  - Effects related to proposed drug indication
  - General pharmacodynamics
  - Drug interactions
- Pharmacokinetics
  - Single dose
  - Repeat dose
  - Distribution in normal and pregnant animals
  - Biotransformation
- Local tissue tolerance
- Environmental toxicity

In general, the registration process in the EU allows one apply to either an overall medicines authority or an individual national authority. Either step is supposed to lead to mutual recognition by all members.

Japan In Japan, the Koseisho is the national regulatory body for new drug. The standard median lethal dose (LD_{50}) test is no longer a regulatory requirement for new medicines in the United States, EU, or Japan. The Japanese guidelines were the first to be amended in accordance with this agreement, with the revised guidelines becoming effective in August 1993. The Japanese may still anticipate that single-dose (acute) toxicity studies should be conducted in at least two species, one rodent and one nonrodent (the rabbit is not accepted as a nonrodent). Both males and females should be included from at least one of the species selected: if the rodent, then a minimum of five per sex; if the nonrodent, at least two per sex. In nonrodents, both the oral

and parenteral routes should be used, and normally the clinical route of administration should be employed. In nonrodents, only the intended route of administration need be employed; if the intended route of administration in humans is intravenous, then use of this route in both species is acceptable. An appropriate number of doses should be employed to obtain a complete toxicity profile and to establish any dose–response relationship. The severity, onset, progression, and reversibility of toxicity should be studied during a 14-day follow-up period, with all animals being necropsied. When macroscopic changes are noted, the tissue must be subjected to histological examination.

Chronic and subchronic toxicity studies are conducted to define the dose level, when given repeatedly, that cause toxicity and the dose level that does not lead to toxic findings. In Japan, such studies are referred to as repeated-dose toxicity studies. As with single-dose studies, at least two animal species should be used, one rodent and one nonrodent (rabbit not acceptable). In rodent studies, each group should consist of at least 10 males and 10 females; in nonrodent species, 3 of each sex is deemed adequate. Where interim examinations are planned, however, the number of animals employed should be increased accordingly. The planned route of administration in human subjects is normally explored. The duration of the study will be dictated by the planned duration of clinical use (Table 2.14).

At least three different dose groups should be included, with the goals of demonstrating an overtly toxic dose and a no-effect dose and establishing any dose–response relationship. The establishment of a nontoxic dose within the framework of these studies is more rigorously adhered to in Japan than elsewhere in the world. All surviving animals should also be necropsied, either at the completion of the study or during its extension recovery period, to assess reversal of toxicity and the possible appearance of delayed toxicity. Full histological examination is mandated on all nonrodent animals used in a chronic toxicity study; at a minimum, the highest dose and control groups of rodents must be submitted to a full histological examination.

TABLE 2.14 Required Duration of Dosing in Nonclinical Study to Support Clinical Dosing

Duration of Dosing in Toxicity Study	Duration of Human Exposure
1 Month	Single dose or repeated dosage not exceeding 1 week
3 Months	Repeated dosing exceeding 1 week and to a maximum of 4 weeks
6 Months	Repeated dosing exceeding 4 weeks and to a maximum of 6 months
12 Months[a]	Repeated dosing exceeding 6 months or where this is deemed to be appropriate

[a]Where carcinogenicity studies are to be conducted, the Koseisho had agreed to forego chronic dosage beyond 6 months. *Source*: New Drugs Division Notification No. 43, June 1992.

While the value of repeated-dose testing beyond 6 months has been questioned (Lumley and Walker, 1992), such testing is a regulatory requirement for a number of agencies, including the FDA and the Koseisho. In Japan, repeated-dose testing for 12 months is required only for new medicines expected to be administered to humans for periods in excess of 6 months (Yakuji Nippo, 1994). At the first ICH held in Brussels, the consensus was that 12-month toxicity studies in rodents could be reduced to 6 months where carcinogenicity studies are required. While not yet adopted in the Japanese guidelines, 6-month repeated-dose toxicity studies have been accepted by the agencies of all three regions. Japan—like the EU—accepts 6 months duration if accompanied by a carcinogenicity study. The United States still requires a 9-month nonrodent study.

With regard to reproductive toxicology, as a consequence of the first ICH, the United States, EU, and Japan agreed to recommend mutual recognition of their respective current guidelines. A tripartite, harmonized guideline on reproductive toxicology has achieved ICH step 4 status and should soon be incorporated into the local regulations of all three regions. This agreement represents a very significant achievement that should eliminate many obstacles to drug registration.

Preclinical Male Fertility Studies Before conducting a single-dose male volunteer study in Japan, it is usually necessary to have completed a preclinical male fertility study (segment I) that has an in-life phase of 10 or more weeks (i.e., 10 weeks of dosing plus follow-up). Although government guidelines do not require this study to be completed before phase I trials begin, the responsible institutional review board or the investigator usually imposes this condition. Japanese regulatory authorities are aware that the segment I male fertility study is of poor predictive value. The rat, which is used in this study, produces a marked excess of sperm. Many scientists therefore believe that the test is less sensitive than the evaluation of testicular weight and histology that constitute part of the routine toxicology assessment

Female Reproductive Studies Before entering a female into a clinical study, it is necessary to have completed the entire reproductive toxicology program, which consists of the following studies:

- *Segment I* Fertility studies in the rat or mouse species used in the segment II program
- *Segment II* Teratology studies in the rat or mouse and the rabbit
- *Segment III* Late gestation and lactation studies in a species used in the segment II studies

Such studies usually take approximately two years. Although the U.S. regulations state the need for completion of segments I and II and the demonstration of efficacy in male patients, where appropriate, before entering females into a clinical program, the current trend in the United States is toward

relaxation of the requirements to encourage investigation of the drug both earlier and in a larger number of females during product development. Growing pressure for the earlier inclusion of women in drug testing may encourage selection of this issue as a future ICH topic. The trend in the United States and the EU toward including women earlier in the critical program has not yet been embraced in Japan, however.

The three tests required in Japan for genotoxicity evaluation are a bacterial gene mutation test, in vitro cytogenetics, and in vivo tests for genetic damage. The Japanese regulations state these tests to be the minimum requirement and encourage additional tests. Currently, Japanese guidelines do not require a mammalian cell gene mutation assay. Harmonization will likely be achieved by the Koseisho recommending all four tests, which will match requirements in the United States and the EU; at present, this topic is at step 1 in the ICH process. The mutagenicity studies should be completed before the commencement of phase II clinical studies.

Guidelines presented at the second ICH are likely to alter the preclinical requirements for registration in Japan; they cover toxicokinetics and when to conduct repeated-dose tissue distribution studies. The former document may improve the ability of animal toxicology studies to predict possible adverse events in humans; currently, there are not toxicokinetic requirements in Japan, and their relevance is questioned by many there. Although there is general agreement on the registration requirement for single-dose tissue distribution studies, implementation of the repeated-dose study requirement has been inconsistent across the three ICH parties.

2.8.3 Safety Pharmacology

Japan was the first major country to require extensive pharmacological profiling on all new pharmaceutical agents as part of the safety assessment profile. Prior to commencement of initial clinical studies, the drug's pharmacology must be characterized in animal models. In the United States and Europe, these studies have been collectively called safety pharmacology studies. For a good general review of the issues surrounding safety pharmacology the reader is referred to Hite (1997). The Japanese guidelines for such characterizations were published in 1991 (New Drugs Division Notification No. 4, January 1991):

- Effects on general activity and behavior
- Effects on the central nervous system
- Effects on the autonomic nervous system and smooth muscle
- Effects on the respiratory and cardiovascular systems
- Effects on the digestive system
- Effects on water and electrolyte metabolism
- Other important pharmacological effects

In the United States, pharmacological studies in demonstration of efficacy have always been required, but specific safety pharmacological studies have never been required. Special situational or mechanistic data would be requested on a case-by-case basis. This is a situation that is changing. In the United States the activities of the Safety Pharmacology Discussion Group, for example, have helped bring attention to the utility and issues surrounding safety pharmacology data. In 1999 and 2000, the major toxicological and pharmacological societal meetings had symposia on safety pharmacological testing. Many major U.S. pharmaceutical companies are in the process of implementing programs in safety pharmacology. The issue has been taken up by ICH and the draft guideline is currently at the initial stages of review. This initial draft (guideline S7) includes core tests in the assessment of CNS, cardiovascular, and respiratory function. Studies will be expected to be performed under GLP guidelines.

2.9 COMBINATION PRODUCTS

Recent years have seen a vast increase in the number of new therapeutic products which are not purely drug, device, or biologic but rather a combination of two or more of these. This leads to a problem of deciding which of the three centers shall have ultimate jurisdiction.

The Center for Devices and Radiological Health (CDRH) is designated the center for major policy development and for the promulgation and interpretation of procedural regulations for medical devices under the act. The CDRH regulates all medical devices, inclusive of radiation-related device, that are not assigned categorically or specifically to CDER. In addition, CDRH will independently administer the following activities (references to "sections" are the provisions of the act):

1. A. Small business assistance programs under Section 10 of the amendments [see Public Law (PL) 94-295]. Both CDER and CDRH will identify any unique problems relating to medical device regulation for small business.
   B. Registration and listing under Section 510, including some CDER-administered device applications. The CDER will receive printouts and other assistance, as requested.
   C. Color additives under Section 706, with review by CDER, as appropriate.
   D. Good Manufacturing Practices Advisory Committee. Under Section 520(f)(3), CDER will regularly receive notices of all meetings, with participation by CDER, as appropriate.
   E. Medical device reporting—The manufacturers, distributors, importers, and users of all devices, including those regulated by CDER, shall

report to CDRH under Section 519 of the act as required. The CDRH will provide monthly reports and special reports as needed to CDER for investigation and follow-up of those medical devices regulated by CDER.

2.9.1 Device Programs That CDER and CDRH Each Will Administer

Both CDER and CDRH will administer and, as appropriate, enforce the following activities for medical devices assigned to their respective centers (references to "sections" are the provisions of the act):

1. A. Surveillance and compliance actions involving general controls violations, such as misbranded or adulterated devices under Sections 301, 501, and 502
   B. Warning letters, seizures, injunctions, and prosecutions under Sections 302, 303, and 304
   C. Civil penalties under Section 303(f) and administrative restraint under Section 304(g)
   D. Nonregulatory activities, such as educational programs directed at users, participation in voluntary standards organizations, and so on
   E. Promulgation of performance standards and applications of special controls under Section 514
   F. Premarket notification, investigational device exemptions including humanitarian exemptions, premarket approval, product development protocols, classification, device tracking, petitions for reclassification, postmarket surveillance under Sections 510(k), 513, 515, 519, 520(g) and (m), and 522, and the advisory committees necessary to support these activities
   G. Banned devices under Section 516
   H. FDA-requested and firm-initiated recalls whether under Section 518 or another authority and other Section 518 remedies such as recall orders
   I. Exemptions, variances, and applications of CGMP regulations under Section 520(f)
   J. Governmentwide quality assurance program
   K. Requests for export approval under Sections 801(e) and 802

Coordination The centers will coordinate their activities in order to assure that manufacturers do not have to independently secure authorization to market their product from both centers unless this requirement is specified in Section VII.

Submissions Submissions should be made to the appropriate center, as specified herein, at the following addresses: Food and Drug Administration, Center for Drug Evaluation and Research, Central Document Room (Room 2-14), 12420 Parklawn Drive, Rockville, Maryland 20852 or Food and Drug

Administration, Center for Devices and Radiological Health, Document Mail Center (HFZ-401), 1390 Piccard Drive, Rockville, Maryland 20850.

For submissions involving medical devices and/or drugs that are not clearly addressed in this agreement, sponsors are referred to the product jurisdiction regulations (21 CFR Part 3). These regulations have been promulgated to facilitate the determination of regulatory jurisdiction but do not exclude the possibility for a collaborative review between the centers.

2.9.2 Center Jurisdiction

The following subsections provide details concerning status, market approval authority, special label/regulatory considerations, investigational options, and intercenter consultations for the categories of products specified. Section VII provides the general criteria that CDRH and CDER will apply in reaching decisions as to which center will regulate a product.

A. 1. (a) Device with primary purpose of delivering or aiding in the delivery of a drug that is distributed without a drug (i.e., unfilled)

Examples

Devices that calculate drug dosages

Drug delivery pump and/or catheter infusion pump for implantation iontophoresis device

Medical or surgical kit (e.g., tray) with reference in instructions for use with specific drug (e.g., local anesthetic)

Nebulizer

Small-particle aerosol generator (SPAG) for administering drug to ventilated patient

Splitter block for mixing nitrous oxide and oxygen

Syringe, jet injector, storage and dispensing equipment

Status Device and drug as separate entities

Market Approval Authority CDRH and CDER, respectively, unless the intended use of the two products, through labeling, creates a combination product

Special Label/Regulatory Considerations The following specific procedures will apply depending on the status of the drug delivery device and drugs that will be delivered with the device:

(i) It may be determined during the design or conduct of clinical trials for a new drug that it is not possible to develop adequate performance specifications data on those characteristics of the device that are required for the safe and effective use of the drug. If this is the case, then drug labeling cannot be written to contain information that makes it possible for the user to substitute a generic, marketed device for the device used during development to use with the marketed drug. In these situations, CDER will be the lead center for regulation of the device under the device authorities.

(ii) For a device intended for use with a category of drugs that are on the market, CDRH will be the lead center for regulation for the device under the device authorities. The effects of the device use on drug stability must be addressed in the device submission, when relevant. An additional showing of clinical effectiveness of the drug when delivered by the specific device will generally not be required. The device and drug labeling must be mutually conforming with respect to indication, general mode of delivery (e.g., topical, IV), and drug dosage/schedule equivalents.

(iii) For a drug delivery device and drug that are developed for marketing to be used together as a system, a lead center will be designated to be the contact point with the manufacturer(s). If a drug has been developed and marketed and the development and study of device technology predominate, the principal mode of action will be deemed to be that of the device, and CDRH would have the lead. If a device has been developed and marketed and the development and study of drug predominate, then, correspondingly, CDER would have the lead. If neither the drug nor the device is on the market, the lead center will be determined on a case-by-case basis.

Investigation Options IDE or IND as appropriate

Intercenter Consultation CDER, when the lead center, will consult with CDRH if CDER determines that a specific device is required as part of the NDA process. CDRH as lead center will consult with CDER if

(a) the device is intended for use with (but not already containing) a marketed drug and the device creates a significant change in the intended use, mode of delivery (e.g., topical, IV), or dose/schedule of the drug, or

(b) the device with primary purpose of delivering or aiding in the delivery of a drug and distributed containing a drug (i.e., "pre-filled delivery system").

Examples

Nebulizer

Oxygen tank for therapy and over-the-counter (OTC) emergency use

Prefilled syringe

Transdermal patch

Status Combination product

Market Approval Authority CDER using drug authorities and device authorities as necessary

Special Label/Regulatory Considerations None

Investigation Options IND

Intercenter Consultations Optional

2. Device incorporating a drug component with the combination product having the primary intended purpose of fulfilling a device function

   Examples
   Bone cement containing antimicrobial agent
   Cardiac pacemaker lead with steroid-coated tip
   Condom, diaphragm, or cervical cap with contraceptive or antimicrobial agent (including virucidal) agent
   Dental device with fluoride
   Dental wood wedge with hemostatic agent
   Percutaneous cuff (e.g., for a catheter or orthopedic pin) coated/impregnated with antimicrobial agent
   Skin closure or bandage with antimicrobial agent
   Surgical or barrier drape with antimicrobial agent
   Tissue graft with antimicrobial or other drug agent
   Urinary and vascular catheter coated/impregnated with antimicrobial agent
   Wound dressing with antimicrobial agent

Status Combination product

Market Approval Authority CDRH using device authorities

Special Label/Regulatory Considerations These products have a drug component that is present to augment the safety and/or efficacy of the device.

Investigation Options IDE

Intercenter Consultation Required if a drug or the chemical form of the drug has not been legally marketed in the United States as a human drug for the intended effect

3. Drug incorporating a device component with the combination product having the primary intended purpose of fulfilling a drug function

   Examples
   Skin-prep pads with antimicrobial agent
   Surgical scrub brush with antimicrobial agent

Status Combination product

Market Approval Authority CDER using drug authorities and, as necessary, device authorities

Special Label/Regulatory Considerations Marketing of such a device requires a submission of an NDA with safety and efficacy data on the drug component or that it meet monograph specifications as generally recognized as safe (GRAS) and generally recognized as effective (GRAE). Drug requirements (e.g., CGMPs, registration and listing, experience reporting) apply to products.

Investigation Options IND

Intercenter Consultation Optional

4. (a) Device used in the production of a drug either to deliver directly to a patient or for use in the producing medical facility (excluding use in a registered drug manufacturing facility)

Examples
Oxygen concentrators (home or hospital)
Oxygen generator (chemical)
Ozone generator

Status Device

Market Approval Authority CDER, applying both drug and device authorities

Special Label/Regulatory Consideration May also require an NDA if the drug produced is a new drug. Device requirements (e.g., CGMPs, registration and listing, experience reporting) will apply to products.

Investigation Options IDA or NDA as appropriate

Intercenter Consultation Optional

(b) Drug/device combination product intended to process a drug into a finished package form

Examples
Device that uses drug concentrates to prepare large-volume parenterals
Oxygen concentrator (hospital) output used to fill oxygen tanks for use within that medical facility

Status Combination product

Market Approval Authority CDER, applying both drug and device authorities

Special Label/Regulatory Considerations Respective drug and device requirements (e.g., CGMPs, registration and listing, experience reporting) will apply.

Investigation Options IDE or NDA as appropriate

Intercenter Consultation Optional but will be routinely obtained

B. 1. Device used concomitantly with a drug to directly activate or to augment drug effectiveness

Examples
Biliary lithotriptor used in conjunction with dissolution agent
Cancer hyperthermia used in conjunction with chemotherapy
Current generator used in conjunction with an implanted silver electrode (drug) that produces silver ions for an antimicrobial purpose
Materials for blocking blood flow temporarily to restrict chemotherapy drug to the intended site of action

Ultraviolet and/or laser activation of oxsoralen for psoriasis or cutaneous T-cell lymphoma

Status Device and drug as separate entities

Market Approval Authority CDRH and CDER, respectively

Special Label/Regulatory Considerations The device and drug labeling must be mutually conforming with respect to indications, general mode of delivery (e.g., topical, IV), and drug dosage/schedule equivalence. A lead center will be designated to be the contact point with the manufacturer. If a drug has been developed and approved for another use and development and study of device technology predominate, then CDRH would have the lead. If a device has been developed and marketed for another use and development and study of drug action predominate, then CDER would have the lead. If neither the drug nor the device is on the market, the lead center will be determined on a case-by-case basis. If the labeling of the drug and device create a combination product, as defined in the combination product regulations, then the designation of the lead center for both applications will be based upon a determination of the product's primary mode of action.

Investigation Options IDE or IND as appropriate

Intercenter Consultations Required

2. Device kits labeled for use with drugs that include both device(s) and drug(s) as separate entities in one package with the overall primary intended purpose of the kit fulfilling a device function

   Examples

   Medical or surgical kit (e.g., tray) with drug component

Status Combination product

Market Approval Authority CDRH, using device authorities, is responsible for the kit if the manufacturer is repackaging a market drug. Responsibility for overall packaging resides with CDRH. CDER will be consulted as necessary on the use of drug authorities for the repackaged drug component.

Special Label/Regulatory Consideration Device requirements (e.g., CGMPs, registration and listing, experience reporting) apply to kits. Device manufacturers must assure that manufacturing steps do not adversely affect drug components of the kit. If the manufacturing steps do affect the marketed drug (e.g., the kit is sterilized by irradiation), an ANDA or NDA would also be required with CDRH as the lead center.

Investigation Options IDA or IND as appropriate

Intercenter Consultation Optional if ANDA or NDA not required

C. Liquids, gases, or solids intended for use as devices (e.g., implanted, or components, parts, or accessories to devices)

Examples

Dye for tissues used in conjunction with laser surgery to enhance absorption of laser light in target tissue
Gas mixtures for pulmonary function-testing devices
Gases used to provide "physical effects"
Hemodialysis fluids
Hemostatic devices and dressings
Injectable silicon, collagen, and Teflon
Liquids functioning through physical action applied to the body to cool or freeze tissues for therapeutic purposes
Liquids intended to inflate, flush, or moisten (lubricate) indwelling device (in or on the body)
Lubricants and lubricating jellies
Ophthalmic solutions for contact lenses
Organ/tissue transport and/or perfusion fluid with antimicrobial or other drug agent, that is, preservation solutions
Powders for lubricating surgical gloves
Sodium hyaluronate or hyaluronic acid for use as a surgical aid
Solution for use with dental "chemical drill"
Spray on dressings not containing a drug component

Status Device

Market Approval Authority CDRH

Special Label/Regulatory Considerations None

Investigation Options IDE

Intercenter Consultation Required if the device has direct contact with the body and the drug or the chemical form of the drug has not been legally marketed as a human drug

D. Products regulated as drugs

Examples

Irrigation solutions
Purified water or saline in prefilled nebulizers for use in inhalation therapy
Skin protectants (intended for use on intact skin)
Sun screens
Topical/internal analgesic-antipyretic

Status Drug

Market Approval Authority CDER

Special Label/Regulatory Considerations None

Investigation Options IND

Intercenter Consultations Optional

E. Ad hoc jurisdictional decisions
Examples

	Status	Center
Motility marker constructed of radiopaque plastic	Device	CDRH
Brachytherapy capsules, needles, etc., that are radioactive and may be removed from the body after radiation therapy has been administered	Device	CDRH
Skin markers	Device	CDRH

Status Device or drug
Market Approval Authority CDRH or CDER as indicated
Special Label/Regulatory Considerations None
Investigation Options IDE or IND as appropriate
Intercenter Consultation Required to assure agreement on drug/device status

General Criteria Affecting Drug/Device Determination

The following represent the general criteria that will apply in making device/drug determinations.

A. *Device Criteria*
1. A liquid, powder, or other similar formulation intended only to serve as a component, part, or accessory to a device with a primary mode of action that is physical in nature will be regulated as a device by CDRH.
2. A product that has the physical attributes described in 201(h) (e.g., instrument, apparatus) of the act and does not achieve its primary intended purpose through chemical action within or on the body or by being metabolized will be regulated as a device by CDRH.
3. The phrase "within or on the body" as used in 201(h) of the act does not include extra corporeal systems or the solutions used in conjunction with such equipment. Such equipment and solutions will be regulated as devices by CDRH.
4. An implant, including an injectable material, placed in the body for primarily a structural purpose even though such an implant may be absorbed or metabolized by the body after it has achieved its primary purpose will be regulated as a device by CDRH.
5. A device containing a drug substance as a component with the primary purpose of the combination being to fulfill a device function is a combination product and will be regulated as a device by CDRH.

6. A device (e.g., machine or equipment) marketed to the user, pharmacy, or licensed practitioner that produces a drug will be regulated as a device or combination product by CDER. This does not include equipment marketed to a registered drug manufacturer.
7. A device whose labeling or promotional materials make reference to a specific drug or generic class of drugs unless it is prefilled with a drug ordinarily remains a device regulated by CDRH. It may, however, also be subject to the combination products regulation.

B. *Drug Criteria*

1. A liquid, powder, tablet, or other similar formulation that achieves its primary intended purpose through chemical action within or on the body or by being metabolized, unless it meets one of the specified device criteria, will as regulated as a drug by CDER.
2. A device that serves as a container for a drug or a device that is a drug delivery system attached to the drug container where the drug is present in the container is a combination product that will be regulated as a drug by CDER.
3. A device containing a drug substance as a component with the primary purpose of the combination product being to fulfill a drug purpose is a combination product and will be regulated as a drug by CDER.
4. A drug whose labeling or promotional materials makes reference to a specific device or generic class of devices ordinarily remains a drug regulated by CDER. It may, however, also be subject to the combination products regulation.

2.10 CONCLUSIONS

In summary, we have touched upon the regulations that currently control the types of preclinical toxicity testing done on potential human pharmaceuticals and medical device products. We have reviewed the history, the law, the regulations themselves, the guidelines, and common practices employed to meet regulatory standards. Types of toxicity testing were discussed, as were the special cases pertaining to, for example, biotechnology products.

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Yakuji Nippo (1994). *Guidelines for Toxicity Study of Drugs Manual*. Yakuji Nippo, Tokyo.

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3

Prior Art and Its Use in Safety Assessment Process

The appropriate starting place for the safety assessment of any new chemical entity, particularly a potential new drug, is to first determine what is already known about the molecule, its structural and therapeutics class analogues (pharmacological analogues being agents with assumed similar pharmacological mechanisms), and the disease one seeks to treat. Such a determination requires the fullest possible access and review of the available literature. Here we try to at least overview the range of approaches to gathering such data (Table 3.1). In using this information, one must keep in mind that there is both an initial requirement to build a data file or database and a continuing need to update such a database on a regular basis, seving as part of the project record. Updating a database requires not merely adding to what is already there but also discarding out-of-date (i.e., now known to be incorrect) information and reviewing the entire structure for connections and organization.

Such data are first used in selecting which possible compounds should be carried forward in development as a possible new drug [as illustrated in Figure 3.1 and explored in detail in Gad (2005)].

3.1 CLAIMS

Claims are what is said in labeling and advertising and may be either of a positive (therapeutic or beneficial) or negative (lack of an adverse effect)

Drug Safety Evaluation, Second Edition, by Shayne Cox Gad

TABLE 3.1 Sources of Prior Art

Internet
FDA: Inactive Ingredients for Currently Marketed Drug Products, http://www.accessdata.fda.gov/scripts/cder/iig/index.cfm
Proprietory databases
Medline/toxline/journals
Book (monographs and edited)
Personal network/meetings
Obscure databases

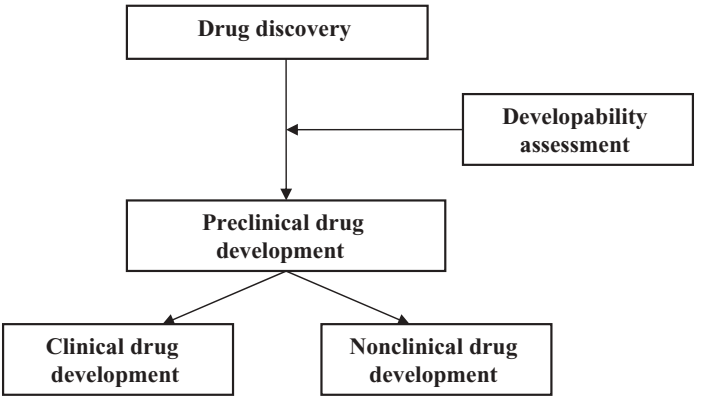

Figure 3.1 *Prior art in assessing pharmaceutical developability.*

nature. The positive or efficacy claims are not usually the direct concern of the toxicologist, though it must be kept in mind that such claims both must be proved and can easily exceed the limits of the statutory definition of a device, turning the product into a drug or combination product. Negative claims such as "nonirritating" or "hypoallergenic" also must be proved and are generally the responsibility of the product safety professional to provide proof for. There are special tests for such claims.

3.2 TIME AND ECONOMIES

The final factors of influence or arbitrator of test conduct and timing are the requirements of the marketplace, the resources of the organization, and the economic worth of the product. Plans for filings with regulatory agencies and for market launches are typically set before actual testing (or final stage development) is undertaken, as the need to be in the marketplace in a certain time frame is critical. Such timing and economic issues are beyond the scope of this volume but must be considered.

3.3 PRIOR KNOWLEDGE

The first step in any new literature review is to obtain as much of the following information as possible:

1. Correct chemical identity, including molecular formula, Chemical Abstracts Service (CAS) registry number, common synonyms, trade names, and a structural diagram. Gosselin et al. (1984) and Ash and Ash (1995, 2007) are excellent sources of information on existing commercial products and their components and uses.
2. Chemical composition (if a mixture) and major impurities.
3. Production and use information.
4. Chemical and physical properties (physical state, vapor pressure, pH, solubility, chemical reactivity, etc.).
5. Any structurally related chemical substances that are already on the market or in production.
6. Known or presumed pharmacological properties.

Collection of the above information is not only important for hazard assessment (high vapor pressure would indicate high inhalation potential, just as high and low pH would indicate high irritation potential), but the prior identification of all intended use and exposure patterns may provide leads to alternative information sources; for example, drugs to be used as antineoplastics or antibiotics may already have extensive toxicology data obtainable from government or private sources. A great deal of the existing toxicity information (particularly information on acute toxicity) is not available in the published or electronic literature because of concerns about the proprietary nature of this information and the widespread opinion that it does not have enough intrinsic scholarly value to merit publication. This unavailability is unfortunate, as it leads to a lot of replication of effort and expenditure of resources that could be better used elsewhere. It also means that an experienced toxicologist must use an informal search of the unpublished literature of his colleagues as a supplement to searches of the published and electronic literature.

There are now numerous published texts that should be considered for use in literature-reviewing activities. An alphabetic listing of 24 of the more commonly used hard-copy sources for safety assessment data is provided in Table 3.2. Obviously, this is not a complete listing and consists of only the general multipurpose texts that have a wider range of applicability for toxicology. Texts dealing with specialized classes of agents (e.g., disinfectants) or with specific target organ toxicity (neurotoxins and teratogens) are generally beyond the scope of this text. Parker (1988) should be consulted for details on the use of these texts. Wexler (2008), Parker (1988), and Sidhu et al. (1989) should be consulted for more extensive listings of the literature and computerized databases. Such sources can be off direct (free) Internet sources (where one must

TABLE 3.2 Key Safety Assessment Reference Texts

Abraham, Donald J. (Ed.) (2003). *Burger's Medicinal Chemistry and Drug Discovery*. John Wiley & Sons, New York.

American Conference of Government Industrial Hygienists (2000). *Documentation of the Threshold Limit Values and Biological Exposure Indices*, 7th ed.—2002 Supplement. ACGIH.

Ash, M., and Ash, I. (1995). *Food Additives, Electronic Handbook*. Gower, Brookfield, VT.

Ash, M., and Ash, I. (2007). *Pharmaceuticals Additives*, 2nd ed., *Electronic Handbook*. Gower, Brookfield, VT.

Barnhart, E. R. (2007). *Physician's Desk Reference*. Medical Economics Company, Oradell, NJ.

Bingham, E., et al. (Eds.) (2001). *Patty's Toxicology*, 5th ed. John Wiley & Sons, New York.

Budavari, E., et al. (Eds.) (2006). *The Merck Index*, 15th ed. Merck and Company, Inc., Rahway, NJ.

Center for Disease Control (2003). *Registry of Toxic Effects of Chemical Substances (RTECS)*. National Institute for Occupational Safety and Health.

Cronin, E. (1980). *Contact Dermatitis*. Churchill Livingston, Edinburgh.

Dart, R. C., et al. (Eds.) (2004). *Medical Toxicology*, 3rd ed., Lippincott Williams & Wilkins, Baltimore, MD.

Deichmann, W., and Gerard, H. (1996). *Toxicology of Drugs and Chemicals*. Academic, New York.

Dipiro, J. T., et al. (Eds.) (2002). *Pharmacotherapy, a Pathophysiologic Approach*, 5th ed., McGraw Hill, New York.

Finkel, A. J. (1983). *Hamilton and Hardy's Industrial Toxicology*, 4th ed. John Wright PSG, Boston.

Ford, M. D., et al. (2001). *Clinical Toxicology*. W.B. Saunders Company, Philadelphia.

Gosselin, R. E., Smith, R. P., Hodge, H. C. (1984). *Clinical Toxicology of Commercial Products*, 5th ed. Wilkins and Williams, Baltimore, MD.

Grant, Morton W., and Schuman, Joel S. (1993). *Toxicology of the Eye*. Charles C. Thomas, Springfield, IL.

Haddad, Lester M., et al. (1998). *Clinical Management of Poisoning and Drug Overdose*, 3rd ed. Saunders, Philadelphia.

Klaassen, Curtis D. (Ed.) (2007). *Casarett & Doull's Toxicology: The Basic Science of Poisons*, 6th ed. McGraw-Hill, New York.

Lewis Sr., Richard J. (Ed.) (1991). *Carcinogenically Active Chemicals: A Reference Guide*. Van Nostrand Reinhold, New York.

Lewis Sr., Richard J. (Ed.) (2000). *Sax's Dangerous Properties of Industrial Materials*, 10th ed., 3 Volume Set. John Wiley & Sons, New York.

National Toxicology Program (2000). Nineteenth Annual Report on Carcinogens. PB 85-134633. Department of Health and Human Services, Washington, DC.

O'Neil, M. J., et al. (Eds.) (2006). *The Merck Index*, 14th ed. Merck & Co., Whitehouse Station, NJ.

Proctor, N. H., and Hughes, J. P. (1978). *Chemical Hazards of the Workplace*, J. B. Lippincott, Philadelphia.

Sax, N. I. (2000). *Dangerous Properties of Industrial Materials*, 10th ed. Van Nostrand Reinhold, New York.

Schardein, J. (2000). *Chemically Induced Birth Defects*. Marcel Dekker, NY.

Shephard, Thomas J. (2007). *Catalog of Teratogenic Agents (Shepard's Catalog)*, 11th ed. Johns Hopkins University Press, Baltimore, MD.

Sweetman, S. C. (2007). *Martindale: The Complete Drug Reference*, Pharmaceutical Press, Chicago.

U.S. Department of Health and Human Services (2002). *Report on Carcinogens*, 10th ed. Public Health Service, National Toxicology Program. (http://ehp.niehs.nih.gov/roc/toc10.html)

Thomas Healthcare (2007). Physician's Desk Reference, 61st ed. Thomson Healthcare, Montvale, NJ. (http://www.pdr.net)

Wexler, Philip (Ed.) (2005). *Encyclopedia of Toxicology*. Elsevier Ltd., Oxford.

Wiley-Interscience (2007). *Wiley Handbook of Current and Emergency Drug Therapies*, 8 Volumes. John Wiley & Sons, Hoboken, N.J.

beware of GIGO: garbage in, garbage out), commercial databases, and package products, to mention just the major categories. Appendix C provides addresses for major free Internet sources.

3.4 MISCELLANEOUS REFERENCE SOURCES

There are some excellent published information sources covering some specific classes of chemicals, for example, heavy metals, plastics, resins, or petroleum hydrocarbons. The National Academy of Science series *Medical and Biologic Effects of Environment Pollutants* covers 10–15 substances considered to be environmental pollutants. *CRC Critical Reviews in Toxicology* is a well-known scientific journal that over the years has compiled over 20 volumes of extensive literature reviews of a wide variety of chemical substances. A photocopy of this journal's topical index will prevent one from overlooking information that may be contained in this important source. Trade organizations such as the Fragrance Industry Manufacturers Association and the Chemical Manufacturers Association have extensive toxicology databases from their research programs that are readily available to toxicologists of member companies. Texts that deal with specific target organ toxicity—neurotoxicity, hepatotoxicity, or hematotoxicity—often contain detailed information on a wide range of chemical structures. Published information sources like the *Target of Organ Toxicity* series (Taylor and Francis, now halfway through a second round of new editions) or a few examples of publications that contain important information on many industrial chemicals may be useful either directly or by analogy. Upon discovery that the material one is evaluating may possess target organ toxicity, a cursory review of these types of texts is warranted.

In the last decade, for many toxicologists the online literature search has changed from an occasional, sporadic activity to a semicontinuous need. Usually, non-toxicology-related search capabilities are already in place in many companies. Therefore, all that is needed is to expand the information source to include some of the databases that cover the types of toxicology information one desires. However, if no capabilities exist within an organization, one can approach a university, consultant, or private contract laboratory and utilize their online system at a reasonable rate. It is even possible to access most of these sources from home using a personal computer. The major available online databases are as follows:

A. *National Library of Medicine* The National Library of Medicine (NLM) information retrieval service contains the well-known and frequently used Medline, Toxline, and Cancerlit databases. Databases commonly used by toxicologists for acute data in the NLM service are the following:
   1. Toxline (Toxicology Information Online) is a bibliographic database covering the pharmacological, biochemical, physiological, environmen-

tal, and toxicological effects of drugs and other chemicals. It contains approximately 1.7 million citations, most of which are complete with abstract, index terms, and CAS registry numbers. Toxline citations have publication dates of 1981 to the present. Older information is on Toxline 65 (pre-1965 through 1980).

2. Medline (Medical Information Online) is a database containing approximately 7 million references to biomedical journal articles published since 1966. These articles, usually with an English abstract, are from over 3000 journals. Coverage of previous years (back to 1966) is provided by back files, searchable online, that total some 3.5 million references.
3. Toxnet (Toxicology Data Network) is a computerized network of toxicologically oriented data banks. Toxnet offers a sophisticated search and retrieval package that accesses the following three subfiles:
   a. Hazardous Substances Data Bank (HSDB) is a scientifically reviewed and edited data bank containing toxicological information enhanced with additional data related to the environment, emergency situations, and regulatory issues. Data are derived from a variety of sources, including government documents and special reports. This database contains records for over 4100 chemical substances.
   b. Toxicology Data Bank (TDB) is a peer-reviewed data bank focusing on toxicological and pharmacological data, environmental and occupational information, manufacturing and use data, and chemical and physical properties. References have been extracted from a selected list of standard source documents.
   c. Chemical Carcinogenesis Research Information System (CCRIS) is a National Cancer Institute–sponsored database derived from both short- and long-term bioassays on 2379 chemical substances. Studies cover carcinogenicity, mutagenicity, promotion, and cocarcinogenicity.
4. Registry of Toxic Effects of Chemical Substances (RTECS) is the NLM's online version of the National Institute for Occupational Safety and Health's (NIOSH) annual compilation of substances with toxic activity. The original collection of data was derived from the 1971 Toxic Substances Lists. RTECS data contain threshold limit values, aquatic toxicity ratings, air standards, National Toxicology Program carcinogenesis bioassay information, and toxicological/carcinogenic review information. The NIOSH is responsible for the file content in RTECS and for providing quarterly updates to NLM: RTECS currently covers toxicity data on more than 106,000 substances.

B. *The Merck Index* *The Merck Index* is now available online for up-to-the minute access to new chemical entities.

3.5 SEARCH PROCEDURE

As mentioned in the introduction, chemical composition and identification information should already have been obtained before the chemical is to be

searched. With most information retrieval systems this is a relatively straightforward procedure. Citations on a given subject may be retrieved by entering the desired free text terms as they appear in titles, key words, and abstracts of articles. The search is then initiated by entering the CAS number and/or synonyms. If you are only interested in a specific target organ effect—for instance, carcinogenicity—or specific publication years, searches can be limited to a finite number of abstracts before requesting the printout.

Often it is unnecessary to request a full printout (author, title, abstract). You may choose to review just the author and title listing before selecting the abstracts of interest. In the long run, this approach may save you computer time, especially if the number of citations being searched is large.

Once you have reviewed the abstracts, the last step is to request photocopies of the articles of interest. Extreme caution should be used in making any final health hazard determination based solely on an abstract or nonprimary literature source.

3.6 MONITORING PUBLISHED LITERATURE AND OTHER RESEARCH IN PROGRESS

Although there are a few other publications offering similar services, the *Life Sciences* edition of *Current Contents* is the publication most widely used by toxicologists for monitoring the published literature. *Current Contents* monitors over 1180 major journals and provides a weekly listing by title and author. Selecting out those journals you wish to monitor is one means of selectively monitoring the major toxicology journals.

Aids available to the toxicologist for monitoring research in progress are quite variable. The National Toxicology Program's (NTP) *Annual Plan for Fiscal Year XXXX* highlights all the accomplishments of the previous year and outlines the research plans for the coming year. *Annual Plan* contains all projects in the president's proposed fiscal year budget that occur within the National Cancer Institute/National Institutes of Health, National Institute of Environmental Health Sciences/National Institutes of Health, National Center for Toxicological Research/Food and Drug Administration, and NIOSH/Centers for Disease Control. this report includes a list of all the chemicals selected for testing in research areas that include but are not limited to mutagenicity, immunotoxicity, teratoly/reproduction, neurotoxicity, pharmacokinetics, subchronic toxicity, and chronic toxicity/carcinogenicity.

Annual Plan also contains a bibliography of NTP publications from the previous year. A companion publication is the 1999 NTP *Review of Current DHHS, DOE, and EPA Research Related to Toxicology*. Similar to *Annual Plan*, this document provides detailed summaries of both proposed and ongoing research.

Another mechanism for monitoring research in progress is by reviewing abstracts presented at the annual meetings of professional societies such as the Society of Toxicology, Teratology Society, Environmental Mutagen Society,

and American College of Toxicology. These societies usually have their abstracts prepared in printed form; for example, the current *Toxicologist* contains over 1700 abstracts presented at the annual meeting. Copies of the titles and authors of these abstracts are usually listed in the societies/respective journals, which, in many cases, would be reproduced and could be reviewed through *Current Contents*.

3.7 NEW SOURCES

Scientists today are more aware than ever before of the existence of what has been called the "information revolutions." At no other time in recent history has so much information become available from so many different "traditional" resources—including books, reviews, journals, and meetings—as well as personal computer–based materials such as databases, alerting services, optical-disk-based information, and news media.

The good news for toxicologists interested in the safety of chemical entities of all types is that numerous new computer-based information products are available that can be extremely useful additions to current safety and toxicology libraries. These tools enable one to save considerable time, effort, and money while evaluating the safety of chemical entities.

The primary focus of this section is on the description and applications of the recent innovations of newly emerging information services based on the personal computer (PC).

3.8 KINDS OF INFORMATION

The kinds of information described here are found on three types of PC media—floppy, CD-ROM, and laser disks. The products run the gamut of allowing one to assess current developments on a weekly basis as well as carry out more traditional reviews of historical information. The general types of information one can cover include basic pharmacology, preclinical toxicology, competitive products, and clinical safety.

The specific products discussed are as follows: two floppy disk–based products called Current Contents on Diskette and Focus On Global Change; five CD-ROM products called Toxic Release Inventory, Material Safety Data Sheets, CCINFOdisc, Pollution/Toxicology, and Medline Ondisc; and a laser disk product entitled the Veterinary Pathology Slide Bank. We provide a brief synopsis of the major features of each as well as a description of their integration into a functional, PC-based toxicology information center (TIC).

When such a TIC is established, one will find that some unusual benefits accrue. One now has immediate and uninterrupted access to libraries of valuable and comprehensive scientific data. This access is free of "online" constraints and designed to be user friendly, with readily retrievable information

available 24 hours a day, 7 days a week. The retrieved information can also usually be manipulated in electronic form, so one can use it in reports and/or store it in machine-readable form as ASCII files.

The minimal hardware requirements, which are certainly adequate for all items discussed here, are an IBM or IBM-compatible PC equipped with at least 640K RAM, a single floppy disk drive, at least a 40-Mbyte hard-disk drive, a CD-ROM drive, a VGA color monitor, and a printer. The basic point here is that hardware requirements are minimal and readily available. In the case of the laser disk products, a laser disk drive and high-resolution (VGA) monitor are also required.

3.8.1 PC-Based Information Products: Floppy Disk Based

We currently have ready access to a rapidly growing variety of relevant information resources. From a current awareness perspective, an excellent source of weekly information is the floppy disk–based product called Current Contents on Diskette (CCOD). Several versions are available; however, the Life Sciences version is most appropriate for this review because of its coverage, on a weekly basis, of over 1200 journals describing work in the biological sciences. One will note that the product has several useful features, including very quick retrieval of article citations as well as several output options (including either hard-copy or electronic storage of references as well as reprint requests).

3.8.2 PC-Based Information Products: CD-ROM Media

The gradual emergence of this technology during the past several years has recently blossomed with the introduction of several CD-ROM products that deal with safety issues surrounding the toxicology and safety of chemicals. CD-ROM media with such information can generally be characterized by two major advantages: They are relatively easy to use and are amazingly quick in retrieving data of interest.

Toxic Release Inventory (TRI) Before embarking on a discussion of products describing health, toxicology, and safety issues, it is well to be aware of a new, pilot CD-ROM version of the U.S. Environmental Protection Agency's (EPA) 1987 Toxic Chemical Release Inventory and Hazardous Substances Fact Sheets. This TRI resource, which contains information regarding the annual inventory of hundreds of named toxic chemicals from certain facilities (since 1987) as well as the toxicological and ecological effects of chemicals, is available from the National Technical Information Service (NTIS), U.S. Department of Commerce, Springfield, Virginia 22161.

The list of toxic chemicals subject to reporting was originally derived from those designed for similar purposes by the states of Maryland and New Jersey. As such, over 300 chemicals and categories are noted. (After appropriate rule

making, modifications to the list can be made by the EPA.) The inventory is designed to inform the public and government officials about routine and accidental releases of toxic chemicals to the environment.

The CD-ROM version of the database can be efficiently searched with a menu-driven type of software called Search Express. It allows one to search with Boolean expressions as well as individual words and/or frequency of "hits" as a function of the number of documents retrieved on a given topic. Numerous searchable fields have been included, allowing one to retrieve information by a variety of means—for example, the compound name; the chemical registry number; the amount of material released into the air, water, or land; the location of the site of release; and the Standard Industrial Classification (SIC) code of the releasing party. One can also employ ranging methods with available numeric fields and sorting of output.

It is hoped that this shared information will help to increase the awareness, concern, and action by individuals to ensure a clean and safe environment. The TRI database is a significant contribution to that effort and the CD-ROM version is a superb medium with which to widely publicize and make accessible the findings.

Material Safety Data Sheets (MSDSs) The MSDS CD-ROM is a useful resource that contains over 33,000 MSDSs on chemicals submitted to the Occupational Safety and Health Administration (OSHA) by chemical manufacturers. This resource contains complete MSDS information as well as other important information such as the chemical formula, structure, physical properties, synonyms, registry number, and safety information.

Users can easily search the CD-ROM by employing the Aldrich catalog number, CAS number, chemical name, or molecular formula. One can also export the chemical structures to some supported software for subsequent inclusion into work-processing programs. The product is available from Aldrich Chemical Company, 940 West St. Paul Avenue, Milwaukee, Wisconsin 54233.

Canadian Centre for Occupational Health and Safety (CCINFO) This set of four CD-ROM disks contains several valuable databases of information that are updated on a quarterly basis: MSDS, CHEM Data, Occupational Health and Safety (OHS) Source, and OHS Data. The MSDS component currently contains over 60,000 MSDSs supplied by chemical manufacturers and distributors. It also contains several other databases [RIPP, RIPA, Pest Management Research Information System (PRIS)], one of which (PRIS) even includes information on pest management products, including their presence and allowable limits in food.

A second disk in the series (CHEM Data) contains comprehensive information from CHEMINFO, Registry of Toxic Effects of Chemical Substances (RTECS) and Chemical Evaluation Search and Retrieval System (CESARS) data bases as well as recommendations on Transport of Dangerous Goods (TDG)/Hazardous Materials (49 CFR).

The third and fourth disks include OHS information. These disks contain databases on resource organizations, resource people, case law, jurisprudence, fatalities, mining incidents, and ADISCAN. Furthermore, information on noise levels, and National Institute for Occupational Safety and Health (NIOSHTEC) nonionizing radiation levels, and a Document Information Directory System is readily retrievable. These CD-ROM materials are available from the Canadian Center for Occupational Health and Safety, 250 Main Street East, Hamilton, Ontario L8N 1H6.

Pollution and Toxicology (POLTOX) This CD-ROM library also focuses our attention on environmental health and safety concerns. Scientists working in any industry or capacity that deals with toxic or potentially toxic chemicals will find it very useful. It allows one access to seven major databases in this field in a single search through its use of "linking" features in its software. The distributors of this product have provided us with a spectrum of information dealing with toxic substances and environmental health.

The collection of these databases include five that are available exclusively from Cambridge Scientific Abstracts (CSA)—Pollution Abstracts, Toxicology Abstracts, Ecology Abstracts, Health and Safety Science Abstracts, and Aquatic Pollution and Environmental Quality. The abstracts come from journals or digests published by CSA on important issues, including environmental pollution, toxicological studies of industrial chemicals, ecological impacts of biologically active chemicals, as well as health, safety, and risk management in occupational situations. The POLTOX CD-ROM contains over 200,000 records from these sources since 1981.

POLTOX also contains two other useful databases—Toxline (described earlier) and the Food Science and Technology Abstracts (FSTA) libraries. The FSTA component is a reasonably comprehensive collection of information regarding toxicological aspects of compounds found in food, including contamination, poison, and carcinogenic properties. The CD-ROM product is available from Compact Cambridge, 7200 Wisconsin Avenue, Bethesda, Maryland 20814.

Medline The Medline database, which comes from the NLM, is a superb, indispensable reference library that is particularly strong in its wide coverage of research activities in the biomedical literature. It also encompasses the areas of clinical medicine, health policy, and health care services. Each year, over 300,000 articles are reviewed and indexed into the database. The full bibliographic citations of these articles, usually including the abstract of the published work, are available from numerous vendors in CD-ROM format and are usually updated on a monthly basis.

Information can be accessed from Medline in a variety of ways: by author, title, subject, CAS registration number, keyword, publication year, and journal title. Medline Ondisc is the CD-ROM product we employ (from Dialog Information Services, 3460 Hillview Ave., Palo Alto, CA 94304). It allows one access

to the full Medline files back to 1984. Each year from that time until 1988 is covered on a single CD-ROM disk; starting in 1989, each disk covers only a six-month time period. The information is accessed through either an easily employed "menu-driven" system or a more standard online type of "command language."

Gower Publishing (Brookfield, VT) has published a series of "electronic handbooks" providing approved ingredient information on materials used in cosmetics, personal care additives, food additives, and pharmaceuticals. Academic Press, through its Sci-Vision branch, launched (in 2000) an ambitious service of CD ROM-based toxicity database products which are structure and substructure searchable.

It is worth nothing that the CD-ROM-based system has been seamlessly integrated with both (proprietary) recordkeeping and communications software so that one can optionally monitor the use of the online services and easily continue searching in the Dialog "online" environment after using the CD-ROM-based Medline library. Another very useful feature includes the storage of one's search logic so that repetitive types of searches, over time, for example, can be done very easily.

3.8.3 PC-Based Information Products: Laser Disk

International Veterinary Pathology Slide Bank (IVPSB) This application represents an important complementary approach toward training and awareness using laser disk technology. The IVPSB provides a quality collection of transparencies, laser videodisks, and interactive computer/videodisk training programs. In particular, the videodisk contains over 21,000 slides from over 60 contributors representing 37 institutions from 6 countries. These slides are accessible almost instantaneously because of the tremendous storage capacity and rapid random-search capabilities of the videodisk through the interactive flexibility of the computer. The information available is of particular interest to toxicologists and pathologists because the visuals illustrate examples of gross lesions of infectious diseases, regional diseases, clinical signs or external microscopy, histopathology, normal histology, cytology and hematology, and parasitology.

The laser disk, a catalog of the entrees, a computer database, and selected interactive programs can be obtained from Dr. Wayne Crowell, Department of Veterinary Pathology, University of Georgia, Athens, Georgia 30602.

3.9 CONCLUSIONS

This brief overview of some of the readily available PC-based information resources will, hopefully, encourage more widespread use of this type of technology. Toxicologists and pathologists, in particular, can avail themselves of these useful resources in a way that was simply not possible just a few years

ago. The information one needs to make decisions is now far more accessible to many more of us for relatively reasonable expenditures of money for software and hardware.

An effective approach to provide maximal access to these resources is to set up a TIC, which consists of the earlier noted PC hardware and single, centrally available copies of the noted floppy disk, CD-ROM-based, and laser disk products. By employing a menu-based system (available commercially or by shareware) to access the respective products, one can usually provide entry into each of the products discussed here with a single keystroke.

As time goes on, one can grow with the system by considering networking the CD-ROM-based resources and/or setting up other, strategically located TICs on one's campus. The important concept here is that we wish to make the superb "new" PC-based information products as available as we can to interested scientists.

A critical part of the strategy for delivery of information to the end user is that one can anticipate marked increased usage of the more traditional, hard-copy-based resources of the centralized library. The tools described here are frequently complementary to the pivotal library-based information center. What one can anticipate, however, is a much more focused use of hard-copy-based information.

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4

Screens in Safety and Hazard Assessment

4.1 INTRODUCTION

In biological research, screens are tests designed and performed to identify agents or organisms having a certain set of characteristics that will either exclude them from further consideration or cause them to be selected for closer attention. In pharmaceutical safety assessment, our use of screens is usually negative (i.e., no activity is found)—agents or objects possessing certain biochemical activities are considered to present enough of a hazard that they are not studied further or developed as potential therapeutic agents without compelling reasons (in cases of extreme benefit such as life-saving qualities).

In the broadest terms what is done in preclinical (and, indeed, in phase I clinical) studies can be considered a form of screening (Zbinden et al., 1984). What varies is the degree of effectiveness of (or our confidence in) each of the tests used. As a general rule, though we think of the expensive and labor-intensive "pivotal" studies required to support regulatory requirements (e.g., 4-week-to-1-year toxicity, carcinogenicity, and segment I–III studies) as definitive, in fact, they are highly effective (or at least we generally so believe) but not necessarily efficient screens.

Though toxicologists in the pharmaceutical industry are familiar with the broad concepts of screening, they generally do not recognize the applicability of screens. The principles underlying screening are also not generally well

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recognized or understood. The objective behind the entire safety assessment process in the pharmaceutical industry is to identify those compounds for which the risk of harming humans does not exceed the potential benefit to them.

In most cases this means that if a test or screen identifies a level of risk that we have confidence in (our "activity criterion"), then the compound that was tested is no longer considered a viable candidate for development. In this approach, what may change from test to test is the activity criterion (i.e., our basis for and degree of confidence in the outcome). We are interested in minimizing the number of false negatives in safety assessment. Anderson and Hauck (1983) should be consulted for statistical methods to minimize false-negative results.

Figure 4.1 illustrates how currently decisions are more likely to be made on a multidimensional basis, which creates a need for balance among (1) degree of benefit, (2) confidence that there *is* a benefit (efficacy is being evaluated in "models" or screens at the same time safety is), (3) type of risk (with, e.g., muscle irritation, mutagenicity, acute lethality, and carcinogenicity having various degrees of concern attached to them), and (4) confidence in and degree of risk. This necessity for balance is commonly missed by many who voice opposition to screens because "they may cause us to throw out a promising compound based on a finding in which we have only (for example) 80% confidence." Screens, particularly those performed early in the research and development process, should be viewed as the biological equivalent of exploratory data analysis. They should be very sensitive, which by definition means that they will have a lot of "noise" associated with them. Screens generally do not establish that an agent is (or is not) a "bad actor" for a certain endpoint. Rather, they confirm that if interest in a compound is sufficient, a more definitive test (a confirmatory test) is required, which frequently will provide a basis for selecting between multiple candidate compounds.

4.2 CHARACTERISTICS OF SCREENS

The terminology involved in screen design and evaluation and the characteristics of a screen should be clearly stated and understood. The characteristics of screen performance are defined as:

- Sensitivity: the ratio of true positives to total actives
- Specificity: the ratio of true negatives to total inactives
- Positive accuracy: the ratio of true to observed positives
- Negative accuracy: the ratio of true to observed negatives
- Capacity: the number of compounds that can be evaluated
- Reproducibility: the probability that a screen will produce the same results at another time (and, perhaps, in some other laboratory)

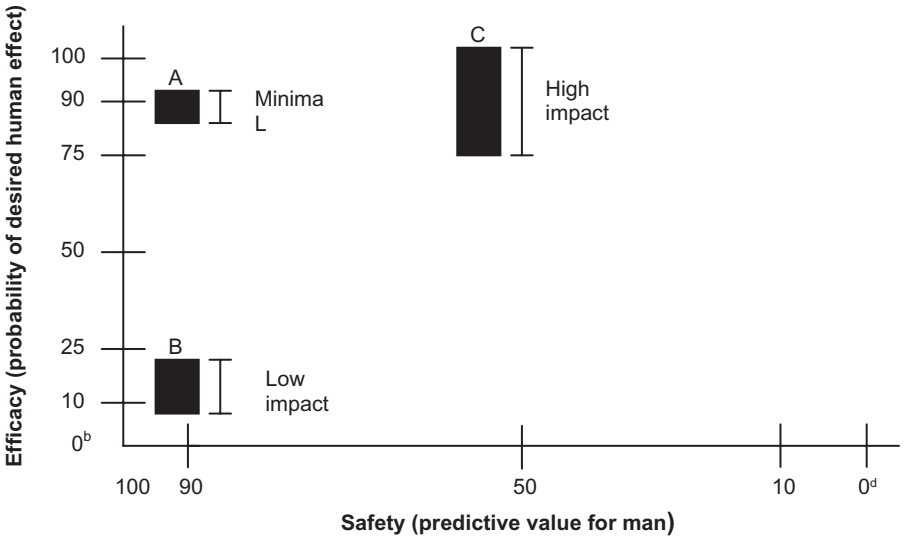


Figure 4.1 *Decision making for pharmaceutical candidates based on outcome of screening tests. (a) A 100% probability of efficacy means that every compound that has the observed performance in the model(s) used has the desired activity in man. (b) A 0% probability of efficacy means that every compound that has the observed performance in the model(s) used does not have the desired activity in man. (c) A 100% probability of a safety finding means that such a compound would definitely cause this toxicity in man. (d) A 0% probability means this will never cause such a problem in man. Note: These four cases (*a, b, c, *and* d*) are almost never found.*

The height of the "impact" column refers to the relative importance ("human risk") of a safety finding. Compound A has a high probability of efficacy but also a high probability of having some adverse effect in man. But if that adverse effect is of low impact—say, transitory muscle irritation for a life-saving antibiotic—A should go forward. Likewise, B, which has a low probability of efficacy and a high probability of having an adverse effect with moderate impact, should not be pursued. Compound C is at a place where the high end of the impact scale should be considered. Though there is only a 5% probability of this finding (say, neurotoxicity or carcinogenicity) being predictive in man, the adverse effect is not an acceptable one. Here a more definitive test is called for or the compound should be dropped.

These characteristics may be optimized for a particular use if we also consider the mathematics underlying them and "errors."

A brief review of the basic relationships between error types and power starts with considering each of five interacting factors (Gad, 1982a, 1982b, 1999) that serve to determine power and define competing error rates:

- α: the probability of our committing a type I error (a false positive)
- β: the probability of our committing a type II error (a false negative)
- Δ: the desired sensitivity in a screen (such as being able to detect an increase of 10% in mutations in a population)
- σ: the variability of the biological system and the effects of chance errors
- n: the necessary sample size needed to achieve the desired levels of each of these factors

We can, by our actions, generally change only this portion of the equation, since n is proportional to $\sigma/(\alpha, \beta, \text{and } \Delta)$.

The implications of this are therefore that (1) the greater σ is, the larger n must be to achieve the desired levels of α, β, and/or Δ, and (2) the smaller the desired levels of α, β, and/or Δ, if n is constant, the larger σ must be.

What are the background response level and the variability in our technique? As any good toxicologist will acknowledge, matched concurrent control (or standardization) groups are essential to minimize within-group variability as an "error" contributor. Unfortunately, in in vivo toxicology test systems, large sample sizes are not readily attainable, and there are other complications to this problem that we shall consider later.

In an early screen, a relatively large number of compounds will be tested. It is unlikely that one will stand out so much as to have greater statistical significance than all the other compounds (Bergman and Gittins, 1985). A more or less continuous range of activities will be found instead. Compounds showing the highest (beneficial) or lowest (adverse) activity will proceed to the next assay or tier of tests in the series and may be used as lead compounds in a new cycle of testing and evaluation.

The balance between how well a screen discovers activities of interest versus other effects (specificity) is thus critical. Table 4.1 presents a graphic illustration of the dynamic relationship between discovery and discrimination.

Both discovery and discrimination in screens hinge on the decision criterion that is used to determine if activity has or has not been detected. How sharply such a criterion is defined and how well it reflects the working of a screening system are two of the critical factors driving screen design.

An advantage of testing many compounds is that it gives the opportunity to average activity evidence over structural classes or to study quantitative structure–activity relationships (QSARs). Quantitative structure–activity relationships can be used to predict the activity of new compounds and thus reduce the chance of in vivo testing on negative compounds. The use of

TABLE 4.1 Discovery and Discrimination of Toxicants

	Actual Activity of Agent Tested	
Screen Outcome	Positive	Negative
Positive	*a*	*b*
Negative	*c*	*d*

Notes: Discovery (sensitivity) $= a/(a+c)$, where a = all toxicants found positive
$a+c$ = all toxicants tested
Discrimination (specificity) $= d/(b+d)$, where d = all nontoxicants found negative
$b+d$ = all nontoxicants tested

QSARs can increase the proportion of truly active compounds passing through the system.

It should be remembered that maximization of the performance of a series of screening assays requires close collaboration among the toxicologist, chemist, and statistician. Screening, however, forms only part of a much larger research and development context. Screens thus may be considered the biological equivalent of exploratory data analysis (EDA). In fact, EDA methods, provide a number of useful possibilities for less rigid and yet utilitarian approaches to the statistical analysis of the data from screens and are one of the alternative approaches presented and evaluated here (Tukey, 1977; Redman, 1981; Hoaglin et al., 1983, 1985). Over the years, the author has published and consulted on a large number of screening studies and projects. These have usually been directed at detecting or identifying potential behavioral toxicants or neurotoxicants, but some have been directed at pharmacological, immunotoxic, and genotoxic agents (Gad, 1988, 1989a).

The general principles or considerations for screening in safety assessments are as follows:

1. Screens almost always focus on detecting a single point of effect (such as mutagenicity, lethality, neurotoxicity, or developmental toxicity) and have a particular set of operating characteristics in common.
2. A large number of compounds are evaluated, so ease and speed of performance (which may also be considered efficiency) are very desirable characteristics.
3. The screen must be very sensitive in its detection of potential effective agents. An absolute minimum of active agents should escape detection; that is, there should be very few false negatives (in other words, the type II error rate or β level should be low). Stated yet another way, the signal gain should be way up.
4. It is desirable that the number of false positives be small (i.e., there should be a low type I error rate or α level).
5. Items 2–4, which to some degree are contradictory, require that involved researchers agree on a set of compromises, starting with the acceptance of a relatively high α level (0.10 or more), that is, a higher noise level.
6. In an effort to better serve item 1, safety assessment screens frequently are performed in batteries so that multiple endpoints are measured in the same operation. Additionally, such measurements may be repeated over a period of time in each model as a means of supporting item 2.
7. The screen should use small amounts of compound to make item 1 possible and should allow evaluation of materials that have limited availability (such as novel compounds) early in development.
8. Any screening system should be validated initially using a set of blind (positive and negative) controls. These blind controls should also be

evaluated in the screening system on a regular basis to ensure continuing proper operation of the screen. As such, the analysis techniques used here can then be used to ensure the quality or modify the performance of a screening system.

9. The more that is known about the activity of interest, the more specific the form of screen that can be employed. As specificity increases, so should sensitivity. However, generally the size of what constitutes a meaningful change (that is, Δ) must be estimated and is rarely truly known.
10. Sample (group) sizes are generally small.
11. The data tend to be imprecisely gathered (often because researchers are unsure what they are looking for) and therefore possess extreme within-group variability or modify test performance.
12. Proper dose selection is essential for effective and efficient screen design and conduct. If insufficient data are available, a suitably broad range of doses must be evaluated (however, this technique is undesirable on multiple grounds, as has already been pointed out).

Much of the mathematics involved in calculating screen characteristics came from World War II military-based operations analysis and research, where it was important in the design of radar, antiair, and antisubmarine warfare systems and operations (Garrett and London, 1970).

4.2.1 Uses of Screens

The use of screens first occurs most to pharmaceutical scientists in pharmacology (Martin et al., 1988). Early experiences with the biological effects of a new molecule are almost always in some form of efficacy or pharmacology screen. The earliest of these tend to be with narrowly focused models, not infrequently performed in vitro. The later pharmacology screens, performed in vivo to increase confidence in the therapeutic potential of a new agent or to characterize its other activities [cardiovascular, central nervous system (CNS), etc.], can frequently provide some information of use in safety assessment also (even if only to narrow the limits of doses to be evaluated), and the results of these screens should be considered in early planning. In the new millennium, requirements for specific safety pharmacology screens have been promulgated. Additionally, since the late 1990s two new areas of screening have become very important in pharmaceutical safety assessment. The first is the use of screens for detecting compounds with the potential to cause fatal cardiac arrhythmias. These are almost always preceded by the early induction of a prolongation of the Q–T interval. While this should be detected in electrocardiograms (ECGs) performed in repeat-dose canine studies, several early screens [such as the human Ether-à-go-go Related Gene (hERG)] are more rapid and efficient (though not conclusive) for selecting candidate compounds for further development.

The other area is the use of microassays in toxicogenomic screening—early detection of the potential for compounds to alter gene expressions with adverse consequences (Pennie, 2000; Nuwaysir et al., 1999).

Safety assessment screens are performed in three major settings—discovery support, development (what is generally considered the "real job" of safety assessment), and occupational health/environmental assessment testing. Discovery support is the most natural area of employment of screens and is where effective and efficient screen design and conduct can pay the greatest long-range benefits. If compounds with unacceptable safety profiles can be identified before substantial resources are invested in them—and structures modified to maintain efficacy while avoiding early safety concerns—then long-term success of the entire research and development effort is enhanced. In the discovery support phase, one has the greatest flexibility in the design and use of screens. Here screens truly are used to select from among a number of compounds.

Examples of the use of screens in the development stage are presented in some detail in the next section.

The use of screens in environmental assessment and occupational health is fairly straightforward. On the occupational side the concerns (as addressed in Chapter 11 of this volume) address the potential hazards to those involved in making the bulk drug. The need to address potential environmental concerns covers both true environmental items (e.g., aquatic toxicity) and potential health concerns for environmental exposure of individuals. The resulting work tends to be either regulatorily defined tests (for aquatic toxicity) or defined endpoints such as dermal irritation and sensitization, which have been (in a sense) screened for already in other nonspecific tests.

The most readily recognized examples of screens in toxicology are those that focus on a single endpoint. The traditional members of this group include genotoxicity tests, lethality tests (particularly recognizable as a screen when in the form of limit tests), and tests for corrosion, irritation (both eye and skin), and skin sensitization. Others that fit this same pattern, as will be shown, include the carcinogenicity bioassay (especially the transgenic mouse models) and developmental toxicity studies.

The "chronic" rodent carcinogenicity bioassay is thought of as the "gold standard" or definitive study for carcinogenicity, but, in fact, it was originally designed (and functions) as a screen for strong carcinogens (Page, 1977). It uses high doses to increase its sensitivity in detecting an effect in a small sample of animals. The model system (be it rats or mice) has significant background problems of interpretation. As with most screens, the design has been optimized (by using inbred animals, high doses, etc.) to detect one type of toxicant—strong carcinogens. Indeed, a negative finding does not mean that a material is not a carcinogen but rather than it is unlikely to be a potent one.

Many of the studies done in safety assessment are multiple-endpoint screens. Such study types as a 90-day toxicity study or immunotox/neurotox screens are designed to measure multiple endpoints with the desire of increasing both

sensitivity and reliability (by correspondence/correlation checks between multiple data sets).

4.2.2 Types of Screens

There are three major types of screen designs: single stage, sequential, and tiered. Both the sequential and tiered are multistage approaches, and each of these types also varies in terms of how many parameters are measured. These three major types have the following characteristics:

Single Stage A single test will be used to determine acceptance or rejection of a test material. Once an activity criterion (such as X score in a righting reflex test) is established, compounds are evaluated based on being less than X (i.e., negative) or equal to or greater than X (i.e., positive). As more data are accumulated, the criterion should be reassessed.

Sequential Two or more repetitions of the same test are performed, one after the other, with the severity of the criterion for activity being increased in each sequential stage. This procedure permits classification of compounds into a set of various ranges of potencies. As a general rule, it appears that a two-stage procedure, by optimizing decision rules and rescreening compounds before declaring compounds "interesting," increases both sensitivity and positive accuracy; however, efficiency is decreased (or is throughput rate).

Tier (or Multistage) In this procedure, materials found active in a screen are reevaluated in one or more additional screens or tests that have greater discrimination. Each subsequent screen or test is both more definitive and more expensive.

For purposes of our discussion here, we will primarily focus on the single-stage system, which is the simplest. The approaches presented here are appropriate for use in any of these screening systems, although establishment of activity criteria becomes more complicated in successive screens. Clearly, the use of multistage screens presents an opportunity to obtain increased benefits from the use of earlier (lower order) screening data to modify subsequent screen performance and the activity criterion.

4.2.3 Criterion: Development and Use

In any early screen, a relatively large number of compounds will be evaluated with the expectation that a minority will be active. It is unlikely that any one will stand out so much as to have greater statistical significance that all the other compounds based on a formal statistical test. A more or less continuous range of activities will be found. Compounds displaying a certain degree of activity will be identified as "active" and handled as such. For safety screens, those which are "inactive" go on to the next test in a series and may be used

as lead compounds in a new cycle of testing and evaluation. The single most critical part of the use of screens is how to make the decision that activity has been found.

Each test or assay has an associated activity criterion. If the result for a particular test compound meets this criterion, the compound is active and handled accordingly. Such a criterion could have a statistical basis (e.g., all compounds with observed activities significantly greater than the control at the 5% level could be tagged). However, for early screens, a statistical criterion may be too strict, given the power of the assay, resulting in a few compounds being identified as active. In fact, a criterion should be established (and perhaps modified over time) to provide a desired degree of confidence in the predictive value of the screen.

A useful indicator of the efficiency of an assay series is the frequency of discovery of truly active compounds. This is related to the probability of discovery and to the degree of risk (hazard to health) associated with an active compound passing a screen undetected. These two factors in turn depend on the distribution of activities in the series of compounds being tested and the chances of rejecting and accepting compounds with given activities at each stage.

Statistical modeling of the assay system may lead to the improvement of the design of the system by reducing the interval between discoveries of active compounds. The objectives behind a screen and considerations of (1) costs for producing compounds and testing and (2) degree of uncertainty about test performance will determine desired performance characteristics of specific cases. In the most common case of early toxicity screens performed to remove possible problem compounds, preliminary results suggest that it may be beneficial to increase the number of compounds tested, decrease the numbers of animals (or other test models) per assay, and increase the range and number of doses. The result will be less information on more structures, but there will be an overall increase in the frequency of discovery of active compounds (assuming that truly active compounds are entering the system at a random and steady rate).

The methods described here are well suited to analyzing screening data when the interest is truly in detecting the absence of an effect with little chance of false negatives. There are many forms of graphical analysis methods available, including some newer forms that are particularly well suited to multivariate data (the type that are common in more complicated screening test designs). It is intended that these aspects of analysis will be focused on in a later publication.

The design of each assay and the choice of the activity criterion should, therefore, be adjusted, bearing in mind the relative costs of retaining false positives and rejecting false negatives (Bickis, 1990). Decreasing the group sizes in the early assays reduced the chance of obtaining significance at any particular level (such as 5%), so that the activity criterion must be relaxed, in a statistical sense, to allow more compounds through. At some stage, however, it becomes too expensive to continue screening many false positives, and the

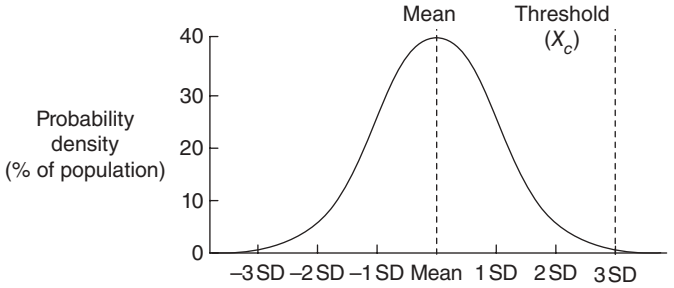


Figure 4.2 *Setting thresholds using historical control data. The figure shows a Gaussian ("normal") distribution of screen parameters; 99.7% of the observations in the population are within three standard deviations (SD) of the historic mean. Here the threshold (i.e., the point at which a datum is outside of normal) was set at* X_c *= mean + 3 SD. Note that such a screen is one sided.*

criteria must be tightened accordingly. Where the criteria are set depends on the acceptable noise levels in a screening system.

Criteria can be as simple (lethality) or as complex (a number of clinical chemical and hematological parameters) as required. The first step in establishing them should be an evaluation of the performance of test systems that have not been treated (i.e., negative controls). There will be some innate variability in the population, and understanding this variability is essential to selling some "threshold" for "activity" that has an acceptably low level of occurrence in a control population. Figure 4.2 illustrates this approach.

What endpoints are measured as inputs to an activity criterion are intrinsic in the screen system but may be either direct (i.e., having some established mechanistic relationship to the endpoint that is being predicted in humans, such as gene mutations as predictive of carcinogenicity) or correlative. Correlated variables (such as many of those measured in in vitro systems) are "black-box" predictors—compounds causing certain changes in these variables have a high probability of having a certain effect in humans, though the mechanisms (or commonality of mechanism) is not established. There is also, it should be noted, a group of effects seen in animals the relevance of which in humans is not known. This illustrates an important point to consider in the design of a screen—one should have an understanding (in advance) of the actions to be taken given each of the possible outcomes of a screen.

4.2.4 Analysis of Screening Data

Screening data present a special case that, due to their inherent characteristics, is not well served by traditional approaches (Gad, 1988, 1989a,b,c).

Why? First consider which factors influence the power of a statistical test. Gad (1988) established the basic factors that influence the statistical performance of any bioassay in terms of its sensitivity and error rates. Recently, Healy (1987) presented a review of the factors that influence the power of a

study (the ability to detect a dose-related effect when it actually exists). In brief, the power of a study depends on six aspects of study design:

- Sample size
- Background variability (error variance)
- Size of true effect to be detected (i.e., objective of the study)
- Type of significance test
- Significance level
- Decision rule (the number of false positives one will accept)

There are several ways to increase power—each with a consequence:

Action	Consequence
Increase the sample size	Greater resources required
Design test to detect larger differences	Less useful conclusions
Use a more powerful significance test	Stronger assumptions required
Increase the significance level	Higher statistical false-positive rate
Use one-tailed decision rule	Blind to effects in the opposite direction

Timely and constant incorporation of knowledge of test system characteristics and performance will reduce background variability and allow sharper focus on the actual variable of interest. There are, however, a variety of nontraditional approaches to the analysis of screening data.

Univariate Data

Control Charts The control chart approach (Montgomery, 1985), commonly used in manufacturing quality control in another form of screening (for defective product units), offers some desirable characteristics.

By keeping records of cumulative results during the development of screen methodology, an initial estimate of the variability (such as standard deviation) of each assay will be available when full-scale use of the screen starts. The initial estimates can then be revised as more data are generated (i.e., as we become more familiar with the screen).

The following example shows the usefulness of control charts for control measurements in a screening procedure. Our example test for screening potential muscle strength suppressive agents measures reduction of grip strength by test compounds compared with a control treatment. A control chart was established to monitor the performance of the control agent to (1) establish the mean and variability of the control and (2) ensure that the results of the control for a given experiment are within reasonable limits (a validation of the assay procedure).

As in control charts for quality control, the mean and average range of the assay were determined from previous experiments. In this example, the screen had been run 20 times previous to collecting the data shown. These initial data showed a mean grip strength X of 400 g and a mean range R of 90 g. These values were used for the control chart (Figure 4.3). The subgroups are of size 5. The action limits for the mean and range charts were calculated as follows:

$$X \pm 0.58R = 400 \pm 0.58 \times 90 = 348\text{–}452 \quad \text{(from } X \text{ chart)}$$

Then, using the upper limit (du) for $n = 5$,

$$2.11R = 2.11 \times 90 = 190 \quad \text{(upper limit for range)}$$

Note that the range limit, which actually established a limit for the variability of our data, is in fact a "detector" for the presence of outliers (extreme values).

Such charts may also be constructed and used for proportion or count types of data. By constructing such charts for the range of control data, we may then

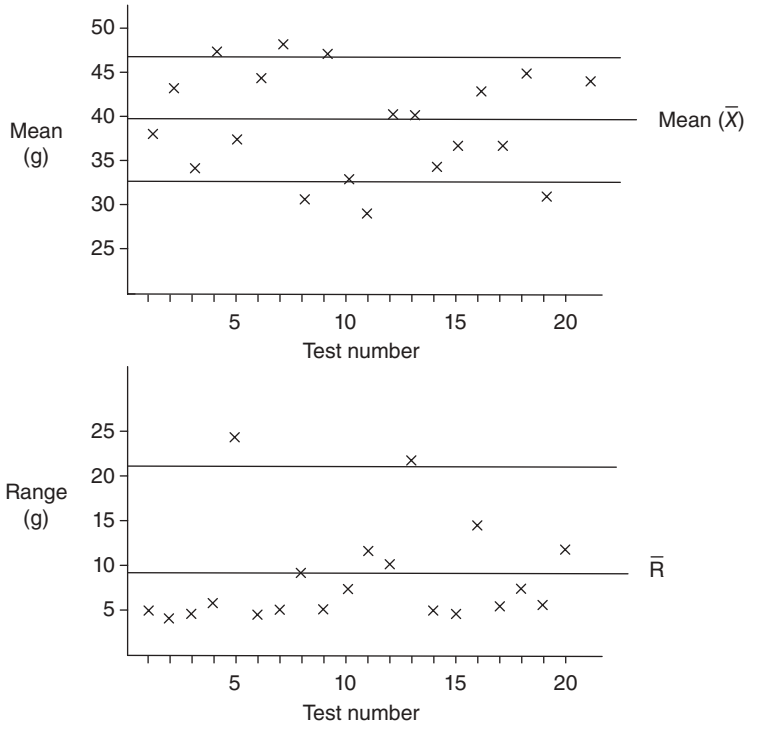


Figure 4.3 *Example of a control chart used to "prescreen" data (actually, explore and identify influential variables) from a portion of a functional observational battery.*

use them as rapid and efficient tools for detecting effects in groups being assessed for that same activity endpoint.

Central Tendency Plots The objective behind our analysis of screen data is to have a means of efficiently, rapidly, and objectively identifying those agents that have a reasonable probability of being active. Any materials that we so identify may be further investigated in a more rigorous manner, which will generate data that can be analyzed by traditional means. In other words, we want a method that makes out-of-the-ordinary results stand out. To do this we must first set the limits on "ordinary" (summarize the control case data) and then overlay a scheme that causes those things that are not ordinary to become readily detected ("exposed," in EDA terms) (Velleman and Hoaglin, 1981; Tufte, 1983). One can then perform "confirmatory" tests and statistical analysis (using traditional hypothesis-testing techniques), if so desired.

If we collect a set of control data on a variable (say scores on our observations of the righting reflex) from some number of ordinary animals, we can plot it as a set of two histograms (one for individual animals and the second for the highest total score in each randomly assigned group of five animals), such as those shown in Figure 4.4 (the data for which came from 200 actual control animals).

Such a plot identifies the nature of our data, visually classifying them into those that will not influence our analysis (in the set shown, clearly scores of zero fit into this category) and those that will critically influence the outcome of an analysis. In so doing, the position of control ("normal") observations is

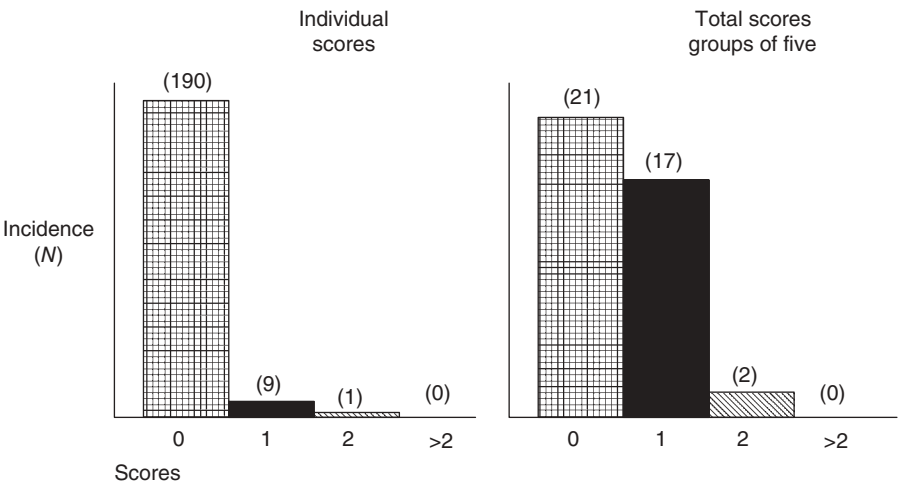


Figure 4.4 *Plotting central tendency. Possible individual scores for righting reflexes may range from 0 to 8 (Gad, 1982a). Group total scores would thus range from 0 to 40. (Shown are the number of groups that contain individual scores in the individual categories.)*

readily revealed as a "central tendency" in the data (hence the name for this technique).

We can (and should) develop such plots for each of our variables. Simple inspection makes clear that answers having no discriminatory power (zero values in Figure 4.4) do not interest us or influence our identifying of an outlier in a group and should simply be put aside or ignored before continuing with the analysis. This first stage, summarizing the control data, thus gives us a device for identifying data with discriminatory power (extreme values), allowing us to set aside the data without discriminatory power.

Focusing our efforts on the remainder, it becomes clear that although the incidence of a single, low, nonzero observation in a group means nothing, total group scores of 2 or more occurred only 5% of the time by chance. So we can simply perform an extreme-value screen on our "collapsed" data sets, looking for total group values or individual values that are beyond our acceptance criteria.

The next step in this method is to develop a histogram for each ranked or quantal variable by both individual and group. "Useless" data (those that will not influence the outcome of the analysis) are then identified and dropped from analysis. Group scores may then be simply evaluated against the baseline histograms to identify those groups with scores divergent enough from control to be either true positives or acceptably low incidence false positives. Additional control data can continue to be incorporated in such a system over time, both increasing the power of the analysis and providing a check on screen performance.

Multivariate Data The traditional acute, subchronic, and chronic toxicity studies performed in rodents and other species also can be considered to constitute multiple-endpoint screens. Although the numerically measured continuous variables (body weight, food consumption, hematology values) generally can be statistically evaluated individually by traditional means, the same concerns of loss of information present in the interrelationship of such variables apply. Generally, traditional multivariate methods are not available, efficient, sensitive, or practical (Young, 1985).

Analog Plot The human eye is extremely good at comparing the size, shape, and color of pictorial symbols (Anderson, 1960; Andrews, 1972; Davison, 1983; Schmid, 1983; Cleveland and McGill, 1985). Furthermore, it can simultaneously appreciate both the minute detail and the broad pattern.

The simple way of transforming a table of numbers to a sheet of pictures is by using analog plots. Numbers are converted to symbols according to their magnitude. The greater the number, the larger the symbol. Multiple variables can be portrayed as separate columns or as differently shaped or colored symbols (Wilk and Gnanadesikan, 1986).

The conversion requires a conversion chart from the magnitude of the number to the symbol size. The conversion function should be monotonic (e.g., dose, and the measured responses should each change in one direction accord-

ing to a linear, logarithmic, or probit function). Log conversion will give more emphasis to differences at the lower end of the scale, whereas a probit will stabilize the central range of response (16–84%) of a percentage variable. For example, for numbers x, symbol radius r, and plotting scaling factor k, a log mapping will give

$$x = \begin{cases} 1 & r = k \\ 10 & r = 2k \\ 100 & r = 3k \end{cases}$$

To compare different variables on the same sheet requires some form of standardization to put them on the same scale. Also, a choice must be made between displaying the magnitude of the numbers and their significance (Kruskal, 1964; Kass, 1980). Two possibilities are:

- Express each mean as a percentage change from a control level or overall mean (*means plot*)
- Calculate effects for meaningful contrasts (*contrasts plot*)

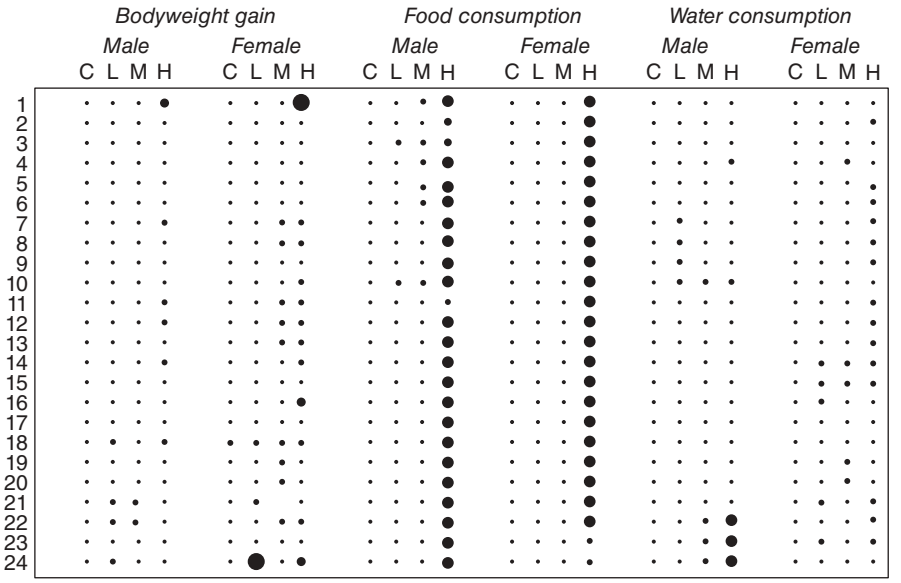
The analog plot chart in Figure 4.5 shows relationships for five measures on a time-versus-dose basis, allowing ready evaluation of interrelationships and patterns.

A study using 50 rats of each sex in each of five groups (two controls and three increasing doses) measured body weight and food and liquid consumption every week or month for two years. This resulted in 3 variables × 2 sexes × 5 groups × 53 times × 50 animals. Means alone constituted some 1600 four-digit numbers.

Body weight gains from the period immediately preceding each consumption measurement were used since these were less correlated. For each variable and at each time, the sums of squares for group differences were divided into four meaningful contrasts:

Control A versus control B
Control A + B versus low
Control A + B + low versus medium
Control A + B + low + medium versus high

To make the variables comparable, the sums of squares were standardized by the within-group standard deviations. Contrast involving doses can be compared with the contrast for the difference between the controls, which should be random. The clearest feature is the high-dose effect for food consumption. However, this seems not to be closely correlated with changes in body weight gains. Certain changes can be seen at the later measurement times, probably because of dying animals.



Note: Square root relationship

Key: C = control A vs. B.
L = low vs. A + B.
M = medium vs. A + B + low.
H = high vs. A + B + low + medium

Figure 4.5 *Analog plot for dose–response contrasts. One of many possible approaches to graphically presenting multidimensional data. In this case, various effects—day of dosing, dose response, and magnitude of response—are simultaneously portrayed, with the size of each circle being proportional to the magnitude of the measured value.*

There are numerous approaches to the problem of capturing all the information in a set of multi-endpoint data. When the data are continuous in nature, approaches such as the analog plot can be used (Chernoff, 1973; Chambers et al., 1983). A form of control chart also can be derived for such uses when detecting effect rather than exploring relationships between variables is the goal. When the data are discontinuous, other forms of analysis must be used. Just as the control chart can be adapted to analyzing attribute data, an analog plot can be adapted. Other methods are also available.

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5

Formulations, Routes, and Dosage Design

The perfect drug would be along the lines of Paul Eurlich's "magic bullet": As illustrated in Figure 5.1, a drug molecule is readily administered, completely absorbed, moves to the desired therapeutic target site, does what it is supposed to, and is completely eliminated. The most pressing (and rewarding) area for current drug development is optimizing the therapeutic target delivery part of this process. One of the key steps in the nonclinical and clinical formulation of the drug is the choice of the inactive ingredients (excipients). Excipients are essential components of drug products in the United States, and one must adequately address the safety of the proposed exposure to the excipients in those products. The specific safety data that may be needed will vary depending upon the clinical situation, including such factors as the duration, level, and route of exposure (i.e., means of patient drug administration).

Many guidances exist to aid in the development of pharmaceutical drugs, but very few guidances exist to aid in the safety evaluation of pharmaceutical excipients. The U.S. Food and Drug Administration (FDA)/Center for Drug Evaluation and Research (CDER) adopted, in 2005, the guidance for industry "Nonclinical Studies for Development of Pharmaceutical Excipients," which focuses on the development of safety profiles to support use of new excipients as components of drug or biological products.

A similar guidance was published by the International Pharmaceutical Excipients Council (IPEC), "Excipient Safety Evaluation Guidance," in 1995

Drug Safety Evaluation, Second Edition, by Shayne Cox Gad

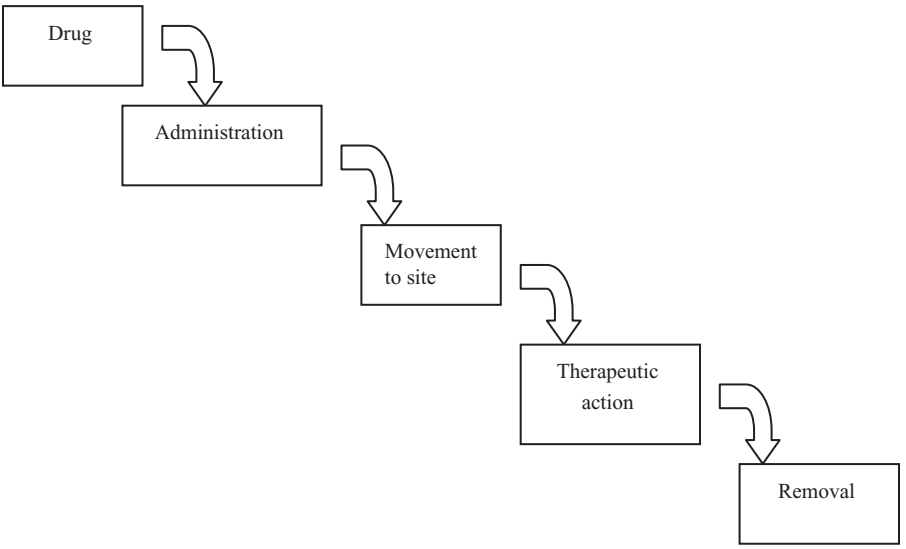

Figure 5.1 *Magic bullet: desired course of drug therapeutic development cycle.*

(updated in 2002). These guidelines are presented in a tiered approach of recommended data that should be available on an excipient to provide a pharmaceutical formulator with a rational basis for including a new excipient in a drug formulation.

The objective of the current proposal is to provide a rational approach to cover the field between "nothing is needed" and "full testing." The final aim of these safety evaluation guidelines for excipients is to provide an important element in the acceptability of a new excipient by health authorities independently of the approval of a specific drug formulation.

The three essential requirements of the active pharmaceutical ingredients (API) principles are compared with those of excipients. Fundamental for both are quality and safety. The requirement of therapeutic efficacy for drugs is replaced by that of functionality for excipient, defined as "the physical, physicochemical and biopharmaceutical properties" of the same.

Throughout the development process for pharmaceuticals, formulation development is proceeding with several objectives in mind. The importance of each of these factors changes over time (Monkhouse and Rhodes, 1998; illustrated in Figure 5.2). First is optimizing the bioavailability of the therapeutic target organ site by the intended clinical route. Clinical route(s) are selected on a number of grounds (nature of the drug, patient acceptance, issues of safety). Second is minimizing any safety concerns. This means not just systemic toxicity but also local tissue tolerance at the site of application. Third is optimizing stability of the drug active ingredient. Its activity and integrity must be maintained for long enough to be made effectively available to patients. Early

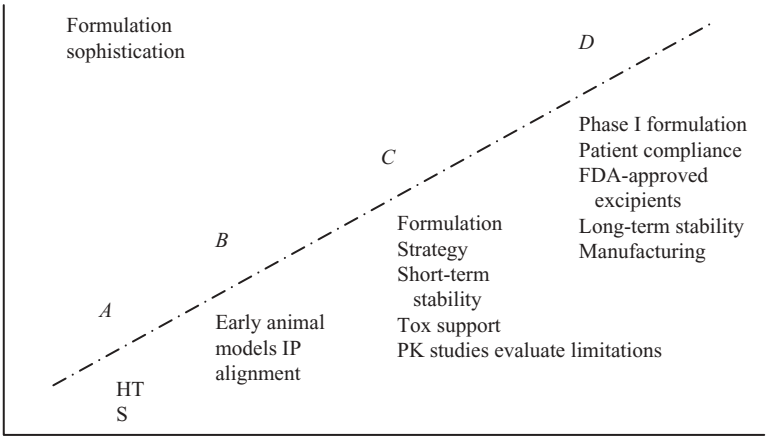

Figure 5.2 *Formulation development during course of drug development.*

on in preclinical development, simplicity and maximized bioavailability are essential. Early single-dose studies in animals are the starting place and usually bear no relationship to what is used later.

Formulations used to administer potential drugs undergoing development occupy an unusual place in pharmaceutical safety assessment compared to the rest of the industrial toxicology. Eventually, a separate function in the pharmaceutical company developing a drug will develop a specific formulation that is to be administered to people—a formulation that optimizes the conditions of absorption and stability for the drug entity (Racy, 1989). The final formulation will need to be assessed to see if it presents any unique local or short-term hazards, but as long as its nonactive constituents are drawn from the approved formulary lists, no significant separate evaluation of their safety is required preclinically. These short-term hazards can, of course, alter the toxicity of the drug under study.

Simultaneous with this development of an optimized clinical formulation, however, preclinical evaluations of the safety of the drug moiety must be performed. Separate preclinical formulations (which generally are less complex than the clinical ones) are developed, sometimes by a formulation group and other times by the toxicology group itself. These preclinical formulations will frequently include much higher concentrations of the drug moiety being tested than do any clinical formulations. The preclinical formulations are developed and evaluated with the aim of reproducibly delivering the drug (if at all possible by the route intended in humans), maintaining drug stability through an optimum period of time and occluding the observed effects of the drug with vehicle effects to the minimum extent possible. And these preclinical formulations are not restricted to materials that will (or even can) be used in final clinical formulations.

In pivotal studies, the actual blood levels of active moiety that are achieved will be determined so that correlations to later clinical studies can be made.

The formulations that are developed and used for preclinical studies are sometimes specific for the test species to be employed, but their development always starts with consideration of the route of exposure that is to be used clinically and, if possible, in accordance with a specified regimen of treatment (mirroring the intended clinical protocol as much as possible). One aspect of both nonclinical and clinical formulation and testing which prevents an important but often overlooked aspect of pharmaceutical safety assessment is the special field of excipients. These will be considered at the end of this chapter.

Among the cardinal principles of both toxicology and pharmacology is that the means by which an agent comes in contact with or enters the body (i.e., the route of exposure or administration) does much to determine the nature and magnitude of an effect. However, a rigorous understanding of formulations, routes, and their implications to the design and analysis of safety studies is not widespread. And in the day-to-day operations of performing studies in animals, such an understanding of routes, their manipulation, means and pitfalls of achieving them, and the art and science of vehicles and formulations is essential to the sound and efficient conduct of a study.

As presented in Table 5.1, there are at least 26 potential routes of administration, of which 10 are commonly used in safety assessment and therefore are addressed here.

5.1 MECHANISMS

There are three primary sets of reasons why differences in formulations and the route of administration are critical in determining the effect of an agent of the biological system: (1) local effects, (2) absorption and distribution, and (3) metabolism.

Local Effects Local effects are those that are peculiar to the first area or region of the body to which a test material gains entry or that it contacts. For the dermal route, these include irritation, corrosion, and sensitization. For the parenteral routes, these include irritation, pyrogenicity, sterility, and blood compatibility. In general, the same categories of possible adverse effects (irritation, immediate immune response, local tissue/cellular compatibility, and physicochemical interactions) are the mechanisms of or the basis for concern.

In general, no matter what the route, certain characteristics will predispose a material to have local effects (and, by definition, if not present, tend to limit the possibility of local effects). These factors include pH, redox potential, high molar concentration, and the low flexibility and sharp edges of certain solids.

TABLE 5.1 Potential Routes of Administration

A. Oral routes
   1. Oral (PO)[a]
   2. Inhalation[a]
   3. Sublingual
   4. Buccal

B. Placed into natural orifice in body other than mouth
   1. Intranasal
   2. Intra-auricular
   3. Rectal
   4. Intravaginal
   5. Intrauterine
   6. Intraurethral

C. Parenteral (injected into body or placed under skin)
   1. Intravenous (IV)[a]
   2. Subcutaneous (SC)[a]
   3. Intramuscular (IM)[a]
   4. Intra-arterial
   5. Intradermal (ID)[a]
   6. Intralesional
   7. Epidural
   8. Intrathecal
   9. Intracisternal
   10. Intracardial
   11. Intraventricular
   12. Intraocular
   13. Intraperitoneal (IP)[a]

D. Topical routes
   1. Cutaneous[a]
   2. Transdermal (also called percutaneous)[a]
   3. Ophthalmic[a]

[a]Commonly used in safety assessment.

These characteristics will increase the potential for irritation by any route and, subsequent to the initial irritation, other appropriate regional adaptive responses (for orally administered materials, e.g., emesis and diarrhea).

Absorption and Distribution For a material to be toxic, it must be absorbed into the organism (local effects are largely not true toxicities by this definition).

There are characteristics that influence absorption by the different routes, and these need to be understood by any person trying to evaluate and/or predict the toxicities of different moieties. Some key characteristics and considerations are summarized below by route.

Table 5.2 presents the normal pH ranges for human physiological fluids. These need to be considered in terms of the impact on solubility and stability of a formulation and active drug.

TABLE 5.2 Normal pH Range for Human Physiological Fluids

Medium	Normal pH Range
Tears	7.35–7.45
Saliva	6.0–8.0
Gastric juice	1.5–6.5
Intestinal juice	6.5–7.6
Blood	7.35–7.45
Skin (sweat)	4.0–6.8

A. Oral and rectal routes [gastrointestinal (GI) tract]
   1. Lipid-soluble compounds (nonionized) are more readily absorbed than water-soluble compounds (ionized).
      a. Weak organic bases are in the nonionized, lipid-soluble form in the intestine and tend to be absorbed there.
      b. Weak organic acids are in the nonionized, lipid-soluble form in the stomach and one would suspect that they would be absorbed there, but absorption in the intestine is greater because of time and area of exposure.
   2. Specialized transport systems exist for some moieties: sugars, amino acids, pyrimidines, calcium, and sodium.
   3. Almost everything is absorbed—at least to a small extent (if it has a molecular weight below 10,000).
   4. Digestive fluids may modify the structure of a chemical.
   5. Dilution increases toxicity because of more rapid absorption from the intestine unless stomach contents bind the moiety.
   6. Physical properties are important; for example, dissolution of metallic mercury is essential to allow its absorption.
   7. Age is important; for example, neonates have a poor intestinal barrier.
   8. Effect of fasting on absorption depends on the properties of the chemical of interest.

B. Inhalation (lungs)
   1. Aerosol deposition
      a. Nasopharyngeal—5 μm or larger in humans, less in common laboratory animals
      b. Tracheobronchial—1–5 μm
      c. Alveolar—1 μm
   2. If inhalant is a solid, mucociliary transport from lungs to GI tract may clear it out.
   3. Lungs are anatomically good for absorption.
      a. Large surface area (50–100 m^2)
      b. Blood flow high
      c. Close to blood (10 μm between gas media and blood)

4. Absorption of gases is dependent on solubility of the gas in blood.
   a. Chloroform, for example, has high solubility and is all absorbed, though respiration is limited.
   b. Ethylene has low solubility and only a small percentage is absorbed—blood flow limits absorption.

C. Parenteral routes
1. Irritation at the site of injection is influenced by solubility, toxicity, temperature, and pH of injected solution.
2. Pyrogenicity and blood compatibility are major concerns for intravenously administered materials.
3. Solubility of test material in an aqueous or modified aqueous solution is the chief limitation on how much material may be given intravenously.

D. Dermal routes
1. In general, any factor that increases absorption through the stratum corneum will also increase the severity of an intrinsic response. Unless this factor mirrors potential exposure conditions, it may, in turn, adversely affect the relevance of test results.
2. The physical nature of solids must be carefully considered both before testing and in interpreting results. Shape (sharp edges), size (small particles may abrade the skin due to being rubbed back and forth under the occlusive wrap), and rigidity (stiff fibers or very hard particles will be physically irritating) of solids may all enhance an irritation response and alter absorption.
3. The degree of occlusion (in fact, the tightness of the wrap over the test site) also alters percutaneous absorption and therefore irritation. One important quality control issue in the laboratory is achieving a reproducible degree of occlusion in dermal wrappings.
4. Both the age of the test animal and the application site (saddle of the back vs. flank) can markedly alter test outcome. Both of these factors are also operative in humans, of course, but in dermal irritation tests, the objective is to remove all such sources of variability. In general, as an animal ages, sensitivity to irritation decreases. And the skin on the middle of the back (other than directly over the spine) tends to be thicker (and therefore less sensitive to irritations) than that on the flanks.
5. The sex of the test animals can also alter study results, because both regional skin thickness and surface blood flow vary between males and females.

As a generalization, there is a pattern of relative absorption rates that characterizes the different routes that are commonly employed. This order of absorption (by rate from fastest to slowest and, in a less rigorous manner, by degree of absorption from most to least is intravenous (IV) > inhala-

tion > intramuscular (IM) > intraperitoneal (IP) > subcutaneous (SC) > oral > intradermal (ID) > other.

Metabolism Metabolism is directly influenced both by the region a material is initially absorbed into and by distribution (both the rate and the pattern). Rate determines whether the primary enzyme systems will handle the entire xenobiotic dose or if these are overwhelmed. Pattern determines which routes of metabolism are operative.

Absorption (total amount and rate), distribution, metabolism, and species similarity in response are the reasons for selecting particular routes in toxicology in general. In safety assessment of pharmaceuticals, however, the route is usually dictated by the intended clinical route and dosing regimen. If this route of human exposure is uncertain or if there is the potential for either the number of routes or the human absorption rate and pattern being greater, then common practice becomes that of the most conservative approach. This approach stresses maximizing potential absorption in the animal species (within the limits of practicality) and selecting from among those routes commonly used in the laboratory the ones that get the most material into the animal's system as quickly and completely as possible to evaluate the potential toxicity. Under this approach, many compounds are administered intraperitoneally in acute testing, though there is little or no real potential for human exposure by this route.

Assuming that a material is absorbed, distribution of a compound in early preclinical studies is usually of limited interest. In so-called heavy acute studies (Gad et al., 1984), where acute systemic toxicity is intensive and evaluated to the point of identifying target organs, or in range-finder-type study results, for refining the design of longer term studies, distribution would be of interest. Some factors that alter distribution are listed in Table 5.3.

The first special case is the parenteral route, where the systemic circulation presents a peak level of the moiety of interest to the body at one time, tempered only by the results of a single pass through the liver.

The second special case arises from inhalation exposure. Because of the arrangement of the circulatory system, inhaled compounds (and those administered via the buccal route) enter the full range of systemic circulation without any "first-pass" metabolism by the liver. Kerberle (1971) and O'Reilly (1972) have previously published reviews of absorption, distribution, and metabolism that are relevant to acute testing.

5.2 COMMON ROUTES

Each of the 10 routes most commonly used in safety assessment studies has its own peculiarities, and for each there are practical considerations and techniques ("tricks") that should be either known or available to the practicing toxicologist.

TABLE 5.3 Selected Factors That May Affect Chemical Distribution to Various Tissues

A. Factors relating to chemical and its administration
   1. Degree of binding of chemical to plasma proteins (i.e., agent affinity for proteins) and tissues
   2. Chelation to calcium, which is deposited in growing bones and teeth (e.g., tetracyclines in young children)
   3. Whether chemical distributes evenly throughout body (one-compartment model) or differentially between different compartments (models of two or more compartments)
   4. Ability of chemical to cross blood–brain barrier
   5. Diffusion of chemical into tissues or organs and degree of binding to receptors that are and are not responsible for drug's beneficial effects
   6. Quantity of chemical given
   7. Route of administration or exposure
   8. Partition coefficients (nonpolar chemicals are distributed more readily to fat tissues than are polar chemicals)
   9. Interactions with other chemicals that may occupy receptors and prevent the drug from attaching to receptor, inhibit active transport, or otherwise interfere with drug's activity
   10. Molecular weight of chemical

B. Factors relating to test subject
   1. Body size
   2. Fat content (e.g., obesity affects distribution of drugs that are highly soluble in fats)
   3. Permeability of membranes
   4. Active transport for chemicals carried across cell membranes by active processes
   5. Amount of proteins in blood, especially albumin
   6. Pathology or altered homeostasis that affects any of the other factors (e.g., cardiac failure, renal failure)
   7. Presence of competitive binding substances (e.g., specific receptor sites in tissues bind drugs)
   8. pH of blood and body tissues
   9. pH of urine[a]
   10. Blood flow to various tissues or organs (e.g., well-perfused organs usually tend to accumulate more chemical than less well perfused organs)

[a]The pH of urine is usually more important than the pH of blood.

5.2.1 Dermal Route

For all agents of concern in occupational toxicology (except therapeutics), the major route by which the general population is most frequently exposed is the percutaneous (dermal) route. Brown (1980) has previously reviewed background incidence data on pesticides, for example, that show such exposures to be common. Dermal (or topical) drugs are not as common but are certainly numerous.

Percutaneous entry into the body is really by separate means (Marzulli, 1962; Scheuplein, 1965, 1967):

- Between the cells of the stratum corneum
- Through the cells of the stratum corneum

- Via the hair follicles
- Via the sweat glands
- Via the sebaceous glands

Certain aspects of the material of interest as well as those of the test animals, including absorption (Blank and Scheuplein, 1964), are as follows:

1. Small molecules penetrate skin better than large molecules.
2. Undissociated molecules penetrate skin better than do ions.
3. Preferential solubility of the toxicant in organic solvents indicates better penetration characteristics than preferential solubility in water.
4. The less viscous or more volatile the toxicant, the greater its penetrability.
5. The nature of the vehicle and the concentration of the toxicant in the vehicle both affect absorption (vehicles are discussed later in this chapter).
6. Hydration (water content) of the stratum corneum affects penetrability.
7. Ambient temperature can influence the uptake of toxicant through the skin. The warmer it is, the greater the blood flow through the skin and, therefore, the greater the percutaneous absorption.
8. Molecular shape (particularly symmetry) influences absorption (Medved and Kundiev, 1964).

There are at least two excellent texts on the subject of percutaneous absorption that go into great detail (Brandau and Lippold, 1982; Bronaugh and Maibach, 1985).

5.2.2 Parenteral Route

The parenteral routes include three major ones—IV, IM, and SC—and a number of minor routes (such as intra-arterial) that are not considered here. Administration by the parenteral routes raises a number of special safety concerns in addition to the usual systemic safety questions. These include irritation (vascular, muscular, or subcutaneous), pyrogenicity, blood compatibility, and sterility (Avis, 1985). The background of each of these, along with the underlying mechanisms and factors that influence the level of occurrence of such an effect, is discussed in Chapter 11.

The need for a rapid onset of action (and/or clearance) usually requires that an IV route be used, although at a certain stage of cardiopulmonary resuscitation (for example), the need for an even more rapid effect may require the use of an intracardiac injection. The required site of action may influence the choice of route of administration (e.g., certain radiopaque dyes are given intra-

arterially near the site being evaluated; streptokinase is sometimes injected experimentally into the coronary arteries close to coronary vessel occlusion during a myocardial infarction to cause lysis of the thrombus and therefore reestablish coronary blood flow).

The characteristics of the fluid to be injected will also influence the choice of parenteral routes. The drug must be compatible with other fluids (e.g., saline, dextrose, Ringer's lactate) with which it may be combined for administration to the patient as well as with the components of the blood itself.

There are certain clinical situations in which a parenteral route of administration is preferred to other possible routes. These include the following:

1. When the amount of drug given to a subject must be precisely controlled (e.g., in many pharmacokinetic studies), it is preferable to use a parenteral (usually IV) route of administration.
2. When the "first-pass effect" of a drug going through the liver must be avoided, a parenteral route of administration is usually chosen, although a sublingual route or dermal patch will also avoid the first-pass effect.
3. When one requires complete assurance that an uncooperative subject has actually received the drug and has not rejected it (e.g., via forced emesis).
4. When subjects are in a stupor, coma, or otherwise unable to take a drug orally.
5. When large volumes (i.e., more than a liter) of fluid are injected (such as in peritoneal dialysis, hyperalimentation, fluid replacement, and other conditions). Special consideration of fluid balance must be given to patients receiving large volumes as well as careful consideration of the systemic effects of injection fluid components (e.g., amino acids and their nephrotoxicity).

The three significant parenteral routes we are concerned with here and their specific set of advantages and disadvantages or specific considerations that must be kept in mind will be discussed next.

Intravenous Route The IV route is the most common method of introducing a drug directly into the systemic circulation (Lukas et al., 1971). It has the following advantages:

1. Rapid onset of effect
2. Usefulness in situations of poor GI absorption
3. Avoidance of tissue irritation that may be present IM or other routes (e.g., nitrogen mustard)
4. More precise control of levels of drug than with other routes, especially of toxic drugs, where the levels must be kept within narrow limits

5. Ability to administer large volumes over time by a slow infusion
6. Ability to administer drugs at a constant rate over a long period of time

It also suffers from disadvantages:

1. Higher incidence of anaphylactic reactions than with many other routes
2. Possibility of infection or phlebitis at site of injection
3. Greater pain to patients than with many other routes
4. Possibility that embolic phenomena may occur—either air embolism or vascular clot—as a result of damage to the vascular wall
5. Impossibility of removing or lavaging drug after it is given, except by dialysis
6. Inconvenience in many situations
7. Possibility that rapid injection rates may cause severe adverse reactions
8. Patient dislike of and psychological discomfort with the injection procedure

For IV fluids, it must be determined how the dose will be given (i.e., by bolus or slow injection, intermittent or constant infusion, or constant drip) and whether special equipment will be used to control and monitor the flow. Drugs with short half-lives are usually given by a constant drip or infusion technique. All IV fluids given immediately subsequent to an IV drug must be evaluated for their compatibility with the study drug. Suspensions are generally not given intravenously because of the possibility of blocking the capillaries.

In the IV route, anaphylactic reactions (caused by administration of an agent to an animal previously sensitized to it or to a particularly sensitive species such as a guinea pig) may be especially severe—probably because of sudden, massive antigen–antibody reactions. When the drug is given by other routes, its access to antibody molecules is necessarily slower; moreover, its further absorption can be retarded or prevented at the first sign of a serious allergic reaction.

Embolism is another possible complication of the IV route. Particulate matter may be introduced if a drug intended for IV use precipitates for some reason or if a particular suspension intended for IM or SC use is inadvertently given into a vein. Hemolysis or agglutination of erythrocytes may be caused by injection of hypotonic hypertonic solutions or by more specific mechanisms (Gray, 1978).

Bolus versus Infusion Technically, for all the parenteral routes (but in practice only for the IV route), there are two options for injecting a material into the body. The bolus and infusion methods are differentiated on the single basis of rate of injection, but they actually differ on a wide range of characteristics.

The most commonly exercised option is the bolus, "push," injection, in which the injection device (syringe or catheter) is appropriately entered into the vein and a defined volume of material is introduced through the device. The device is then removed. In this operation, it is relatively easy to restrain an experimental animal and the stress on the animal is limited. Though the person doing the injection must be skilled, it takes only a short amount of time to become so. And the one variable to be controlled in determining dosage is the total volume of material injected (assuming dosing solutions have been properly prepared) (Theeuwes and Yum, 1976). See Chapter 9 for a more complete discussion.

Subcutaneous Route Drugs given by the SC route are forced into spaces between connective tissues, as with IM injections. Vasoconstrictors and drugs that cause local irritation should not be given subcutaneously under usual circumstances, since inflammation, abscess formation, or even tissue necrosis may result. When daily or even more frequent SC injections are made, the site of injection should be continually changed to prevent local complications. Fluids given subcutaneously must have an appropriate tonicity to prevent pain. Care must be taken to prevent injection of the drug directly into veins.

The absorption of drugs from a SC route is influenced by blood flow to the area, as with IM injections. The rate of absorption may be retarded by cooling the local area to cause vasoconstriction, adding epinephrine to the solution for the same purpose (e.g., with local anesthetics), decreasing blood flow with a tourniquet, or immobilizing the area. The opposite effect may be achieved by warming the injection region or by using the enzyme hyaluronidase, which breaks down mucopolysaccharides of the connective tissue matrix to allow the injected solution to spread over a larger area and thus increase its rate of absorption.

Absorption from SC injection sites is affected by the same factors that determine the rate of absorption from IM sites (Schou, 1971). Blood flow through these regions is generally poorer than in muscles, so the absorption rate is generally slower.

The rate of absorption from an SC injection site may be retarded by immobilization of the limb, local cooling to cause vasoconstriction, or application of a tourniquet proximal to the injection site to block the superficial venous drainage and lymphatic flow. In small amounts, adrenergic stimulants, such as epinephrine, will constrict the local blood vessels and therefore slow systemic absorption. Conversely, cholinergic stimulants (such as methacholine) will induce very rapid systemic absorption subcutaneously. Other agents may also alter their own rate of absorption by affecting local blood supply or capillary permeability.

A prime determinant of the absorption rate from an SC injection is the total surface area over which the absorption can occur. Although the SC tissues are somewhat loose and moderate amounts of fluid can be administered, the normal connective tissue matrix prevents indefinite lateral spread

of the injected solution. These barriers may be overcome by agents that break down mucopolysaccharides of the connective tissue matrix; the resulting spread of injected solution leads to a much faster absorption rate.

In addition to fluids, solid forms of drugs may be given by SC injection. This has been done with compressed pellets of testosterone placed under the skin which are absorbed at a relatively constant rate over a long period.

Intramuscular Route The IM route is frequently used for drugs dissolved in oily vehicles or for those in a microcrystalline formulation that are poorly soluble in water (e.g., procaine or penicillin G). Advantages include rapid absorption (often in under 30 min), the opportunity to inject a relatively large amount of solution, and a reduction in pain and local irritation compared with SC injections. Potential complications include infections and nerve damage. The latter usually results from the choice of an incorrect site for injection.

Although the time to peak drug concentration is often on the order of 1–2 h, depot preparations given by IM injection are absorbed extremely slowly. Numerous physiochemical properties of a material given intramuscularly will affect the rate of absorption from the site within the muscle (e.g., ionization of the drug, lipid solubility, osmolality of the solution, volume given). The primary sites used for IM injections in people are the gluteal (buttocks), deltoid (upper arm), and lateral vastus (lateral thigh) muscles, with the corresponding sites in test animals being species specific. The rate of drug absorption and the peak drug levels obtained will often differ between sites used for IM injections because of differences in blood flow between muscle groups. The site chosen for an IM injection in humans and some animals may be a critical factor in whether or not the drug exhibits an effect (Schwartz et al., 1974). Agents injected into the larger muscle masses are generally absorbed rapidly.

Blood flow through muscles in a resting animal is about 0.02–0.07 mL min^{-1} g^{-1} of tissue, and this flow rate may increase many times during exercise, when additional vascular channels open. Large amounts of solution can be introduced intramuscularly, and there is usually less pain and local irritation than is encountered by the SC route. Ordinary aqueous solutions of chemicals are usually absorbed from an IM site within 10–30 min, but faster or slower absorption is possible, depending on the vascularity of the site, the ionization and lipid solubility of the drug, the volume of the injection, the osmolality of the solution, animal temperature, and other variables. Small molecules are absorbed directly into the capillaries from an IM site, whereas large molecules (e.g., proteins) gain access to the circulation by way of the lymphatic channels (Ballard, 1968). Radiolabeled compounds of widely differing molecular weights (maximum 585) and physical properties have been shown to be absorbed from rat muscle at virtually the same rate, about 16% per minute (i.e., the absorption process is limited by the rate of blood flow).

Drugs that are insoluble at tissue pH or that are in an oily vehicle form a depot in the muscle tissue, from which absorption proceeds very slowly.

Intraperitoneal Route Kruger et al. (1962) demonstrated the efficiency of absorption of some chemicals injected intraperitoneally, while Lukas et al. (1971) showed that compounds administered intraperitoneally are absorbed primarily through the portal circulation.

A prime practical consideration in the use of the IP route for acute testing should be the utilization of aseptic techniques to preclude bacterial or viral contamination. If these are not exercised, the resulting infected and compromised animals cannot be expected to produce either valid or reproducible indications or actual chemical toxicity.

Compounds that are very lipophilic will be quickly absorbed systemically by the IP route but not by the IM or SC route.

5.2.3 Oral Route

The oral route is the most commonly used route for the administration of drugs both because of ease of administration and because it is the most readily accepted route of administration. Although the dermal route may be as common for occupational exposure, it is much easier to accurately measure and administer doses by the oral route.

Enteral routes technically include any that will put a material directly into the GI tract, but the use of enteral routes other than oral (such as rectal) is rare in toxicology. Though there are a number of variations of technique and peculiarities of animal response that are specific to different animal species, there is also a great deal of commonality across species in methods, considerations, and mechanisms.

Mechanisms of Absorption Ingestion is generally referred to as oral or peroral (PO) exposure and includes direct intragastric exposure in experimental toxicology. The regions for possible agent action and absorption from PO absorption should, however, be considered separately (Hogben et al., 1959; Bates and Gibaldi, 1970; Gad 2007, 2008).

Because of the rich blood supply to the mucous membranes of the mouth (buccal cavity), many compounds can be absorbed through them. Absorption from the buccal cavity is limited to nonionized, lipid-soluble compounds. Buccal absorption of a wide range of aromatic and aliphatic acids and basic drugs in human subjects has been found to be parabolically dependent on log P, where P is the octanol–water partition coefficient. The ideal lipophilic character (log P_0) for maximum buccal absorption has also been shown to be in the range 4.2–5.5 (Schranker et al., 1957; Lien et al., 1971). Compounds with large molecular weights are poorly absorbed in the buccal cavity, and, since absorption increases linearly with concentration and there is generally no difference between optical enantiomorphs of several compounds known to be absorbed from the mouth, it is believed that uptake is by passive diffusion rather than by active transport chemical moieties.

A knowledge of the buccal absorption characteristics of a chemical can be important in a case of accidental poisoning. Although an agent taken into the

mouth will be voided immediately on being found objectionable, it is possible that significant absorption can occur before any material is swallowed.

Unless voided, most materials in the buccal cavity are swallowed. No significant absorption occurs in the esophagus and the agent passes on to enter the stomach. It is common practice in safety assessment studies to avoid the possibility of buccal absorption by intubation (gavage) or by the administration of the agent in gelatin capsules designed to disintegrate in the gastric fluid.

Absorption of chemicals with widely differing characteristics can occur at different levels in the GI tract (Schranker, 1960). The two factors primarily influencing this regional absorption are (1) the lipid–water partition characteristics of the undissociated toxicant and (2) the dissociation constant pK_a, which determines the amount of toxicant in the dissociated form.

Therefore, weak organic acids and bases are readily absorbed as uncharged lipid-soluble molecules, whereas ionized compounds are absorbed only with difficulty, and nonionized toxicants with poor lipid solubility characteristics are absorbed slowly. Lipid-soluble acid molecules can be absorbed efficiently through the gastric mucosa, but bases are not absorbed in the stomach.

In the intestines the nonionized form of the drug is preferentially absorbed and the rate of absorption is related to the lipid–water partition coefficient of the toxicant. The highest pK_a value for a base compatible with efficient gastric absorption is about 7.8 and the lowest pK_a for an acid is about 3.0, although a limited amount of absorption can occur outside these ranges (Share et al., 1971). The gastric absorption and the intestinal absorption of a series of compounds with different carbon chain lengths follow two different patterns. Absorption from the stomach increases as the chain lengthens from methyl to *n*-hexyl, whereas intestinal absorption increases over the range methyl to *n*-butyl and then diminishes as the chain length further increases. Houston et al. (1974) concluded that to explain the logic of optimal partition coefficients for intestinal absorption it was necessary to postulate a two-compartment model with a hydrophilic barrier and a lipoidal membrane and that if there is an acceptable optimal partition coefficient for gastric absorption it must be at least 10 times greater than the corresponding intestinal value.

Because they are crucial to the course of an organism's response, the rate and extent of absorption of biologically active agents from the GI tract also have major implications for the formulation of test material dosages and also for how production (commercial) materials may be formulated to minimize potential accidental intoxications while maximizing the therapeutic profile.

There are a number of separate mechanisms involved in absorption from the GI tract, and these will be discussed below.

Passive Absorption The membrane lining of the tract has a passive role in absorption. As toxicant molecules move from the bulk water phase of the intestinal contents into the epithelial cells, they must pass through two membranes in series, one the layer of water and the other the lipid membrane of the microvillar surface (Wilson and Dietschy, 1974). The water layer may be

the rate limiting factor for passive absorption into the intestinal mucosa, but it is not rate limiting for active absorption. The concentration gradient and the physiochemical properties of the drug and the lining membrane are the controlling factors. Chemicals that are highly lipid soluble are capable of passive diffusion, and they pass readily from the aqueous fluids of the gut lumen through the lipid barrier of the intestinal wall and into the bloodstream. The interference in the absorption process by the water layer increases with increasing absorbability of the substances in the intestine (Winne, 1978).

Aliphatic carbamates are rapidly absorbed from the colon by passive uptake (Wood et al., 1978), and it is found that there is a linear relationship between log k_a and log P for absorption of these carbamates in the colon and the stomach, whereas there is a parabolic relationship between these two values for absorption in the small intestine. The factors to be considered are

Octanol–buffer partition coefficient P
Absorption rate constant k_a
Time t
Half-life $t^{1/2} = \ln 2/k_a$

Organic acids that are extensively ionized at intestinal pH's are absorbed primarily by simple diffusion.

Facilitated Diffusion Temporary combination of the chemical with some form of "carrier" occurs in the gut wall, facilitating the transfer of the toxicant across the membranes. This process is also dependent on the concentration gradient across the membrane, and there is no energy utilization in making the translocation. In some intoxications, the carrier may become saturated, making this the rate-limiting step in the absorption process.

Active Transport As above, the process depends on a carrier but differs in that the carrier provides energy for translocation from regions of lower concentration to regions of higher concentration.

Pinocytosis This process, by which particles are absorbed, can be an important factor in the ingestion of particulate formulations of chemicals (e.g., dust formulations, suspensions of wettable powders); however, it must not be confused with absorption by one of the above processes, where the agent has been released from particles.

Absorption via Lymphatic Channels Some lipophilic chemicals dissolved in lipids may be absorbed through the lymphatics.

Convective Absorption Compounds with molecular radii of less than 4 nm can pass through pores in the gut membrane. The membrane exhibits a molecular sieving effect.

Characteristically, within certain concentration limits, if a chemical is absorbed by passive diffusion, then the concentration of toxicant in the gut and the rate of absorption are linearly related. However, if absorption is mediated by active transport, the relationship between concentration and rate of absorption conforms to Michaelis–Menten kinetics and a Lineweaver–Burk plot (i.e., reciprocal of rate of absorption plotted against reciprocal of concentration), which graphs as a straight line.

Differences in the physiological chemistry of GI fluids can have a significant effect on toxicity. Both physical and chemical differences in the GI tract can lead to species differences in susceptibility to acute intoxication. The antihelminthic pyrvinium chloride has an identical median lethal dose (LD_{50}) value when administered intraperitoneally to rats and mice (approximately 4 mg kg^{-1}); when administered orally, however, the LD_{50} value in mice was found to be 15 mg kg^{-1}, while for the rat, the LD_{50} values were 430 mg kg^{-1} for females and 1550 mg kg^{-1} for males. It is thought that this is an absorption difference rather than a metabolic difference (Ritschel et al., 1974).

Most exogenous chemical absorbed from the GI tract must pass through the liver via the hepatic–portal system (leading to the so-called first-pass effect) and, as mixing of the venous blood with arterial blood from the liver occurs, consideration and caution are called for in estimating the amounts of chemical in both the systemic circulation and the liver itself.

Despite the GI absorption characteristics discussed above, it is common for absorption from the alimentary tract to be facilitated by dilution of the toxicant. Borowitz et al. (1971) have suggested that the concentration effects they observed in atropine sulfate, aminopyrine, sodium salicylate, and sodium pentoparbital were due to a combination of rapid stomach emptying and the large surface area for absorption of the drugs.

Major structural or physiological differences in the alimentary tract (e.g., species differences or surgical effects) can give rise to modifications of toxicity. For example, ruminant animals may metabolize toxicants in the GI tract in a way that is unlikely to occur in nonruminants.

The presence of bile salts in the alimentary tract can affect absorption of potential toxicants in a variety of ways, depending on their solubility characteristics.

Factors Affecting Absorption Test chemicals are given most commonly by mouth. This is certainly the most convenient route, and it is the only one of practical importance for self-administration. Absorption, in general, takes place along the whole length of the GI tract, but the chemical properties of each molecule determine whether it will be absorbed in the strongly acidic stomach or in the nearly neutral intestine. Gastric absorption is favored by an empty stomach, in which the chemical, in undiluted gastric juice, will have good access to the mucosal wall. Only when a chemical would be irritating to the gastric mucosa is it rational to administer it with or after a meal. However, the antibiotic griseofulvin is an example of a substance with poor water solubility

the absorption of which is aided by a fatty meal. The large surface area of the intestinal villi, the presence of bile, and the rich blood supply all favor intestinal absorption of griseofulvin and physiochemically similar compounds.

The presence of food can impair the absorption of chemicals given by mouth. Suggested mechanisms include reduced mixing, complexing with substances in the food, and retarded gastric emptying. In experiments with rats, prolonged fasting has been shown to diminish the absorption of several chemicals, possibly by deleterious effects upon the epithelium of intestinal villi.

Chemicals that are metabolized rapidly by the liver cannot be given for systemic effect by the enteral route because the portal circulation carries them directly to the liver. For example, lidocaine, a drug of value in controlling cardiac arrhythmias, is absorbed well from the gut but is completely inactivated in a single passage through the liver.

The principles governing the absorption of drugs from the GI lumen are the same as for the passage of drugs across biological membranes elsewhere. Lower degree of ionization, high lipid–water partition coefficient of nonionized form, and small atomic or molecular radii of water-soluble substances all favor rapid absorption. Water passes readily in both directions across the wall of the GI lumen. Sodium ion is probably transported actively from lumen into blood. Magnesium ion is very poorly absorbed and therefore acts as a cathartic, retaining an osmotic equivalent of water as it passes down the intestinal tract. Ionic iron is absorbed as an amino acid complex at a rate usually determined by the body's need for it. Glucose and amino acids are transported across the intestinal wall by specific carrier systems. Some compounds of high molecular weight (polysaccharides and large proteins) cannot be absorbed until they are degraded enzymatically. Other substances cannot be absorbed because they are destroyed by GI enzymes—insulin, epinephrine, and histamine are examples. Substances that form insoluble precipitates in the GI lumen or that are insoluble either in water or in lipid clearly cannot be absorbed.

Absorption of Weak Acids and Bases Human gastric juice is very acid (about pH 1), whereas the intestinal contents are nearly neutral (actually very slightly acid). The pH difference between plasma (pH 7.4) and the lumen of the GI tract plays a major role in determining whether a drug that is a weak electrolyte will be absorbed into plasma or excreted from plasma into the stomach or intestine. For practical purposes, the mucosal lining of the GI tract is impermeable to the ionized form of a weak acid or base, but the nonionized form equilibrates freely. The rate of equilibration of the nonionized molecule is directly related to its lipid solubility. If there is a pH difference across the membrane, then the fraction ionized may be considerably greater on one side than on the other. At equilibrium, the concentration of the nonionized moiety will be the same on both sides, but there will be more total drug on the side where the degree of ionization is greater. This mechanism is known as *ion trapping*. The energy for sustaining the unequal chemical potential of the acid

or base in question is derived from whatever mechanism maintains the pH difference. In the stomach, this mechanism is the energy-dependent secretion of hydrogen ions.

Consider how a weak electrolyte is distributed across the gastric mucosa between plasma (pH 7.4) and gastric fluid (pH 1.0). In each compartment, the Henderson–Hasselbalch equation gives the ratio of acid–base concentrations. The negative logarithm of the acid dissociation constant is designated here by the symbol pK_a rather than the more precisely correct pK^1:

$$\mathrm{pH} = \mathrm{p}K_a + \log\left(\frac{\text{base}}{\text{acid}}\right)$$

$$\log\left(\frac{\text{base}}{\text{acid}}\right) = \mathrm{pH} - \mathrm{p}K_a$$

$$\frac{\text{Base}}{\text{Acid}} = \text{antilog}(\mathrm{pH} - \mathrm{p}K_a)$$

The implications of the above equations are clear. Weak acids are readily absorbed from the stomach. Weak bases are not absorbed well; indeed, they would tend to accumulate within the stomach at the expense of agent in the bloodstream. Naturally, in the more alkaline intestine, bases would be absorbed better, acids more poorly.

It should be realized that although the principles outlines here are correct, the system is dynamic, not static. Molecules that are absorbed across the gastric or intestinal mucosa are removed constantly by blood flow; thus, simple reversible equilibrium across the membrane does not occur until the agent is distributed throughout the body.

Absorption from the stomach, as determined by direct measurements, conforms, in general, to the principles outlined above. Organic acids (as indeed many of the drug molecules) are absorbed well since they are all almost completely nonionized at the gastric pH; indeed, many of these substances are absorbed faster than ethyl alcohol, which had long been considered one of the few compounds that were absorbed well from the stomach. Strong acids whose pK_a values lie below 1, which are ionized even in the acid contents of the stomach, are not absorbed well. Weak bases are absorbed only negligibly, but their absorption can be increased by raising the pH of the gastric fluid.

As for bases, only the weakest are absorbed to any appreciable extent at normal gastric pH, but their absorption can be increased substantially by neutralizing the stomach contents. The quaternary cations, however, which are charged at all pH values, are not absorbed at either pH.

The accumulation of weak bases in the stomach by ion trapping mimics a secretory process; if the drug is administered systemically, it accumulates in the stomach. Dogs given various drugs intravenously by continuous infusion to maintain a constant drug level in the plasma had the gastric contents

sampled by means of an indwelling catheter. The results showed that stronger bases ($pK_a > 5$) accumulated in stomach contents to many times their plasma concentrations; the weak bases appeared in about equal concentrations in gastric juice and in plasma. Among the acids, only the weakest appeared in detectable amounts in the stomach. One might wonder why the strong bases, which are completely ionized in gastric juice and whose theoretical concentration ratios (gastric juice/plasma) are very large, should nevertheless attain only about a 40-fold excess over plasma. Direct measurements of arterial and venous blood show that essentially all the blood flowing through the gastric mucosa is cleared of these agents; obviously, no more chemical can enter the gastric juice in a given time period than is brought there by circulation. Another limitation comes into play when the base pK_a exceeds 7.4; now a major fraction of the circulating base is cationic and a decreasing fraction is nonionized, so the effective concentration gradient for diffusion across the stomach wall is reduced.

The ion-trapping mechanism provides a method of some forensic value for detecting the presence of alkaloids (e.g., narcotics, cocaine, amphetamines) in cases of death suspected to be due to overdosage of self-administered drugs. Drug concentrations in gastric contents may be very high even after parenteral injection.

Absorption from the intestine has been studied by perfusing drug solutions slowly through rat intestine in situ and by varying the pH as desired. The relationships that emerge from such studies are the same as those for the stomach, the difference being that the intestinal pH is normally very near neutrality. As the pH is increased, the bases are absorbed better, the acids more poorly. Detailed studies with a great many drugs in unbuffered solutions revealed that in the normal intestine acids with $pK_a > 3.0$ and bases with $pK_a < 7.8$ are very well absorbed; outside these limits the absorption of acids and bases falls off rapidly. This behavior leads to the conclusion that the "virtual pH" in the microenvironment of the absorbing surface in the gut is about 5.3; this is somewhat more acidic than the pH in the intestinal lumen is usually considered to be.

Absorption from the buccal cavity has been shown to follow exactly the same principles as those described for absorption from the stomach and intestine. The pH of human and canine saliva is usually about 6. Bases in people are absorbed only on the alkaline side of their pK_a, that is, only in the nonionized form. At normal saliva pH, only weak bases are absorbed to a significant extent.

Bioavailability and Thresholds The difference between the extent of availability (often designated solely as bioavailability) and the rate of availability is illustrated in Figure 5.3, which depicts the concentration–time curve for a hypothetical agent formulated into three different dosage forms. Dosage forms A and B are designed so that the agent is put into the blood circulation at the same rate but twice as fast as for dosage form C. The times at which

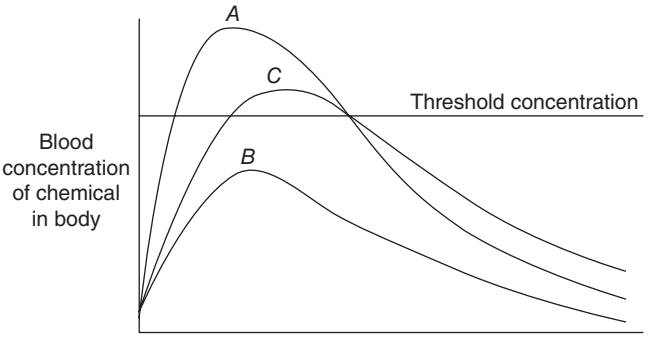

Figure 5.3 *Blood concentration–time curves illustrating how changes in rate and extent of chemical availability can influence duration of action and efficacy of dose of agent. The designated line indicates the threshold concentration (T_c) of the agent in the body that will evoke a response. Case* A *is absorbed rapidly and completely. This product produces a prompt and prolonged response. The agent in case* B *is absorbed at the same rate as that in case* A *but is only 50% as available. There will be no response from this dose of the agent because* T_c *is not reached. The agent in case* C *is absorbed at one-half the rate seen in cases* A *and* B *but is 100% available.*

agent concentrations reach a peak are identical for dosage forms A and B and occur earlier than the peak time for dosage form C. In general, the relative order of peak times following the administration of different dosage forms of the drug corresponds to the rates of availability of the chemical moiety from the various dosage forms. The extent of availability can be measured by using either chemical concentrations in the plasma or blood or amounts of unchanged chemical in the urine. The area under the blood concentration–time curve for an agent can serve as a measure of the extent of its availability. In Figure 5.3, the areas under curves *A* and *C* are identical and twice as great as the area under curve *B*. In most cases, where clearance is constant, the relative areas under the curves or the amount of unchanged chemical excreted in the urine will quantitatively describe the relative availability of the agent from the different dosage forms. However, even in nonlinear cases, where clearance is dose dependent, the relative areas under the curves will yield a measurement of the rank order of availability from different dosage forms or from different routes of administration.

Because there is usually a critical concentration of a chemical in the blood that is necessary to elicit either a pharmacological or toxic effect, both the rate and extent of input or availability can alter the toxicity of a compound. In the majority of cases, the duration of effects will be a function of the length of time the blood concentration curve is above the threshold concentration; the intensity of the effect for many agents will be a function of the elevation of the blood concentration curve above the threshold concentration.

Thus, the three different dosage forms depicted in Figure 5.3 will exhibit significant differences in their levels of "toxicity." Dosage form B requires that twice the dose be administered to attain blood levels equivalent to those for

dosage form A. Differences in the rate of availability are particularly important for agents given acutely. Dosage for A reaches the target concentration earlier than chemical from dosage form C; concentrations from A reach a higher level and remain above the minimum effect concentration for a longer period of time. In a multiple-dosing regimen, dosage forms A and C will yield the same average blood concentrations, although dosage form A will show somewhat greater maximum and lower minimum concentrations.

For most chemicals, the rate of disposition or loss from the biological system is independent of rate of input once the agent is absorbed. Disposition is defined as what happens to the active molecule after it reaches a site in the blood circulation where concentration measurements can be made (the systemic circulations, generally). Although disposition processes may be independent of input, the inverse is not necessarily true, because disposition can markedly affect the extent of availability. Agents absorbed from the stomach and the intestine must first pass through the liver before reaching the general circulation (Figure 5.4). Thus, if a compound is metabolized in the liver or excreted in bile, some of the active molecule absorbed from the GI tract will be inactivated by hepatic processes before it can reach the systemic circulation and be distributed to its sites of action. If the metabolizing or biliary excreting capacity of the liver is great, the effect on the extent of availability will be substantial. Thus, if the hepatic blood clearance for the chemical is large relative to hepatic blood flow, the extent of availability for this chemical will be low when it is given by a route that yields first-pass metabolic effects. This

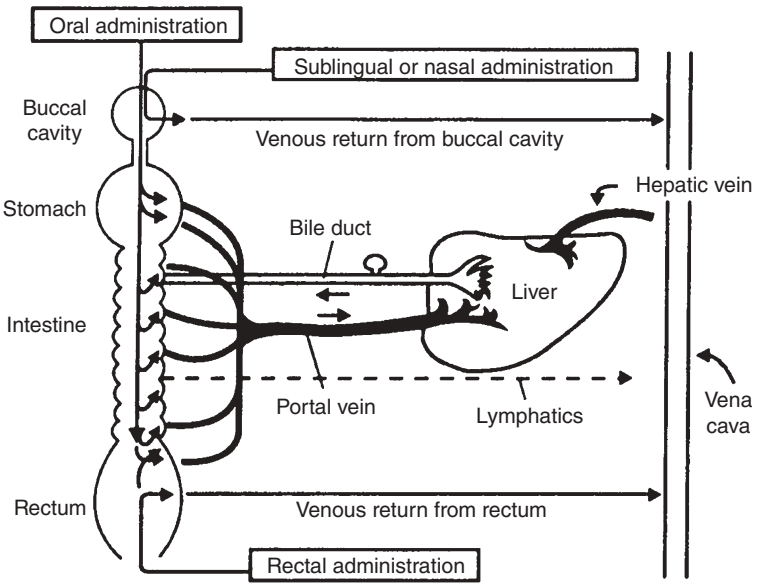

Figure 5.4 *Diagrammatic representation of entry of drug moieties into body from variety of routes, with subsequent passage into bloodstream and out of body.*

decrease in availability is a function of the physiological site from which absorption takes place, and no amount of modification to dosage form can improve the availability under linear conditions. Of course, toxic blood levels can be reached by this route of administration if larger doses are given.

It is important to realize that chemicals with high extraction ratios (i.e., greater extents of removal by the liver during first-pass metabolism) will exhibit marked intersubject variability in bioavailability because of variations in hepatic function or blood flow or both. For the chemical with an extraction ratio of 0.90 that increases to 0.95, the bioavailability of the agent will be halved, from 0.10 to 0.05. These relationships can explain the marked variability in plasma or blood drug concentrations that occurs among individual animals given similar doses of a chemical that is markedly extracted. Small variations in hepatic extraction between individual animals will result in large differences in availability and plasma drug concentrations.

The first-pass effect can be avoided, to a great extent, by use of the sublingual route and by topical preparations (e.g., nitroglycerine ointment), and it can be partially avoided by using rectal suppositories. The capillaries in the lower and middle sections of the rectum drain into the interior and middle hemorrhoidal veins, which in turn drain into the inferior vena cava, thus bypassing the liver. However, suppositories tend to move upward in the rectum into a region where veins that lead to the liver predominate, such as the superior hemorrhoidal vein. In addition, there are extensive connections between the superior and middle hemorrhoidal veins, and thus probably only about 50% of a rectal dose can be assumed to bypass the liver. The lungs represent a good temporary clearing site for a number of chemical (especially basic) compounds by partition into lipid tissues as well as serve a filtering function for particulate matter that may be given by IV injection. In essence, the lung may cause first-pass loss by excretion and possible metabolism for chemicals input into the body by the non-GI routes of administration.

Biological (test subject) factors that can influence absorption of a chemical from the GI tract are summarized in Table 5.4.

There are also a number of chemical factors that may influence absorption from the GI tract. These are summarized in Table 5.5.

Techniques of Oral Absorption There are three major techniques for oral delivery of drugs to test animals. The most common way is by gavage, which requires that the material be in a solution or suspension for delivery by tube to the stomach. Less common materials may be given as capsules (particularly to dogs) or in diet (for longer term studies). Rarely, oral studies may also be done by inclusion of materials in drinking water.

Test materials may be administered as solutions or suspensions as long as they are homogeneous and delivery is accurate. For traditional oral administration (gavage), the solution or suspension can be administered with a suitable stomach tube or feeding needle (“Popper” tube) attached to a syringe. If the dose is too large to be administered at one time, it can be divided into

TABLE 5.4 Test Subject Characteristics That Can Influence GI Tract Absorption[a]

A. General and inherent characteristics
   1. General condition of subject (e.g., starved versus well fed, ambulatory versus supine)
   2. Presence of concurrent diseases (i.e., diseases may either speed or slow gastric emptying)
   3. Age
   4. Weight and degree of obesity
B. Physiological function
   1. Status of subject's renal function
   2. Status of subject's hepatic function
   3. Status of subject's cardiovascular system
   4. Status of subject's GI motility and function (e.g., ability to swallow)
   5. pH of gastric fluid (e.g., affected by fasting, disease, food intake, drugs)
   6. Gastrointestinal blood flow to area of absorption
   7. Blood flow to areas of absorption for dose forms other than those absorbed through GI routes
C. Acquired characteristics
   1. Status of subject's anatomy (e.g., previous surgery)
   2. Status of subject's GI flora
   3. Timing of drug administration relative to meals (i.e., presence of food in GI tract)
   4. Body position of subject (e.g., lying on side slows gastric emptying)
   5. Psychological state of subject (e.g., stress increases gastric emptying rate and depression decreases rate)
   6. Physical exercise of subject may reduce gastric-emptying rate
D. Physiological principles
   1. Food enhances gastric blood flow, which should theoretically increase rate of absorption
   2. Food slows the rate of gastric emptying, which should theoretically slow the rate of passage to the intestines where the largest amounts of most agents are absorbed. This should decrease the rate of absorption for most agents. Agents absorbed to a larger extent in the stomach will have increased time for absorption in the presence of food and should be absorbed more completely than in fasted patients
   3. Bile flow and secretion are stimulated by fats and certain other foods. Bile salts may enhance or delay absorption depending on whether they form insoluble complexes with drugs or enhance the solubility of agents
   4. Changes in splanchnic blood flow as a result of food depend on direction and magnitude of the type of food ingested
   5. Presence of active (saturable transport mechanisms places a limit on the amount of a chemical that may be absorbed

[a]The minimization of variability due to these factors rests on the selection of an appropriate animal model, careful selection of healthy animals, and use of proper techniques.

equal subparts with 2–4 h between each administration; however, this subdivided dosing approach should generally be avoided.

Test chemicals placed into any natural orifice exert local effects and, in many instances, systemic effects as well. The possibility of systemic effects occurring when local effects are to be evaluated should be considered.

For routes of administration in which the chemical is given orally or placed into an orifice other than the mouth, clear instructions about the correct administration of the chemical must be provided. Many cases are known of oral pediatric drops for ear infections being placed into the ear, and vice versa (ear drops being swallowed) in humans. Errors in test article administration

TABLE 5.5 Chemical Characteristics of Drug That May Influence Absorption

A. Administration of chemical and its passage through the body
   1. Dissolution characteristics of solid dosage forms, which depend on formulation in addition to the properties of the chemical itself (e.g., vehicle may decrease permeability of suspension or capsule to water and retard dissolution and diffusion).
   2. Rate of dissolution in GI fluids. Chemicals that are inadequately dissolved in gastric contents may be inadequately absorbed.
   3. Chemicals that are absorbed into food may have a delayed absorption.
   4. Carrier-transported chemicals are more likely to be absorbed in the small intestine.
   5. Route of administration.
   6. Chemicals undergo metabolism in the GI tract.

B. Physiochemical properties of chemicals
   1. Chemicals that chelate metal ions in food may form insoluble complexes and will not be adequately absorbed.
   2. pH of dosing solutions—weakly basic solutions are absorbed to a greater degree in the small intestine.
   3. Salts used.
   4. Hydrates or solvates.
   5. Crystal form of chemical (e.g., insulin).
   6. "Pharmaceutical" form (e.g., fluid, solid, suspension).
   7. Enteric coating.
   8. Absorption of quaternary compounds (e.g., hexamethonium, amiloride) is decreased by food.
   9. Molecular weight of chemical (e.g., when the molecular weight of a drug is above about 1000, absorption is markedly decreased).
   10. pK_a (dissociation constant).
   11. Lipid solubility (i.e., a hydrophobic property relating to penetration through membranes).
   12. Particle size of chemical in solid dosage form—smaller particle sizes will increase the rate and/or degree of absorption if dissolution of the chemical is the rate-limiting factor in absorption. Chemicals that have a low dissolution rate may be made in a micronized form to increase their rate of dissolution.
   13. Particle size of the dispersed phase in an emulsion.
   14. Type of disintegrating agent in the formulation.
   15. Hardness of a solid (granule, pellet, or tablet) (i.e. related to amount of compression used to make tablet) or capsule if they do not disintegrate appropriately.

are especially prevalent when a chemical form is being used in a nontraditional manner (e.g., suppositories that are given by the buccal route).

Administration of a drug in capsules is a common means of dosing larger test animals (particularly dogs). It is labor intensive (each capsule must be individually prepared, though robotic systems are now available for this), but capsules offer the advantages that neat drug may be used (no special formulation need be prepared, and the questions of formulation or solution stability are avoided), the dogs are less likely to vomit, and the actual act of dosing requires less labor than using a gavage tube. Capsules may also be used with primates, though they are not administered as easily.

Incorporation of a drug in the diet is commonly used for longer term studies (particularly carcinogenicity studies, though the method is not limited to these). Dosing by diet is much less labor intensive than any other oral dosing

methodology, which is particularly attractive over the course of a long (13-week, 1-year, 18-month, or 2-year) study.

The most critical factor to dietary studies is the proper preparation of the test chemical–diet admixtures. The range of physical and chemical characteristics of test materials requires that appropriate mixing techniques be determined on an individual basis. Standard practices generally dictate the preparation of a premix to which is added appropriate amounts of feed to achieve the proper concentrations.

Dietary preparation involving liquid materials frequently results in either wet feed in which the test article does not disperse or formation of "gumballs"—feed and test material that form discernible lumps and chemical "hotspots." Drying and grinding of the premix to a free-flowing form prior to mixing the final diets may be required; however, these actions can affect the chemical nature of the test article.

Solid materials require special techniques prior to or during addition to diets. Materials that are soluble in water may be dissolved and added as described above for liquids. Non-water-soluble materials may require several preparatory steps. The test chemical may be dissolved in corn oil, acetone, or other appropriate vehicle prior to addition to the weighed diet. When an organic solvent such as acetone is used, the mixing time for the premix should be sufficient for the solvent to evaporate. Some solids may require grinding in a mortar and pestle with feed added during the grinding process.

Prior to study initiation, stability of the test chemical in the diet must be determined over a test period at least equivalent to the time period during which animals are to be exposed to a specific diet mix. Stability of test samples under the conditions of the proposed study is preferable. Labor and expense can be saved when long-term stability data permit mixing of several weeks (or a month) of test diet in a single mixing interval.

Homogeneity and concentration analysis of the test article–diet admixture are performed by sampling at three or four regions within the freshly mixed diet (e.g., samples from the top, middle, and bottom of the mixing bowl or blender).

A variety of feeders are commercially available for rats and mice. These include various-sized glass jars and stainless steel or galvanized feed cups, which can be equipped with restraining lids and food followers to preclude significant losses of feed due to animals digging in the feeders. Slotted metal feeders are designed so that animals cannot climb into the feed, and they also contain mesh food followers to prevent digging.

Another problem sometimes encountered is palatability—the material may taste so strongly that animals will not eat it. As a result, palatability, stability in diet, and homogeneity of mix must all be ensured prior to the initiation of an actual study.

Inclusion in drinking water is rarely used for oral administration of human drugs to test animals, though it sees more frequent use for the study of environmental agents.

Physiochemical properties of the test material should be a major consideration in selecting drinking water as a dosing matrix. Unlike diet preparation or preparation of gavage dose solutions and suspensions where a variety of solvents and physical processes can be utilized to prepare a dosable form, preparations of drinking water solutions are less flexible. Water solubility of the test chemical is the major governing factor and is dependent on factors such as pH, dissolved salts, and temperature. The animal model itself sets limitations for these factors (acceptability and suitability of pH and salt-adjusted water by the animals as well as animal environmental specifications such as room temperature).

Stability of the test chemical in drinking water under study conditions should be determined prior to study initiation. Consideration should be given to conducting stability tests on test chemical–drinking water admixtures presented to some test animals. Besides difficulties of inherent stability, changes in chemical concentrations may result from other influences. Chemicals with low vapor pressure can volatilize from the water into the air space located above the water of an inverted water bottle; thus, a majority of the chemical may be found in the "dead space," not in the water.

Certain test chemicals may be degraded by contamination with microorganisms. A primary source of these microorganisms is the oral cavity of rodents. Although rats and mice are not as notorious as the guinea pig in spitting back into water bottles, significant bacteria can pass via the sipper tubes and water flow restraints into the water bottles. Sanitation and sterilization procedures for water bottles and sipper tubes must be carefully attended to.

Many technicians may not be familiar with terms such as sublingual (under the tongue), buccal (between the cheek and gingiva), otic, and so on. A clear description of each of these nontraditional routes (i.e., other than gavage routes) should be discussed with technicians, and instructions may also be written down and given to them. Demonstrations are often useful to illustrate selected techniques of administration (e.g., to use an inhaler or nebulizer). Some chemicals must be placed by technicians into body orifices (e.g., medicated intrauterine devices such as Progesterset).

5.2.4 Minor Routes

The minor routes see some use in safety assessment and four are briefly presented here.

Perocular Route The administration of drugs or accidental exposure of chemicals to the eyes is not commonly a concern in systemic toxicity due to the small surface area exposed and the efficiency of the protective mechanisms (i.e., blink reflex and tears). As long as the epithelium of the eyes remains intact, it is impermeable to many molecules, but, if the toxicant has a suitable polar–nonpolar balance, penetration may occur (Kondrizer et al., 1959; Swan and White, 1972).

Holmstedt (1959) and Brown and Muir (1971) have reviewed perocular absorption of pesticides. More recently, Sinow and Wei (1973) have shown that the quarternary herbicide paraquat can be lethal to rabbits if applied directly to the surface of the eyes. Parathion, in particular, is exceedingly toxic when administered via the eye—a concern that must be kept in mind for the protection of pesticide applicators.

Rectal Administration Since a number of therapeutic compounds are given in the form of suppositories, an indication of the toxicity after rectal administration is sometimes required. Toxicity studies and initial drug formulations of such compounds are usually performed by the oral route and the rectal formulation comes late in development and marketing. In view of the difference between laboratory animals and humans in the anatomy and microflora of the colon and rectum, animal toxicity studies late in drug development are of limited value. However, in cases where an indication of potential rectal hazard or bioavailability is required, the compound may be introduced into the rectum of the rat using an oral dosing needle to prevent tissue damage. To avoid the rapid excretion of the unabsorbed dose, anesthetized animals should be used and the dose retained with an inert plug or bung (such as a cork).

Drugs (and therefore test chemicals) are occasionally administered by rectum, but most are not as well absorbed here as they are from the upper intestine. Aminophylline, used in suppository form for the management of asthma, is one of the few drugs routinely given in this way. Inert vehicles employed for suppository preparations include cocoa butter, glycerinated vehicles, gelatin, and polyethylene glycol. Because the rectal mucosa is irritated by nonisotonic solutions, fluids administered by this route should always be isotonic with plasma (e.g., 0.9% NaCl).

Vaginal Administration Though not a common one, some materials do have routine exposure by this route (e.g., spermicides, tampons, douches, and antibiotics) and therefore must be evaluated for irritation and toxicity. The older preferred models used rabbits and monkeys (Eckstein et al., 1969), but more recently a model that uses rats has been developed (Staab et al., 1987). McConnell (1973) clearly described the limitations, particularly of volume of test material, involved in such tests.

Nasal Administration A route that has gained increasing popularity of late for pharmaceutical administration in humans is the intranasal route. The reasons for this popularity are the ease of use (and therefore ready patient acceptance and high compliance rate), the high degree and rate of absorption of many substances (reportedly for most substances up to 1000 molecular weight; McMartin et al., 1987), and avoidance of the highly acid environment in the stomach and first-pass metabolism in the liver (particularly important for some of the newer peptide moieties) (Attman and Dittmer, 1971). The only special safety concerns are the potential for irritation of the mucous

TABLE 5.6 General Guidelines for Maximum Dose Volumes by Route

Route	Volume ($mL\,kg^{-1}$) Should Not Exceed	Notes
Oral	20	Fasted animals
Dermal	2	Limit is accuracy of dosing per available body surface
Intravenous	1	Over 5 min
Intramuscular	0.5	At one site
Perocular	0.01 mL	
Rectal	0.5	
Vaginal	0.2 mL in rat 1 mL in rabbit	
Inhalation	$2\,mg\,L^{-1}$	
Nasal	0.1 mL per nostril in monkey or dog	

Source: Baker et al., 1979; Garramone, 1986.

membrane and the rapid distribution of administered materials to the central nervous system (CNS).

A number of means may be used to administer materials nasally—nebulizers and aerosol pumps being the most attractive first choices. Accurate dose administration requires careful planning, evaluation of the administration device, and attention to technique.

Volume Limitations by Route In the strictest sense, absolute limitations on how much of a dosage form may be administered by any particular route are determined by specific aspects of the test species or dosage form. But there are some general guidelines (determined by issues of humane treatment of animals, accurate deliver of dose, and such) that can be put forth. These are summarized in Table 5.6. The chapter Appendix and Section 5.3 should, of course, be checked to see if there is specific guidance due to the characteristics of a particular vehicle.

5.2.5 Route Comparisons and Contrasts

The first part of this chapter described, compared, and contrasted the various routes used in toxicology and presented guidelines for their use. There are, however, some exceptions to the general rules that the practicing toxicologist should keep in mind.

The relative ranking of efficacy of routes that was presented earlier in the chapter is not absolute; there can be striking exceptions. For example, though materials are usually much quicker acting and more potent when given by the oral route than by the dermal one, this is not always the case. In the literature, Shaffer and West (1960) reported that tetram as an aqueous solution was more toxic when applied dermally than when given orally to rats. The LD_{50} values reported were as follows [LD_{50} ($mg\,kg^{-1}$) of tetram; 95% confidence limits]:

Rat	Oral ($mg\,kg^{-1}$)	Percutaneous ($mg\,kg^{-1}$)
Male	9 (7–13)	2 (1–3)
Female	8 (6–11)	2 (1–3)

The author has in the past experienced this same phenomenon. Several materials that were found to be relatively nontoxic orally were extremely potent by the dermal route (differences in potency of more than an order of magnitude have been seen at least twice).

A general rule applicable to routes and vehicles should be presented here:

Vehicles can mask the effects of active ingredients. Particularly for clinical signs, attention should be paid to the fact that a number of vehicles (e.g., propylene glycol) cause transient neurobehavioral effects that may mask similar short-lived (though not necessarily equally transient and reversible) effects of test materials.

5.3 FORMULATION OF TEST MATERIALS

One of the areas that is overlooked by virtually everyone in toxicology testing and research yet is of crucial importance is the use of vehicles in the formulation of test chemicals for administration to test animals. For a number of reasons, a drug of interest is rarely administered or applied as is ("neat"). Rather, it must be put in a form that can be accurately given to animals in such a way that it will be absorbed and not be too irritating. Most laboratory toxicologists come to understand vehicles and formulation, but to the knowledge of the author, guidance on the subject is limited to a short chapter on formulations by Fitzgerald et al. (1983). There is also a very helpful text on veterinary dosage forms by Blodinger (1982).

Regulatory toxicology in the United States can be said to have arisen, due to the problem of vehicles and formulation, in the late 1930s, when attempts were made to formulate the new drug sulphanilamide. This drug is not very soluble in water, and the U.S. firm Massengill produced a clear, syrupy elixir formulation that was easy to take orally. The figures illustrate how easy it is to be misled. The drug sulphanilamide is not very soluble in glycerol, which has an LD_{50} in mice of $31.5\,g\,kg^{-1}$, but there are other glycols that have the characteristic sweet taste and a much higher solvent capacity. Ethylene glycol has an LD_{50} of $13.7\,g\,kg^{-1}$ in mice and $8.5\,g\,kg^{-1}$ in rats, making it slightly more toxic than diethylene glycol, which has an LD_{50} in rats of $20.8\,g\,kg^{-1}$, similar to that for glycerol. The drug, which is itself inherently toxic, was marketed in a 75% aqueous diethylene glycol–flavored elixir. Early in 1937 came the first reports of deaths, but the situation remained obscure for about six months until it became clear that the toxic ingredient in the elixir was the diethylene glycol. Even as late as March 1937, Haag and Ambrose were reporting that the glycol was excreted substantially unchanged in dogs, suggesting that it was likely to

be safe (Hagenbusch, 1937). Within a few weeks, Holick (1937) confirmed that a low concentration of diethylene in drinking water was fatal to a number of species. Hagenbusch (1937) found that the results of necropsies performed on patients who had been taking 60–70 mL of the solvent per day were similar to those of rats, rabbits, and dogs taking the same dose of solvent with or without the drug. This clearly implicated the solvent, although some authors considered that the solvent was simply potentiating the toxicity of the drug. Some idea of the magnitude of this disaster may be found in the paper of Calvary and Klump (1939), who reviewed 105 deaths and a further 2560 survivors who were affected to varying degrees, usually with progressive failure of the renal system. It is easy to be wise after the event, but the formulator fell into a classic trap, in that the difference between acute and chronic toxicity had not been adequately considered. In passing, the widespread use of ethylene glycol itself as an antifreeze has led to a number of accidental deaths, which suggests that the lethal dose in humans is around 1.4 mL kg^{-1}, or a volume of about 100 mL. In the preface to the first United States Pharmacopeia (USP), published in 1820, there is the statement that "it is the object of the Pharmacopoeia to select from among substances which possess medical power, those, the utility of which is most fully established and best understood; and to form from them preparations and compositions, in which their powers may be exerted to the greatest advantage." This statement suggests that the influence that formulation and preparation may have on the biological activity of a drug (and on nonpharmaceutical chemicals) has been appreciated for a considerable time.

Available and commonly used vehicles and formulating agents are reviewed, along with basic information on their characteristics and usages, in the appendix at the end of the chapter. There is a general presumption that those excipients and formulating agents listed in the USP or the *Inactive Ingredient Guide* prepared by the FDA (www.accessdata.fda.gov) are safe to use and without biological effect. This may not always be the case in either experimental animals (see appendix to this chapter) or humans (see Weiner and Bernstein, 1989) either directly or in how they alter absorption of and response to the active ingredient.

There are some basic principles to be observed in developing and preparing test material formulations. These are presented in Table 5.7.

Bioavailability is defined as the fraction of the dose reaching either the therapeutic target organ or tissue or the systemic circulation as unchanged compound following administration by any route. For an agent administered orally, bioavailability may be less than unity for several reasons. The chemical may be incompletely absorbed. It may be metabolized in the gut, the gut wall, the portal blood, or the liver prior to entry into the systemic circulation (see Figure 5.4). It may undergo enterohepatic cycling with incomplete reabsorption following elimination into the bile. Biotransformation of some chemicals in the liver following oral administration is an important factor in the pharmacokinetic profile, as will be discussed further. Bioavailability measures following oral administration are generally given as the percentage of the dose available to the systemic circulation.

TABLE 5.7 Basic Principles in Developing Formulations

A. Preparation of the formulation should not involve heating of the test material anywhere near the point where its chemical or physical characteristics are altered.
B. If the material is a solid and it is to be assessed for dermal effects, its shape and particle size should be preserved. If intended for use in humans, topical studies should be conducted with the closest possible formulation to that to be used on humans.
C. Multicomponent test materials (mixtures) should be formulated so that the administered form accurately represents the original mixture (i.e., components should not be selectively suspended or taken into solution).
D. Formulation should preserve the chemical stability and identity of the test material.
E. The formulation should be such as to minimize total test volumes. Use just enough solvent or vehicle.
F. The formulation should be easy to administer accurately.
G. pH of dosing formulations should be between 5 and 9, if possible.
H. Acids or bases should not be used to divide the test material (for both humane reasons and to avoid pH partitioning in either the gut or the renal tubule).
I. If a parenteral route is to be employed, final solutions should be as nearly isotonic as possible. Do not assume a solution will remain such upon injection into the bloodstream. It is usually a good idea to verify that the drug stays in solution upon injection by placing some drops into plasma.
J. Particularly if use is to be more than a single injection, steps (such as filtration) should be taken to ensure suitable sterility.

As the components of a mixture may have various physiochemical characteristics (solubility, vapor pressure, density, etc.), great care must be taken in preparing and administering any mixture so that what is actually tested is the mixture of interest.

Examples of such procedures are making dilutions (not all components of the mixture may be equally soluble or miscible with the vehicle) and generating either vapors or respirable aerosols (not all the components may have equivalent volatility or surface tension, leading to a test atmosphere that contains only a portion of the components of the mixture).

By increasing or decreasing the viscosity of a formulation, the absorption of a toxicant can be altered (Ritschel et al., 1974). Conversely, the use of absorbents to diminish absorption has been used as an antidote therapy for some forms of intoxication. Using the knowledge that rats cannot vomit, there have been serious attempts at making rodenticides safer to nontarget animals by incorporating emetics into the formulations, but this has had only a limited success. Gaines (1960) used in vivo liver perfusion techniques to investigate the apparent anomaly that the carbamate Isolan was more toxic when administered to rats percutaneously than when administered orally. It has been shown that these results, a manifestation of different formulations, have been used for the two routes of exposure (oral and percutaneous) in estimating the LD_{50} values using a common solvent, *n*-octanol. It was found that Isolan was significantly more toxic by the oral route than by the percutaneous route; by regression analysis it was found that at no level of lethal dose values was the reverse correct.

Although the oral route is the most convenient, there are numerous factors that make it unpredictable. Absorption by this route is subject to significant variation from animal to animal and even in the same individual animal at different times. Considerable effort has been spent by the pharmaceutical industry to develop drug formulations with absorption characteristics that are both effective and dependable. Protective enteric coatings for pharmaceuticals were introduced long ago to retard the action of gastric fluids and then disintegrate and dissolve after passage of a tablet into the human intestine. The purposes of these coatings for drugs are to protect the active ingredient, which would be degraded in the stomach, to prevent nausea and vomiting caused by local gastric irritation (also a big problem in rodent studies, where over a long time period gastric irritation frequently leads to forestomach hyperplasia), to obtain higher local concentrations of the active ingredient intended to act locally in the intestinal tract, to produce a delayed biological effect, or to deliver the active ingredient to the intestinal tract for optimal absorption there. Such coatings are generally fats, fatty acids, waxes, or other such agents, and all of these intended purposes for drug delivery can readily be made to apply for some toxicity studies. Their major drawback, however, is the marked variability in time for a substance to be passed through the stomach. In humans, this gastric emptying time can range from minutes to as long as 12 h. One would expect the same for animals, as the limited available data suggest is the case. Similar coating systems, including microencapsulation (see Melnick et al., 1987), are available for and are currently used in animal toxicity studies.

The test chemical is unlikely to be absorbed or excreted unless it is first released from its formulation. It is this stage of the process that is the first and most critical step for the activity of many chemicals. If the formulation does not release the chemical, the rest of the process becomes somewhat pointless.

It might be argued that the simplest way around the formulation problem is to administer any test as a solution in water, thereby avoiding the difficulties altogether. However, since multiple, small, accurately measured doses of a chemical are required repeatedly, reproducible dilutions must be used. Also, the water itself is to be regarded as the formulation vehicle, and the test substance must be water soluble and stable in solution, which many are not. If we take into account the need for accuracy, stability, and optimum performance in vivo, the problem can become complex.

Direct connection between observed toxicity and formulation components is uncommon and it is usually assumed that vehicles and other nontest chemical components are innocuous or have only transitory pharmacological effects. Historically, however, this has certainly not been the case. Even lactose may have marked toxicity in individual test animals (or humans) who are genetically incapable of tolerating it.

The initial stage of drug release from the formulation, in terms of both the amount and the rate of release, may exercise considerable influence at the clinical response level. A close consideration of the formulation parameters

of any chemical is therefore essential during the development of any new drug, and, indeed, there are examples where formulations of established drugs also appear to require additional investigation.

The effects of formulation additives on chemical bioavailability from oral solutions and suspensions have been well reviewed by Hem (1973). He pointed out how the presence of sugars in a formulation may increase the viscosity of the vehicle. However, sugar solutions alone may delay stomach-emptying time considerably when compared to solutions of the same viscosity prepared with celluloses, which may be due to the sugar's effect on osmotic pressure. Sugars of different types may also have an effect on fluid uptake by tissues and this, in turn, correlates with the effect of sugars such as glucose and mannitol on drug transport.

Surfactants have been explored widely for their effects on drug absorption, in particular using experimental animals (Gibaldi and Feldman, 1970; Gibaldi, 1976). Surfactants alter dissolution rates (of lipid materials), surface areas of particles and droplets, and membrane characteristics, all of which affect absorption.

Surfactants may increase the solubility of the drug via micelle formation, but the amounts of material required to increase solubility significantly are such that at least orally the laxative effects are likely to be unacceptable. The competition between the surfactant micelles and the absorption sites is also likely to reduce any useful effect and make any prediction of net overall effect difficult. However, if a surfactant has any effect at all, it is likely to be in the realm of agents that help disperse suspensions of insoluble materials and make them available for solution. Natural surfactants, in particular bile salts, may enhance absorption of poorly soluble materials.

The effective surface area of an ingested chemical is usually much smaller than the specific surface area that is an idealized in vitro measurement. Many drugs whose dissolution characteristics could be improved by particle size reduction are extremely hydrophobic and may resist wetting by GI fluids. Therefore, the GI fluids may come in intimate contact with only a fraction of the potentially available surface area. The effective surface area of hydrophobic particle can often be increased by the addition of a surface-active agent to the formulation, which reduces the contact angle between the solid and the GI fluids, thereby increasing effective surface area and dissolution rate.

Formulations for administering dermally applied toxicants present different considerations and problems. The extent of penetration and speed with which a biologically active substance penetrates the skin or other biological membrane depends on the effect that the three factors—vehicle, membrane, and chemical—exert on the diffusion process. It is now accepted that they together represent a functional unit that controls the penetration and location of the externally applied chemicals in the deeper layers of the skin or membrane layer. The importance of the vehicle for the absorption process has been neglected until recently. One of the few requirements demanded of the vehicle has been that it act as an inert medium that incorporates the test chemical in the most homogeneous distribution possible. In addition, chemical stability

and good cosmetic appearance have been desirable. Most formulation in toxicology are based on empirical experience.

The chemical incorporated in a vehicle should reach the surface of the skin at a suitable rate and concentration. If the site of action lies in the deeper layers of the epidermis or below, the substance must cross the stratum corneum if the skin is intact. Both processes, diffusion from the dosage form and diffusion through the skin barriers, are inextricably linked. They should be considered simultaneously and can be influenced by the choice of formulation.

The thesis that all lipid-soluble compounds basically penetrate faster than water-soluble ones cannot be supported in this absolute form. A lipophilic agent can penetrate faster or slower or at the same rate as a hydrophilic agent, depending on the vehicle used.

Disregarding such chemical-specific properties as dissociation constants (in the case of ionic compounds), particle size, and polymorphism, as well as side effects of viscosity, binding to vehicle components, complex formulation, and the like, the following formulation principles arise:

(a) Optimization of the concentration of chemical capable of diffusion by testing its maximum solubility
(b) Reduction of the proportion of solvent to a degree that is adequate to keep the test material still in solution
(c) Use of vehicle components that reduce the permeability barriers

These principles lead to the conclusion that each test substance requires an individual formulation. Sometimes different ingredients will be required for different concentrations to obtain the maximum rate of release. No universal vehicle is available for any route, but a number of approaches are. Dosage preparation laboratories should be equipped with glassware, a stirring hot plate, a sonicator, a good homogenizer, and a stock of the basic formulating material, as detailed at the end of this chapter.

5.3.1 Dermal Formulations

Preparing formulations for application to the skin has special considerations associated with it, which, in the case of human pharmaceuticals, has even led to a separate book (Barry, 1983).

The physical state of the skin is considerably affected by external factors such as relative humidity, temperature, and air movement at the skin surface. If this contact is broken (e.g., by external applications of ointments or creams), it is reasonable to assume that the new skin will change in some way, sometimes to an extent that creates new conditions of permeability for the test material. This would be the case, for example, if the stratum corneum becomes more hydrated than normal due to the topical delivery form. Temperature might also have an effect, as is the case when any constituents of the vehicle

affect the inner structure of the skin through interactions with endogenous skin substances. Often several of these processes occur together. Figure 5.5 shows the relationship between water content and relative humidity.

Since this is a question of interaction between the vehicle and the skin (and the latter cannot be viewed as an inert medium), the composition of the vehicle itself may be altered (e.g., by incorporation of skin constituents or through loss of volatile components).

The first contact between vehicle and skin occurs on the skin's surface. The first phase of interaction undoubtedly begins with the lipid mantle in the case of so-called normal skin. If the skin has been damaged by wounds, the surface can form a moist milieu of serious exudate, resulting in abnormal wetting properties. Normally it is impregnated with oily sebaceous secretions and horny fat, presenting a hydrophobic surface layer. Water will not spread out as a film but will form droplets, while bases with a high affinity to the skin surface constituents spread spontaneously into a film and can wet. In the case of a base low viscosity, the degree of wetting can often be determined by measuring the angle of contact. If the preparation wets the skin surface, is drawn by capillary action from the visible area into the large inner surface of the stratum corneum, and is transported away into the interior, then it is said that the ointment or cream penetrates well. Spreading and wetting are purely surface phenomena, not penetration in the strict sense. If the skin shows a high content of its own lipids, spreading is limited. It is also reduced if the value of the surface tension of the skin (o_s) decreases compared to the value of the interfacial force between the skin and subject liquid ($y_{s/1}$) and the surface tension of the subject liquid (o_1), as is the case with aqueous bases. Addition of amphiphilic compounds decreases o_1 and $y_{s/1}$ and thus spreadability increases.

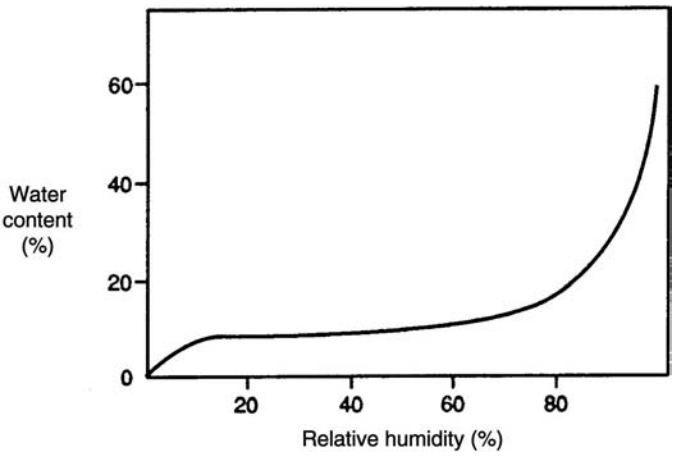

Figure 5.5 *Sorption isotherms of water vapor as function of relative humidity, composition of constituents, and water content in stratum corneum.*

How much the endogenous emulsifying substances of the fatty film, such as cholesterol esters and fatty acid salts, affect this spreading process is not clear. They can probably promote the emulsification of hydrophobic substances with water. Whether the sebaceous and epidermal lipids alone are sufficient to emulsify water and so form a type of emulsive film remains controversial. However, it is assumed that they, together with appropriate vehicle components, improve the spreading of the applied vehicle and that this effect can be potentiated by mechanical means such as intensive rubbing in. Good spreadability ensures that the active ingredient is distributed over a large area.

High local concentrations are avoided and, at the same time, close contact is made between the chemical and the upper layers of the skin.

In grossly simplified terms, hydrogels, suspensions, and oil–water emulsions behave on the skin surface similarly to aqueous solutions. By contrast, pastes and water–oil emulsions act like oil. The ability of an organic solvent to stick or wet depends on its specific properties (e.g., its viscosity and its surface tension).

At present, information concerning alterations in vehicle composition on the skin surface is sparse. However, two possible extremes are conceivable. On the one hand, if the vehicle has a high vapor pressure, it often completely evaporates shortly after application. On the other hand, the vehicle may remain on the skin surface in an almost completely unchanged composition (e.g., highly viscous Vaseline or similar thick covering systems). Between these two extremes lie the remaining types of vehicles.

The first situation applies for the short-chain alcohols, acetone, or ether. After their evaporation, the drug remains finely dispersed on or in the skin at 100% concentration.

If individual components evaporate, the structure of the vehicle changes and, under certain circumstances, also the effective drug concentration. Oil–water emulsions lose water rapidly, giving rise to the well-known cooling effect. If evaporation continues, the dispersed oil phase coalesces and forms a more or less occlusive film on the skin, together with the emulsifier and the drug. Of course, it is possible that a certain hydrophilic proportion of the drug is then present in suspended form or at least can react with charged molecules and is thus removed from the diffusion process at the start. At the same time, it is to be expected that soluble constituents of the skin are incorporated so that a new system can be formed on the surface and the adjoining layers of skin. Comparable transformations probably also occur after application of water–oil emulsions, providing one realizes that the water evaporates more slowly, the cooling effect is less strong, and, due to the water–oil character of the molecule, the occlusive effect can be more marked because of the affinity of the oily components for the skin.

By contrast, Vaseline and similar highly viscous, lipid bases from the outset form an impenetrable layer, virtually unaffected by external factors or effects emanating from the skin itself. Interactions with the skin lipids are only likely at the boundary between ointment and skin.

The evaporation of the water from the skin into the atmosphere is a continuous process. It can be increased or decreased by the use of suitable vehicles. An evaporation increase will always occur if the water vapor from the vehicle is taken away more quickly than water can diffuse from the deeper layers into the stratum corneum. This applies in principle to all hydrophilic bases, particularly for systems with an oil–water character that, after loss of most of their own water, develop a true draining effect that can lead to the drying out of the underlying tissue. How much the penetration of hydrophilic drugs can be proved with the help of oil–water systems depends on the solution properties of the rest of the components in the skin. Generally, such compounds can only seldom reach deeper layers. It is equally difficult to show an adequate release of water from hydrophilic systems to a dry skin. If any such effects do occur, they are short term and are quickly overtaken by opposing processes. The same seems to apply to most traditional moisturizers such as glycerin and propylene glycol (Powers and Fox, 1957; Rieger and Deems, 1974). They can also cause a large rise in the rate of evaporation, depending on the relative humidity, and thus increase the transepidermal loss of water. It is probably impossible to prevent this drying out without preparations having some occlusive properties.

In contrast, vehicles that are immiscible with water and those with a high proportion of oils have occlusive effects. They reduce both insensible perspiration and the release of sweat. The sweat collects as droplets at the opening of the glands but does not spread as a film between the hydrophobic skin surface and the lipophilic base because the free surface energy of the vehicle–skin interface is smaller than that between water and skin. If a lipophilic layer of vehicle is present, this is not spontaneously replaced by the water–skin layer if sweat is secreted.

The horny layer consists of about 10% extracellular components such as lipids, proteins, and mucopolysaccharides. Around 5% of the protein and lipids form the cell wall. The majority of the remainder is present in the highly organized cell contents, predominantly as keratin fibers, which are generally assigned an α-helical structure. They are embedded in a sulfur-rich amorphous matrix, enclosed by lipids that probably lie perpendicular to the protein axis. Since the stratum corneum is able to take up considerably more water than the amount that corresponds to its volume, it is assumed that this absorbed fluid volume is mainly located in the region of these keratin structures.

Some insight into where on the relative humidity continuum water molecules are absorbed can be gained from equilibrium isotherms (Ziegenmeyer, 1982) (Figure 5.5), which show a characteristic sigmoidal shape. At low relative humidity, water is first absorbed at specific skin sites, probably in the region of the peptide compounds and the various polar side chains. At higher moisture content, layers of water form on the skin. By using Zimm–Lundberg cluster theory (Zimm and Lundberg, 1956), additional information can be obtained about the nature of the absorbed water.

Because of thick intertwining protein fibers in the cell and in the area of the cell membrane, cell structure is rigid and remains so but is altered by the osmotic effect of the penetrating water. The uptake of water entails a continual shifting of the cell matrix, which gradually develops elastic opposing forces that increasingly resist further expansion. An equilibrium is reached if both forces balance each other. In the case of water, it takes quite a long time to completely hydrate the cell. This process can, however, be shortened if there are components present with a solvent effect diffusing out of a basic vehicle. The duration and degree of swelling depends on the affinity of all the dissolved substances for the tissue and on the size of the maximum possible elastic reaction, which stabilizes cell structure.

5.3.2 Interactions between Skin, Vehicle, and Test Chemical

The diffusion coefficient of the hydrated stratum corneum is larger than that of dry skin. Therefore, hydration increases the rate of passage of all substances that penetrate the skin. If the hydrated keratin complex is represented by a biphasix system, then it can be considered to exist as a continuous region covered with layers of water and intervening layers of lipids. Nonpolar compounds are predominantly dissolved in the nonpolar lipid matrix and diffuse through it. Polar substances, by contrast, pass through the aqueous layers. The diffusion of water and low-molecular-weight, hydrophilic molecules through these layers of water is more difficult than a corresponding free diffusion in an aqueous solution. This could, under certain circumstances, be due to a higher degree of organization of water in the protein structures (than in plasma or the free state), in the sense that this water is only available as the driving force of the diffusion process to a limited degree.

The degree of hydration can be controlled by the choice of vehicle. Lipophilic paraffin bases are available, but vehicles such as water–oil emulsions are more acceptable since they are less occlusive and offer ease of formulation.

In principle, temperature can also have an effect on penetration, which may be exerted on the basic vehicle if it contains temperature-sensitive components (e.g., nonionic tensides). Room and body temperatures can be enough to change the hydrophilic–lipophilic balance and thus possibly change the entire system. It has long been known that increasing temperature can considerably reduce diffusional resistance and thereby increase the rate of penetration of substances. In practice, however, this effect is of no importance. Of course, skin temperature will be increased a few degrees by occlusion because of the prevention of sweating and restriction of heat radiation. However, compared to the increase in penetration achieved by the simultaneous hydration process, this effect is insignificant.

Additives aimed at accelerating penetration always attempt to enable diffusion of pharmacologically active compounds into or through the stratum corneum without damaging it and without causing undesirable systemic effects. Although attempts have been made to limit these effects, this goal has not

been achieved as yet. There are numerous substances that decrease the diffusional resistance of the skin, such as propylene glycol, tensides, parotic substances such as urea, dimethylsulfoxide (DMSO), dimethylformamide (DMF), and various other organic solvents, mostly of medium chain length. They all improve the penetration of dissolved agents, but only at the cost of the integrity of skin structure, raising the question of the degree of damage and reversibility.

If the substances have passed the stratum corneum, they also generally diffuse into the living part of the epidermis, reach the circulation, and then have systemic effects depending on the amount absorbed. Because these are often constituents of formulations, one generally expects them to have little direct influence on skin penetration. However, their amphiphilic properties allow them to form new systems with the body's constituents and even to change the physical state of water in the skin. By this means, a pathway is cleared for other hydrophilic substances to gain entry into the general circulation.

Most of a permeability enhancer (such as a tenside) is bound to the stratum corneum. It is assumed that the underlying mechanism of the process involves interactions with keratin structures. Positively and negatively charged ionic groups of proteins have been suggested as binding sites for ionic substances. Ion pairs could also form. On the other hand, hydrophobic areas are present that bind with the uncharged part of the enhancers. The total free binding energy of molecules to keratin is made up of the contributions arising from electrostatic and nonpolar interactions. Nonpolar interactions increase with the chain length of the molecule. This would be the reason why predominantly anionic molecules of medium chain length exert stronger effects on the keratin structure than those of shorter chain length (Dominguez et al., 1977).

In order to reach the interior of the tightly enmeshed keratin, the molecule must overcome the elastic energy of the polypeptide matrix. The energy necessary to do this is proportional to the volume of the penetrating molecule. The larger the volume, the more difficult it will be for the molecule to approach the various binding sites of proteins in the interior of the keratin complex. Thus, the size of the penetrating molecule is subject to certain limits. If more molecules are present than can become bound, it is possible that a few of them will reach the living layers of the epidermis, as has been described for several anionic, mostly medium-chain enhancer molecules such as tensides. It remains unclear whether this is a consequence of pure saturation or if other interactions are involved (e.g., with structural lipids or hydrophilic materials from the intercellular lipids).

The extent to which the vehicle can affect the entire diffusion process can be shown by an example. In a four-component system of 40% oil, 40% water, and 20% of an emulsifying agent and coemulsifier, alteration of only the proportion of emulsifier to coemulsifier leads to systems of completely different colloidal–chemical structures, which can be labeled as creams, gels, or microemulsions.

Dermal administration presents fewer logistic difficulties than oral administration. Liquids can be administered as supplied and powders or solids can be moistened with saline to form a thick paste or slurry or can be applied dry and moistened with saline. Solid materials (e.g., sheets of plastic, fabric) can also be administered dermally. Liquid materials or slurries are applied directly to the skin, taking care to spread the material evenly over the entire area or as much of the area as can reasonably be covered and then covering with a strip of gauze. If a large amount of material is being administered and the abdominal skin will be exposed, it is sometimes necessary to apply material to the gauze and to the skin. Dry materials are weighed out, then placed on the gauze strip and moistened with physiological saline (generally 15 mL) so that they adhere to the gauze. The gauze is then wrapped around the animal. This porous gauze dressing is then held in place by an additional wrapping, generally of an impervious material, to create an "occlusive" covering. This occlusion enhances penetration and prevents ingestion or evaporation of the test material.

Another recently developed approach is the use of plastic containment capsules (modified Hill Top Chambers) for administration of well-measured doses in a moisturized microenvironment (Derelanko et al., 1987).

Finally, it should be noted that for some agents (contrary to the general rule) decreasing the concentration of chemical in a vehicle may increase its apparent intrinsic toxicity.

5.3.3 Oral Formulations

The physical form of a material destined for oral administration often presents unique challenges. Liquids can be administered as supplied or diluted with an appropriate vehicle, and powders or particulates can often be dissolved or suspended in an appropriate vehicle. However, selection of an appropriate vehicle is often difficult. Water and oil (such as vegetable oils) are used most commonly. Materials that are not readily soluble in either water or oil can frequently be suspended in a 1% aqueous mixture of methylcellulose. Occasionally, a more concentrated methylcellulose suspension (up to 5%) may be necessary. Materials for which appropriate solutions or suspensions cannot be prepared using one of these three vehicles often present major difficulties.

Limited solubility or suspendability of a material often dictates preparation of dilute mixtures that may require large volumes to be administered. The total volume of liquid dosing solution or suspension that can be administered to a rodent is limited by the size of its stomach. However, because rats lack a gagging reflex and have no emetic mechanism, any material administered will be retained. Guidelines for maximum amounts to be administered are given in Table 5.6.

Limitations on total volume therefore present difficulties for materials that cannot easily be dissolved or suspended. The most dilute solutions that can be administered for a limit-type test (5000 mg kg^{-1}), using the maximum volumes

shown in Table 5.6, generally are 1% for aqueous mixtures and 50% for other vehicles.

Although vehicle control animals are not required for commonly used vehicles (water, oil, methylcellulose), most regulations require that the biological properties of a vehicle be known and/or that historical data be available. Unfortunately, the best solvents are generally toxic and thus cannot be used as vehicles. Ethanol and acetone can be tolerated in relatively high doses but produce effects that may complicate interpretation of toxicity associated with the test material alone. It is sometimes possible to dissolve a material in a small amount of one of these vehicles and then dilute the solution in water or in oil.

Gels and resins often present problems because of their viscosity at room temperature. Warming these materials in a water bath to a temperature of up to 50 °C will frequently facilitate mixing and dosing. However, it is important to ascertain that no thermal degradation occurs and that actually administered formulations be at or near body temperature.

Other possibilities for insoluble materials are to mix the desired amount of material with a small amount of the animal's diet or to use capsules. The difficulty with the diet approach is the likelihood that the animal will not consume all of the treated diet or that it may selectively not consume chunks of test material. Use of capsules, meanwhile, is labor intensive. In rare cases, if all of these approaches fail, it may not be possible to test a material by oral administration. In capsules, particle size is generally inversely related to solubility and bioavailability. However, milling of solids may adversely affect their chemical nature and/or pose issues of safety.

If necessary, the test substance should be dissolved or suspended as a suitable vehicle, preferably in water, saline, or an aqueous suspension such as 0.5% methylcellulose in water. If a test substance cannot be dissolved or suspended in an aqueous medium to form a homogenous dosage preparation, corn oil or another solvent can be used. The animals in the vehicle control group should receive the same volume of vehicle given to animals in the highest dose group.

The test substance can be administered to animals at a constant concentration across all dose levels (i.e., varying the dose volume) or at a constant dose volume (i.e., varying the dose concentration). However, the investigator should be aware that the toxicity observed by administration in a constant concentration may be different from that observed when given in a constant dose volume. For instance, when a large volume of corn oil is given orally, GI motility is increased, causing diarrhea and decreasing the time available for absorption of the test substance in the GI tract. This situation is particularly true when a highly lipid-soluble chemical is tested.

If an organic solvent is used to dissolve the chemical, water should be added to reduce the dehydrating effect of the solvent within the gut lumen. The volume of water or solvent–water mixture used to dissolve the chemical should be kept low, since excess quantities may distend the stomach and cause rapid gastric emptying. In addition, larger volumes of water may carry the chemical

through membrane pores and increase the rate of absorption. Thus, if dose-dependent absorption is suspected, it is important that the different doses are given in the same volume of solution.

Larger volumes than those detailed earlier may be given, although nonlinear kinetics seen under such circumstances may be due to solvent-induced alteration of intestinal function. The use of water-immiscible solvents such as corn oil (which are sometimes used for gavage doses) should be avoided, since it is possible that mobilization from the vehicle may be rate limiting. Magnetic stirring bars or homogenizers can be used in preparing suspensions. Sometimes a small amount of a surfactant such as Tween 80, Span 20, or Span 60 is helpful in obtaining a homogenous suspension.

A large fraction of such a material may quickly pass through the GI tract and remain unabsorbed. Local irritation by a test substance generally decreases when the material is diluted. If the objective of the study is to establish systemic toxicity, the test substance should be administered in a constant volume to minimize GI irritation that may, in turn, affect its absorption. If, however, the objective is to assess the irritation potential of the test substance, then it should be administered undiluted.

5.3.4 Parenteral Formulations

Parenteral dose forms include aqueous, aqueous organic, and oily solutions, emulsions, suspensions, and solid forms for implantation. These parenterals need to be sterile and pyrogen free; they are, if possible, buffered close to normal physiological pH and preferably are isotonic with the body fluids.

The preparation of parenteral dosage forms of approved and potential drugs for animals is the same as for humans. Turco and King (1974) provide a comprehensive review of the subject, which though written with human therapeutics in mind contains very little that is not applicable to animals. Sterility, lack of pyrogenicity, blood compatibility, and low to no irritation at the point of injection are biological requirements; there are also a corresponding set of physicochemical requirements.

Parenteral products are usually given to humans when an immediate effect is needed, when a patient is unable to accept medication by the oral route, or when the drug will be ineffective by the oral route. These conditions apply to animals used in safety evaluation.

Parenteral products can be easily administered to confined or restrained animals, leaving no doubt that the animal received its medication.

To be acceptable, a SC or IM formulation should cause only a minimum of irritation and no permanent damage to the tissues and be systemically distributed and active when administered by this route. The ideal parenteral product is an aqueous solution isotonic with the body fluids with a pH between 7 and 8. When the drug lacks sufficient aqueous solubility, a suspension may be considered; however, in most cases, the bioavailability of the drug may be affected

and encapsulation by the body at the site of injection is extremely likely. The solubility of the drug in water may be improved by the addition of cosolvents such as alcohol, propylene glycol, polyethylene glycol, dimethylacetamide, DMSO, or DMF. The resulting solution must have additional tolerance for water so that the drug will not precipitate at the site of injection when the solution is diluted by body fluids. If precipitation occurs at the site of injection, the absorption of the drug may be delayed or even completely inhibited.

Water-miscible solvents alone can be used when the drug is chemically unstable in the presence of water. The number of solvents available for this purpose is extremely limited. The classic review of this subject was made in 1963 (Spiegel and Noseworthy), and some 30 years later, no additional solvents are available. This is unlikely to change in the near future due to the extensive effort necessary to determine the safety of a solvent used as a vehicle. When a nonaqueous vehicle is used, one can invariably expect some degree of pain upon injection, and subsequent tissue destruction is possible. This damage may be due to the heat of solution as vehicle mixes with body fluids, it may be associated with tissues rejecting the solvent, or it may be an inherent property of the solvent.

Fixed oils of vegetable origin and their esters may be used as parenteral vehicles for some drugs, particularly steroidal hormones. While an oleaginous vehicle may delay or impair absorption of the drug, this characteristic has been used to advantage with some drugs where a small dose is desired over a long period of time. The formulator must know which species will receive the formulation and the type of equipment used in its administration. A product intended for a dog or primate is usually given to a single animal at a time. Conventional glass or disposable syringes will be used with a 20- or 22-gauge needle, which may impede the flow of the liquid, especially when an oleaginous vehicle is used. Impedance is usually compensated for by using small animals, since the volume of injection is small and no more than one injection is normally given at one time.

The viscosity of the solution will influence its acceptability when automatic injection equipment is used. If many animals are injected at one time, a viscous solution that requires a great deal of force to eject will rapidly tire the user. When the automatic injector is refilled from a reservoir, a viscous solution will be slow to fill the volumetric chamber. The subjective aspect of measuring the ease of expelling a dose can be eliminated by constructing an apparatus that will measure the pressure needed to expel a dose (Groves, 1966). An objective means of measuring ease will allow the formulator to vary the composition of the injection and measure any improvement in injectability. For example, the addition of a wetting agent can be investigated and, if improvement is seen, the level of use can be optimized.

A parenteral product in a multidose vial must contain a preservative to protect the contents of the vial against contamination during repeated withdrawal of dose aliquots.

5.4 DOSING CALCULATIONS

One of the first things a new technician (or graduate student) must learn is how to calculate dose. Generally, administered doses in systemic toxicity studies are based on the body weight of the animal (expressed as either weight or volume—for liquids—of the test substance per kilogram of body weight of the animal), although some would maintain that surface area may be a more appropriate basis on which to gauge individual dose. The weight (or dose) of the test substance is often expressed in milligrams or grams of active ingredient if the test substance is not pure (i.e., if it is not 100% active ingredient).

Ideally, only the 100% pure sample should be tested; however, impurity-free samples are difficult to obtain and preparation of formulations (as previously discussed) is frequently essential. The toxicity of impurities or formulation components should be examined separately if the investigator feels that they may contribute significantly to the toxicity of the test substance.

If the test substance contains only 75% active ingredient and the investigator chooses a constant dose volume of $10\,mL\,kg^{-1}$ body weight across all dose levels, it will be more convenient to prepare a stock solution such that, when $10\,mL\,kg^{-1}$ of this stock solution is given to the animal, the dose will be the desired one (say $500\,mg\,kg^{-1}$ of active ingredient). The concentration of this stock solution would be (500 mg/10 mL)/0.75 = 66.7 mg of the test substance per milliliter of diluent.

Aliquots of the test substance for other dose levels can then be prepared by dilution of the stock solution. For example, the solution concentration for a dose level of $250\,mg\,kg^{-1}$ is (200 mg/10 mL)/0.75 = 26.7 mg of the test substance per milliliter of diluent.

This solution can be prepared by diluting the stock solution 25 times; that is, for each milliliter of the 26.7-$mg\,mL^{-1}$ solution to be prepared,

$$\frac{(26.7\,mg\,mL^{-1})(1\,mL)}{66.7\,mg\,mL^{-1}} = 0.400\,mL \text{ of stock solution}$$

This amount should be diluted to a final volume of 1 mL with the vehicle.

The other way to express a relative dose in animals or humans is to do so in terms of body surface area. There are many reasons for believing that the surface area approach is more accurate for relating doses between species (Schmidt-Nielsen, 1984)—and especially between test animals and humans—but this is still a less common approach in safety assessment, although it is the currently accepted norm in a couple of areas—carcinogenesis and chemotherapy, for example.

5.5 CALCULATING MATERIAL REQUIREMENTS

One of the essential basic skills for the efficient design and conduct of safety assessment studies is to be able to accurately project compound requirements

for the conduct of a study. In theory, this simply requires plugging numbers into a formula such as

$$(A \times B \times C \times D) \times 1.1 = \text{total compound requirement}$$

where A = number of animals in each study group
B = *sum* of doses of the dose groups (such as $0.1 + 0.3 + 1.0\,\text{mg kg}^{-1} = 1.4\,\text{mg kg}^{-1}$)
C = number of doses to be delivered (usually length of study in days)
D = average body weight per animal (assuming dosing is done on per-body-weight basis)
1.1 = safety factor (in effect, 10%) to allow for spillage, etc.

As an example of this approach, consider a study that calls for 10 dogs per sex per group ($A = 10 \times 2 = 20$) to receive 0, 10, 50, or $150\,\text{mg kg}^{-1}\,\text{day}^{-1}$ ($B = 10 + 50 + 150 = 210\,\text{mg kg}^{-1}$) for 30 days ($C = 30$). On average, our dogs of the age range used weigh 10 kg ($D = 10\,\text{kg}$). Our compound need is then $(20 \times 210\,\text{mg kg}^{-1} \times 30 \times 10\,\text{kg}) \times 1.1 = 1.386\,\text{kg}$.

The real-life situation is a bit more complicated, since animal weights change over time, diet studies have doses dependent on daily diet consumption, the material may be a salt but dosage should be calculated on the basis of the parent compound, and not all animals may be carried through the entire study.

For rats and mice (where weight change is most dramatic and diet studies most common), Table 5.8 presents some reliable planning values for compound requirements during diet studies.

5.6 EXCIPIENTS

Excipients are usually thought of as inert substances (such as gum arabic and starch) that form the vehicle or bulk of the dosage form of a drug. They are, of course, both much more complicated than this and not necessarily inert. A better definition would be that of the USP (2007) and National Formulary (NF), which defined excipients as any component other than the active substances (i.e., drug substances or DSs) intentionally added to the formulation of a dosage form. These substances serve a wide variety of purposes: enhancing stability, adding bulking, increasing and/or controlling absorption, providing or masking flavor, coloring, and serving as a lubricant in the manufacturing process. They are, in fact, essential for the production and delivery of marketed drug products. As will soon be made clear, they are regulated both directly and as part of the drug product (DP). For the pharmaceutical manufacturers, using established and accepted excipients [such as can be found in Hawley, 1971; Budavari, 1989; Smolinske 1992 or American Pharmaceutical Association (APhA), 1994—though these lists are not complete] is much preferred.

TABLE 5.8 Standardized Total Compound Requirements for Rodent Diet Studies[a]

	Total Compound Requirement (g) per dose ($mg\,kg^{-1}\,day^{-1}$)					
Length of Study	1	3	10	30	100	300
Rat[b]						
2 Weeks	0.2	0.4	1.2	4	10.6	32
4 Weeks	0.43	0.7	2.5	7.5	25	75
13 Weeks	0.8	2.6	8.5	25.5	85	260
52 Weeks	7	21	70	210	0.7[c]	2.1[c]
2 Years	15	45	150	450	1.5[c]	4.5[c]
Mouse						
2 Weeks	0.03	0.06	0.22	0.65	2.2	6.4
4 Weeks	0.08	0.14	0.8	1.4	8	14
13 Weeks	0.14	0.42	1.4	4.2	14	42
18 Months	0.85	2.5	8.5	25	85	250

[a]Based on 10 animals per sex per group for the length of the study that are 6–8 weeks old at study initiation. Animals are weighed to determine body weights.
[b]Sprague-Dawley rats (body weights and compound requirements for Fischer's would be less).
[c]In kilograms.

However, both pharmaceutical manufacturers and the companies which supply excipients must from time to time utilize (and therefore develop, evaluate for safety, and get approved) new excipients.

5.6.1 Regulation of Excipients

Table 5.9 lists the relevant sections of Code of Federal Regulations (CFR) 21 which govern excipients. Under Section 201(g)(1) of the Federal Food, Drug, and Cosmetic Act (FDCA; 1), the term *drug* is defined as:

> (A) articles recognized in the official *United States Pharmacopeia*, official *Homeopathic Pharmacopeia of the United States*, or official *National Formulary*, or any supplement to any of them; and (B) Articles intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease in man or other animals; and (C) Articles (other than food) intended to affect the structure of any function of the body of man or other animals; and (D) Articles intended for use as a component of any articles specified in clause (A), (B), or (C).

An excipient meets the definitions as listed in (A) and (D) above.

In 21 CFR 210.3(b)(8)(2), an "inactive ingredient means any component other than an active ingredient." According to the CFR, the term *inactive ingredient* includes materials in addition to excipients. According to 21 CFR 201.117:

> Inactive ingredients: A harmless drug that is ordinarily used as an inactive ingredient, such as a coloring, emulsifier, excipient, flavoring, lubricant, preservative,

TABLE 5.9 U.S. Code of Federal Register References to Excipients

Subject	Reference	Content
General	21 CFR 210.3(b)(8)	Definitions
	21 CFR 201.117	Inactive ingredients
	21 CFR 210.3(b)(3)	Definitions
Over-the-counter drug products	21 CFR 330.1(e)	General conditions for general recognition as safe, effective, and not misbranded
	21 CFR 328	Over-the-counter drug products intended for oral ingestion that contain alcohol
Drug master files	21 CFR 314.420	Drug master files
Investigational new drug application	21 CFR 312.23(a)(7)	IND content and format
New drug application	21 CFR 312.31	Information amendments
	21 CFR 314.50(d)(1)(ii)(a)	Content and format of an application
	21 CFR 314.70	Supplements and other changes to an approved application
Abbreviated new drug application	21 CFR 314.94(a)(9)	Content and format of an abbreviated application
	21 CFR 314.127	Refusal to approve an abbreviated new drug application
	21 CFR 314.127(a)(8)	Refusal to approve an abbreviated new drug application
Current good manufacturing practice	21 CFR 211.84(d)	Testing an approval or rejection of components, drug product containers, and closures
	21 CFR 211.165	Testing and release for distribution
	21 CFR 211.180(b)	General requirements
	21 CFR 211.80	General requirements
	21 CFR 211.137	Expiration dating
Listing of drugs	21 CFR 207	Registration of procedures of drugs and listing of drugs in commercial distribution
	21 CFR 207.31(b)	Additional drug listing information
	21 CFR 207.10(e)	Exceptions for domestic establishments
Labeling	21 CFR 201.100(b)(5)	Prescription drugs for human use
	21 CFR 201.20	Declaration of presence of FD&C yellow no. 5 and/or FD&C yellow no. 6 in certain drugs for human use
	21 CFR 201.21	Declaration of presence of phenylalanine as component of aspartame in over-the-counter and prescription drugs for human use
	21 CFR 201.22	Prescription drugs containing sulfites; required warning statements

> or solvent in the preparation of other drugs shall be exempt from Section 502(f)(1) of the Act. This exemption shall not apply to any substance intended for a use which results in the preparation of a new drug, unless an approved new-drug application provides for such use.

Excipients also meet the definition of component in the good manufacturing practice (GMP) regulations in 21 CFR 210.3(b)(3): "Component means any ingredient intended for use in the manufacture of a drug product, including those that may not appear in such drug product."

The NF admissions policy in the *United States Pharmacopeia 30/National Formulary 25* defines the word *excipient* (3): "An excipient is any component other than the active substance(s), intentionally added to the formulation of a dosage form. It is not defined as an inert commodity or an inert component of a dosage form."

Similar to all other drugs, excipients must comply with the adulteration and misbranding provisions of the FDCA (Katdare and Chaubal, 2006). Under Section 501(a), an excipient shall be deemed to be adulterated if it consists in whole or in part of any filthy, putrid, or decomposed substance or if it has been prepared, packed, or held under unsanitary conditions whereby it may have been contaminated with filth or whereby it may have been rendered injurious to health. An excipient is adulterated if the methods used in or the facilities or controls used for its manufacture, processing, packing, or holding do not conform to or are not operated or administered in conformity with current GMPs to assure that such drug meets the requirements of the act as to safety and has the identity and strength and meets the quality and purity characteristics which it purports or is represented to possess. In addition, under Section 501(b), an excipient shall be deemed to be adulterated if it purports to be or is represented as a drug the name of which is recognized in an official compendium, and its strength differs from or its quality or purity falls below the standards set forth in such compendium.

In 2005, the FDA promulgated new guidance on the selection and use of excipients in nonclinical and clinical studies. FDA compliance officials require the use of inactive ingredients that meet compendial standards when standards exist and either have previous use in FDA-approved pharmaceuticals or may be qualified as "novel" excipients (with studies as summarized in Table 5.10). The CDER maintains an inactive ingredient committee whose charter includes the evaluation of the safety of inactive ingredients on an as-needed basis, preparation of recommendations concerning the types of data needed for excipients to be declared safe for inclusion in a drug product, and other related functions.

From a regulatory standpoint, the FDA's concern regarding safety involves the toxicity, degradants, and impurities of excipients, as discussed in other chapters in this book. In addition, other chapters of this book address types of toxicity concerns, toxicity-testing strategies, and exposure and risk assessment of excipients.

TABLE 5.10 Summary of Toxicological Studies Recommended for New Pharmaceutical Excipients Based on Route of Exposure

Tests	Oral	Mucosal	Transdermal	Dermal/Topical	Parenteral	Inhalation/Intranasal	Ocular
Appendix 1—base set	R	R	R	R	R	R	R
Acute oral toxicity	R	R	R	R	R	R	R
Acute dermal toxicity	C	C	C	C	C	R	C
Acute inhalation toxicity	R	R	R	R	R	R	R
Eye irritation	R	R	R	R	R	R	R
Skin irritation	R	R	R	R	R	R	R
Skin sensitization	R	R	R	R	R	R	R
Acute parenteral toxicity	—	—	—	—	R	—	—
Application site evaluation	—	—	R	R	R	R	—
Pulmonary sensitization	—	—	—	—	—	R	—
Phototoxicity/photoallergy	—	—	R	R	—	—	—
Ames test	R	R	R	R	R	R	R
Micronucleus test	R	R	R	R	R	R	R
ADME—intended route	R	R	R	R	R	R	R
28-Day toxicity (2 species)—intended route	R	R	R	R	R	R	R
Appendix 2							
90-Day toxicity (most appropriate species)	R	R	R	R	R	R	R
Developmental toxicity (rat and rabbit)	R	R	R	R	R	R	R
Additional assays	C	C	C	C	C	C	C
Genotoxicity assays	R	R	R	R	R	R	R
Appendix 3							
Chronic toxicity (rodent, nonrodent)	C	C	C	C	C	C	C
Photocarcinogenicity	—	—	C	C	—	—	—
Carcinogenicity	C	C	C	C	C	C	—

Note: R, required; C, conditionally required.

Excipients must be safe for their intended use. Under 21 CFR 330.1(e), over-the-counter (OTC) human drugs that are generally recognized as safe and effective and not misbranded may only contain inactive ingredients if they are suitable and if the amounts administered are safe and do not interfere with the effectiveness of the drug or with required tests or assays. Color additives may be used in accordance with the provisions of the FDCA and the regulations of 21 CFR Parts 70–82. The FDA proposed that, to be considered as suitable within the meaning of 21 CFR 330.1(e), each inactive ingredient in an OTC human drug product should perform a specific function (5). The proposed regulation defined *safe and suitable* to mean that the inactive ingredient meets various conditions as mentioned in the foregoing. Over-the-counter drug manufacturers are responsible for assuring that these conditions are met. There is no formal approval mechanism (Levi, 1963).

In the United States, the safety and suitability of excipients used in new drugs are considered as part of the new drug application (NDA) process. There is no separate and independent review and approval system for excipients. There are no specific regulations or guidelines that specify the requirements needed to gain approval of a new drug that contains a new excipient. Generally, pharmaceutical companies choose excipients that previously have been approved for commercial use in other NDAs. The FDA's *Inactive Ingredient Guide*, discussed later in this chapter, contains a listing of inactive ingredients present in approved drug products. There is currently no way of gaining a listing for an excipient in the guide independent of the NDA route. The FDA reviews the status of an excipient in food as information to support its use in drug products. Factors relative to the use of an excipient, such as dosing regimen and route of administration, are also reviewed. Advances in excipient technology and drug dosage from technology have created a need for a separate regulatory approval process for new excipients. The USP published IPEC's Excipient Safety Evaluation Guidelines as Information Chapter 1074, Excipient Biological Safety Evaluation Guideline.

Information on existing or new excipients can be described and provided to the FDA in an NDA directly. Alternatively, the manufacturers of excipients may prepare and submit type IV drug master files (DMFs) to support the use of an excipient in one or more NDAs. The DMFs are discussed in FDA regulations under 21 CFR 314.420 and the FDA-issued Guidance for Drug Master Files. When authorized by the DMF submitter (i.e., the excipient manufacturer) and cross-referenced by an NDA submitter, the FDA reviews the DMF to make determinations on the safety, manufacture, and quality of the excipient use in the new drug that is the subject of the then-pending NDA. The DMF becomes active when reviewed in conjunction with the review and approval of an NDA.

The USP/NF provides a listing of excipients by categories in a table according to the function of the excipient in a dosage form, such as tablet binder, disintegrant, and such. An excellent reference for excipient information is the *Handbook of Pharmaceutical Excipients* (APhA, 2007). Additionally, Gad

et al. (2006) provide an excellent and extensive database of nonclinical formulation components and either acceptable maximum usage levels by species route and duration of study.

Excipients have historically not been subjected to extensive safety testing because they have been considered a priori to be biologically inactive and therefore nontoxic. Many, if not most, excipients used are approved food ingredients, the safety of which has been assured by a documented history of safe use or appropriate animal testing. Some of the excipients are generally recognized as safe (GRAS) food ingredients. The excipient is an integral component of the finished drug preparation and, in most countries, is evaluated as part of this preparation. There has been no apparent need to develop specific guidelines for the safety evaluation of excipients, and most developed countries do not have specific guidelines. However, as drug development has become more complex and/or new dosage forms have developed, improved drug bioavailability has become more important. It was noted that the available excipients were often inadequate, new pharmaceutical excipients specifically designed to meet the challenges of delivering new drugs were needed, and these are being developed. The proper safety evaluation of new excipients has now become an integral part of drug safety evaluation.

In the absence of official regulatory guidelines, safety committees of the IPEC in the United States, Europe, and Japan developed guidelines for the proper safety evaluation of new pharmaceutical excipients (IPEC, 1997). The committees critically evaluated guidelines for the safety evaluation of food ingredients, cosmetics, and other products as well as textbooks and other appropriate materials. Before initiating a safety evaluation program for a new pharmaceutical excipient, it is advisable to address the following:

1. Chemical and physical properties and functional characterization of the test material
2. Analytical methods that are sensitive and specific for the test material and that can be used to analyze for the test material in animal food used in the feeding studies or in the vehicle used for other studies
3. Available biological, toxicological, and pharmacological information on the test material and related materials (which involves a thorough search of the scientific literature)
4. Intended conditions of use, including reasonable estimates of exposure
5. Potentially sensitive segments of the population

As discussed in Chapter 1, a comprehensive and critical search of the scientific literature on the test material and related materials is essential before the start of any testing program.

As pharmaceutical excipients are assumed to be biologically nonreactive, dose–response relations cannot always be established. An acceptable alternative is to use a maximum attainable or maximum feasible dose. This is the

highest dose possible that will not compromise the nutritional or health status of the animal. Table 5.11 summarizes the maximum or limit doses for various types of studies by different routes of exposure. For example, 2000 mg kg^{-1} body weight of an orally administered test material is the maximum dose recommended for a testing strategy that has been developed for new pharmaceutical excipients that takes into consideration the physicochemical nature of the product and the potential route(s) and duration of exposures, both through its intended use as part of a drug product and through workplace exposure during manufacturing. The number and types of studies recommended in this tiered approach are based on the duration and routes of potential human exposure. Thus, the longer the exposure to the new pharmaceutical excipient, the more studies are necessary to assure safety. Table 5.10 summarizes the entire set of toxicological studies recommended for new pharmaceutical excipients (Wiener and Katkoskie, 1999; IPEC, 1997).

Tests have been outlined for each exposure category to assure safe use of the time period designated. The tests for each exposure category assure the safe use of the new pharmaceutical excipient of the time frame specified for the specific exposure category. Additional tests are required for longer exposure times.

The base set required for all excipients is detailed in Table 5.12. These are sufficient, however, only for those excipients intended for use for up to two weeks in humans.

TABLE 5.11 Limit Doses for Toxicological Studies

Nature of Test	Species	Limit Dose[a]
Acute oral	Rodent	2000 mg kg^{-1} bw
Acute dermal	Rabbit Rat	2000 mg kg^{-1} bw
Acute inhalation[b]	Rat	5 mg L^{-1} air for 4 h or maximum attainable level under conditions of study
Dermal irritation	Rabbit	0.5 mL liquid 0.5 g solid
Eye irritation	Rabbit	0.1 mL liquid 100 mg solid
14-day/28-day oral repeated dosing; 90-day subchronic	Rodent, nonrodent	1000 mg kg^{-1} bw^{-1} day
14-day/28-day oral repeated dosing; 90-day subchronic	Rat, rabbit	1000 mg kg^{-1} bw^{-1} day
Chronic toxicity, carcinogenicity	Rats, mice	5% maximum dietary concentration for nonnutrients
Reproduction	Rats	1000 mg kg^{-1} bw^{-1} day
Developmental toxicity (teratology)	Mice, rats, rabbits	1000 mg kg^{-1} bw^{-1} day

[a]mg kg^{-1} bw, milligrams of test material dosed per kilogram of body weight to test species.

[b]Acute inhalation guidelines that indicate this limit dose are U.S. Environmental Protection Agency Toxic Substance Health Effect Test Guidelines, Oct. 1984; (PB82-232984) Acute Inhalation Toxicity Study; the OECD Guidelines of the Testing of Chemicals, Vol. 2, Section 4; Health Effects, 403, Acute Inhalation Toxicity Study, May 12, 1982; and the *Official Journal of the European Communities*, L383A, Vol. 35, Dec. 29, 1992, Part B.2 (adapted from Wiener and Katkoskie, 1999).

TABLE 5.12 Base Set Studies for Single Dose Up to 2 Weeks Exposure in Humans

Test	Purpose
Acute oral toxicity	To determine potential acute toxicity–lethality following single oral dose
Acute dermal toxicity	To determine potential acute toxicity–lethality following single dermal dose
Acute inhalation toxicity	To determine potential acute toxicity–lethality following single 4-h inhalation exposure to test atmosphere containing new pharmaceutical excipient (aerosol, vapor, or particles)
Eye irritation	To determine potential to produce acute irritation or damage to eye
Skin irritation	To determine potential to produce acute irritation or damage to skin
Skin sensitization	To determine potential to induce skin sensitization reactions
Ames test	To evaluate potential mutagenic activity in bacterial reverse mutation system with and without metabolic activation
Micronucleus test	To evaluate clastogenic activity in mice using polychromatic erythrocytes
ADME—intended route	To determine extent of absorption, distribution, metabolism, and excretion by intended route of exposure following single dose and repeated doses
28-Day toxicity—intended route	To assess repeated-dose toxicity in male and female animals of two species following dosing for 28 days by intended route of exposure

TABLE 5.13 Studies for Intermediate Duration (28 Day to 3 Months) Exposure to Humans

Test	Purpose
90-Day Toxicity—intended route (Rodent and nonrodent)	To assess the repeated-dose toxicity in male and female animals of two species following daily dosing for 90 days by the intended route of administration
Developmental Toxicity	To assess the effects of dosing of pregnant female animals by the intended route during the period of organogenesis

If exposure to the new pharmaceutical excipient is expected to occur for longer than two but no more than six weeks, additional toxicological studies are required, as shown in Table 5.13. The longer the expected human exposure, the more extensive will be the toxicological studies to assure safety. A tiered approach assures that those tests necessary to ensure safety for the expected duration of human exposure are conducted. Thus, to assure safe use for greater than two weeks but no more than six weeks in humans, subchronic toxicity and developmental toxicity studies are required. To assure safe use for greater than six continuous weeks, chronic or oncogenicity studies are conditionally required, as per Table 5.14. This means long-term studies should be considered for prolonged human exposures but may not be absolutely required. A thorough scientific review of the data generated in the base set and Appendix 2 studies should be undertaken. From a critical evaluation by a competent toxi-

TABLE 5.14 Appendix 3 Studies for Repeated Chronic Exposure in Humans

Test	Purpose
Chronic toxicity	To assess toxicity following chronic (lifetime) exposure by route of intended exposure
Oncogenicity	To assess potential to induce tumors by intended route of exposure
One-generation reproduction	To assess potential reproductive and developmental toxicity in males and females by intended route of exposure

cologist, the results of the physicochemical properties of the test material, the 28- and 90-day tests, the ADME-PK (absorption distribution metabolism and excretion pharmacokinetics) acute and repeated-dose tests, and the developmental toxicity test(s), a final determination can be made on the value of chronic toxicity or oncogenicity studies.

For example, if no toxicity is observed at a limit dose of 1000 mg kg^{-1} body weight per day following the 90-day toxicity study, no genotoxicity was found, and the ADME-PK profile indicates that the material is not absorbed and is completely excreted unchanged in the feces, then it is likely that a chronic study is not necessary. The decision to conduct chronic studies should be determined on a case-by-case basis using scientific judgment. It will be interesting to observe how this scheme may change in light of the International Conference on Harmonisation (ICH).

APPENDIX PRIMARY POTENTIAL FORMULATION COMPONENTS FOR NONCLINICAL TOXICITY STUDIES

Excipient/Vehicle	Chemical Abstract Service Number	Chemical Name	Animal Studies
2-Hydroxypropyl-β-cyclodextrin	128446-35-5		Rat, primate, mouse, rabbit, dog
Acacia	9000-01-5	Acaciae gummi	Rat, primate
Acetate, sodium	127-09-3	Acetic acid sodium salt	Rat
Acetic acid	64-19-7	Ethanolic acid	Rat, mouse
Acetone	67-64-1	2-Propanone	Rat, mouse, guinea pig, rabbit
Acetylmethylamine in water	79-16-3	*N*-Methylacetamide	
Alginic acid	9005-32-7	Norgine	Rat

Excipient/Vehicle	Chemical Abstract Service Number	Chemical Name	Animal Studies
Anecortave acetate	7753-60-8	—	Rat
Benzoic acid	65-85-0	Benzoic acid	Rat
β-Cyclodextrin	7585-39-9	β-Dextrin	Rat, primate
BHT	128-37-0	Butylated hydroxytoluene	
Canola oil	120962-03-0	Canbra oil	Dog
Capryol 90	31565-12-5	Propylene glycol monocaprylate	Rat, dog, rabbit
Captisol	182410-00-0	β-Cyclodextrin sulfobutyl ether, sodium salt (CDSBE)	Rat, primate, mouse
Carboxymethylcellulose (CMC)	9000-11-7	acetic acid; 2,3,4,5,6-pentahydroxyhexanal	Primate, rat
Carboxymethylcellulose calcium	9050-04-8	Calcium CMC	Dog
Carboxymethylcellulose sodium	9004-32-4	Carmellose sodium	Rabbit
Cavasol W7	128446-35-5	2-Hydroxypropyl cycloheptaamylose	
Cetyl alcohol	36653-82-4	Hexadecan-1-ol	Mouse
Citrate buffer	77-92-9	sodium citrate–citric acid buffer	Dog, rat
Citric acid buffer	77-92-9		Rat
CMC with dimethicone	9004-32-4 9006-65-9	Carboxymethylcellulose sodium trimethyltrimethylsilyloxysilane	
Coconut oil	8001-31-8	N/A	
Collagen matrix	9007-34-5	Collagen human	Primate, rabbit
Corn oil	8001-30-7	corn germ oil, glyceridic	Dog, rat, mouse, rabbit, chick embryo
Cremophore EL	61791-12-6	Polyoxyl castor oil	Dog, rat
Cyclohexane	110-82-7	Hexahydrobenzene; hexamethylene; hexanaphthene	Rat, rabbit
DAM PEG (polyethyllene Glycol)			Dog, rat
Dextrose	50-99-7	D-Glucose, anhydrous; dextrosol	Dog, rat
Diethyleneglycolmonoethylether	111-90-0		Primate
DMSO	67-68-5	Dimethylsulfoxide	Dog, rat, guinea pig, primate, mouse, rabbit
Dulbecco's modified PBS			Rat
EDTA	60-00-4	Ethylenediamineetraacetic acid	

Excipient/Vehicle	Chemical Abstract Service Number	Chemical Name	Animal Studies
Ethanol	64-17/5	Ethyl alcohol	Dog, rat, primate, mouse
Gelucire 44/14	121548-04-7	PEG-32 glyceryl laurate	Rabbit, rat, dog
Gelucire 50/13	121548-05-8	G-50-13	Rabbit, rat, dog
Glucose	50-99-7	Dextrose	Dog, rat, primate
Glycerol	56-81-5	Glycerine	Rat, guinea pig, mouse, rabbit
Gum tragacanth	9000-65-1		Mouse
Gum xanthane	11138-66-2		
Hydroxypropyl β-cyclodextrin	94035-02-6		Dog, rat
Hydroxypropyl cellulose	9004-64-2	Methocel	Rat
Hydroxypropyl methylcellulose	9004-65-3	Benecel MHPC, hypromellose	Dog, rat, mouse
Isopropyl alcohol	67-63-0	*sec*-Propyl alcohol	Rabbit
Isopropyl myristate	110-27-0	Crodamol IPM	Rabbit
Labrafil M1944	62563-68-2	Labrafil	Dog
Labrasol	85536-07-8	Polyglycolyzed glycerides	Rat, dog, rabbit
Lauroglycol	27194-74-7	Lauric acid, monoester with propane-1,2-diol	Rabbit, rat
Lactose	63-42-3(anhy)	*O*-β-D-Galactopyranosyl-(1->4)-α-D-glucopyranose	Primate
Lanolin	8006-54-0	Lanolin	Rabbit
L-Ascorbic acid	50-81-7	Cevatine, Cevex, Cevital	Rat
Maltitol solution	9053-46-7	Liquid maltitol	Rat
Maltol	118-71-8	3-Hydroxy-2-methyl-4*H*-pyran-4-one	Guinea pig, rabbit
Mannitol	69-65-8	D-Mannitol	Primate
Methane sulfonic acid	75-75-2	Methylsulfonic acid	
Methyl cellulose	9004-67-5	Cellulose methyl ester	Rat, guinea pig, primate, mouse, rabbit, dog
Miglyol 810	85409-09-2	Caprylic, capric triglycerides	
Mineral oil	8012-95-1	Liquid paraffin	Rat, mouse, dog
Neobee 1053	73398-61-5	Medium-chain triglycerides	
N-Methylpyrrolidone (Pharmasolv)	872-50-4	1-Methyl-2-pyrrolidinone	

Excipient/Vehicle	Chemical Abstract Service Number	Chemical Name	Animal Studies
PBS (phosphate-buffered saline)			Rat, primate, mouse
Peanut oil	8002-03-7	Arachis oil, Fletcher's	Rat
PEG 300	25322-68-3	Polyethylene glycol #300	Guinea pig, mouse, rabbit
PEG 400	25322-68-3	Polyethylene glycol #400	Rat, minipig, guinea pig, mouse
Petrolatum	8009-03-8	Yellow soft paraffin	Rabbit
Poloxamer	9003-11-6	Lutrol	Rat, mouse
Polysorbate 80	9005-65-6	Polyoxyethylene (20) sorbitan monooleate	
Povidone	9080-59-5	2-methoxy-6-methyl-phenol	Rat
Propylene glycol	57-55-6	1,2-Dihydroxypropane	Rat, minipig, mouse, dog
Rameb 7.5%		Randomly methylated-β-cyclodextrins	Primate
Sesame oil	8008-74-0	Sesame oil	Rat, mouse, rabbit, dog
Sodium acetate trihydrate buffer	6131-90-4		Primate
Sodium chloride	7647-14-5	Salt, halite	Dog, rat, primate, mouse, rabbit
Sodium phosphate	7558-80-7		Dog, rat
Solutol® HS15/purified water	70142-34-6	Polyethylene glycol-15-hydroxystearate	
Succinate, sodium	150-90-3	Succinic acid Sodium salt	
Tartaric acid	87-69-4	D-Tartaric acid; 2,3-dihydroxybutanedioic acid	Rat, rabbit
Transcutol	111-90-0	2-(2-Ethoxyethoxy)ethanol	Cat, rabbit, rat
Trisodium citrate dihydrate	6132043		Dog, rat, mouse
Tween 20	9005-64-5	Polysorbate 20 NF	Rat, mouse
Tween 80	9005-65-6	armotan pmo-20, Tween(R) 80	Rat, primate, mouse, dog
Xylitol	87-99-0	Xylite	Primate

Source: Gad et al., 2006.

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6

Single-Dose (Acute) and Pilot (DRF) Toxicity Testing in Drug Safety Evaluation

6.1 INTRODUCTION

Acute toxicity testing is the defining and evaluation of the toxic syndrome (if any) produced by a single or a few doses over the course of a day, such as twice or three times per day (bid or tid, in the case of continuously infused intravenous formulation in a 24-h course of treatment) of a drug. Historically, the main focus of these tests has been lethality determinations and the identification of overt signs and symptoms of overdosage. For a complete historical perspective, see Deichmann and Gerarde (1969), Piegorsh (1989), Auletta (1998), Gad and Chengelis (1999), or Rhodes (2000). A more enlightened and modern view holds that, especially for pharmaceutical agents, lethality in animals is a relatively poor predictor of hazard (other than lethality) in humans (Gad and Chengelis, 1999). The current trend is toward gaining increasing amounts of more sophisticated data from these tests. The various types of acute study designs, their utility in pharmaceutical product testing, their limitation, and the resultant sample data are discussed in this chapter.

For new product approvals (and first in human clinically trials), single-dose toxicity studies are required by regulatory authorities though this requirement

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is being challenged in the European communities [Osterberg, 1983; U.S. Food and Drug Administration (FDA), 1996, 2006]. In the pharmaceutical industry, acute toxicity testing has uses other than simply product safety determinations. First, as in other industries, acute toxicity determinations are part of industrial hygiene or occupational health environmental impact assessments. These requirements demand testing not only for finished products but frequently of intermediates as well. These issues and requirements, however, are discussed in that content in Chapter 2 and are not directly addressed here.

Another use, now almost abandoned except in natural product–derived drugs (Pendergast, 1984), is in quality control testing or batch release testing. The latter was once a mandated part of the standardization process for antibiotics, digoxin, and insulin in the U.S. Pharmacopeia. While, perhaps, this type of testing is part of a broad safety picture, it is not typically part of a "preclinical" safety package used to make decisions on whether to market a new chemical entity or on what the allowable clinical dosage shall be. These uses also therefore are not discussed here. The emphasis in this volume, rather, is on tests used to elucidate the toxicity of new chemical entities, not the safely of finished drug preparations. These tests fall into three general categories: (1) range-finding studies, used primarily to set dosages for initial subchronic or acute testing; (2) complete "heavy" or expanded acute toxicity tests, used to thoroughly describe the single-dose toxicity of a chemical or to support the opening of an explanatory or phase-zero investigational new drug (IND); and (3) screening tests, used to select candidates for development.

6.2 RANGE-FINDING STUDIES

Range finders or pilots [now also commonly called dose range finders (DRFs)] are not normally done completely under the auspices of the Good Laboratory Practices Act. They are not used to generate data to support decisions on human safety; rather, they are used to allow successful dose selection for definitive toxicity studies. These dosage-level determinations can be used in acute studies, in in vivo genotoxicity studies, or subchronic studies. As discussed by Gad and Chengelis (1999), however, there can be a great deal of difference between the acute toxic dosage and subchronic daily dosage of a drug. Therefore, acute range-finding studies currently most common include a component (or second phase) whereby a second set of animals will receive a short-term treatment (up to seven days) with the drug in question. Accordingly, the definition of "acute" in this chapter is stretched to include "subacute" (lower than acute level) dosing of very short duration.

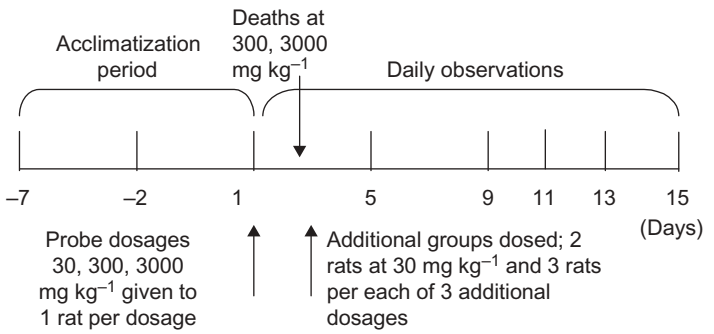
6.2.1 Lethality Testing

Often, in range-finding tests, the endpoint is simply to determine the maximum dosage of a drug that can be given without killing an animal. There are numer-

ous designs available for obtaining this information that minimize the expenditure of animals and other resources.

Classical LD_{50} The median lethal dose (LD_{50}) test has a rich and controversial history (it is one of a number of tests that raises the ire of the animal welfare movement) (Trevan, 1927; Rowan, 1981; LeBeau, 1983). In pharmaceutical development, however, there is rarely a need or requirement for an LD_{50}. In general, a complete, precisely calculated LD_{50} consumes more resources than is generally required for range-finding purposes. The reader is referred to Chapter 7 of Gad and Chengelis (1999) for a complete discussion of this test.

Dose Probes Dose probe protocols (see Figure 6.1) are of value when one needs the information supplied by a traditional protocol but has no preliminary data from which to choose dosages. In this acute protocol, one animal is dosed at each of three widely spaced dosages, where the top dosage is generally the maximum deliverable. The method works best if the dosages are separated by constant multiples (e.g., 3000, 300, and 30 mg kg^{-1}—a logarithmic progression). Subsequent dosages are selected on the basis of the results from these probe animals. If none of these animals dies, the protocol defaults to a limit test (described below), and two more animals are dosed at the top dosage to confirm the limit.



Results

Dosage	Mortality
30 mg kg^{-1}	0/3
60	1/3
120	2/3
240	2/3

LD_{50} = 115 mg kg^{-1} (moving-average method)

Figure 6.1 *Example of typical dosage probe protocol.*

A dose probe can develop into a more thorough lethality determination. If one or two animals die, then two additional dosages between the lethal and nonlethal dosages are chosen and three animals are treated per dosage for defining acute lethality. Selection of these dosages is often a matter of personal judgment. If, for example, one wishes to apply the moving-average method of calculation, these subsequent dosages can be either even fractions of the top dosage or even multiples of the low dosage. In either case, two to three animals are dosed at the initial dose and three to four animals are dosed at each of the two to three new dosages. The results should be three to four groups of three to four animals each, which should probably provide sufficient data for calculating the LD_{50} and the slope of the curve. Probing can also be used to define the dosages for subchronic tests. Instead of selecting additional doses for an acute study, one can use the results from the probe to select two dosages for a short (e.g., five-day) daily dosing regimen (see the later section entitled "'Rolling' Acute Tests").

In a few instances, all the animals may die following the first day of dosing. In that case, the probe activity continues on day 2 with two more animals dosed at two widely spaced lower dosages (i.e., 3 and 0.3 mg kg^{-1}). This regimen could continue daily until a nonlethal dosage is identified. Unless one has grossly misestimated the toxicity of the test substance, it is unlikely that the probing process would take more than three days. Carrying our example into three days of dosing would have resulted in probing in the range 3 µg kg^{-1}–3 g kg^{-1}, and it is a rare chemical that is lethal at less than 3 µg kg^{-1}. Once a nonlethal dosage is identified, additional animals and/or dosages can be added, as discussed above.

There are two disadvantages to dose probe studies. First, delayed deaths pose difficulties. Hence, all animals should be observed for at least seven days after dosing (though most deaths occur within three days). Second, if the follow-up dosages are not lethal, the next decision point is ill defined. Should more animals be dosed at some different dosage? The resulting data sets may be cumbersome and difficult to analyze by traditional statistical methods. Alternatively (and this is true regardless of protocol design), if no "partial response" (mortality greater than zero but less than 100%) dosage is identified, one can simply conclude that the LD_{50} is between two dosages, but the data do not permit the calculation of the LD_{50} or the slope of the curve. This can happen if the dosage response is fairly steep.

Lörke (1983) has developed a similar protocol design. His probe (or dose range) experiment consists of three animals per dosage at 10, 100, and 1000 mg kg^{-1}. The results of the experiment dictate the dosages for the second round of dosing, as shown in Table 6.1. Animals were observed for 14 days after dosing. Lörke (1983) compared the results obtained when one to five animals were used per dosage group for the second test. He concluded that using only one animal per group gives unreliable results in only 7% of chemicals tested. Hence, the Lörke design can produce reasonable estimates of lethal dosages using 14 or fewer animals. Schutz and Fuchs (1982) have pro-

TABLE 6.1 Dosage Selection for Two-Step Dose-Probing Protocol Design

Mortality by Dose[a]			Dosages ($mg\,kg^{-1}$) for Definitive Experiment as Determined by Results of Probe			
$10\,mg\,kg^{-1}$	$100\,mg\,kg^{-1}$	$1000\,mg\,kg^{-1}$				
0/3	0/3	0/3		1600	2900	5000
0/3	0/3	1/3	600	1000[b]	1600	2900
0/3	0/3	2/3	200	400	800	1600
0/3	0/3	3/3	140	225	370	600
0/3	1/3	3/3	50	100[b]	200	400
0/3	2/3	3/3	20	40	80	160
0/3	3/3	3/3	15	25	40	60
1/3	3/3	3/3	5	10[b]	20	40
2/3	3/3	3/3	2	4	8	16
3/3	3/3	3/3	1	2	4	8

[a]Number of animals that died/number of animals used.
[b]The results from the probe is inserted for these doses.
Source: Lörke, 1983.

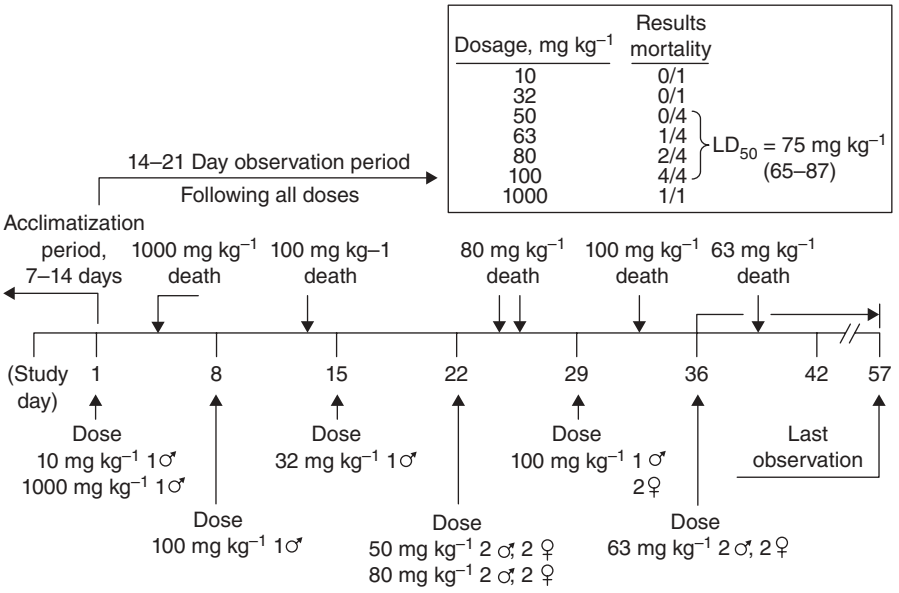


Figure 6.2 *Example of dose probe method with delayed deaths. From Schultz and Fuchs (1982).*

posed a dose probe protocol that adequately deals with delayed deaths (Figure 6.2). All animals are observed for seven days before subsequent dosages are given. Dosing is initiated at two widely delivered dosages using one rate for each dosage. A third probe dosage is determined pending the outcome of the first two probes. A fourth may also be used. After that groups of three to four

animals are used at subsequent dosages either as part of a "para-acute" dosing regimen to select or confirm dosages for a subchronic study or to continue with the definition of an acute lethality curve.

Up/Down Method Using classical or traditional acute lethality protocols, 15–30 animals per curve may be required to calculate a singe LD_{50}. This is because the method relies on the analysis of group responses. The up/down method can provide lethality information by analyzing the responses on an individual animal basis using appropriate statistical maximum-likelihood methods (Bruce, 1985). Deichmann and LeBlanc (1943) published an early method that provided an estimate of lethality using no more than 6 animals. All animals were dosed at the same time. The dosage range was defined as 1.5 times a multiplication factor (e.g., 1.0, 1.5, 2.2, 3.4, 5.1 mL kg^{-1}). The approximate lethal dose (ALD), as they defined it, was the highest dose that did not kill the recipient animal. The resultant ALD differed from the LD_{50} (as positive calculated by the probit method from more complete data sets) by –22 to +33%.

The Deichmann method proved to be too imprecise (Muller and Kley, 1982). Later, Dixon and Wood (1948), followed by Brownlee et al. (1953), developed the method in which one animal was exposed per dosage, but subsequent dosages were adjusted up or down by some constant factor depending on the outcome of the previous dosage. In this method (Figure 6.3), which has been developed more extensively by Bruce (1985), individual animals are dosed at different dosages on successive days. If an animal dies, the dosage for the next animal is decreased by a factor of 1.3. Conversely, if an animal lives, the next dosage is increased by a factor of 1.3. The process is continued until

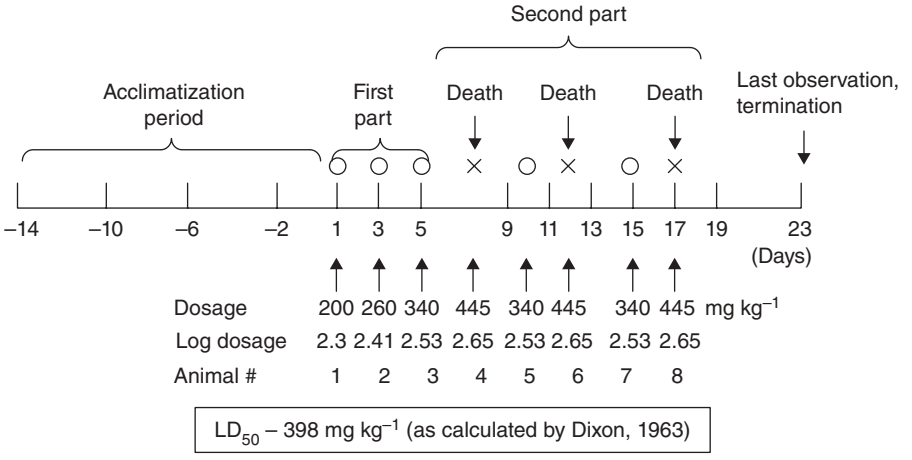


Figure 6.3 *Example of typical up/down acute lethality protocol.*

five animals have been dosed after a reversal of the first observation. Alternatively, one can use the tables developed by Dixon (1965). This design can be used not only for range-finding purposes but also to define an LD_{50} if this value is needed. In general, only six to nine animals are required—unless the initial dosages are grossly high or low. When compared to the LD_{50} obtained by other more classical protocols, excellent agreement is obtained with the up/down method (Bruce, 1985). As with classical protocols, sexes should be tested separately. However a further reduction in the numbers of animals used can be accomplished if one is willing to accept that females are of the same or increased sensitivity as males, as is the case approximately 85–90% of the time (Gad and Chengelis, 1999).

There are three main disadvantages to using the up/down method. The first is regulatory, the second procedural, and the third scientific. First, many regulatory guidelines simply have a requirement for the use of traditional protocols. Some also specify the method of calculation. Second, the sequential dosing design is inappropriate for substances that cause delayed deaths. As reported by various authors (Gad et al., 1984; Bruce, 1985), delayed deaths (beyond two days after dosing) are rare but not known. They are most prevalent when animals are dosed by the intraperitoneal route with a chemical that causes peritonitis. Death secondary to severe liver or gastrointestinal damage may also take over two days to occur. To guard against possible spurious results, all animals should be maintained and observed for at least seven days after dosing. If delayed deaths occur, the original data set must be corrected and the LD_{50} recalculated. A substantial number of delayed deaths could result in a data set from which an LD_{50} cannot be calculated, in which case the test should be rerun.

"Pyramiding" Studies Using this type of design (Figure 6.4), one can obtain information about lethality with the minimum expenditure of animals. A minimum of two animals are dosed throughout the study, usually on alternate days (e.g., Monday, Wednesday, and Friday), but the dosage at session may be 1, 3, 10, 30, 100, 300, 1000, and 3000 $mg\,kg^{-1}$ or 10, 20, 40, 80, 160, 320, 640, and 1280 $mg\,kg^{-1}$. One is literally stepping up, or pyramiding, the lethality–dosage curve. Dosing continues in this fashion until one or both animals die or until some practical upward limit is reached. For drugs, there is no longer a need to go higher than 1000 $mg\,kg^{-1}$ for rodents or nonrodents. An alternative, but similar, design is the "leapfrog" study (Figure 6.5). This consists of two groups of two animals each. They are dosed on alternating days, but the dosages are increased each day. Extending the example of the pyramiding regiment, group 1 would receive 10, 60, and 120 $mg\,kg^{-1}$, while group 2 would be given 30, 100, and 120 $mg\,kg^{-1}$. This design is of value when one has to complete the range-finding activity in a short period of time. Because these designs utilize few animals, they are commonly used for assessing lethality in nonrodent species. An exploratory study typically uses an animal of each sex.

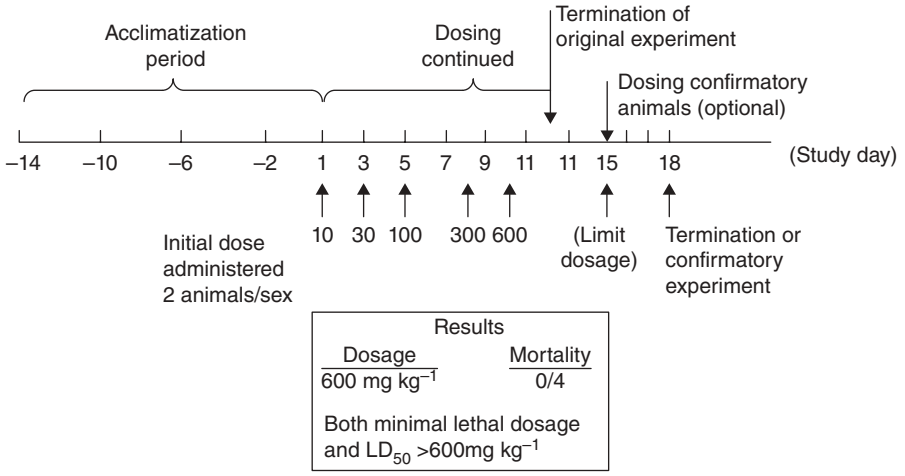


Figure 6.4 *Example of typical pyramiding dose protocol.*

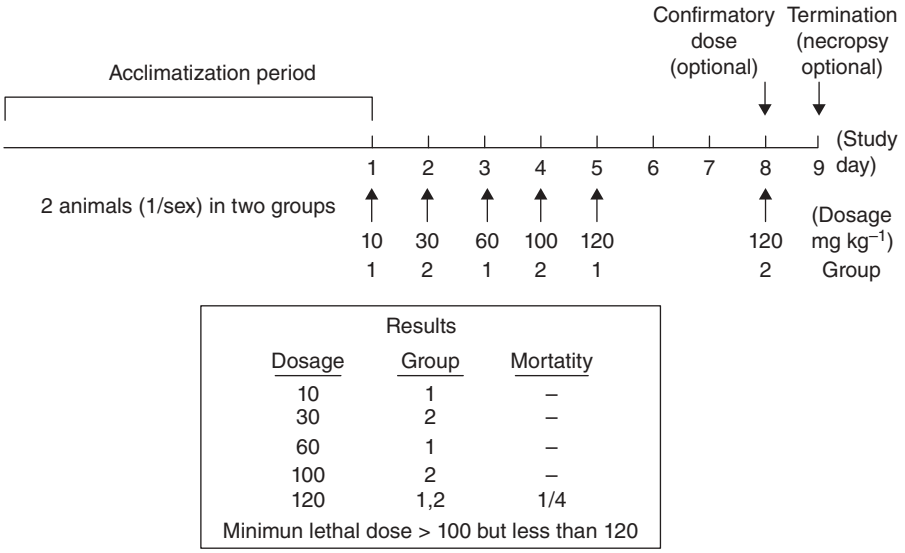


Figure 6.5 *Example of typical "leapfrog" dosing protocol.*

There are three conclusions that can be reached on the basis of data from a pyramiding dosage study. First, if none of the animals die, then both the threshold or minimum lethal dosage (MLD) and the LD_{50} are greater than the top or limit dosage. Second, if all animals die at the same dosage, then both the MLD and the LD_{50} are reported as being between the last two dosages given. This not uncommon finding is an indication that the lethality curve has a steep slope. Third, one animal may die at one dosage, and remaining deaths

occur at a subsequent dosage. In this care, the MLD is between the lowest nonlethal dosage and the dosage at which the first death occurred, while the LD_{50} is reported as being between this latter dosage and the dosage at which the last animal dies. A frequently employed variation with nonrodents is, if lethality is not observed, the animals are dosed for five or seven consecutive days at the highest observed tolerated dose. This "phase B" study portion serves to provide more confidence in selecting the top dose in subsequent repeat-dose studies.

There are some disadvantages to the pyramiding dose protocol. First, it cannot produce a lethality curve or provide for the calculation of an LD_{50}. Second, this method cannot identify delayed deaths. If an animal, for example, dies 1 h after the second dosage, one has no way of determining whether it was actually the second dosage or a delayed effect of the first. For this reason it is of little value to observe the animals for any more than a few days after the last dosage. Third, if the test article has an unusually long half-life, bioaccumulation can lead to an underestimation of the acute lethal dosage. By contract, the pharmacological accommodation can lead to a spuriously high estimate of lethality. Depending on the importance of the finding, one may want to confirm that the results obtained at the highest dosage administered were dosing two naïve animals at the same dosage. Fortunately, the minimum 48-h period between dosing sessions will minimize such effects. Because of this design feature, it may take as long as three weeks to complete the dosing sequence. However, as there is generally no need for a one- to two-week postdosing observation or holding period, the actual study may not take significantly more time than a test of more traditional design.

Keep in mind that the objective of such studies is to gain information about lethality and gross tolerance. For nonrodents (especially monkeys), if none of the animals die or demonstrate obvious signs of toxicity, little would be gained by euthanizing and necropsying such animals. They can be saved and used again, following a reasonable "washout" period, to assess the lethality, toxicity, or safety pharmacology of a different chemical. In the hands of a skilled toxicologist, such adaptive reuse of animals is a cost-effective way to minimize overall usage.

Limit Tests There are relatively innocuous drugs that are simply not potently lethal. The limit test (Figure 6.6) provides the simplest protocol for determining the lethality or such substances. The limit test is designed to obtain clearance at a specific dosage based on the assumption that what may occur at a higher dosage is not of practical relevance. Thus, one dosage only is studied. This limit "dosage" can be set on the basis of the chemical or physical properties of the test article (or vehicle) or on the basis of an upward safety margin. If the preparation is highly acidic (pH < 3), large intravenous dose would be expected to cause systemic acidosis as well as local irritation but will yield little relevant toxicology information, as such a preparation would never be approved for clinical use. Alternately, if the anticipated human dosage of a

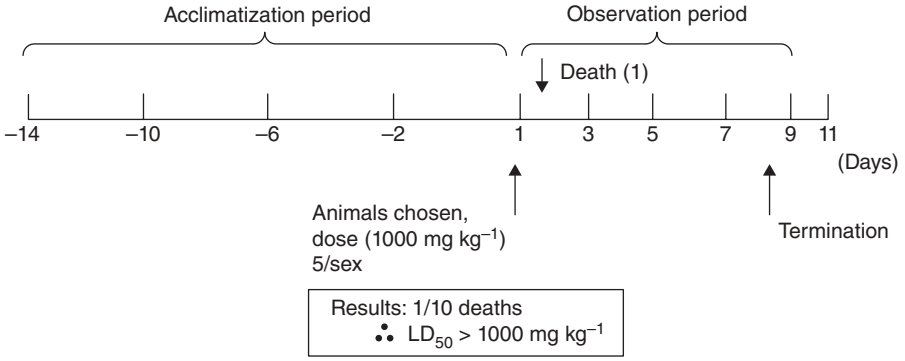


Figure 6.6 *Example of typical limit test protocol.*

drug is 0.3 mg kg^{-1}, there is probably little reason to test dosages in excess of 300 mg kg^{-1} (1000 times the expected human dosage). In general, there is never any reason to use dosages of 5 g kg^{-1} or greater and rarely any reason to exceed 3 g kg^{-1}.

There are three possible outcomes to a limit test. If none of the animals die, then the conclusion is that the MLD is greater than the limit dosage. If fewer than 50% of the animals die, then the conclusion is that the LD_{50} is greater than the limit dosage. If more than 50% of the animals die, then one has a problem. Depending on the reasons for performing the test, one could reset the limit and repeat the study or one could assess lethality by a different protocol. Alternatively, the change in the limit could reflect a change in the chemical or biological properties of the test substance that should be evaluated further.

Fixed-Dose Procedure The fixed-dose design (Figure 6.7) was proposed by the British Toxicology Society (1984). It is designed to supply the data needed for classification or labeling purposes. It is essentially a three-step limit test.

Five rats per sex are given 50 mg kg^{-1}. If survival is less than 90%, a second group of animals is given 5 mg kg^{-1}. If survival is again less than 90%, the substance is classified as "very toxic"; otherwise, it is classified as "toxic."

If, after the 50-mg kg^{-1} dose, survival is 90% but there is evident toxicity, no further dosages are given and the substance is classified as "harmful." If, on the other hand, there is no evident toxicity at 50 mg kg^{-1}, another group of rats is given 500 mg kg^{-1}. If there is again 90% survival and no evident toxicity, the substance is given "unclassified" or "slightly toxic" status.

The fixed-dose procedure is relatively new and apparently results in a large decrease in animal usage. It is also noteworthy in that it utilizes not only lethality but also "evident toxicity," which, in all likelihood, refers to obvious signs of central nervous system (CNS) effect, such as seizures or prostration. Whether or not this protocol design becomes widely accepted by various regulatory agencies remains to be established.

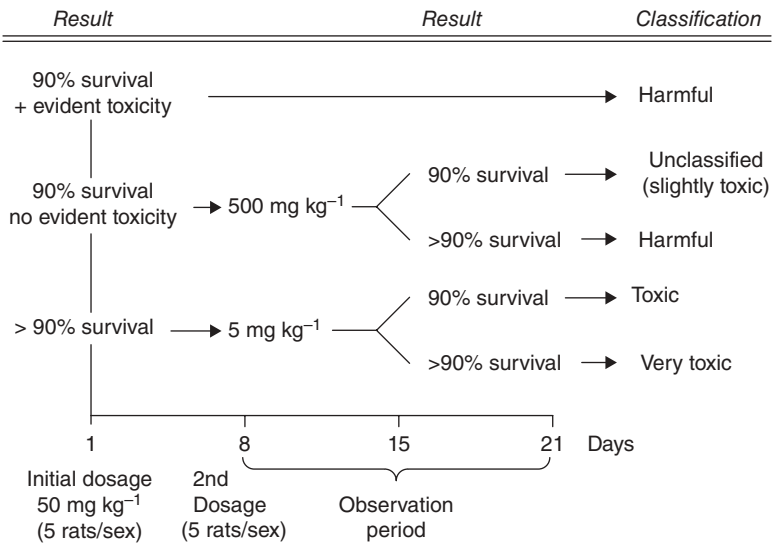


Figure 6.7 *British Toxicology Society fixed-dose procedure.*

The potential utility of the fixed-dose procedure was demonstrated in an international validation study in which the acute oral toxicity of 20 different chemicals was evaluated using both the fixed-dose and classical LD_{50} procedures. Thirty-three laboratories in 11 different countries were involved in the validation project, and the results have been published (van den Heuvel et al., 1990). The results demonstrated that the fixed-dose procedure produced consistent evaluations of acute toxicity that were not subject to significant interlaboratory variation and provided sufficient information for hazard identification and risk assessment based on signs of toxicity (clinical signs, time to onset, duration, outcome, etc.). The fixed-dose procedure used fewer animals than the classical LD_{50} tests and generally required less time to complete. Because of the emphasis on toxicity (rather than mortality) and the use of fewer animals, the fixed-dose procedure could be considered a more "humane" or animal-sparing design than the classical LD_{50} test. When the results of the fixed-dose and LD_{50} tests were compared for hazard-ranking purposes (Table 6.2), comparable results were obtained. Thus, it would appear that the fixed-dose procedure has utility and has been recommended late in 2000 for broad regulatory adaptation by ICVAM (Interagency Coordinating Committee on the Validation of Alternative Methods).

"Rolling" Acute Test The rolling acute test is a combination protocol that is designed to find a tolerated dose to use for a subchronic toxicity test. The first segment can be either a dose probe or an up/down or pyramiding type of study to define the MLD. In the second segment, three to five animals are dosed for a short period of time—five to seven days. The objective of this

TABLE 6.2 Comparison of Toxicity Classification Based on LD_{50} versus Fixed-Dose Procedure

Test Chemical	Toxicity Classification Based on LD_{50}	Fixed Dose: Number of Laboratories Classifying Chemical			
		Very Toxic	Toxic	Harmful	Unclassified
Nicotine	Toxic	—	23	3	—
Sodium	Harmful	—	1	25	—
Ferrocene	Harmful/unclassified	—	—	3	
2-Chloroethyl alcohol	Toxic	—	19	7	—
Sodium arsenite	Toxic	—	25	1	—
Phenyl mercury acetate	Toxic	2	24	—	—
p-Dichlorobenzene	Unclassified	—	—	—	26
Fentin hydroxide	Toxic	—	8	17	1
Acetanilide	Harmful	—	—	4	22
Quercetin dihydrate	Unclassified	—	—	—	26
Tetrachlorvinphos	Unclassified	—	—	1	25
Piperidine	Harmful	—	2	24	—
Mercuric chloride	Toxic	—	25	1	—
1-Phenyl-2-thiourea	Toxic/harmful	12	12	2	—
4-Aminophenol	harmful	—	—	17	9
Naphthalene	Unclassified	—	—	—	26
Acetonitrile	Harmful	—	—	4	22
Aldicarb (10%)	Very toxic	22	—	—	—
Resorcinol	Harmful	—	—	25	1
Dimethyl formamide	Unclassified	—	—	—	26

Source: van der Heuvel et al., 1990.

design is to compensate for the fact that cumulative toxicity can occur at substantial differences in acute and subchronic toxic dosages. One can be easily misled by selecting subchronic dosages based entirely on acute lethality data. An example is a drug tested where it was found that $360\,mg\,kg^{-1}$ was acutely nonlethal and the MLD was $970\,mg\,kg^{-1}$. The dosages selected for the four-week subchronic study were 50, 100, 200, and $400\,mg\,kg^{-1}\,day^{-1}$. The top-dose animals all died within a week. Substantial mortality occurred at $200\,mg\,kg^{-1}$ and evident toxicity was present at $50\,mg\,kg^{-1}$. A no-effect dosage was not identified, so the entire test had to be repeated with a different dosage structure. The rolling acute structure is a quick and relatively simple "sanity" check that permits one to avoid making such mistakes.

6.2.2 Using Range-Finding Lethality Data in Drug Development: Minimum Lethal Dosage

Range-finding data are often used early in drug development to make preliminary safety estimates. The LD_{50} is simply a calculated point on a curve. The

shape or slope of this curve is also an important characteristic of the test substance. However, unless one does a great deal of acute toxicity testing, the difference between a slope of 1.5 and a slope of 4 has very little meaning. Further, for safety considerations, the dosage that kills 50% of the animals is not as important as the dosage at which lethality first becomes apparent (i.e., the threshold dosage or MLD). For example, if the oral LD_{50}s of two different drugs (A and B) were 0.6 and 2.0 g kg^{-1}, respectively, what would we conclude about the relative safety of these compounds? Further, let us assume that the estimated human dosage of drug A is 0.5 mg kg^{-1} and of drug B is 5 mg kg^{-1}. Do our conclusions concerning the relative safety of these two drugs change? In fact, the LD_{50}s of both drugs are so high that both are considered only slightly toxic (0.5–5.0 g kg^{-1}). One can also compute the lethality safety margin or index (LSI, equal to LD_{50}/EHD, where EHD is the estimated human dose) for these two drugs; both indices are so large (1200 for A and 400 for B) that there is still no toxicologically relevant difference between the two drugs. Let us now assume that the lethality curve for substance A is very steep, such that 0.4 g kg^{-1} causes death in a very small percentage of animals—it is, in fact, the lowest dose administered that causes death. This is the MLD or estimated MLD (EMLD). Let us now assume that the lethality curve for B is very shallow, such that its MLD is also 0.4 g kg^{-1}. Does this change our safety considerations of these two drugs? One can calculate a new more conservative safety index (MLD/EHD) of 800 for A and 80 for B. As a very general rule of thumb, an index for lethality of less than 100 is cause for mild concern, one less than 10 is cause for caution, and one less than 1 should be cause for extreme caution. In the case of our two hypothetical drugs, the development of drug B should be approached with more caution than that of drug A, despite the fact that B has a higher LD_{50}. This is demonstrated in Figure 6.8. There are drugs sold over the counter, however, that have lethality safety indices of less than 10. For example, the MLD of indomethacin in rats is 3.7 mg kg^{-1} (from data reported by Schiantarelli and Cadel, 1981), while the maximum recommended human dose is 200 mg (2.9 mg kg^{-1} for a 70-kg person); hence, indomethacin has an LSI of 1.3. Such a finding is only cause for some caution but does not in and of itself justify restricting the use or sale of a drug. Hence, because it results in a more conservative safety factor and also takes into consideration the slope of the lethality curve, the use of the MLD rather than the LD_{50} is recommended in calculating acute safety indices.

A number of different safety factors and therapeutic indices have been proposed in the literature. Despite their similarity, some distinction should be made between these two. A therapeutic index applies only to drugs and is the ratio between a toxic dosage (TD or LD: the toxic endpoint does not always have to be death) and the pharmacologically effective dosage (ED) in the same species. A safety index can be calculated for all xenobiotics, not just drugs. A safety index is the ratio of likely human exposure (or dosage) and the dosage that causes death or other forms of toxicity in the most sensitive experimental animal species. The most conservative (lethality) safety index (LSI) is obtained by dividing the maximum estimated human dosage or

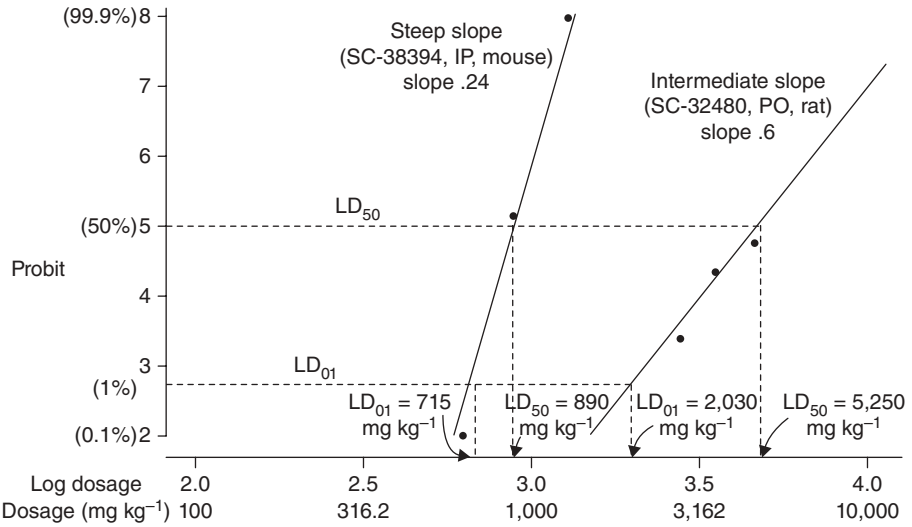


Figure 6.8 *Examples of probit-log dosage–response curves illustrating differences in slope curves and relationship between slope, LD_{50}, and LD_{01}.*

exposure by either the minimum lethal dosage or the maximum nonlethal dosage.

Minimum Lethal Dosage Protocols Stating that the MLD is preferable to the LD_{50} for safety considerations is one thing; trying to determine what a specific MLD may be or could be is another. There are no commonly used experimental designs that have the MLD as an endpoint. Assuming a log dose response, the MLD may become a function of group size. Theoretically, if enough animals are dosed, at least one animal could die at any reasonable dosage. There are, however, practical considerations that can and should be applied to determining an MLD. As a practical rule of thumb, we recommend that the estimated LD_{01}—the dose that would be expected to kill 1% of the experimental animals exposed—be used as an estimate of the MLD. If one already has sufficient data to describe a lethality curve, an LD_{01} can be calculated as easily as the LD_{50}. This is often the case with acute toxicity data obtained to support regulatory submission.

How is the MLD calculated without a complete lethality curve? A modified pyramiding dosage design may be the most appropriate approach. With this design, groups of animals are treated with stepwise increases in dosage until death occurs or a limit dosage is attained. If one has no idea as to what the initial dosage should be or how to graduate the dosages, a dose-probing experiment can be conducted. If the dose-probing experiment produces no deaths, two to three more animals can be dosed at the limit dose to confirm the results; the lethality determination is now complete. If the probe experiment does

produce death, then the additional dosages can be graduated between the lowest lethal and the highest nonlethal dosages. A typical progression may proceed as follows (Figure 6.9): On day 1 of the study, three probe animals are dosed at 10, 100, and 1000 mg kg^{-1}. The animal at 100 mg kg^{-1} dies within a few hours of dosing. The two remaining animals are dosed at 300 mg kg^{-1} on day 3. Neither dies. They are then dosed at 500 mg kg^{-1} on day 5. One dies. Three additional animals should be dosed on day 7 or 8 at a dosage in between (i.e., 400 mg kg^{-1} is a good estimate of the maximum nonlethal dosage, or MNLD). While different by definition, there is usually not a great deal of distance between the MLD and the MNLD, as this example illustrates. In fact, even for a well-characterized lethality curve, the confidence limits for the LD_{01} will be quite broad and encompass both the MLD and MNLD.

Malmfors and Teiling (1983) have proposed a similar method for determining what they also termed the MNLD. Rather than initiating the study with probe animals, their design calls for three consecutive pyramiding-type studies with the steps becoming increasingly smaller. For example, two animals will be sequentially dosed at 2, 200, and 2000 mg kg^{-1}. If death occurs at 2000 mg kg^{-1}, a new pair of animals is initiated at 200 mg kg^{-1}, and sequential dosages are increased by a factor of 1.8 until death occurs. Then another pair of animals is initiated at the highest nonlethal dosage, and successive dosages are increased by a factor of 1.15. The result of this exercise will be two dosages, one apparently nonlethal and the other lethal. Six animals are dosed at each dosage. If none die at the lower dosage and one dies at the higher dose, then the lower dose is considered to be the MNLD. At least 24 h between dosing rounds are recommended. While this method may have some utility, there are some disadvantages. First, the recommended limiting dosage of 6.5 g kg^{-1} is too high. Second, 24 h between doses may be too short a period to allow for recovery. Third, even with only 24 h between doses, this is a time-consuming procedure—it may take up to two weeks to complete the dosing. Finally, it does not decrease the number of animals needed, since it may use 18–20 animals.

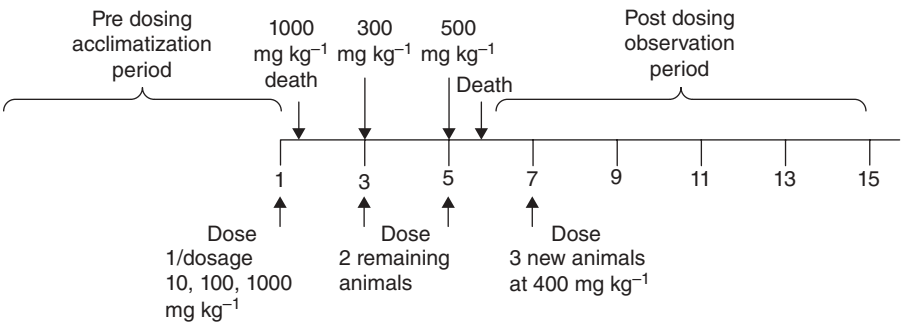


Figure 6.9 *Example of MLD pyramiding dose design.*

Dose probing is not generally used for nonrodents (rather, the pyramiding dose scheme is) and the initiating dosage is normally in the range of 1–5 times the projected human clinical dosage. The limit is generally in the area of 1 g kg^{-1} or 100–200 times the human dosage, whichever is less. The normal study will include two animals of each sex treated with the test article. For simple lethality, there is seldom any need to include control animals. If the projected human dosage is 4 mg kg^{-1}, for example, the initial dosage in an MLD range finder in dogs will be 20 mg kg^{-1} and succeeding dosages will increase stepwise at half-log intervals; thus, 20-, 60-, 200-, and 600-mg kg^{-1} doses are separated by at least 48 h. The MLD is simply reported as being between the highest observable nonlethal and the lowest lethal dosages, or at greater than the limit dosage—in this case, 600 mg kg^{-1}. Studies should not be done with nonrodents solely for determining lethality, because this would not be an appropriate use of time and animals. Generally, these studies should also include some combination of extensive physical examinations, such as electrocardiograms (ECGs) and rectal temperatures, careful observations of behavior and activity, and extensive clinical laboratory workups after each dose.

The pyramiding dose study is not without disadvantages. The small number of animals used can cause simple random variation resulting in misestimation of lethality. It is a well-accepted statistical maxim that the smaller the sample size, the greater the impact of any random variation (error or outlier) on the population characteristic. This may be especially true for a nonrodent species where experimental animals are drawn from an outbred population. Second, the pyramiding dose regimen can permit the development of tolerance. For example, pyramiding dosage studies were conducted to range find dosages for a two-week study on 1,4-benzodiazepine. Lethality in dogs was observed at 600 mg kg^{-1} in the pyramiding study. For the subsequent subchronic study, the top dose was set at 300 mg kg^{-1}; both dogs died of CNS depression of the first day of dosing.

6.3 ACUTE SYSTEMIC TOXICITY CHARACTERIZATION

Acute systemic toxicity studies are performed to more completely define the acute toxicity of a drug. They are more extensive and time consuming than range-finding tests or screens and are normally the type of study done to satisfy regulatory requirements or to provide a more thorough early characterization or prediction of toxicity (McClain, 1983). In pharmaceutical development, rarely would an acute test be sufficient to support registration, but it could support exploratory INDs first in a human single-dose study (FDA, 2006) or a single human dose study of an imaging agent and it may be required as part of an overall package. These protocols may resemble range-finding tests, but they call for collection of more data. A list of the types of data that can be obtained in well-conducted acute toxicity tests is given in Table 6.3. Given that these studies usually include control groups, the classical or tradi-

TABLE 6.3 Information, Including Lethality, That Can be Gained in Acute Toxicity Testing

Lethality/mortality
LD_{50} with confidence limits
Shape and slope of lethality curves
Estimation of maximum nonlethal dose or minimum lethal dose (LD_{01})
Time to dose estimates
Clinical signs
Times of onset and recovery
Thresholds
Agonal vs. nonagonal (i.e., do signs occur only in animals that die?)
Specific vs. general responses
Separation of dose–response curves from lethality curves
Body weight changes
Actual loss vs. decreased gain
Recovery
Accompanied by changes in feed consumption
Changes in animals that die vs. those that survive
Target organ identification
Gross examinations
Histological examinations
Clinical chemical changes
Hematological changes
Specialized function tests
Immunocompetency
Neuromuscular screening
Behavioral screening
Pharmacokinetic considerations
Different routes of administration yielding differences in toxicity
Plasma levels of test article
Areas under the curves, volume of distribution, half-life
Metabolic pattern of test article
Distribution to key organs
Relationship between plasma levels and occurrence of clinical signs

tional design is the most common because it allows for the most straightforward statistical analyses. In addition, while the use of staggered dosing days for different groups is still a fairly common practice, data analyses may be more sensitive if all animals are dosed on the same day, requiring that one have preliminary range finder data that permit selection of appropriate dosages. Studies of more than one species and/or more than one route should be limited to those instances where they are required by statute.

In general, traditionally designed acute toxicity tests can be divided into three types that can be called the minimal acute toxicity test, the complete acute toxicity test, and the supplemented acute toxicity test. Of these, the minimal protocol is by far the most common and is discussed first. The other two represent increasing orders of complexity as additional parameters of measurement are added to the basic minimal study.

6.3.1 Minimal Acute Toxicity Test

An example of a typical minimal acute toxicity test protocol is shown in Figure 6.10. This study resembles a traditional lethality test in terms of the number of groups and the number of animals per group. Standard protocols consist of three or four groups of treated animals and one group of control animals, each group consisting of five animals per sex per dosage (OECD, 1991). Traditionally, the emphasis in these types of studies was on determining the LD_{50}, time to death, slope of the lethality curve, and prominent clinical signs, as illustrated by the data reported by Jenner et al. (1964). More recent designs specify, in addition to lethality and clinical observations, that body weights be recorded during the study and gross necropsies performed at the end of the postdosing observation period. For an excellent example of a well-performed acute toxicity evaluation the reader is referred to the paper by Peterson et al. (1987) of the acute toxicity of the alkaloids of *Lupinus angustifolius*, in which the LD_{50}s, time to death, clinical signs, body weight effect, and gross necropsy findings were all discussed. For pharmaceuticals, where acute toxicity data for more than one species are often required, these studies will be done as batteries on both rats and mice. In addition, because many drugs will be given by more than one route to human patients, these batteries will include groups treated by two different routes. Thus, an acute study on a pharmaceutical agent will often result in eight "curves"—one per route per species per sex. For tests on nonrodent species, as required for pharmaceuticals, a different design is used (discussed later).

The animals should be acclimated to laboratory conditions for 7–14 days prior to dosing. For acute toxicity tests, this pretreatment period should be more than just a holding period. Animals should be checked daily for signs of ill health and/or abnormal behavior. Body weights may also be determined. These data should be used to exclude abnormal animals from the test. Such data also provide an additional basis for interpreting the data gathered during

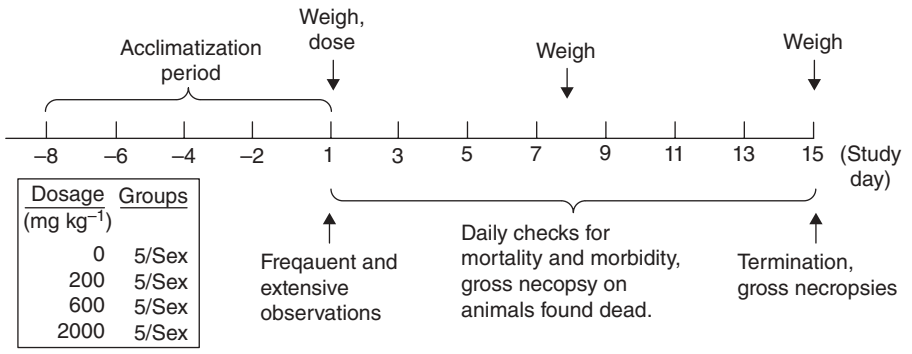


Figure 6.10 *Example of minimal acute toxicity protocol.*

the postdosing period. Finally, these activities acclimate the animals to the frequent handling that is a necessary part of an acute toxicity test.

In selecting dosages for an acute systemic toxicity study, the same general guidelines apply as with lethality testing:

1. There is little to be gained from testing dosages that are so high that the physical rather than biological properties become prominent. Generally, little additional information is gained by pushing dosages past $2\,g\,kg^{-1}$. The usual regulatory limit for pharmaceuticals is now $1.5\,g\,kg^{-1}$.
2. The highest dosage should be no larger than 100–300 times the anticipated human dosage.
3. Widely spaced dosages are better than narrowly spaced dosages.

This latter point is particularly true in an acute toxicity test on a drug, because pharmacologically based clinical signs may occur at dosages considerably lower than those that cause death. Also, as discussed by Sperling (1976) and Gad (1982), the effects at high dosages may mask the effects that would be observed at low dosages. As human beings are more likely to be exposed to lower dosages than experimental animals, these low-dosage effects may be important parameters to define.

Historically, it has been stated in various regulatory communications that a well-conducted acute toxicity test should contain sufficient data to calculate an LD_{50}. This is no longer necessarily the case. Simpler, less resource-intensive range-finding protocols should be used for defining lethality. Because it is rare that an extensive acute protocol would be attempted without preliminary lethality data, the lethality objectives of acute systemic testing are not always critical. Ideally, the highest dosage should elicit marked toxicity (such as lethality), but it does not need to kill all of the animals to satisfy one's need to show due diligence in stressing the test system. If one already has sufficient preliminary data to suspect that the top dosage will be nonlethal or otherwise innocuous, the test can be conducted as a limit test, consisting of one treated group and one control group.

Clinical Signs The nonlethal parameters of acute toxicity testing have been extensively reviewed by Sperling (1976) and Balazs (1970, 1976). Clinical observations or signs of toxicity are perhaps the most important aspect of a minimal acute toxicity test because they are the first indicators of drug- or chemical-related toxicity or morbidity, and they are necessary in the interpretation of other data collected. For example, body weight loss (or a reduction in body weight gain) would be expected if an animal had profound CNS depression lasting several hours.

With regard to clinical signs and observations, there are some basic definitions that should be kept in mind. Symptomatology is the overall manifestation of toxicity. Signs are overt and observable events (Brown, 1983). Symptoms

are the subjective impressions of a human patient (e.g., headache) and cannot be described or reported by speechless animals (Balazs, 1970). Clinical signs can be reversible or irreversible. Reversible signs are those that dissipate as the chemical is cleared from the body or tolerance develops (Chan et al., 1982) and are generally not accompanied by permanent organ damage. Irreversible signs are those that do not dissipate and are generally accompanied by organ or tissue damage. Signs can also represent a normal biological or pharmacological response (Chan et al., 1982). For example, an antidepressant would be expected to cause decreased activity and some ataxia. These symptoms are generally reversible and can lead to secondary, nonspecific signs—nonspecific in that any number of agents or stimuli can evoke the same response and secondary in that they are probably not due (at least, one has no evidence to determine otherwise) to the direct action of the test article. Responses can also be abnormal in that they are not due to a homeostatic process. The increases in serum urea and creatinine due to kidney damage, for example, are abnormal responses. These are often irreversible, but this is not always the case, depending on the repair capacity or functional reserves of the target organ. These abnormal responses may also be called primary effects because they reflect the direct action of a test article. Agonal signs are those occurring immediately prior to or concomitantly with death. They are obviously irreversible, but not necessarily reflective of a specific effect of a test article. For example, regardless of the cause, labored breathing will occur in a moribund animal. It is therefore important to distinguish between signs that occur in animals that die and those that do not. It should also be kept in mind that agonal signs may mask (make it difficult or impossible) to observe other signs, including those clearly seen at lower doses.

In their simplest form, clinical observations are those done on an animal in its cage or, preferably, in an open plane, such as on the top of a counter or laboratory cart. These are considered passive observations. One can gain even more information by active examination of the animal, such as the animal's response to stimulation. Fowler and Rutty (1983) divide their clinical evaluation of toxicity into those signs scored by simple observations (e.g., ataxia), those scored by provocation (e.g., righting reflex), those scored in the hand (e.g., mydriasis), and those scored by monitoring (e.g., rectal temperature). Cage pans should always be examined for unusually large or small amounts of excreta or excreta of abnormal color or consistency. A list of typical observations is summarized in Table 6.4. A more extensive table has been prepared by Chan et al. (1982). Given the fact that the number of different signs displayed is not infinite and that some signs are simply easier to discern than others, most clinical signs are referable to the CNS (e.g., lack of activity), the gastrointestinal (GI) tract (e.g., diarrhea), or the general autonomic nervous system (e.g., increased salivation or lacrimation). This is illustrated by an actual example set of data from acute toxicity studies summarized in Table 6.5.

Other signs can be detected by a well-trained observer but are nonetheless less common than those described above. Respiratory distress can be diag-

TABLE 6.4 Clinical Observations in Acute Toxicity Tests

Organ System	Observation and Examination	Common Signs of Toxicity
CNS and somatomotor	Behavior	Unusual aggressiveness, unusual vocalization, restlessness, sedation
	Movements	Twitch, tremor, ataxia, catatonia, paralysis, convulsion
	Reactivity to various stimuli	Irritability, passivity, anesthesia, hyperesthesia
	Cerebral and spinal reflexes	Sluggishness, absence of reflex
	Muscle tone	Rigidity, flaccidity
Autonomic nervous system	Pupil size	Miosis, mydriasis
Respiratory	Nostrils	Discharge (color vs. uncolored)
	Character and rate	Bradypnea, dyspnea, Cheyne–Stokes breathing, Kussmaul breathing
Cardiovascular	Palpation of cardiac region	Thrill, bradycardia, arrhythmia, stronger or weaker beat
Gastrointestinal	Events	Diarrhea, constipation
	Abdominal shape	Flatulence, contraction
	Feces consistency and color	Unformed, black or clay colored
Genitourinary	Vulva, mammary glands	Swelling
	Penis	Prolapse
	Perineal region	Soiled
Skin and fur	Color, turgor, integrity	Reddening, flaccid skinfold, eruptions, piloerection
Mucous membranes	Conjunctiva, mouth	Discharge, congestion, hemorrhage, cyanosis, jaundice
Eye	Eyelids	Ptosis
	Eyeball	Exophthalmos, nystagmus
	Transparency	Opacities
Others	Rectal or paw skin temperature	Subnormal, increased
	Injection site	Swelling
	General condition	Abnormal posture, emaciation

Source: Balazs, 1970.

nosed by examining the animal's breathing motions and listening for breathing noises. Cardiovascular signs are generally limited to pallor, cyanosis, and/or hypothermia. Changes in cardiac function can be difficult to detect in small animals and generally consist of "weak" or "slow" breathing. Arrhythmias can be difficult to detect because the normal heart rate in a rodent is quite rapid. ECGs are difficult to record from rodents on a routine basis. Therefore, the assessment of potential acute cardiovascular effect of a drug or chemical is usually restricted to a nonrodent species, usually the dog.

Given the subjective nature of recognizing clinical signs, careful steps must be taken to ensure uniformity (is the animal depressed or prostrated?) of

TABLE 6.5 Summary of Clinical Observations from Actual Acute Toxicity Tests

Drug (Route)	Indication	Acute Clinical Signs[a]
SC-37407 (PO)	Analgesic (opiate)	Reduced motor activity, mydriasis, reduced fecal output, hunched posture, convulsions (tonic), ataxia
SC-35135 (PO)	Arrhythmias	Reduced motor activity, lost righting reflex, tremors, dyspnea, ataxia, mydriasis
SC-32840 (PO)	Intravascular thrombosis	Reduced motor activity, ataxia, lost righting reflex, closed eyes, red/clear tears
SC-31828 (PO)	Arrhythmias	Reduced activity, dyspnea, ataxia, lost righting reflex, red/clear tears
SC-25469 (PO)	Analgesic (nonopiate)	Reduced motor activity, ataxia, lost righting reflex, dyspnea, convulsions (clonic)

[a]The five or six most frequent signs in descending order of occurrence.

observation so that the data can be analyzed in a meaningful fashion. There are three ways of achieving this. First, signs should be restricted to a predefined list of simple descriptive terms, such as those listed in Table 6.4 or in Appendix B. Second, if a computerized data acquisition system is unavailable, the use of standardized forms will add uniformity to the observation and recording processes. An example of such a form is shown in Figure 6.11. Third, technicians should be trained in studies (not intended for regulatory submission) using material of known toxicity, so that all personnel involved in such evaluations are using the same terminology to describe the same signs.

Animals should be observed continuously for several hours following dosing. Times of observation should be recorded as well as the actual observations. After the first day of the study, observations generally need only to consist of brief checks for sign remission and the development of new signs of morbidity. Data should be collected in such a way that the following could be concluded for each sign: (1) estimated times of onset and recovery, (2) the range of threshold dosages, and (3) whether signs are directly related (primary) to the test article. An example of clinical signs provoked by a specific drug is given in Table 6.6. Incidences are broken down by dosage group and sex. These data illustrate the fact that mortality can censor (preclude) the occurrence of clinical signs. Note that reduced fecal output was a more frequent observation at the intermediate dosages because most of the animals died at the higher dosages.

Therapeutic ratios are traditionally calculated using the dose of the lowest observed adverse effect. A more sensitive therapeutic ratio could be calculated using the ED_{50} (effective dose) for the most prominent clinical sign. However, while it may be possible to describe a dosage–response curve (which may, in fact, have a different slope than the lethality curve) for a clinical sign and calculate the ED_{50}, in practice this is rarely done. It is more common for the approximate threshold dosages or no observable effect levels (NOELs) to be reported. A typical minimal acute toxicity study can be summarized as shown in Table 6.7.

Acute observation record

(Days, other than Study Day 1, on which no signs are observed are recorded on the Log of Animal Observations)

Species _____ Sex _____ Route _____ Dose level _____ Animals coded* _____ Date dosed _____

Study day												
Observations: Time												
Date												
No signs observed												
Reduced motor activity												
Ataxia												
Lost righting reflex												
Convulsions ( )												
Mydriasia												
Death												
Observer												

Page of notes:

*An. Code _____ *An. ID _____

*Animal code for recording observations

Read and understood

_____ Date

Figure 6.11 *Example of form for recording clinical observations in acute systemic toxicity studies.*

TABLE 6.6 Example of Clinical Observations Broken Down by Dosage Group and Sex in Acute Toxicity Study of Drug SC-37407[a]

	Dose Levels ($mg\,kg^{-1}$) by Sex									
	0		50		160		500		1600	
Signs Observed	M	F	M	F	M	F	M	F	M	F
Reduced motor activity	—	—	—	—	—	—	5/5	5/5	4/5	4/5
Mydriasis	—	—	—	—	3/5	4/5	4/5	5/5	5/5	5/5
Reduced fecal output	—	—	5/5	5/5	3/5	5/5	—	1/5	—	—
Hunched posture	—	—	—	—	—	1/5	3/5	3/5	—	—
Convulsions (tonic)	—	—	—	—	—	—	5/5	1/5	5/5	3/5
Ataxia	—	—	—	—	—	—	5/5	4/5	2/5	1/5
Tremors	—	—	—	—	—	—	1/5	2/5	1/5	—
Death	0/5	0/5	0/5	0/5	0/5	0/5	5/5	4/5	5/5	5/5

[a]Signs observed in rats treated orally (no. exhibiting sign within 14 days after treatment/no. treated). A dash indicates the sign was not observed at that dose level.

TABLE 6.7 Minimal Acute Toxicity Study Summary of Drug SC-34871

Species (Route)	Dose ($mg\,kg^{-1}$)	Dead/ Dosed	LD_{50} ($mg\,kg^{-1}$)	Signs Observed	Treatment to Death Intervals
Rat (PO)	2400	0/10	>2400[a]	None	None
Rat (IV)	16	0/10	Approximately 67	Reduced motor activity at $50\,mg\,kg^{-1}$; convulsions, dyspnea, lost righting reflex at $160\,mg\,kg^{-1}$	0–2 h
	50	2/10			
	160	10/10			
Mouse (PO)	500	0/10	>2400	None	None
	1600	0/10			
	2400	0/10			
Mouse (IV)	50	1/10	120 (75–200)[b]	Reduced motor activity, ataxia at $160\,mg\,kg^{-1}$; tremors, convulsions, dyspnea at $500\,mg\,kg^{-1}$	0–2 h
	160	6/10			
	500	10/10			

[a]Limit dosage.
[b]Fiducial limits.

6.3.2 Complete Acute Toxicity Testing

An example of the next-level test, the complete acute toxicity test, is given in Figure 6.12. As stated by Dayan (1983), the value of doing more than the minimal test will depend on the nature of subsequent testing. The complete protocol is designed to provide for a more in-depth search for target organs than the minimal protocol. This type of study, which has been well described by Gad and co-workers (1984), is similar in design to a minimal acute toxicity study but includes feed consumption data, more frequent body weight determinations, and more detailed and frequent clinical sign assessment. Groups should consist of at least 10 animals per group; 5 per sex per dosage should then be sacrificed 24–48 h for more immediate examination of any pathological changes induced by the test article. The remaining animals will be sacrificed at the end of the two-week period and examined for pathological changes. Blood will be collected at both sacrifices for clinical chemistry and/or hematology determinations. It should be noted that this design bears a striking resemblance to the design specified for an "expanded acute" study as required under the exploratory IND guidance (FDA, 2006), as shown in Figure 6.13.

Body Weight Considerations Body weight and feed consumption are frequently determined parameters in toxicity testing. To an extent, the ability of

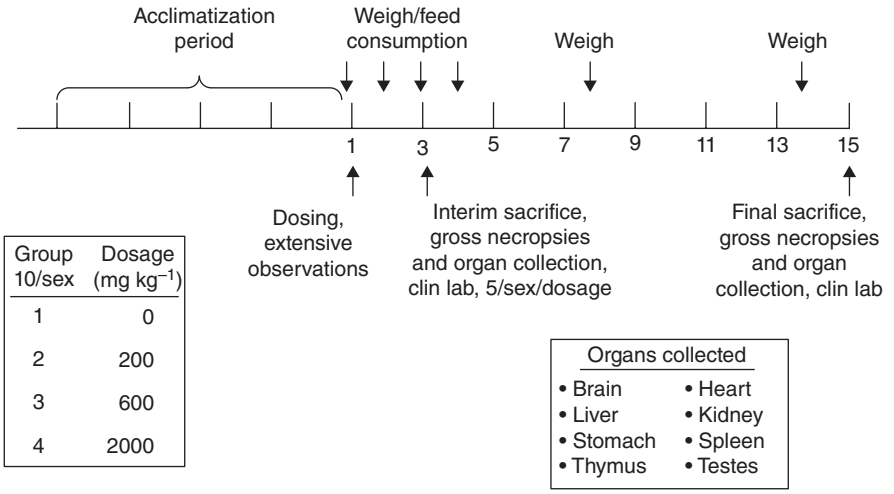
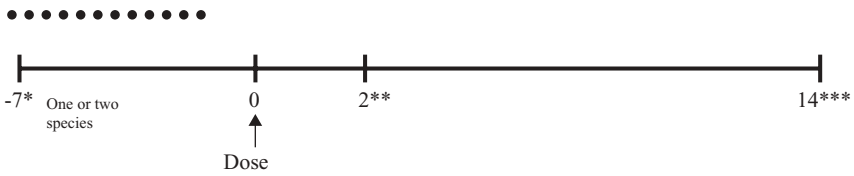


Figure 6.12 *Example of complete acute toxicity protocol.*



* One species only (selections justified with in vitro data) or two species

** Clinical chemistry & hematology, gross necropsy & histopathology—interim groups & histopathology

*** Clinical chemistry & hematology, gross necropsy & histopathology—in terminal groups

Also body weights (days 1, 2, 4, 7, & 14)
Clinical observations (daily)
Doses—100x what you want to do in clinic (microdoses)

Figure 6.13 *Exploratory IND enabling acute study. From Gad et al. (1984).*

an animal to gain or maintain weight may be considered a sensitive but nonspecific indicator of health. While this is true in subchronic or chronic studies, its relevance in acute studies must be carefully considered. In most protocols, body weights are determined on day 1 (prior to dosing), day 7, and day 14, which are the days mandated by most regulatory guidelines. Despite being common, the design is not well founded: If an animal has not died within seven days postdosing, it has probably recovered and its body weight may not be noticeably different from controls by day 14. A complete protocol addresses this problem by specifying more frequent body weight determinations (daily for the first three to five days of the observation period) so that not only can

initial decreases (if they occur) be detected, but recovery can also be charted. Feed consumption measurements should be made at the same times, because it is difficult to determine the causes behind body weight changes in the absence of feed consumption data. Body weight loss accompanied by normal feed consumption implies something very different than body weight loss (or lack of gain) accompanied by lack of feed consumption. In the absence of feed consumption data, however, changes in body weight should still be considered indicative of a change in an animal's health status.

Yet another reason why body weight determinations are of questionable value in acute studies has to do with the statistical analysis of the data. Deaths may substantially alter group size and complicate analysis. The death of two of five animals causes a 40% decrease in group size and a substantial diminution of the power of any statistical test. In addition, the resulting data sets are censored: Comparisons will often be between the control group, a dosage group where all the animals survive, and a high-dosage group where less than 50% of the animals survive to the end of the observation period. One has to question the utility of body weight changes if they occur at dosages that are acutely lethal. The data in Table 6.8 illustrate this point. Body weight changes tended to occur only at dosages that were acutely lethal. Additionally, one would suspect that the censoring of body weights in groups where death occurs is not random; that is, the animals that die are most likely those that are most sensitive, while those that survive are the most resistant or robust. This problem can be addressed by building exclusionary criteria into a protocol. For example, one could statistically analyze body weight data in groups that only had less than 50% mortality.

Minimal rather than complete protocols tend to be more common in the acute testing of pharmaceutical agents. Drugs will almost always be subjected to at least one subchronic study. Body weight and feed consumption determinations are a standard feature of such studies. Additionally, changes in body weight and feed consumption are more likely in a subchronic than an acute study because the animals are dosed continuously between body weight determinations.

Pathology Considerations One of the objectives of any well-conducted toxicity study is to identify target organs. There is some question, however, concerning the utility of extensive pathological assessments as part of an acute study. Gross necropsies are generally the minimum assessments requested by most regulatory bodies. Hence, minimal protocols will include necropsies on all animals found dead and those sacrificed following the postdosing observation period. An example of necropsy findings is given in Table 6.9. This table illustrates that gross necropsy observations on acute studies rarely predict the toxicity that will be seen when the chemical is given for longer periods of time. This is not surprising, because most drug-related histological lesions are the result of chronicity; that is, discernible lesions tend to result from the cumulative effect of dosages that are acutely well tolerated.

TABLE 6.8 Examples of Body Weight Changes in Rats from Minimal Acute Toxicity Studies

Drug (Route)	Dosage (mg kg^{-1})	BWT Change (g)[a]	Mortality
SC-32561			
PO	0	45 ± 4	0/10
	5000	39 ± 10	0/10
IP	0	43 ± 4	0/10
	500	43 ± 9	0/10
	890	44 ± 11	0/10
	1600	6 ± 14[b]	2/10
	2800	24 ± 20[b]	3/10
SC-36250			
PO	0	38 ± 10	0/10
	5000	34 ± 10	0/10
IP	0	34 ± 6	0/10
	670	50 ± 8[b]	2/10
	890	46 ± 8[b]	3/10
	1200	45 ± 4	4/10
	1400	35[c]	9/10
SC-36602			
IV	0	38 ± 9	0/10
	58	38 ± 3	0/10
	67	36 ± 7	2/10
	77	49 ± 5[b]	3/10
	89	41 ± 7	7/10
PO	0	38 ± 5	0/10
	2100	41 ± 5	3/10
	2800	38 ± 5	7/10
	3700	26 ± 6	7/10

[a]Mean ± standard deviation. Body weight (BWT) changes in grams for each group during the first week of the postdosing observation period.
[b]Statistically different from control (0 dosage group), $p \leq 0.05$.
[c]Only one animal survived, so there is no standard deviation.

The data in Table 6.9 also demonstrate that substantial gross macroscopic findings are rare in minimal acute studies and seldom suggestive of a specific effect. There are several reasons for the lack of specificity. The first is the rather limited nature of gross observations, in that they are limited to broad descriptive terms (size, shape, color, etc.). Second, for animals found dead, it is difficult to separate the chemically associated effects from agonal and/or autolytic changes. Finally, it is difficult to come to a conclusion about the nature of a gross lesion without histological assessment.

If there are any identifiable gross lesions, they often differ between animals that die and those that survive to the end of the observation period. The reason for these differences is very simple. An animal that dies less than 24 h after chemical exposure probably has not had sufficient time to develop a well-defined lesion. As mentioned earlier, most deaths occur within 24 h. Animals that survive for the two-week observation period have probably totally recov-

TABLE 6.9 Examples of Gross Necropsy Findings from Acute Toxicity Studies

Drug	Acute Gross Pathology	Subchronic Target Organs[a]
SC-36602	Distended stomach and intestine, bloody fluid in intestine, congested lung, pale liver	None
SC-38394	None	Liver, testes, bone marrow, thymus, kidney
SC-32840	None	Heart, stomach, kidney, bladder
SC-25469	Peritonitis (IP route only)	None
SC-36250	Peritonitis (IP route only)	Adrenal, liver, thyroid
SC-27166	None	Liver

[a]Organs that showed any evidence of test-article-related changes in repeated-dose studies of two weeks or longer duration.

ered and rarely have apparent lesions. Hence, the animals that provide the best chance to identify test-article-specific lesions are those that die in the region of 24–96 h postdosing. This is, in fact, one of the problems with acute pathology data—that is, comparing animals found dead with those sacrificed at a different time and comparing both to controls. As mentioned, a complete protocol, where groups of animals are sacrificed 24 to 96 h after dosing, at least partially solves this problem.

Many guidelines suggest microscopic confirmation of gross lesions "when necessary"; however, these are seldom done because of the autolytic nature of many of the tissues collected from animals found dead. Additionally, the practice of collecting and examining only gross lesions is difficult to justify because it does not permit in-depth comparisons. Pathological findings are most easily interpreted when the same organs are collected and examined from all animals on a test regardless of the circumstances of death. Elsberry (1986) recommends that the GI tract, kidney, heart, brain, liver, and spleen be specifically examined routinely in acute studies. Given the timing issues discussed in the previous paragraph, the amount of effort may not be worth the result. In an attempt to address these problems, Gad and co-workers (1984) have developed a complete protocol that includes groups of satellite animals that are sacrificed 48 h after exposure, necropsied, and a standardized organ list collected, weighed, and prepared for histological assessment. This list routinely includes the "first-line" organs: brain, thyroid, liver, kidneys, heart, and adrenals. The same organs are collected from all other animals, that is, those that die as a result of the toxicity as well as control animals. Additional tests can be included if one has a specific concern. For example, the structure of a test article may suggest that it has anticholinesterase potential. Therefore, one could include serum pseudocholinesterase determinations in the clinical laboratory package, as is frequently done for organophosphate and carbamate structures.

6.3.3 Supplemented Acute Studies

An example of the third-level acute toxicity test, a supplemented study, is given in Figure 6.14. Such tests are rarely performed but are of use when one wishes to obtain data other than descriptive toxicity data. For example, the addition of satellite groups of animals to be dosed with a radiolabeled compound to gain pharmacokinetic information will turn a "complete" study into a "supplemented" one. Another common practice is the addition of other examinations or measurements to gain more information about a potential target organ. An example of this would be recording ECGs in rats, which is too complicated and time consuming to do on a routine basis but should be considered if the heart is a potential target organ. One way of describing such a study is that it is a complete toxicity study carrying a specific screen "piggyback."

An excellent example of a supplemented protocol is that described by Gad and colleagues (1984). A neuromuscular screen was developed (Gad, 1982) and incorporated into their routine acute toxicity protocol for testing nonpharmaceuticals. Doing so allowed for the more systematic and quantifiable examination of effects of the CNS than reliance on simple clinical observations. The neuromuscular screen consists of a battery incorporating standard clinical observations plus some behavioral assessment techniques already described in the literature. These are summarized in Table 6.10. This screen has been further developed to become the now regulatorily required FOB (functional observational battery). An advantage of this screen is that it uses noninvasive techniques and therefore will require the use of no additional animals. If an animal is displaying signs of severe CNS depression 2 h postdosing, little useful data will be gathered by examining behavior. In testing a pharmaceutical it is probably better practice to apply the neuromuscular screen on days 2, 7, and 14 postdosing in an attempt to identify more subtle or lingering effects and to

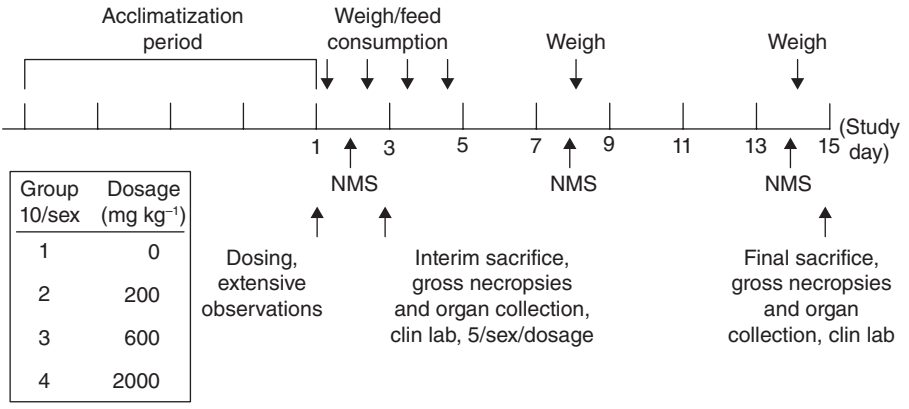


Figure 6.14 *Design and conduct of supplemented (or "heavy") acute systemic toxicity study. The figure illustrates the approach to such a study when it is to serve as the definitive systemic toxicity study for some period of time.*

TABLE 6.10 Neuromuscular Screen Observations

Observation	Nature of Data Generated[a]	Correlates to Which Neutral Component[b]
Locomotor activity	S/N	M/C
Righting reflex	S	C/M
Grip strength (forelimb)	N	M
Body temperature	N	C
Salivation	Q	P
Startle response	Q	S/C
Respiration	S	M/P/C
Urination	S	P/M
Mouth breathing	Q	S
Convulsions	S	C
Pineal response	Q	Reflex
Piloerection	Q	P/C
Diarrhea	S	GI tract/P/M
Pupil size	S	P/C
Pupil response	Q	P/C
Lacrimation	Q	S/P
Impaired gait	S	M/C
Stereotypy	Q	C
Toe pinch	S	S (surface pain; spinal reflex)
Tail pinch	S	S (deep pain)
Wire maneuver	S	C/M
Hind-leg splay	N	P/M
Positional passivity	S	S/C
Tremors	S	M/C
Extensor thrust	S	C/M
Positive geotropism	Q	C
Limb rotation	S	M/C

[a]Data quantal (Q), scalar (S), or interval (N). Quantal data are characterized by being of an either/or variety, such as dead/alive or present/absent. Scalar data allow one to rank something as less than, equal to, or greater than other values, but one cannot exactly quantitate the difference between such rankings. Interval data are continuous data where one can assign (theoretically) an extremely accurate value to a characteristic that can be precisely related to other values in a quantitative fashion.
[b]Peripheral (P), sensory (S), muscular (M), or central (C).

chart recovery from these effects. For drugs that produce no observable CNS effect following dosing, the neuromuscular screen can be done a few hours postdosing. The more extensive and detailed nature of the data generated by the neuromuscular screen permits more confidence in the conclusion that the test article had no effect on the CNS. Any suspect target organ can be investigated in a similar fashion. Depending on the invasiveness of the supplementary techniques, satellite groups may or may not need to be added to the study. Care must be taken in this regard to prevent the study from becoming too cumbersome and too complicated to conduct. It may be better to address some questions as separate studies. For this reason, one should not attempt to address more than one supplemental question in any one study.

6.3.4 Acute Toxicity Testing with Nonrodent Species

The designs described thus far for acute toxicity testing generally assume that the test species being used is a rodent. Nonrodent species are also used for acute toxicity testing. Many regulatory bodies require acute testing in at least one nonrodent species. The animals most often used are the dog, pig, or monkey. Veterinary products will also be tested in the target species. For example, a flea collar intended for cats must be tested in cats. While the rabbit is not technically a rodent, it is the species of choice for a variety of tests for assessing acute oral or intravenous toxicity and is considered a rodent for regulatory purposes. The section is written with the dog and monkey in mind. Clearly, there are some profound differences between these species and rodents with regard to handling, husbandry, and dosing. Here we focus on the design differences in toxicity testing in large species.

For financial, procurement, and ethical reasons, acute systemic toxicity tests on nonrodents are not performed using traditionally designed animal-intensive protocols. The minimal acute study requires 30–50 animals. Complete and supplemented studies will usually require even more. At a cost of $1500 per beagle dog, $1200 for minipig, or $3500 per monkey, the animal costs alone are enough to make such studies with these species prohibitively expensive. Vivarium space and husbandry costs are also much higher with nonrodent species that with rodents. Nonrodents also require a much longer prestudy quarantine period than rodents: at least 6–8 weeks for dogs and pigs and 18–24 weeks for monkeys. Treatment during the quarantine period is more extensive than that given rodents. The animals should be given frequent physical examinations, including complete clinical laboratory panels and appropriate tests for common illnesses and parasites. Special care must be taken with monkeys not only because they can be vectors of human disease but also because they can contract human diseases and a sick animal can compromise study outcome. All these factors dictate that these animals should be used sparingly. Hence, it is most common to study acute systemic toxicity in nonrodent animals using a pyramiding dosage design. The typical study will consist of two treated animals per sex and two control animals per sex for a total of eight animals. A typical protocol is shown in Figure 6.15.

The use of fewer but larger animals permits more extensive observations of each individual. Following each dose, animals can be given complete physical examinations that include palpations, behavioral checks, spinal reflex checks, pupillary light reflexes, respiration rate, ECG recording, and rectal temperature measurement. Blood samples can also be collected following each dose to determine standard clinical chemistry and hematology profiles. Hence, while fewer animals are used with the pyramiding dosage protocol, more information per animal is collected.

The small number of animals used in a pyramiding dosage study makes it difficult to do standard statistical comparisons. This difficulty can be overcome to a certain extent by taking advantage of two design aspects of the pyramiding

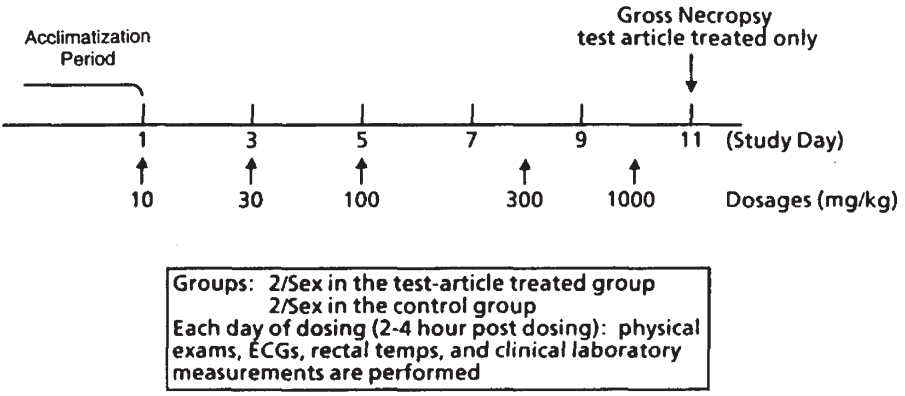


Figure 6.15 *Example of pyramiding dose study for acute toxicity testing in nonrodent species.*

protocol. First, pretreatment data can and should be obtained on all animals for all parameters examined or determined. In-study comparisons should be made both to pretreatment data and to concurrent control animals. Such comparisons can be made not only on the basis of absolute numbers but also on the magnitude of any changes from pretreatment values. Second, all animals should be measured repeatedly throughout the study. Hence, to reflect a true drug-related effect, the magnitude of change should increase following each dose (though one must be aware of the potential for the development of tolerance as induction of metabolism). This is in fact the only way one can make any dosage–response or threshold conclusions using the pyramiding protocol.

Seldom are drugs tested in nonrodent animals via routes other than the intended or likely routes of human exposure. Hence, the most common routes in these types of protocols are oral, intravenous, and respiratory. Rarely is a test article given to nonrodent species by the intraperitoneal route. Routes are discussed elsewhere in detail (Gad and Chengelis, 1999), but some discussion is appropriate here because of design considerations. Test articles are normally given orally by capsule to dogs and pigs and by gavage to monkeys. Nonrodents have to be restrained if dosed by gavage, making the process very labor intensive. This is minimized by the small number of animals specified by the pyramiding protocol. In contrast, because of the differences in size, it is much easier to deliver a test article intravenously to nonrodents than to rodents. For topical studies, the rabbit is the nonrodent choice because it is easier to prevent a rabbit from grooming the delivery site, and considerably less material is required to deliver a comparable dose to a rabbit than a dog or pig. Acute dermal studies are not, however, usually done with a pyramiding study design but rather as a limit dose study

The biggest problem with the pyramiding protocol is the development of tolerance. If no toxicity is observed, the chemical could be innocuous or animals could have developed tolerance during the study. The escalating dosage feature of the pyramiding protocol is an excellent vehicle for fostering the development of tolerance. One can check this by dosing additional naïve animals at the limit dosage to confirm, as it were, a negative result. Another problem, which is most peculiar to the dog, is emesis. Oral administration of a large amount of almost any material will cause a dog to vomit. This is always somewhat of a surprise to toxicologists whose prior experience is primarily with rodents, which cannot vomit. One should pay close attention to dogs the first hour after capsule delivery. If the dog vomits up most of the dose, the actual dosage absorbed could be grossly overestimated. This can be a particular problem if one is using the results of a pyramiding dosage study to set the dosages for a repeated-dose study. Dogs can develop tolerance to the emetic effect of a set dosage. When this occurs, absorption and resulting blood concentrations of a test article can increase dramatically, resulting in more florid toxicity than expected on the basis of the pyramiding study. Another problem is that emesis can result in secondary electrolyte changes—especially decreases in chloride—that may be mistaken for a direct effect of the test article. If emesis is a severe problem, one can study toxicity in a different nonrodent species or divide larger dosages into two or three divided dosages on the day of dosing.

As with traditionally designed rodent studies, the pathology component of pyramiding studies usually consists of gross necropsies followed by (when appropriate and necessary) histological assessment of gross lesions. Unfortunately, this study design does not permit the establishment of a dose–response relationship with regard to gross necropsy findings. In addition, the small number of animals makes definitive conclusions difficult. Usually, gross lesions are defined in absolute terms with few comparisons to control animals. Suspected target organs should be further investigated in subsequent subchronic studies or in rigorous and specific mechanistic studies. Because of the limited value of the pathology data generated by the pyramiding protocol, control animals should not be terminated but rather should be saved for reuse.

6.3.5 Factors That Can Affect Acute Tests

Many investigations into the sources of variability in acute toxicity testing have been conducted, and these have been reviewed by Elsberry (1986). The factors causing the greatest interstudy variation included lack of specifications for sex, strain, age, and weight range. When clearly defined, detailed protocols were used and interlaboratory variation was found to be minimal. Hence, it is equally important that the details of the protocol be well described and followed. It is not appropriate to draw dosage–response conclusion by comparing

groups that differ substantially in age or that have been fed, fasted, or otherwise manipulated differently. Guidelines for standardization of acute toxicity testing were proposed by the interagency regulatory liaison group [Interagency Regulatory Liaison Group (IRLG), 1981; Elsberry, 1986]. These do not differ markedly from those mandated by the Toxic Substance Control Act of 1986 (Gad and Chengelis, 1999).

Number, Size, and Sex of Dosage Groups The precision with which lethality and signs of toxicity are described will depend on the number of groups (hence, dosages) and the number of animals in each group. Between 1940 and 1980, the standard was to use from four to six dosages with 10 animals per dosage. The current emphasis is on limiting the number of animals used for acute testing, particularly with recognition of the limited value of "precise" lethality data (Gad and Chengelis, 1999). Retrospective analyses by DePass (1989) and Olson et al. (1990) have demonstrated that decreasing group size to two to three animals generally has little impact on overall study results. Hence, the number and size of dosage groups will depend, to an extent, on the methods of statistical analysis. The classic statistical methods for analyzing lethality data (or, indeed, any quantal dosage–response data) were published between 1930 and 1960 and have been extensively reviewed by Armitage and Allen (1959) and Morrison et al. (1968). These methods are mentioned here with regard to the demand they make on protocol design—specifically, the number of dosage groups, the spacing of the dosages, and the number of animals per dosage group. The probit and moving-average methods are most commonly used today. In general, all methods of calculation and computation are more precise if the designs are symmetrical (i.e., the dosages are evenly spaced and the group sizes are equal). The probit method, first developed by Bliss (1935, 1957) and later refined by Finney (1971, 1985), is considered to be the most precise, but it requires at least two groups of partial responses (i.e., mortality greater than zero but less than 100%). This may require dosing more than three groups until this criterion is met. It also deals ineffectively with groups that had either zero or 100% mortality. (The most common correction for these groups is to substitute 0.1% for zero and 99.7% for 100%.) The moving-average method, first described by Thompson and Weil (1952), does not require partial responses, deals effectively with complete responses, and therefore can produce an acceptable estimate of an LD_{50} with as few as three groups of three to five animals each. The moving-average method can also be used to design the experiment. Groups can be dosed in a sequential fashion as in a pyramiding study, with each step dictated by the moving-average method. Once evidence of toxicity is observed, further dosing is discontinued. This method requires that the dosages be separated by a constant geometric factor (e.g., 2, 4, and 8 $mg\,kg^{-1}$) and that groups be of equal size. Weil (1952) and later Gad and Chengelis (1999) and Gad (2007) published tables that allow for the easy calculation of the LD_{50} using $K = 3$ (where K is the number of dosage groups minus 1). The LD_{50} for $K < 3$ can be easily calculated without

the aid of tables. In addition, methods for estimating the confidence limits of this calculated LD_{50} have also been published (Gad and Chengelis, 1999). Traditionally, the moving-average method has not been extensively used because, while it yielded an estimate of the LD_{50}, it did not give the slope of the (probit-transformed) lethality curve. However, Weil (1983) has published a method for calculating a slope from the same data. Hence, an estimate of the LD_{50} and slope can be obtained from as few as three groups of three to five animals per group, provided that at least one group shows a response less than 50% and another shows a response greater than 50%.

The Litchfield and Wilcoxon (1949) plotting method was once commonly used. It is certainly a valid method, and it poses no more restrictions on study design than those imposed by the probit method. The Litchfield–Wilcoxon method has become a victim of technology as modern, hand-held calculators and the ready availability of simple computer programs have made other methods more convenient to run. However, at least one software company has adopted the Litchfield–Wilcoxon method for its acute toxicity protocol package.

The normit–chi square, developed by Berkson (1955), is also sometimes used. Like the probit method, the normit–chi square does not absolutely require equally spaced dosages or equal group sizes, but it does require at least one partial response. Hence, fewer dosage groups may be needed with the normit–chi square method than with the probit method. According to Waud (1972), the correction for including complete responses is better than that used for probit analysis but is still "tainted." His method supposedly deals adequately with complete responses, but it is extremely complex and, probably for this reason, is rarely used.

In an early paper, Karber (1931) published a simple method (often described but rarely cited) for calculating LD_{50}. It does not require that dosages be equally spaced, but widely divergent dosages will lead to a biased result. The method was originally described for groups of equal size, but groups of slightly varying sizes can be used, provided they do not differ by more than a few animals each. In this case, mean group size can be inserted into Karber's formula with little change in accuracy. The formula is very simple, and one can calculate an acceptable estimate of LD_{50} quickly with only a few arithmetic computations. This method, unlike those mentioned above, does not allow for calculating the confidence limit or slope of the probit response curve. Hence, if these calculated parameters are not sought, the Karber method allows one a bit more freedom in picking dosages.

While much has been written about the influence of gender on acute lethality, most authors now agree that there are seldom any substantial differences in LD_{50} due to sex (DePass et al., 1984; Gad and Chengelis, 1999). In those instances where there is a sex-related difference, females tend to be more sensitive than males (approximately 85% of the time). If one is willing to accept this amount of uncertainty, only one sex needs to be tested. Alternatively, as few as two to three animals per sex per dosage can be used. Schutz

and Fuchs (1982) have demonstrated that, by pooling sexes, there are seldom any substantial differences in the LD_{50} calculations between groups consisting of five per sex versus three per sex. If there are no substantial differences between sexes (i.e., 70% mortality for males and 80% for females at a dosage), the results from pooling the sexes can provide a pooled LD_{50}. For most safety considerations, an LD_{50} derived on this basis will be acceptable and will result in the use of fewer animals.

6.3.6 Selection of Dosages

In setting dosages for acute studies a few commonsense rules have to be applied. First, the intrinsic biological and chemical activity of the test article must be considered. Zbinden and Flury-Roversi (1981) have documented several cases where lethality was of no biological relevance. The oral lethality of tartaric acid, for example, is due to the caustic action of a high concentration of acid in the GI tract. In these instances, limit tests are more appropriate tests. Additionally, it is uncommon that a completely unknown chemical will be tested. Factors such as known pharmacological profile; chemical or physical characteristics including molecular weight, particient coefficient, and the like; and the toxicity of related chemicals should be considered. For example, it is likely that a polymeric, poorly soluble molecule will not be bioavailable at an initial dosage of $100\,mg\,kg^{-1}$. A full understanding of all available data will permit one to pick dosages with more confidence and thereby save both time and resources.

Second, no protocol will yield high-quality data if all dosages given cause 100% lethality. Therefore, one is best advised to pick widely spaced, rather than closely spaced, dosages. In general, the best dosage regimen includes a dose that will definitely produce a high incidence of severe toxicity, another that will produce marginal toxicity, and one in between. If this pattern is obtained, adding more groups does not generally change the results. This point is illustrated by the data in Table 6.11. For two drugs, an LD_{50} of $300\,mg\,kg^{-1}$ was obtained using six groups of 10 mice each. Essentially the same result was obtained if the second, fourth, and sixth groups were eliminated and not used in the calculations. Behrens (1929) noted this phenomenon almost 60 years ago.

Widely spaced dosages also decrease the likelihood of nonnormotonic data, where mortality does not necessarily increase with dosage (see Table 6.12). This can occur when the test chemical has a shallow dose–response curve and the group size is small (three to four animals). Wile it is possible to calculate an LD_{50} from such data, the slope and confidence limits will be inaccurate. Nonmonotonic data can also occur if the lethality is indeed biphasic. If one suspects that this is occurring, additional dosages should be examined. For safety considerations, only the first part of the curve, the lowest LD_{50}, is of importance.

TABLE 6.11 Sample Data Sets: LD_{50} Calculations Using Fewer Dosages[a]

SC-27166		Theophylline	
Dosage ($mg\,kg^{-1}$)	Mortality	Dosage ($mg\,kg^{-1}$)	Mortality
100	0/10	280	0/10
180	0/10	320	3/10
240	4/10	370	5/10
320	7/10	430	9/10
560	9/10	500	10/10
1000	10/10	670	10/10
LD_{50} = 300		LD_{50} = 300	
Using Every Other Dosage			
100	0/10	280	0/10
240	4/10	370	5/10
560	9/10	500	10/10
LD_{50} = 290		LD_{50} = 290	

[a]Adult male mice; drugs given by gavage.

TABLE 6.12 Sample Data Sets: Homogeneous versus Heterogeneous Data

Homogeneous[a] (Normotonic)		Hetereogeneous[b] (Nonnormotonic)	
Dosage ($mg\,kg^{-1}$)	Mortality	Dosage ($mg\,kg^{-1}$)	Mortality
300	0/20	620	0/10
600	1/20	1600	2/10
800	10/20	2100	8/10
1000	17/20	2800	5/10
		3700	8/10
		5000	8/10

[a]Data from study of SC-31828 using adult rats of both sexes.
[b]Data from study of SC-3894 using adult male rats.

Timing The greatest precision in any lethality curve is obtained when the number of experimental variables is kept to a minimum. Hence, it is best if all the animals used for determining a specific curve are dosed on the same day and, if possible, at the same time of day, which limits age-related and diurnal effects. If a total of only 15 animals are being dosed, this is not a difficult task for a single well-trained technician. However, if the test substance is of unknown lethality, it is imprudent to deliver all doses on the same day. It is common practice for a single dosage group to be treated on the first day of an experiment and the dosages for the second and third groups to be adjusted pending the results of the first group. Generally, most acute deaths will occur within 24 h of dosing. Delayed deaths (those occurring more than 24 h after

dosing) are relatively rare and generally restricted to the 72-h period following dosing (Gad et al., 1984; Bruce, 1985). Hence, waiting for 24 h between doses will generally yield sufficient data to allow the choice of the next dosage. For example, if all but one of the animals dosed in the first groups dies, there is no doubt that the next dosage should be adjusted downward considerably, whether or not the final animal eventually dies. All the dosing for a single curve can be completed in three days. If a test article is being tested in traditional protocols (with two species, two routes, separate sexes), the two initial groups by a route can be treated on the first day of the dosing period and the second route initiated on the next day. Subsequent dosages can be adjusted on alternate days. Little real impact on the results will occur if there are two to three days between dosing sets. After that, however, the increasing age of the animals may result in a change in sensitivity. As reviewed by Balazs (1976), for example, the ratios of the LD_{50}s obtained in adult animals to the LD_{50}s obtained in neonates can vary from 0.002 to 160. One can use longer observation periods between dosing days if separate animal orders are timed for delivery to ensure that all animals dosed are closer in age. As a rule of thumb, the animals should not differ in age by more that 15%; hence, the younger the animals, the small the age window.

6.4 SCREENS

Screens are generally not safety studies in the regulatory sense. These are the studies done, as the name implies, to examine several chemicals in order either to select those with the most desirable properties for development or to eliminate those that have undesirable properties. There is nothing novel about screening; the process has been an integral part of pharmaceutical research for decades (Irwin, 1962). In a pioneering paper, Smyth and Carpenter (1944) described a screening process for gathering preliminary toxicity data for a new chemical. In their discussion they clearly state the underlying rationale for toxicity screening:

> Opinions upon the toxicity, hazards of manufacture, and fields for safe use must be expressed regarding many chemicals which will never be produced in quantity. Large expenditures of time and money upon securing these basic conclusions is not justified. Later, when a few of the new compounds are obviously going to be made commercially, more detailed studies can be undertaken.

Screens are designed for speed, simplicity, and minimal resource expenditure. They are designed to answer positive, single-sided questions. For example, the lack of an effect in an initial screen does not mean that toxicity will not be manifested with a different formulation or in a different species. It is for this reason that screen should not, as stated by Zbinden et al. (1984), be seen as replacements for thorough safety testing. An acute toxicity screen can be

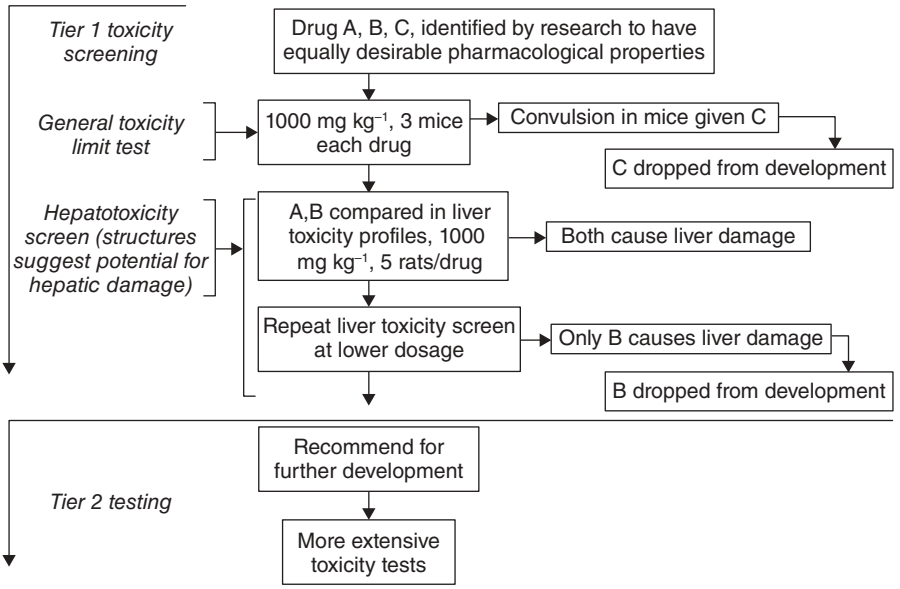


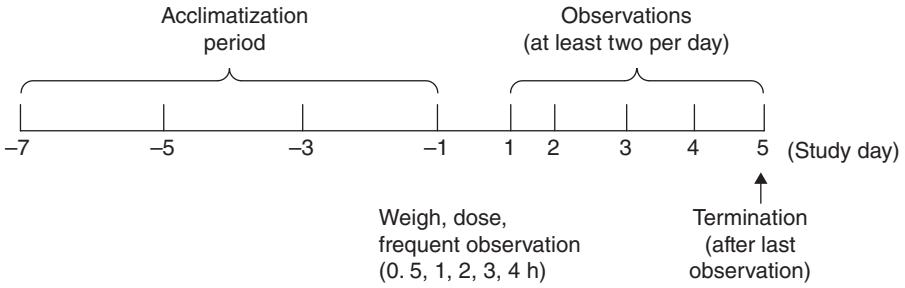
Figure 6.16 *Example of use of screens in selecting drug candidates for development.*

the first leg in a decision tree or tier-testing process for selecting a chemical or drug candidate for development. An example of this process is given in Figure 6.16.

6.4.1 General Toxicity Screens

There are two types of acute toxicity screens. In the general toxicity screen, animals (often, for economic reasons, mice) are exposed to two or three predefined dosages of chemical. No more than three mice per dosage are necessary and no control group is required. An example of this type of protocol is shown in Figure 6.17. The animals are carefully observed for mortality and obvious signs of toxicity, such as convulsions, but no attempt should be made to quantify the severity of a response. There is seldom any need to have an observation period of more than four to five days. Because of the quantal nature of the data, interpretation is straightforward. There are four possible outcomes: (1) no death or signs of toxicity seen at dosages up to X mg kg^{-1}; (2) no deaths but evident signs of toxicity seen at X mg kg^{-1}; (3) evident signs of toxicity at X mg kg^{-1}; (4) deaths and evident signs of toxicity both occurring at X mg kg^{-1}. General toxicity screens may also provide the preliminary information for picking the dosages for more definitive acute studies.

There are two ways to apply the data from toxicity screens to the development of a drug or chemical. On a relative basis, the drugs under consideration can be ranked according to screen results and the one that appears to be the



		Results	
Group	*Dosage*	*Deaths*	*Signs summary*
3 mice	100 mg kg^{-1}	0/3	None
3 mice	300	0/3	CNS depression
3 mice	1000	0/3	CNS depression, agonal changes

Figure 6.17 *Example of general toxicity screen.*

least toxic can be chosen for future development. Alternatively, decisions can be made on an absolute basis. All candidates that are positive below a certain dosage are dropped, and all those that are negative at or above that dosage will continue to the next tier of testing. If absolute criteria are used, the screen need be done only at the critical dosage. If only one dosage is examined, the test is a limit test. A limit test of this kind is the simplest form of toxicity screen and, depending on the nature of subsequent testing, it is highly recommended.

Fowler and his colleagues (1979) have described a rat toxicity screen (illustrated in Figure 6.18) that is more extensive and detailed than the one shown in Figure 6.17. It includes two rounds of dosing. In the first round, up to 12 rats are (singly) exposed to six different dosages by two different routes for the purpose of defining the maximally tolerated dose (MTD). In the second round of dosing, 16 rats are dosed at two-thirds (0.66) of the MTD and sacrificed on a serially timed basis for blood sample collections to determine test article concentrations and for clinical laboratory tests. These features make this design too complicated, time consuming, and expensive to run as an initial screen. This design is better suited as a second-tier screen to provide a more extensive follow-up study for a more limited screen. Fowler et al. contend that their screen disclosed most toxicity uncovered by more conventional studies. This screen was most successful in defining acute CNS, liver, or kidney toxicity (Fowler et al., 1979). Lesions that require long-term exposure, such as those generally involving the eyes, may not be detected in this type of screen.

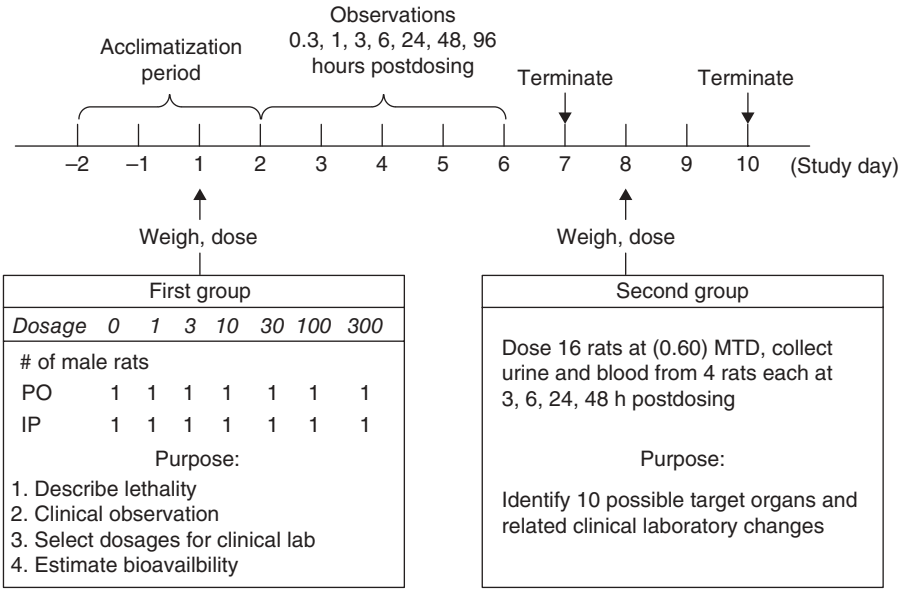


Figure 6.18 *Example of rat toxicity screen for drugs.*

Up/down or pyramiding designs can be used for general toxicity screens, but this is not a common approach because of the time involved. In addition, if several chemicals are being compared, an up/down study where death occurs at different dosages can be too complicated to run. It is much easier to test several chemicals at the same time using a limit test design. Because only individual animals are dosed, these designs can be used when there is a very limited amount of test article available and/or there are few prior data on which to base an expected toxic dosage.

Hazelette and colleagues (1987) have described a rather novel pyramiding dosage screen that they term the rising-dose tolerance (RDT) study (illustrated in Figure 6.19). The study, which uses a subacute rather than an acute dosing regimen, can also be used as a range-finding study design. The rats are exposed for four days to the initial dosage followed by three days of recovery before the next four-day dosing period at the next highest dosage. This process is repeated for the three dosing cycles. Plasma and urine samples are collected for clinical chemistry and urinalysis as well as test article determinations. Necropsies and microscopic examinations are performed. While this study design is novel, it appears to provide considerable acute data. It is also possible that this design could generate sufficient data to plan a pivotal subchronic study and therefore replace a traditional two-week study, resulting in considerable savings of time and animals. This is not a simple study and, therefore, is

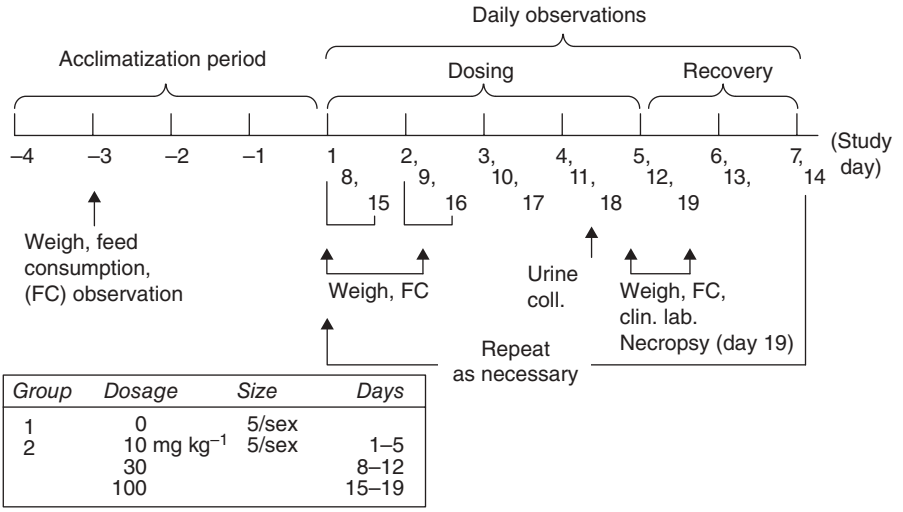


Figure 6.19 *Example of rising-dose tolerance test [no pharmacokinetic (PK) groups].*

inappropriate as an initial screen, but it would appear to be appropriate for a second-tier test.

6.4.2 Specific Toxicity Screening

The second type of acute toxicity screen is the specific toxicity test. This type of test is done when one has a specific toxicological concern, for example, when prior structure–activity data suggest that a family of chemicals can be hepatotoxic. A screen to select the chemical with the least hepatotoxic potential is then in order. These tests are also done, as described by Zbinden (1984), to look for a specific toxicological effect that may be easily overlooked in a routine safety study. Zbinden gives, as an example, screens that are designed to detect specific lesions to the hemostatic process. As pointed out by Irwin (1962) over three decades ago, such tests have their greatest power if more than one measure of specific target organ toxicity is used. Dayan (1983) refers to this technique as a matrix of measurements. In a liver toxicity screen, for example, liver weights (both absolute and relative), gross necropsy examinations, and a battery of serum enzyme assays should all be part of the protocol. As a general rule, because of the time and expense involved, screens should be designed to minimize the use of histopathological techniques. The dosages can be standardized or set on the basis of the results of a generalized toxicity screen. Specific toxicity screens can be the next-level test in the decision

tree process for selecting a candidate for development (as illustrated in Figure 6.16).

The number of animals and the number of dosages are highly dependent on the type of data gathered. A few rules of thumb should be followed: (1) Keep it lean. Each additional group, animal, or test article added to a protocol makes the study exponentially more difficult to conduct; simplicity is one of the most important features of a screen. (2) The more parameters examined, the fewer the number of animals required. (3) If normal limits of a test parameter are relatively broad (e.g., serum glucose), more animals will be required than if the parameter is normally tightly controlled (e.g., prothrombin time). In general, 3 is the minimum and 10 is the maximum number of animals required per group. Further, if a single chemical is examined per study, no more than three groups will be required. If more than one chemical is included in the study, then a single dosage (limit) group per chemical is the best design.

Strictly speaking, an acute toxicity study is conducted to examine the effect of a single dose of a single compound. In designing specific toxicity screens, however, deviation from this principle is permissible if it increases screen sensitivity. For example, the sensitivity of mice to many indirect hepatotoxins will be enhanced by prior treatment with phenobarbital. Hence, the sensitivity of a hepatotoxicity screen will be enhanced if the mice are pretreated for three days with phenobarbital.

The screen should be validated for consistency of response with both positive and negative control articles. A positive control article is one that is known to reliably produce the toxic syndrome the screen is designed to detect. Concurrent control groups are not required with each replicate. Rather, control groups should be evaluated on some regular basis to ensure that screen performance is stable. Because a screen relies on a biological system, it is not a bad idea to test the control benchmarks, particularly the positive ones, on a routine period basis. Not only does that give one increased confidence in the screen, but it also provides a historical base against which to compare the results of new test articles. Zbinden and colleagues (1984) refer to the positive control as the reference compound, and they have discussed some of the general criteria to be applied in the selection of these compounds. Any changes to the design should trigger revalidation. Any analytical methods should be subjected to PASS (precision, accuracy, sensitivity, and selectivity) validation.

Interpretation of specific toxicity screen data is not as straightforward as that of a general toxicity screen. This is because the data will often be continuous, following a Gaussian, or normal, distribution. This has two ramifications. First, for results around the threshold, it may be very difficult to differentiate between positive and negative responses. Second, for any one parameter, there is a real chance of false statistical significance (type I errors), especially if small numbers of animals are used. This occurrence is one of the reasons why specific toxicity screens should include the determination of more than one variable, since it is unlikely for multiple false positives to occur in the same group of animals. An undetected false positive could lead to the dropping of a promis-

ing candidate in error. False negatives, by contrast, may not be as critical (other than the time lost and the resources spent), because extensive subsequent tests should lead to the more complete description of the test article's toxic properties.

The problems described in the preceding paragraph assume that the screen will include a traditional ("negative," or vehicle) control group and the data from the treated groups will be compared to those of the control group by standard methods. These problems will be minimized if no control group and therefore no traditional statistical comparisons are included. In addition, a decrease in the number of animals used simplifies the study. Data can be interpreted by comparison to a historical control database as described by Zbinden (1984). The threshold, or test criterion X_c, is calculated according to the formula

$$X_c = m + (z)s$$

where m is the population mean, s is the standard deviation, and z is an arbitrary constant. This formula is essentially a method of converting continuous data to quantal data: It is used to determine if individual animals are over the test threshold, not if the group mean is over the threshold. Analysis of screening data by comparison to experience (i.e., historical control data) and an activity criterion are discussed in greater detail in Chapter 4 and by Gad (1989, 2007). The higher the z value, the lower the probability of a false positive, but the lower the sensitivity of the screen. Again, including multiple parameters in the screen helps alleviate this problem. Zbinden has proposed a ranking procedure in which various levels of suspicion (LOS) or a level of certainty (LOC) is assigned to the result of a toxicity screen. This is simply a formalized fashion of stating that the more animals that respond and the greater the severity of the response, the more certainty one has in drawing a conclusion. If relative comparisons are being made, this system provides a framework for ranking test articles and selecting those to continue to the next tier of testing.

With regard to specific toxicity screening, behavioral toxicity screening is an area currently generating a great deal of interest. As reviewed by Hopper (1986), there are several reasons for this interest. First, the Toxic Substance Control Act of 1976 legislatively recognized behavioral measures as essential to examining chemicals for neurotoxic potential. Second, the structure and function of the CNS are not amenable to traditional methods of examination, in that profound behavioral changes can be induced in the absence of any detectable morphological lesions. This large and somewhat controversial subject is outside the scope of this chapter. Specific screening strategies are presented and critically discussed by Hopper (1986). Other recommended references to consult for different perspectives on acute toxicity testing are Rhodes (2000), Brown (1980), and Arnold et al. (1990).

6.5 PILOT AND DRF STUDIES

Between acute studies and the repeat-dose (14- or more typically 28-day) studies (described in Chapter 8), there are those which are necessary to be able to set doses required by regulation to be able to initiate initial clinical studies.

The DRF-style studies are not required by regulation but rather by proper scientific practice. As such, it should be noted that while the results must be included in the documents submitted to regulatory authorities, they do not have to be performed in compliance with good laboratory practices (GLPs). The studies should be conducted with dosing by the route and regimen (dose frequencies) intended for clinical studies. The formulations used should be those to be employed in the 14- or 28-day studies, but the actual drug substance [active pharmaceutical ingredient (API)] lot does not need to be.

For nonrodent species (dog, pig, or primate), dose levels are set based on the maximum tolerated dose identified in the acute pyramiding dose study. The design of the study is presented in Table 6.13.

The design for the rodent pilot study is shown in Table 6.14. Dose levels are set based on results seen in the acute study with the middose typically being the maximum tolerated dose seen in that study.

The hematology, clinical chemistry, and urinalysis measures are as described in Chapter 8.

TABLE 6.13 Seven-Day Nonrodent Pilot Toxicology Study

Animal: 9 Males, 9 Females
Study Design:
Vehicle control 3 males, 3 females
Low dose 3 males, 3 females
High dose 3 males, 3 females
Dosing: Once daily all animals for 7 days
Observations: (Mortality/moribundity) Twice daily
Clinical examination: Daily after dosing
Body weights: Before start and on days 1, 2, 3, 5, and 7
Food consumption: Before start and on days 1, 2, 3, 5, and 7
Physical examinations: Conducted by staff veterinarian on all animals prior to initiation of compound administration and at study termination
Electrocardograms: All animals prior to initiation of compound administration and at study termination
Clinical pathology: Hematology, clinical chemistry, and urinalysis evaluations on all animals pretest and at study termination
Necropsy: All animals on day after last dose
Organ weights: Adrenals, brain, heart, kidneys, liver, lungs, ovaries with oviducts, pituitary, prostate, salivary glands, seminal vesicles, spleen, thyroid with parathyroid, thymus, testes, uterus

TABLE 6.14 7-Day Rodent Pilot Toxicology Study

Study design: main study
Vehicle Control 5 males, 5 females
Low Dose 5 males, 5 females
Mid Dose 5 males, 5 females
High Dose 5 males, 5 females
Dosing: Once daily all animals
Observations: (Mortality/moribundity) Twice daily
Detailed clinical observation: Daily
Functional observational batter: After first dose
Body consumption: Weekly
Clinical pathology: Hematology, clinical chemistry, and urinalysis evaluations on all surviving main study animals at termination (see attachment)
Toxicokinetics: Blood may be collected after first and last dose at six time points
Necropsy: All main study animals, toxicokinetics animals euthanized and discarded
Organ weights: Adrenals, brain, heart, kidneys, liver, lungs, ovaries with oviducts, pituitary, prostate, salivary glands, seminal vesicles, spleen, thyroid with parathyroid, thymus, testes, uterus

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7

Genotoxicity

Genotoxicity encompasses all the potential means by which the genetic material of higher organisms may be damaged, with resulting serious consequences. Most forms of genotoxicity are expressions of mutagenicity—the induction of deoxyribonucleic acid (DNA) damage and other genetic alterations, with changes in one or a few DNA bare pairs (gene mutations). Others are clastogenicity, with gross changes in chromosomal structure (i.e., chromosomal aberrations) or in chromosome numbers. Clearly the potential of any pharmaceutical to cause such damage is a concern.

It has been known for several hundred years that exposure to particular chemicals or complex mixtures can lead to cancer in later life (Doll, 1977), and it has been postulated more recently that chemicals can also induce heritable changes in humans, leading to diseases in the next generation (ICEMC, 1983). There has been accumulating evidence that such changes can arise following damage to DNA and resulting mutations (see, e.g., Bridges, 1976). Therefore, it has become necessary to determine whether widely used drugs or potentially useful new drugs possess the ability to damage DNA. In pharmaceutical development, such information may be used to discard a new candidate drug from further work, to control or eliminate human exposure for a mutagenic industrial compound, or, for a drug, to proceed with development if benefits clearly outweigh risks. Data concerning the genotoxicity of a new drug have become part of the safety package, though the timing of the performance of the tests may vary. They are needed for decision making and to reduce risks that might otherwise be unforeseen.

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TABLE 7.1 Genotoxicity tests recommended by ICH (ICH, 1996)

Genotoxicity Test—ICH	Mutation	Cell Type	Method
A test for gene mutation in bacteria	gene	bacterial	in vitro
In vitro cytogenetic assay using mouse lymphomas tk cells	chromosome	mammalian	in vitro
In vivo test for chromosomal damage using rodent hematopoietic cells	gene	mammalian	in vivo

Note: The International Conference on Harmonization recommends a specific profile of genotoxicity tests for drugs, which is different from those of OECD and ISO. They want to see an in vivo test conducted.

The International Conference on Organisation (ICH) S2B clear guidance in testing requirements is summarized in Table 7.1. Currently (2005) an early stage (step 2) revision S2 is under consideration. This revision, if adapted, would fundamentally change many aspects of how candidate drugs are evaluated for potential genotoxicity. The U.S. Food and Drug Administration (FDA) follows the operative ICH guidance.

The ICH recommends a rather different profile of genotoxicity tests for drugs. It wants to see an in vivo test conducted.

7.1 DNA STRUCTURE

With the exception of certain viruses, the blueprint for all other organisms is contained in code by DNA, a giant macromolecule whose structure allows a vast amount of information to be stored accurately. We have all arisen from a single cell, the fertilized ovum containing two sets of DNA (packaged with protein to form chromatin), one set from our mother, resident in the nucleus of the unfertilized ovum, the second set from our father via the successful sperm. Every cell in the adult has arisen from this one cell and (with the exception of the germ cell and specialized liver cells) contains one copy of these original chromosome sets.

The genetic code is composed of four "letters"—two pyrimidine nitrogenous bases, thymine and cytosine, and two purine bases, guanine and adenine—which can be regarded functionally as arranged in codons (or triplets). Each codon consists of a combination of three letters; therefore, 4^3 (64) different codons are possible. Sixty-one codons code for specific amino acids (three produce stop signals), and as only 20 different amino acids are used to make proteins, one amino acid can be specified by more than one codon. The bases on one strand are connected together by a sugar (deoxyribose) phosphate backbone. DNA can exist in single- or double-stranded form. In the latter state, the two strands are held together by hydrogen bonds between the bases. Hydrogen bonds are weak electrostatic forces involving oxygen and nitrogen atoms. As a strict rule, one fundamental to mutagenesis, the adenine bases on one strand always hydrogen bond to the thymine bases on the sister

strand. Similarly, guanine bases pair with cytosine bases. Adenine and thymine form two hydrogen bonds, and guanine and cytosine form three.

Double-stranded DNA has a unique property in that it is able to make identical copies of itself when supplied with precursors, relevant enzymes, and cofactors. In simplified terms, two strands begin to unwind and separate as the hydrogen bonds are broken. This produces single-stranded regions. Complementary deoxyribonucleotide triphosphates then pair with the exposed bases under the control of a DNA polymerase enzyme.

A structural gene is a linear sequence of codons which codes for a functional polypeptide, that is, a linear sequence of amino acids. Individual polypeptides may have a structural, enzymatic, or regulatory role in the cell. Although the primary structure of DNA is the same in prokaryotes and eukaryotes, there are differences between the genes of these two types of organism, in internal structure, number, and mechanism of replication. In bacteria, there is a single chromosome, normally a closed circle, which is not complexed with protein, and replication does not require specialized cellular structures. In plant and animal cells, there are many chromosomes, each present as two copies, as mentioned earlier, and the DNA is complexed with protein. Replication and cell division require the proteinaceous spindle apparatus. The DNA of eukaryotic cells contains repeated sequences of some genes. Also, eukaryotic genes, unlike prokaryotic genes, have noncoding DNA regions called introns between coding regions called exons. This property means that eukaryotic cells have to use an additional processing step at transcription.

7.1.1 Transcription

The relationship between the DNA in the nucleus and proteins in the cytoplasm is not direct. The information in the DNA molecule is transmitted to the protein-synthesizing machinery of the cell via another informational nucleic acid, called messenger RNA (mRNA), which is synthesized by an enzyme called RNA polymerase. Although similar to DNA, mRNAs are single stranded and possess the base uracil instead of thymine and the sugar ribose rather than deoxyribose. These molecules act as short-lived copies of the genes being expressed.

In eukaryotic cells, the initial mRNA copy contains homologues of both the intron and exon regions. The intron regions are then removed by enzymes located in the nucleus of the cell. Further enzymes splice the exon regions together to form the active mRNA molecules. In both groups of organisms mature mRNA molecules then pass out of the nucleus into the cytoplasm.

7.1.2 Translation

The next process is similar in both eukaryotes and prokaryotes and involves the translation of mRNA molecules into polypeptides. This procedure involves

many enzymes and two further types of RNA: transfer RNA (tRNA) and ribosomal RNA (rRNA). There is a specific tRNA for each of the amino acids. These molecules are involved in the transportation and coupling of amino acids into the resulting polypeptide. Each tRNA molecule has two binding sites, one for the specific amino acid, the other containing a triplet of bases (the "anticodon") which is complementary to the appropriate codon on the mRNA.

Ribosomal RNA is complexed with protein to form a subcellular globular organelle called a ribosome. Ribosomes can be regarded as the "reading head", which allows the linear array of mRNA codons each to base pair with an anticodon of an appropriate incoming tRNA–amino acid complex. The polypeptide chain forms as each tRNA–amino acid comes in to register with the RNA codon and with specific sites on the ribosome. A peptide bond is formed between each amino acid as it passes through the reading head of the ribosome (Venitt and Parry, 1984).

7.1.3 Gene Regulation

Structural genes are regulated by a special set of codons, in particular "promoter" sequences. The promoter sequence is the initial binding site for RNA polymerase before transcription begins. Different promoter sequences have different affinities for RNA polymerase. Some sets of structural genes with linked functions have a single promoter and their coordinate expression is controlled by another regulatory gene called an operator. A group of such genes is called an operon. The activity of the operator is further controlled by a protein called a repressor, since it stops the expression of the whole operon by binding to the operator sequence, preventing RNA polymerase from binding to the promoter. Repressors can be removed by relevant chemical signals or in a time-related fashion.

In the ways described above, only the genes required at a given moment are expressed. This not only helps to conserve the energy of the cell but also is critical for correct cellular differentiation, tissue pattern formation, and formation of the body plan.

7.1.4 DNA Repair

All living cells appear to possess several different major DNA repair processes [for reviews see Walker (1984) and Rossman and Klein (1988)]. Such processes are needed to protect cells from the lethal and mutating effects of heat-induced DNA hydrolysis, ultraviolet light, ionizing radiation, DNA-reactive chemicals, free radicals, and so on. In single-celled eukaryotes such as the yeast *Saccharomyces cerevisiae*, the number of genes known to be involved in DNA repair approaches 100 (Friedberg, 1988). The number in mammalian cells is expected to be at least equal to this and emphasizes the importance of correction of DNA damage.

Excision Repair Some groups of enzymes (light independent) are apparently organized to act cooperatively to recognize DNA lesions, remove them, and correctly replace the damaged sections of DNA. The most comprehensively studied of these is the excision repair pathway.

Briefly, the pathway can be described as follows:

1. *Preincision Reactions* UvrA protein dimers are formed which bind to the DNA at a location distant from the damaged site. The UvrB protein then binds to the DNA–UvrA complex to produce an energy-requiring topological unwinding of the DNA via DNA gyrase. This area of unwinding is then translocated, again using adenosine triphosphate (ATP) as an energy source, to the site of the damaged DNA.
2. *Incision Reactions* The UvrC protein binds to the DNA–UvrA,B complex and incises DNA at two sites—seven bases to the 5′ end and three bases to the 3′ end of the damage.
3. *Excision Reactions* UvrD protein and DNA polymerase 1 excise the damaged bases and then resynthesize the strand using the sister strand as a template. The Uvr complex then breaks down, leaving a restored, but nicked, strand.
4. *Ligation Reaction* The nick in the phosphate backbone is repaired by DNA ligase. A similar excision repair mechanism exists in mammalian cells (see, e.g., Cleaver, 1983). In both cases, the process is regarded as error free and does not lead to the generation of mutations. However, this pathway can become saturated with excessive numbers of damaged DNA sites, forcing the cell to fall back on other repair mechanisms.

7.1.5 Error-Prone Repair

Exposure of *Escherichia coli* to agents or conditions that either damage DNA or interfere with DNA replication results in the increased expression of the so-called SOS regulatory network (Walker, 1984). Included in this network is a group of at least 17 unlinked DNA damage-inducible (*din*) genes. The *din* gene functions are repressed in undamaged cells by the product of the *lexA* gene (Little and Mount, 1982) and are induced when the LexA protein is cleaved by a process that requires modified RecA protein (RecA*), which then acts as a selective protease (Little, 1984). The *din* genes code for a variety of functions, including filamentation and cessation of respiration. Included are the *umuDC* gene products, which are required for so-called error-prone or mutagenic DNA repair (Kato and Shinoura, 1977). The precise biochemical mechanism by which this repair is achieved is still not fully understood. Bacterial polymerase molecules have complex activities, including the ability to "proofread" DNA—that is, to ensure that the base-pairing rules of double-stranded DNA are met. It is hypothesized that Umu proteins may suppress this proofreading activity, so that base mismatches are tolerated (Villani et al.,

1978). Recent evidence suggests that DNA lesions are bypassed, and this bypass step required UmuDC proteins and RecA* protein (Bridges et al., 1987). The net result is that random base insertion occurs opposite the lesion which may result in mutation.

Analogues of the *umuDC* genes can be found in locations other than the bacterial chromosome—for example, plasmid pKM101 (Walker and Dobson, 1979), a derivative of the drug resistance plasmid R46 (Mortelmans and Strocker, 1979), which carried *mucAB* genes (Shanabruch and Walker, 1980; see pp. 879–880). Mutagenic repair, as controlled by *umuDC*, is not universal even among enterobacteria (Sedgwick and Goodwin, 1985). For instance, *Salmonella typhimurium* LT2 does not appear to express mutagenic repair (Walker, 1984). Thus, the usefulness of strains of this species is greatly enhanced by using derivatives containing plasmids with genes coding for error-prone repair (MacPhee, 1973).

7.1.6 Mismatch Repair

Mismatched pairs that break the normal base-pairing rules can arise spontaneously due to DNA biosynthetic errors, events associated with genetic recombination, and the deamination of methylated cytosine (Modrich, 1987). With the latter, when cytosine deaminates to uracil, an endonuclease enzyme, *N*-uracil-DNA glycosylase (Lindahl, 1979), excises the uracil residue before it can pair with adenine at the next replication. However, 5-methyl cytosine deaminates to form thymine and will not be excised by a glycosylase. As a result, thymine exits on one strand paired with guanine on the sister strand, that is, a mismatch. This will result in a spontaneous point mutation if left unrepaired. For this reason, methylated cytosines form spontaneous mutation "hot spots" (Miller, 1985). The cell is able to repair mismatches by being able to distinguish between the DNA strand that exists before replication and a newly synthesized strand.

The mechanism of strand-directed mismatch correction has been demonstrated in *E. coli* (see, e.g., Wagner and Meselson, 1976). In this organism, adenine methylation of d(G–A–T–C) sequences determines the strand on which repair occurs. Thus, parental DNA is fully methylated, while newly synthesized DNA is undermethylated, for a period sufficient for mismatch correction. By this means the organism preserves the presumed correct sequence—that is, that present on the original DNA strand—and removes the aberrant base on the newly synthesized strand. Adenine methylation is achieved in *E. coli* by the *dam* methylase, which is dependent on *S*-adenosylmethionine. Mutants (*dam*) lacking this methylase are hypermutable, as would be expected by this model (Marinus and Morris, 1974).

7.1.7 Adaptive Repair Pathway

The mutagenic and carcinogenic effects of alkylating agents such as ethyl methane sulfonate are due to the generation of O^6-alkylguanine residues in

DNA, which result in point mutations. Bacterial and mammalian cells can repair a limited number of such lesions before DNA replication, thus preventing mutagenic and potentially lethal events taking place.

If *E. coli* are exposed to low concentrations of a simple alkylating agent, a repair mechanism is induced that causes increased resistance to subsequent challenge with a high dose. This adaptation response was first described by Samson and Cairns (1977) and has recently been reviewed by Lindahl et al. (1988). The repair pathway is particularly well understood.

7.1.8 Plasmids

Plasmids are extrachromosomal genetic elements that are composed of circular double-stranded DNA. In bacteria some can mediate their own transfer from cell to cell by conjugation—that is, they contain a set of *tra* genes coding for tubelike structures, such as pili, through which a copy of plasmid DNA can pass during transfer.

Plasmids range in size from 1.5 to 200 million daltons. The number of copies per cell differs from plasmid to plasmid. Copy number relates to control of replication and this correlates with size—that is, small plasmids tend to have large copy numbers per cell. This may relate to a lack of replication control genes (Mortelmanns and Dousman, 1986).

7.1.9 Plasmids and DNA Repair

Many plasmids are known to possess three properties: (1) increased resistance to the bactericidal effects of UV and chemical mutagens, (2) increased spontaneous mutagenesis, and (3) increased susceptibility to UV and chemically induced mutagenesis. Some plasmids possess all three properties; others may possess just one, for example, increased susceptibility to mutagenesis [for a review see Mortelmanns and Dousman (1986)]. Often the profile of activity depends on the DNA repair status of the host cell (Pinney, 1980). Plasmid pKM101 carries DNA repair genes and has been widely in strains in bacterial mutagenicity tests.

7.1.10 Nature of Point Mutations

The word "mutation" can be applied to point mutations, which are qualitative changes involving one or a few bases in base sequences within genes, as described below, as well as to larger changes involving whole chromosomes (and thus many thousands of genes), and even to changes in whole chromosome sets (described in Section 7.2).

Point mutations can occur when one base is substituted for another (base substitution). Substitution of another purine for a purine base or of another pyrimidine for a pyrimidine base is called a transition, while substitutions of purine for pyrimidine or pyrimidine for purine are called transversions. Both types of base substitution have been identified within mutated genes. These

changes lead to a codon change which can cause the "wrong" amino acid to be inserted into the relevant polypeptide and are known as missense mutations. Such polypeptides may have dramatically altered properties if the new amino acid is close to the active center of an enzyme or affects the three-dimensional makeup of an enzyme or a structural protein. These changes in turn can lead to change or reduction in function, which can be detected as a change in phenotype of the affected cells.

A base substitution can also result in the formation of a new inappropriate terminator (or nonsense) codon and is thus known as nonsense mutation. The polypeptide formed from such mutated genes will be shorter than normal and is most likely to be inactive. Owing to the redundancy of the genetic code, about a quarter of all possible base substitutions will not result in an amino acid replacement and will be silent mutations.

Bases can be deleted or added to a gene. As each gene is of a precisely defined length, these changes, if they involve a number of bases that is not a multiple of 3, result in a change in the "reading frame" of the DNA sequence and are thus known as frameshift mutations. Such mutations tend to have a dramatic effect on the polypeptide of the affected gene, as most amino acids will differ from the point of the insertion or deletion of bases onward. Very often a new terminator codon is produced, so, again, short inactive polypeptides will result.

Both types of mutation result in an altered polypeptide, which in turn can have a marked effect on the phenotype of the affected cell. Much use of phenotypic changes is made in mutagenicity tests.

Base substitutions and frameshift changes occur spontaneously and can be induced by radiations and chemical mutagens. It is apparent that the molecular mechanisms resulting in these changes are different in each case, but the potential hazards associated with mutagens capable of inducing the different types of mutation are equivalent.

7.1.11 Suppressor Mutations

In some instances a mutation within one gene can be corrected by a second mutational event at a separate site on the chromosome. As a result, the first defect is suppressed and the second mutation is known as a suppressor mutation. Most suppressor mutations have been found to affect genes encoding for RNAs. Usually the mutation causes a change in the sequence of the anticodon of the tRNA. Thus, if a new terminator or nonsense codon is formed as the first mutation, this can be suppressed by a second mutation forming a tRNA species that now has an anticodon complementary to a termination codon. Thus, the new tRNA species will supply an amino acid at the terminator site on the mRNA and allow translation to proceed. Surprisingly most suppressors of this type do not adversely affect cell growth, which implies that the cell can tolerate translation proceeding through termination signals, producing abnormal polypeptides. An alternative explanation is that the particular DNA

sequences surrounding normal terminator codons result in a reduced efficiency of suppressor tRNAs (Bossi, 1985).

Frameshift suppression is also possible. This can be achieved by a second mutation in a tRNA gene such that the anticodon of a tRNA molecule consists of four bases rather than three—for example, an extra C residue in the CCC anticodon sequence of a glycine tRNA gene. This change will allow correction of a +1 frameshift involving the GGG codon for glycine (Bossi, 1985).

7.1.12 Adduct Formation

The earlier discussion of adaptive repair made reference to the fact that some unrepaired alkylated bases are lethal, owing to interference with DNA replication, while others, such as O^6-methylguanine, lead to mutation if unrepaired. These differences indicate that not all DNA adducts (i.e., DNA bases with additional chemical groups, not associated with normal DNA physiology) are equivalent. In fact, some adducts appear not to interfere with normal DNA functions or are rapidly repaired; others are mutagenic and yet others are lethal. Chemicals that form electrophilic species readily form DNA adducts. These pieces of information are hard won, and the reader is recommended to read reviews of the pioneering work of Brooks and Lawley (see Lawley, 1989) summarizing work identifying the importance of DNA adduct formation with polycyclic hydrocarbons and the importance of "minor" products of base alkylation such as O^6-methylguanine and, in addition, the work of the Millers in linking attack of nucleophilic sites in DNA by electrophiles to mutagenesis and carcinogenesis (Miller and Miller, 1971).

If a DNA adduct involves the nitrogen or oxygen atoms involved in base pairing and the adducted DNA is not repaired, base substitution can result. Adducts can be small, such as the simple addition of methyl or ethyl groups, or they can be very bulky, owing to reaction with multiringed structures. The most vulnerable base is guanine, which can form adducts at several of its atoms (e.g., N^7, C^8, O^6, and exocyclic N^2) (Venitt and Parry, 1984). Adducts can form links between adjacent bases on the same strand (intrastrand crosslinks) and can form interstrand crosslinks between each strand of double-stranded DNA.

The induction of frameshift mutation does not necessarily require covalent adduct formation. Some compounds that have a flat, planar structure, such as particular polycyclic hydrocarbons, can intercalate between the DNA strands of the DNA duplex. The intercalated molecules interfere with DNA repair enzymes or replication and cause additions and deletions of base pairs. The precise mechanism is still unclear, although several mechanisms have been proposed. Hot spots for frameshift mutation often involve sections of DNA where there is a run of the same base—for example, the addition of a guanine to a run of six guanine residues. Such information led to a "slipped mispairing" model for frameshift mutation (Streisinger et al., 1966; Roth, 1974). In this scheme single-strand breaks allow one strand to slip and loop out one or more base pairs, the configuration being stabilized by complementary base pairing

at the end of the single-stranded region. Subsequent resynthesis results ultimately in additions or deletions of base pairs (Miller, 1985).

7.1.13 Mutations Due to Insertion Sequences

The subject of mutations due to insertion sequences is reviewed in Cullum (1985). Studies of spontaneous mutation in *E. coli* detected a special class of mutations that were strongly polar, reducing the expression of downstream genes (Jordan et al., 1967). These genes mapped as point mutations and reverted like typical point mutations. However, unlike point mutations, mutagens did not increase their reversion frequency. Further studies showed that these mutations were due to extra pieces of DNA that can be inserted into various places in the genome. They are not just random pieces of DNA but are "insertion sequences" 0.7–1.5 kb long that can "jump" into other DNA sequences. They are related to transposons, which are insertion sequences carrying easily detected markers such as antibiotic resistance genes, and Mu phages (bacterial viruses).

7.1.14 Link between Mutation and Cancer

The change in cells undergoing normal, controlled cell division and differentiation to cells that are transformed, dividing without check, and are undifferentiated or abnormally differentiated does not appear to occur as a single step—that is, transformation is multistage. Evidence for this comes from in vitro studies, animal models, and clinical observations—in particular, the long latent period between exposure to a carcinogen and the appearance of a tumor in the target tissue. There is much evidence for the sequence of events shown in Figure 7.1 (tumor initiation, promotion, malignant conversion, and progression). Such a scheme provides a useful working model but clearly does not apply to all "carcinogens" in all circumstances.

Study of Figure 7.1 shows that there are several points where genetic change appears to play a role. Such change may occur spontaneously, due to rare errors at cell division such as misreplication of DNA or spindle malfunction, or may be induced by exposure to viruses (e.g., acute transforming retroviruses), ionizing and nonionizing radiations absorbed by DNA (e.g., X-rays, UVC), or particular chemical species capable of covalently interacting with DNA (as discussed earlier) or with vital proteins, such as tubulin, that polymerize to form the cell division spindle apparatus.

7.1.15 Genetoxic versus Nongenotoxic Mechanisms of Carcinogenesis

The previous discussions of oncogene activation and human DNA repair deficiencies provide strong evidence for carcinogenesis via genotoxic mechanisms. However, it has been recognized for many years that cancers can arise

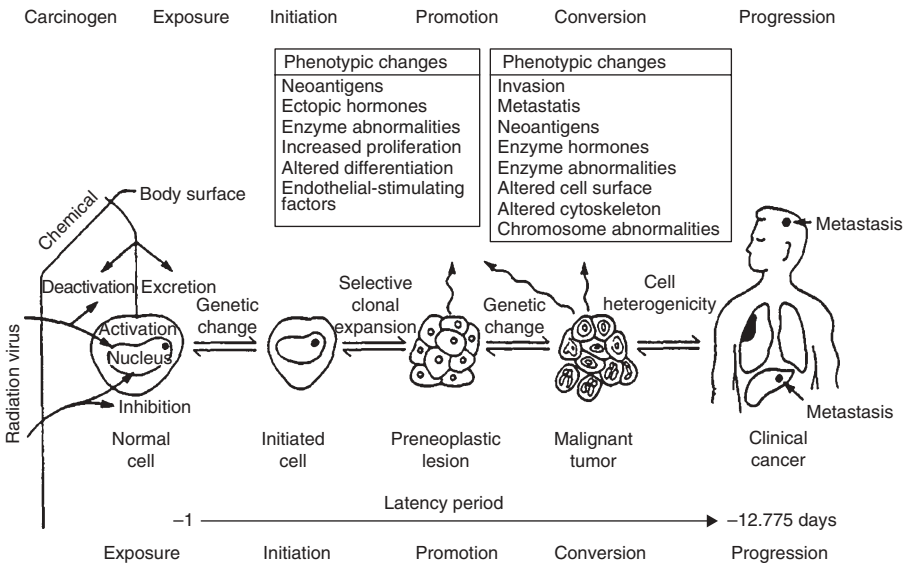

Figure 7.1 *Schematic representation of events leading to neoplasia. [Source: Adapted from Harris et al. (1987).]*

without biologically significant direct or indirect interaction between a chemical and cellular DNA (see, e.g., Gatehouse et al., 1988). The distinction between nongenotoxic and genotoxic carcinogens has recently been brought into a sharper focus following the identification of a comparatively large number of "nongenotoxic" carcinogens by the United States National Toxicology Program (Tennant et al., 1987). These include a wide range of chemicals acting via a variety of mechanisms, including augmentation of high "spontaneous" tumor yields; disruption of normal hormonal homeostasis in hormone-responsive tissues; peroxisome proliferation; and proliferation of urothelial cells following damage via induced kidney stones (Clayson, 1989). This author points out that a major effort is under way to determine whether many of these compounds can elicit similar effects in humans.

Ashby and Tennant (1988) and Ashby et al. (1989) stress the significance of their observations that 16 tissues are apparently sensitive to genotoxic carcinogens, while a further 13 tissues are sensitive to both genotoxic and nongenotoxic carcinogens (Table 7.2). Also, genotoxic carcinogens tend to induce tumors in several tissues of both males and females in both rats and mice. This contrasts with nongenotoxic carcinogens, which may induce tumors at high doses, in one tissue, of one sex, of one species. Although it is most unlikely that all nongenotoxic carcinogens will prove to be irrelevant in terms of human risk, it appears from the analysis above that a proportion of carcinogens identified by the use of near-toxic levels in rodent bioassays are of dubious relevance to the induction of human cancer. For further discussion, see Butterworth and Slaga (1987).

TABLE 7.2 Tissues Sensitive to Genotoxic and/or Nongenotoxic Carcinogens

Tissues Sensitive Primarily to Genotoxins	Tissues Sensitive to Both Genotoxins and Nongenotoxins
Stomach	Nose
Zymbal gland	Mammary gland
Lung	Pituitary gland
Subcutaneous tissue	Integumentary system
Circulatory system	Kidney
Clitoral gland	Urinary bladder
Skin	Liver
Intestine/colon	Thyroid gland
Uterus	Hematopoietic system
Spleen	Adrenal gland
Tunica vaginalis	Pancreas
Bile duct	Seminal vesicle
Ovary	Urinary tract
Haderian gland	Lymphatic system
Preputial gland	
Multiple organ sites	

7.1.16 Genetic Damage and Heritable Defects

Concern about the effects of radiations and chemicals on the human gene pool and the resulting heritable malformations and syndromes have steadily risen during this century. The recognition that changes in morphology would result from changes in the hereditary material due to mutations (from the Latin word *mutare*, "to change") was adopted by de Vries following observations on the evening primrose, *Oenothera* (deVries, 1901). Muller went on to demonstrate that X-rays could induce mutations in the germ cells of the fruit fly *Drosophila melanogaster* (Muller, 1927).

The human gene pool is known to carry many deleterious genes acquired from preceding generations which result in numerous genetic disease. It is clear that these arise as a result of DNA changes affecting particular chromosomes or genes. They can be grouped as follows:

1. Chromosome abnormalities, small changes in either number or structure
2. Autosomal-dominant gene mutations, in which a change in only one copy of the pair of genes is sufficient for the condition to be expressed
3. Autosomal-recessive gene mutations in which both copies of a gene must be mutated for the trait to become manifest
4. Sex-linked conditions, which may also be recessive or dominant, where the mutant gene is on an X chromosome and will be expressed at high frequency in males (XY) and at a much lower frequency in females (XX) if the gene acts in a recessive manner

5. Polygenic mutations, in which the condition results from the interaction of several genes and may include an environmental component

7.1.17 Reproductive Effects

If a potent genotoxin is able to cross the placental barrier, it is very likely to interfere with differentiation of the developing embryo and thus possess teratogenic potential. Indeed, many of the better studied teratogens are also mutagenic (Kalter, 1977). However, mutagens form only one class of teratogens and a large proportion of teratogens are not mutagenic. Alternative mechanisms of teratogenesis include cell death, mitotic delay, retarded differentiation, vascular insufficiency, and inhibited cell migration (Beckman and Brent, 1986).

It is known that more fetal wastage and many spontaneous abortions arise as a result of the presence of dominant lethal mutations in the developing embryo, many of which appear to be due to major chromosomal damage. In addition, impairment of male fertility may also be a consequence of exposure to mutagens.

7.2 CYTOGENETICS

There are various types of cytogenetic change which can be detected in chromosomes. These are structural chromosome aberrations (Cas), numerical changes which could result in aneuploidy, and sister chromatid exchanges (SCEs). Chromosome aberration assays are used to detect the induction of chromosome breakage (clastogenesis) in somatic or germinal cells by direct observation of chromosome damage during metaphase analysis or by indirect observation of micronuclei. Chromosome damage detected in these assays is mostly lethal to the cell during the cell cycle following the induction of the damage. Its presence, however, indicates a potential to induce more subtle chromosome damage which survives cell division to produce heritable cytogenetic changes. Cytogenetic damage is usually accompanied by other genotoxic damage such as gene mutation.

7.2.1 Cytogenetic Damage and Its Consequences

Structural and numerical chromosomal aberrations in somatic cells may be involved in the etiology of neoplasia and in germ cells can lead to perinatal mortality, dominant lethality or congenital malformations in the offspring (Chandley, 1981), and some tumors (Anderson, 1990).

Chromosome defects arise at the level of the individual chromosome or at the level of the chromosomal set, so affecting chromosomal number.

7.2.2 Individual Chromosome Damage

Damage to individual chromosomes consists of breakage of chromatids, which must result from a discontinuity of both strands of the DNA in a chromatid. How mutagens produce chromosome breakage is not totally understood, but DNA lesions which are not in themselves discontinuities will produce breakage of a chromosome as a consequence of their interference with the normal process of DNA replication. In haploid microorganisms and prokaryotes chromosome breaks are usually lethal, but not in diploid eukaryotes. According to Bender et al. (1974), in these organisms chromosome breaks may reconstitute in the same order, probably as a result of an enzyme repair process, resulting in no apparent cytogenetic damage; they may remain unjoined as fragments, which could result in cell death at the next or following mitoses—if, for example, unrejoined fragments are introduced into the zygote via treated germ cells, the embryo may die at a very early stage from a dominant lethal mutation or they may rejoin in a different order form the original one, producing chromosomal rearrangements. There are various types of chromosomal rearrangements:

Reciprocal translocations can result from the exchange of chromosomal segments between two chromosomes and, depending on the position of the centromeres in the rearranged chromosomes, different configurations will result.

1. Asymmetrical exchanges arise when one of the rearranged chromosomes carries both centromeres and is known as dicentric while the other carries none and is acentric. The cell or zygote carrying this anomaly usually dies, death being caused by segregation difficulties of the dicentric or the loss of the acentric fragment at cell division. Such a translocation contributes to dominant lethality.
2. Symmetrical exchanges occur when each rearranged chromosome carries just one centromere. This allows the zygote to develop normally, but when such heterozygotes form germ cells at meiosis, about half of their gametes will be genetically unbalanced, since they have deficiencies and duplications of chromosomal material. The unbalanced gametes which survive produce unbalanced zygotes, which results in death shortly before and after birth, or congenital malformations.

Centric fusions involve the joining together of two chromosomes, each of which has a centromere at or near one end, to produce a single metacentric or submetacentric chromosome. When such translocations are produced in a germ cell and result from breakage and rejoining in the short arms of the two chromosomes, as a consequence of loss of the derived acentric fragments, a genetic deficiency can result. Some Robertsonian translocations are able to survive but others pose a risk. In heterozygotes the two arms of the translocation chromosome may pair with the two separate homologous chromosomes

at meiosis but segregate in a disorderly manner. Some of the resultant germ cells lack copies (nullisomy) or carry two copies (disomy) of one or other of the two chromosomes involved, which results in monosomic or trisomic embryos. Monosomics die early but trisomic embryos, which carry three copies of a chromosome, can survive to birth or beyond. If chromosome 21 is involved in the translocation, it can form a translocation trisomy and produce inherited Down syndrome (this differs from nondisjunctional Down syndrome trisomy).

Deletions and deficiencies are produced when two breaks arise close together in the same chromosome. The two ends of the chromosome join when the fragment between the breaks becomes detached. At the next cell division the unattached piece of chromosome is likely to be lost. Large deletions may contribute to dominant lethality. Small deletions are difficult to distinguish from point mutations. Deletions may uncover preexisting recessive genes. If one gene that is essential for survival is uncovered, it can act as a lethal in a homozygote and as a partial dominant in a heterozygote.

Inversions occur when two breaks occur in the same chromosome. The portion between them is detached and becomes reinserted in the opposite way to its original position, that is, the gene order is reversed. This need not cause a genetic problem, but imbalanced gametes could result in congenital malformation or fetal death.

7.2.3 Chromosome Set Damage

Accuracy of chromosome replication and segregation of chromosomes to daughter cells requires accurate maintenance of the chromosome complement of a eukaryotic cell. Chromosome segregation in meiosis and mitosis is dependent upon the synthesis and functioning of the proteins of the spindle apparatus and upon the attachment and movement of chromosomes on the spindle. The kinetochores attach the chromosomes to the spindle and the centrioles are responsible for the polar orientation of the division apparatus. Sometimes such segregation events proceed incorrectly and homologous chromosomes separate, with deviations from the normal number (aneuploidy) into daughter cells or as a multiple of the complete karyotype (polyploidy). When both copies of a particular chromosome move into a daughter cell and the other cell receives none, the event is known as nondisjunction.

Aneuploidy in live births and abortions arises from aneuploid gametes during germ cell meiosis. Trisomy or monosomy of large chromosomes leads to early embryonic death. Trisomy of the smaller chromosomes allows survival but is detrimental to the health of an affected person—for example, Down syndrome (trisomy 21), Patau syndrome (trisomy 13), and Edward syndrome (trisomy 18). Sex chromosome trisomies (Klinefelter's and XXX syndromes) and the sex chromosome monosomy (XO), known as Turner syndrome, are also compatible with survival.

Aneuploidy in somatic cells is involved in the formation of human tumors. Up to 10% of tumors are monosomic and trisomic for a specific chromosome

as the single observable cytogenetic change. Most common among such tumors are trisomy 8, 9, 12, and 21 and monosomy for chromosomes 7, 22, and Y.

7.2.4 Test Systems

In vivo and in vitro techniques are available to test mutagenic properties to demonstrate presence or lack of ability of the test material to cause mutation or chromosomal damage or cause cancer, as summarized in Table 7.3. The material intended for intimate contact and long exposure should not have any genotoxic properties. The presence of unpolymerized materials and traces of monomers, oligomers, additives, or biodegration products can cause mutations. Mutation can be a point mutation or chromosomal rearrangement caused by DNA damage. Therefore, the material's ability to cause point mutation, chromosomal change, or evidence of DNA damage is tested. As we have seen, correlations exist between mutagenic and carcinogenic properties. Most carcinogens are mutagens, but not all mutagens are human carcinogens.

The Ames salmonella/microsome test is a principal sensitive mutagen screening test. Compounds are tested on the mutants of *S. typhimurium* for reversion from a histidine requirement back to prototrophy. A positive result is seen by the growth of revertant bacteria (which do not require an external

TABLE 7.3 Fifteen Common Assays Described by OECD

	In Vitro	In Vivo
Assays for Gene Mutations		
Salmonella typhimurium reverse mutation assay (Ames test, bacteria) (OECD 471)	✓	
Escherichia coli reverse mutation assay (bacteria) (OECD 472)	✓	
Gene mutation in mammalian cells in culture (OECD 476)	✓	
Drosophila sex-linked recessive lethal assay (fruit fly) (OECD 477)		✓
Gene mutation in *Saccharomyces cerevisiae* (yeast) (OECD 480)	✓	
Mouse spot test (OECD 484)		✓
Assays for Chromosomal and Genomic Mutations		
In vitro cytogenetic assay (OECD 473)	✓	
In vivo cytogenetic assay (OICD 475)		✓
Micronucleus test (OECD 474)		✓
Dominant lethal assay (OECD 478)		✓
Heritable translocation assay (OECD 485)		✓
Mammalian germ cell cytogenetic assay (OECD 483)		✓
Assays for DNA Effects		
DNA damage and repair: unscheduled DNA synthesis in vitro (OECD 482)	✓	
Mitotic recombination in *S. cerevisiae* (yeast) (OECD 481)	✓	
In vitro sister chromatid exchange assay (OECD 479)	✓	

histidine source). A microsomal activation system should be included in this assay. The use of five different bacterial test strains are generally required.

Two mammalian mutagenicity tests are generally required to support the lack of mutagenic or carcinogenic potential. Some well known tests are:

- The L5178Y mouse lymphoma assay (MLA) for mutants at the TK locus
- Induction of recessive lethals in *D. melanogaster*
- Metaphase analysis of cultured mammalian cells and of treated animals
- SCE assay
- Unscheduled DNA synthesis (UDS) assay
- Cell transformation assay
- SOS repair system assay
- Gene mutation in cultured mammalian cells such as Chinese hamster V79 cell/hypoxanthine guanine phosphoribosyl transferase (HGPRT) mutation system

The ICH guidelines specifically require three genotoxicity assays for all drugs (see Table 7.4). The assays should preferably evaluate DNA effects, gene mutations, and chromosomal aberrations, and two of the assays should preferably use mammalian cells. Guidance for providing tests for selection to meet these needs is contained in the Organisation for Economic Co-operation and Devolpment (OECD) guidelines, which include eight in vitro and seven in vivo assays.

7.2.5 In Vitro Test Systems

The principal tests can be broadly categorized into microbial and mammalian cell assays. In both cases the tests are carried out in the presence and

TABLE 7.4 Alternative Test under ICH

Aspect	Comet Assay	DNA Adducts	UDS Test (Liver)	Transgenic Gene Mutation
Test definition (accepted protocol)	Yes	No	Yes	Yes
Regulatory acceptance/ use	Yes	Yes	Yes	Yes
Relevance of endpoint	Moderate	Moderate	Moderate	High
Technical demands	Low moderate	Moderate high	Moderate	High
Widespread use	Yes	No	Yes	No
Applicable to most tissues	Yes	Yes	No	Yes
Dependence of cell turnover	No	No	No	Yes
Cost	Low moderate	Moderate high	Low	high

absence of in vitro metabolic activation enzymes, usually derived from rodent liver.

In Vitro Metabolic Activation The target cells for in vitro mutagenicity tests often possess a limited (often overlooked) capacity for endogenous metabolism of xenobiotics. However, to simulate the complexity of metabolic events that occur in the whole animal, there is a critical need to supplement this activity.

Choice of Species A bewildering variety of exogenous systems have been used for one purpose or another in mutagenicity tests. The choice begins with plant or animal preparations. The attraction of plant systems has stemmed from a desire to avoid the use of animals, where possible, in toxicity testing. In addition, plant systems have particular relevance when certain chemicals are being tested, for example, herbicides.

If animal systems are chosen, preparations derived from fish (see, e.g., Kada, 1981) and birds (Parry et al., 1985) have been used. However, by far the most widely used and validated are those derived from rodents—in particular, the rat. Hamsters may be preferred as a source of metabolizing enzymes when particular chemical classes are being screened—for example, aromatic amines, heterocyclic amines, *N*-nitrosamines, and azo dyes (Prival and Mitchell, 1982; Haworth et al., 1983).

Choice of Tissue The next choice is that of source tissue. Preparations derived from liver are the most useful, as this tissue is a rich source of mixed-function oxygenases capable of converting procarcinogens to genetically active electrophiles. However, many extrahepatic tissues (e.g., kidney, lung) are also known to possess important metabolic capacity which may be relevant to the production of mutagenic metabolites in the whole animal.

Cell-Free versus Cell-Based Systems Most use has been made of cell-free systems—in particular, crude homogenates such as 9000 g supernatant (S9 fraction) from rat liver. This fraction is composed of free endoplasmic reticulum, microsomes (membrane-bound packets of "membrane-associated" enzymes), soluble enzymes, and some cofactors. Hepatic S9 fractions do not necessarily completely reflect the metabolism of the whole organ, in that they mainly possess phase I metabolism (e.g., oxygenases) and are deficient in phase II systems (e.g., conjugation enzymes). The latter are often capable of efficient detoxification, while the former are regarded as "activating." This can be a strength in that S9 fractions are used in screening tests as a surrogate for all tissues in an animals, some of which may be exposed to reactive metabolites in the absence of efficient detoxification. Many carcinogens are organ specific in extrahepatic tissues, yet liver S9 fraction will reveal their mutagenicity. The deficiency of S9 fractions for detoxification can also be a weakness, in that detoxification may predominate in the whole animal, such that the potential carcinogenicity revealed in vitro is not realized in vivo.

Cell-free systems, when supplemented with relevant cofactors, are remarkably proficient, despite their crudity in generating reactive electrophiles from most procarcinogens. However, they provide at best a broad approximation of in vivo metabolism and can fail to produce sufficient quantity of a particular reactive metabolite to be detectable by the indicator cells or they can produce inappropriate metabolites that do not play a role in vivo [for a discussion see Gatehouse and Tweats (1987)].

Some of these problems can be overcome by the use of cell-based systems—in particular, primary hepatocytes. Hepatocytes closely simulate the metabolic systems found in the intact liver and do not require additional cofactors for optimal enzyme activity. However, apart from greater technical difficulties in obtaining hepatocytes as opposed to S9 fraction, hepatocytes can effectively detoxify particular carcinogens and prevent their detection as mutagens. Despite these difficulties, hepatocytes have a role to play in mutagenicity screening in both bacterial and mammalian-based systems (Tweats and Gatehouse, 1988).

Inducing Agents The final choice considered here is whether to use "uninduced" liver preparations or those derived from animals pretreated with an enzyme inducer to promote high levels of metabolic activity. If induced preparations are preferred, which inducer should be used?

It appears that uninduced preparations are of limited use in screening assays, as they are deficient in particular important activities such as cytochrome P-450_{IA1} cytochrome oxygenases. In addition, species and organ differences are most divergent with uninduced enzyme preparations (Brusick, 1987a).

The above differences disappear when induced microsomal preparations are used. A number of enzyme inducers have been used, the most popular being Aroclor 1254, which is a mixture of polychlorinated bipheynyls (as described by Ames et al., 1975). However, concern about the toxicity, carcinogenicity, and persistence of these compounds in the environment has led to the use of alternatives, such as a combination of phenobarbitone (phenobarbital) and β-naphthoflavone (5,6-benzoflavone). This combination results in the induction of a range of monooxygenases similar to that induced by Aroclor 1254 (see, e.g., Ong et al., 1980). More selective inducers such as phenobarbitone (cytochrome P-450_{IIa1}, P-450_{IIB1}) or 3-methylcholanthrene (cytochrome P-450_{IA1}) have also been used.

In summary, genetic toxicity tests with both bacterial and mammalian cells are normally carried out with rat liver cell-free systems (S9 fraction) from animals pretreated with enzyme inducers. However, investigations should not slavishly follow this regimen: There may be sound scientifically based reasons for using preparations from different species or different organs or for using whole cells such as hepatocytes.

Standard Method of S9 Fraction Preparation The following method describes the production of hepatic S9 mix from rats induced with a combina-

tion of phenobarbitone and β-naphthoflavone and is an adaptation of the method described by Gatehouse and Delow (1979).

Male albino rats within the weight range 150–250 g are treated with phenobarbitone sodium 16 mg mL^{-1}, 2.5 mL kg^{-1} in sterile saline and β-naphthoflavone 20 mg mL^{-1} in corn oil. A fine suspension of the latter is achieved by sonicating for 1 h. These solutions are dosed by intraperitoneal injection on days 1, 2, and 3.

Phenobarbitone sodium is normally administered between 0.5 and 2 h prior to β-naphthoflavone.

The animals are killed on day 4 by cervical dislocation and the livers removed as quickly as possible and placed on ice-cold KCI buffer (0.01 M Na_2HPO_4 + KCl 1.15%). The liver is cleaned, weighted, minced, and homogenized (in an Ultra Turrx homogenizer) in the above buffer to give a 25% (w/v) liver homogenate. The homogenate is stored at 4 °C until it can be centrifuged at 9000*g* for 15 min. The supernatant is decanted, mixed, and divided into 2-mL volumes in cryotubes. These are then snap frozen in liquid nitrogen. Storage at –196 °C for up to three months results in no appreciable loss of most P-450 isoenzymes (Ashwood-Smith, 1980).

Quality control of S9 batches is usually monitored by ability to activate compounds known to require metabolism to generate mutagenic metabolites. This is a rather crude approach and more accurate data can be obtained by measuring biochemical paramets—for example, protein, cytochrome P-450 total activity (from crude S9), and related enzyme activities (from purified microsomes) such as 7-ethoxyresorufin-*O*-deethylase and 7-methoxycoumarin-*O*-demethylase—to give an indication of S9 batch-to-batch variation and to set standards for rejecting suboptimal batches (Hubbard et al., 1985). For further details on critical features affecting the use and limitations of S9 fraction, see Gatehouse and Tweats (1987).

S9 Mix The S9 fraction prepared as described above is used as a component in "S9 mix" along with buffers and various enzyme cofactors. The amount of S9 fraction in the S9 mix can be varied, but a "standard" level of 0.1 mL mL^{-1} of S9 mix (or 10% S9) is often recommended for general screening.

No single concentration of S9 fraction in the S9 mix will detect all classes of genotoxic carcinogen with equal efficiency (Gatehouse et al., 1990). Some mutagens, including many polycyclic aromatic hydrocarbons, are activated to mutagens by higher than normal levels of S9 fraction in the S9 mix (see, e.g., Carver et al., 1985).

The mixed-function oxidases in the S9 fraction require NADPH (the reduced form of nicotinamide adenine dinucleotide phosphate), normally generated from the action of glucose-6-phosphate dehydrogenase acting on glucose-6-phosphate and reducing NADP, both of which are normally supplied as cofactors. As an alternative, isocitrate can be substituted for glucose-6-phosphate (to be used as a substrate by isocitrate dehydrogenase) (Linblad and Jackim, 1982). Additional cofactors may be added (e.g., flavin mono-

TABLE 7.5 Composition of Standard S9 Mix

Constituent	Final Concentration in Mix (mM)
Glucose-6-phosphate	5
Nicotinamide adenine dinucleotide phosphate	4
Salt solution	
$MgC1_26H_2O$	8
KC1	33
Phosphate buffer (90.2 M)	100
Distilled water to make up to required volume	
S9 fraction added at 0.1 mL mL^{-1} S9 mix	

Note: For assays using cultured mammalian cells, phosphate buffer and distilled water are replaced by tissue culture medium as high concentrations of Na and K salts are toxic to such cells. The concentration of S9 fraction in the S9 mix varies, depending on the relevant assay (see individual sections). Once prepared, S9 mix should be used as soon as possible, and should be stored on ice until required. S9 fraction, once thawed, should not be refrozen for future use.

nucleotide) when particular classes of compound such as azo dyes are being tested (Prival et al., 1984) or acetyl coenzyme A when aromatic animes such as benzidine are being tested (Kennelly et al., 1984). The composition of a standard S9 mix is given in Table 7.5.

7.2.6 Bacterial Mutation Tests

The study of mutation in bacteria (and bacterial viruses) has had a fundamental role in the science of genetics in the twentieth century. In particular, the unraveling of biochemical anabolic and catabolic pathways, the identification of DNA as the hereditary material, the fine structure of the gene, the nature of gene regulation, and so on, have all been aided by bacterial mutants.

As an offshoot of studies of genes concerned with the biosynthesis of amino acids, a range of *E. coli* (see, e.g., Yanofsky, 1971) and *S. typhimurium* strains (see, e.g., Ames, 1971) with relatively well-defined mutations in known genes became available. Thus, bacteria already mutant at an easily detectable locus are treated with a range of doses of the test material to determine whether the compound can induce a second mutation that directly reverses or suppresses the original mutations. Thus, for amino acid auxotrophs, the original mutation has resulted in loss of ability to grow in the absence of the required amino acid. The second mutation restores prototrophy—that is, the affected cell is now able to grow in the absence of the relevant amino acid if provided with inorganic salts and a carbon source. This simple concept in fact underlines the great strength of these assays, for it provides enormous selective power which can identify a small number off the chosen mutants from a population of millions of unmutated cells and cells mutated in other genes. The genetic target—that is, the mutated DNA bases in the gene in question (or bases in the relevant tRNA genes; see the discussion of suppressor mutations)—can thus be very small, just one or a few bases in length.

An alternative approach is to use bacteria to detect "forward mutations." Genetic systems which detect forward mutations have an apparent advantage in that a wide variety of genetic changes may lead to a forward mutation—for example, point mutation, deletions, and insertions. In addition, forward mutations in a number of different genes may lead to the same change in phenotype; thus, the genetic target is much larger than that seen in most reverse mutation assays. However, if a particular mutagen causes rare specific changes, these changes may be lost against the background of more common events (Gatehouse et al., 1990). Spontaneous mutation rates tend to be relatively high in forward mutation systems. Acquisition of resistance to a toxic chemical (e.g., an amino acid analogue or antibiotic) is a frequently used genetic marker in these systems. For instance, the use of resistance to the antibiotic streptomycin preceded the reversion assays in common use today.

Reversion Tests: Background There are several excellent references describing the background and use of bacteria for reversion tests (Brusick, 1987a,b; Gatehouse et al., 1990). Three different protocols have been widely used: plate incorporation assays, treat and plate tests, and fluctuation tests. These methods are described in detail in the following sections. Fundamental to the operation of these tests is the genetic compositions of the tester strains selected for use.

Genetic Make-Up of Tester Strains The most widely used strains are those developed by Bruce Ames and colleagues which are mutant derivatives of the organism *S. typhimurium*. Each strain carries one of a number of mutations in the operon coding for histidine biosynthesis. In each case the mutation can be reverted either by base change or by frameshift mutations. The genotype of the commonly used strains is shown in Table 7.6.

TABLE 7.6 Genotype of Commonly Used Strains of *Salmonella typhimurium* LT2 and Their Reversion Events

Strain	Genotype	Reversion Events
TA1535	$hisG_{46}$ rfa ◑ gal chlD bio uvrB	Subset of base-pair substitution events
TA100	$hisG_{46}$ ◑ rfa gal chlD bio uvrB (pKM101)	Subset of base-pair substitution events
TA1537	$hisC_{3076}$ ◑ rfa gal chlD bio uvrB	Frameshifts
TA1538	$hisD_{3052}$ ◑ rfa gal chlD bio uvrB	Frameshifts
TA98	$hisD_{3052}$ ◑ rfa gal chlD bio uvrB pKM101)	Frameshifts
TA97	$hisD_{6610}$ $hisO_{1242}$ rfa ◑ gal chl^{D} bio uvrB (pKM101)	Frameshifts
TA102	his · $(\dot{G})_{8476}$ rfa galE (pAQ1) (pKM101)	All possible transitions and transversions; small deletions

Use of Plasmid pKM101 *Salmonella typhimurium* LT2 strains do not appear to possess classical "error-prone" repair as found in *E. coli* strains and some other members of the Enterobacteria (Walker, 1984; Sedgwick and Goodwin, 1985). This is due to a deficiency in *umuD* activity in these *Salmonella* strains (Herrera et al., 1988; Thomas and Sedgewick, 1989). One way to overcome this deficiency and to increase sensitivity to mutagens is to use strains containing a plasmid carrying analogues to the *umuDC* genes, such as are present in the pKM101 pasmid.

Ames Salmonella/Plate Incorporation Method The following procedure is based on that described by Ames and colleagues (Maron and Ames, 1983), with additional modifications:

1. Each selected test strain is grown for 10 h at 37 °C in nutrient broth (Oxoid no. 2) or supplemented minimal media (Vogel-Bonner) on an orbital shaker. A timing device can be used to ensure that cultures are ready at the beginning of the working day.
2. Two-milliliter aliquots of soft agar overlay medium are melted just prior to use and cooled to 50 °C, and relevant supplements added—that is, L-histidine, final concentration 9.55 μg mL^{-1}, and D-biotin, 12 μg mL^{-1}. (*Note:* If *E. coli* WP2 tester strains are used, the only supplement required is tryptophan 3.6 μg mL^{-1}.) The medium is kept semimolten by holding the tubes containing the medium in a hot aluminum dry block held at 45 °C. It is best to avoid water baths as microbial contamination can cause problems.
3. The following additions are made to each tube of top agar: the test article (or solvent control) in solution (10–200 μL), the test strain (100 μL), and, where necessary, S9 mix (500 μL). The test is carried out in the presence and absence of S9 mix. The exact volume of test article or solvent may depend on toxicity or solubility, as described in the preceding section.
4. There should be at least three replicate plates per treatment with at least five test doses plus untreated controls. Duplicate plates are sufficient for the positive and sterility control treatments. The use of twice as many negative control plates as used in each treatment group will lead to more powerful tests from a statistical standpoint (Mahon et al., 1989).
5. Each tube of top agar is mixed and quickly poured onto dried prelabeled Vogel-Bonner basal agar plates.
6. The soft agar is allowed to set at room temperature and the plates are inverted and incubated (within 1 h of pouring) at 37 °C in the dark. Incubation is continued for two to three days.
7. Before scoring the plates for revertant colonies, the presence of a light background lawn of growth (due to limited growth of nonrevertant colonies before the trace of histidine or tryptophan is exhausted) should be

confirmed for each concentration of test article by examination of the plate under low power of a light microscope. At concentrations that are toxic to the test strains, such a lawn will be deplated and colonies may appear that are not true revertants but surviving, nonprototrophic cells. If necessary, the phenotype of any questionable colonies (pseudorevertants) should be checked by plating on histidine or tryptophan-free medium.

8. Revertant colonies can be counted by hand or with an automatic colony counter. Such machines are relatively accurate in the range of colonies normally observed (although regular calibration against manual counts is a wise precaution). Where accurate quantitative counts of plates with large numbers of colonies are required, only manual counts will give accurate results.

7.2.7 Controls

Positive Controls Where possible, positive controls should be chosen that are structurally related to the test article. This increases the confidence in the results. In the absence of structurally related mutagens, the set of positive controls given in Table 7.7 can be used. The use of such controls validates each test run and helps to confirm the nature of each strain. Pagano and Zeiger (1985) have shown that it is possible to store stock solutions of most routinely used positive controls (sodium azide, 2-aminoanthracene, benzo[*a*]phyene, 4-nitroquinoline oxide) at –20 to –80 °C for several months without loss of

TABLE 7.7 Positive Controls for Use in Plate Incorporation Assays

Species	Strain	Mutagen	Concentration (μg plate^{-1})[a]
(a) In absence of S9 mix			
S. typhimurium	TA1535, TA100	Sodium azide	1–5
	TA1538, TA98	Hycanthone methane sulfonate	5–20
	TA1537	ICR 191	1
E. coli	WP2 uvrA	Nifuroxime	5–15
(b) In presence of S9 mix			
E. coli	WP2 uvraA (pKM101)		
S. typhimurium	TA1538, TA1535, TA100, TA90	2-Aminoanthracene	1–10
	TA1537	Neutral red	10–20

[a]The concentration given above will give relatively small increases in revertant count above the spontaneous level. There is little point in using large concentrations of reference mutagens which invariably give huge increases in revertant counts. This would give little information on the day-to-day performance of the assay.

activity. This measure can help reduce potential exposure to laboratory personnel.

Untreated/Vehicle Controls Untreated controls omit the test article but are made up to volume with buffer. The vehicle control is made up to volume with the solvent used to dissolve the test substance. It is preferable to ensure that each of the treated plates contains the same volume of vehicle throughout.

As detailed by Gatehouse and Tweats (1987), the nature and concentration of solvent may have a marked effect on the test result. Dimethylsulfoxide (DMSO) is often used as the solvent of choice for hydrophobic compounds. However, there may be unforeseen effects, such as an increase in mutagenicity of some compounds—for example, *p*-phenylenediamine (Burnett et al., 1982)—or a decrease in mutagenicity of others, such as simple aliphatic nitrosoamines (Yahagi et al., 1977). It is essential to use fresh batches of the highest purity grade available and to prevent decomposition/oxidation on storage. The products after oxidation, for example, both are toxic and can induce base-pair substitutions in both bacterial and mammalian assays. Finally, DMSO and other organic solvents can inhibit the oxidation of different premutagens by microsomal monooxygenases (Wolff, 1977a,b). To reduce the risk of artifactual results, it is essential to use the minimum amount of organic solvent (e.g., <2% w/w/) compatible with adequate testing of the test chemical.

It is important to keep a careful check of the number of mutant colonies present on untreated or vehicle control plates. These numbers depend on the following factors:

1. The repair status of the cell—that is, excision repair-deficient strains tend to have more "spontaneous mutants" than repair-proficient cells.
2. The presence of mutator plasmids. Excision-deficient strains containing pKM101 have a higher spontaneous mutation rate at both base substitution and frameshift loci than excision-proficient strains.
3. The total number of cell divisions that take place in the supplemented top agar. This is controlled by the supply of nutrients—in particular, histidine. Rat liver extracts may also supply trace amounts of limiting nutrients, resulting in a slight increase in the spontaneous yield of mutants in the presence of S9 mix.
4. The size of the initial inoculum. During growth of the starting culture, mutants will arise. Thus, if a larger starting inoculum is used, more of these "preexisting" mutants will be present per plate. In fact, the "plate mutants" arising as described in point 3 predominate.
5. The intrinsic mutability of the mutation in question. In practice the control mutation values tend to fall within a relatively precise range for each strain. Each laboratory should determine the normal range of revertant colonies per plate for each strain.

Deviations in background reversion counts from the normal range should be investigated. It is possible that cross-contamination, variations in media quality, and so on, have occurred that may invalidate particular experiments.

Frequent checks should also be made on the sterility of S9 preparations, media, and test articles. These simple precautions can prevent loss of valuable time and resources.

Evaluation of Results At least two independent assays are carried out for each test article. The criterion for positive response is a reproducible and statistically significant result at any concentration for any strain. When positive results are obtained, the test is repeated using the strain(s) and concentration range with which the initial positive results were observed. This range may be quite narrow for toxic agents.

Several statistical approaches have been applied to the results of plate incorporation assays (Mahon et al., 1989). These authors make a number of important suggestions to maximize the power of statistical analyses; those that relate to the method of analysis are reproduced below:

1. Unless it is obvious that the test agent has had no effect, the data should be plotted to give a visual impression of the form of any dose response and the pattern of variability.
2. Three methods of analysis—linear regression (Gad, 1999; Steel and Torrie, 1960); a multiple-comparison analysis, Dunnett's (1955) method; and a nonparametric analysis, such as Kruskal–Wallis (Gad, 1999)—can all be recommended. Each has its strengths and weaknesses, and other methods are not excluded.
3. Linear regression assumes that variance across doses is constant and that the dose response is linear. If the variance is not approximately constant, then a transformation may be applied or a weighted analysis may be carried out. If the dose scale tends to a plateau, then the dose scale may be transformed. If counts decline markedly at high doses, then linear regression is inappropriate.
4. Dunnett's method, perhaps with a transformation, is recommended when counts decline markedly at one or two high doses. However, when the dose response shows no such decline, other methods may be more powerful.
5. Kruskal–Wallis's nonparametric method avoids the complications of transformations of weighting and is about as powerful as any other method. However, it is inappropriate when the response declines markedly at high dose.

Preincubation Tests Some mutagens are poorly detected in the standard plate incorporation assay, particularly those that are metabolized to short-

lived reactive electrophiles—for example, short-chain aliphatic *N*-nitroso compounds (Bartsch et al., 1984). It is also possible that some metabolites may bind to components within the agar. Such compounds can be detected by using a preincubation method first described by Yahagi et al. (1975) in which the bacteria, test compound, and S9 mix are incubated together in a small volume at 37 °C for a short period (30–60 min) before adding the soft agar and pouring as for the standard assay. In this variation of the test, during the preincubation step, the test compound, S9 mix, and bacteria are incubated in liquid at higher concentrations than in the standard test, and this may account for the increased sensitivity with relevant mutagens. In the standard method the soluble enzymes in the S9 mix, cofactors, and test agent may diffuse into the bottom agar. This can interfere with the detection of some mutagens—a problem that is overcome in the preincubation method (Forster et al., 1980; Gatehouse and Wedd, 1984).

The test is carried out as follows:

1. The strains are cultured overnight, and the inocula and S9 mix are prepared as in the standard Ames test.
2. The soft agar overlays are prepared and maintained at 45 °C prior to use.
3. To each of three to five tubes maintained at 37 °C in a Driblock are added 0.5 mL of S9 mix, 0.1 mL of the tester strain (10–18-h culture), and a suitable volume of the test compound to yield the desired range of concentrations. The S9 mix is kept on ice prior to use.
4. The reaction mixtures are incubated for 1 h at 37 °C.
5. Two milliliters of soft agar is added to each tube. After mixing, the agar and reaction mixture are poured onto previously labeled, dried Vogel–Bonner plates.
6. Once the agar has set, the plates are incubated for two to three days before revertant colonies are scored.

The use of controls is as described for the plate incorporation assay. It is crucial to use the minimum amount of organic solvent in this assay, as the total volume of the incubation mixture is small relative to the solvent component.

This procedure can be modified to provide optimum conditions for particular chemical classes. For instance, preincubation times greater than 60 min plus aeration have been found necessary in the detection of allyl compounds (Neudecker and Henschler, 1985).

***E. coli* Tester Strains** Ames and colleagues have made an impressive contribution to mutagenicity testing by the development of the *Salmonella*/ microsome test and, in particular, its application in the study of environmental mutagens. In genetic terms, *Salmonella* strains are, in some ways, not the best choice (see, e.g., Venitt and Croften-Sleigh, 1981). Unlike the *Salmonella*

strains, *E. coli* B strains such as the WP2 series developed by Bridges, Green, and colleagues (Bridges, 1972; Green and Muriel, 1976) inherently possess the *umuDC*$^+$ genes involved in generating mutations; they are also part rough and thus allow many large molecules to enter the cell.

In addition to being effective general strains for mutagen detection, studies by Wilcox et al. (1990) have shown that a combination of *E. coli* WP2 *trp E* (pKM101), which has a functioning excision repair system for the detection of crosslinking agents, and *E. coli* WP2 *trp E uvrA* (pKM101) can be used as alternatives to *Salmonella* TA102 for the detection of oxidative mutagens. The *E. coli* strains have the advantage of lower spontaneous mutation rate and are somewhat less difficult to use and maintain. The *Salmonella* strains are, however, more commonly employed.

Storage and Checking of Tester Strains Detailed instructions for maintenance and confirmation of the phenotypes of the various tester strains are given in Maron and Ames (1983) and Gatehouse et al. (1990). Permanent master cultures of tester strains should be stored in liquid nitrogen or in dry ice. Such cultures are prepared from fresh nutrient broth cultures to which DMSO is added as a cryopreservative. These cultures are checked for the various characteristics before storage as described below. Cultures for use in individual experiments should be set up by inoculation from the master culture or from a plate made directly from the master culture, not by passage from a previously used culture. Passage in this way will inevitably increase the number of preexisting mutants, leading to unacceptably high spontaneous mutation rates (Gatehouse et al., 1990).

The following characteristics of the tester strains should be confirmed at monthly intervals or if the internal controls of a particular experiment fail to meet the required limits:

- Amino acid requirement.
- Sensitivity to the lethal effects of the high-molecular-weight dye crystal violet for those strains carrying the *rfaE* mutation.
- Increased sensitivity to UV irradiation for those strains carrying the *uvrA* or *uvrB* mutations.
- Resistance to ampicillin for strains carrying pKM101 and resistance to tetracycline for strains carrying pAQ1.
- Sensitivity to diagnostic mutagens. This can be measured very satisfactorily by testing pairs of strains—one giving a strongly positive response, the partner a weak response.

The importance of these checks together with careful experiment-to-experiment controls of spontaneous mutation rates and response to reference mutation rates and response to reference mutagens cannot be overstressed; failure to apply them can result in much wasted effort.

7.2.8 Plate Incorporation Assay

Protocol for Dose Ranging and Selection Before carrying out the main tests, it is necessary to carry out a preliminary toxicity dose-ranging test. This should be carried out following the same basic protocol as the mutation test, except that instead of scoring the number of mutants on, for example, minimal media plates with limiting amounts of a required amino acid, the number of survivors is scored on fully supplemented minimal media. A typical protocol is outlined below:

1. Prepare a stock solution of the test compound at a concentration of $50\,mg\,mL^{-1}$ in an appropriate solvent. It may be necessary to prepare a lower concentration of stock solution, depending on the solubility of the test compound.
2. Make dilutions of the stock solution.
3. To 2.0-mL aliquots of soft agar overlay medium (0.6% agar and 0.5% sodium chloride in distilled water) containing a trace of histidine and excess biotin and maintained at 45 °C in a dry block, add 100 µL of a solution of the test article. Use only one plate per dilution.
4. Mix and pour onto dried Vogel and Bonner minimal medium plates as in an Ames test, including an untreated control and a solvent control, if necessary. The final concentrations of test compound will be 5000, 1500, 500, 150, and $50\,\mu g\,plate^{-1}$.
5. Repeat step 3 using 0.5 mL of 8% S9 mix per 2.0-mL aliquot of soft agar in addition to the test compound and tester strain. The S9 mix is kept on ice during the experiment.
6. Incubate the plates for two days at 37 °C and examine the background lawn of growth with a microscope (8-eyepiece lens, 10-objective lens). The lowest concentration giving a depleted background lawn is regarded as a toxic dose.

This test will also demonstrate excess growth, which may indicate the presence of histidine or tryptophan or their precursors in the test material, which could make testing for mutagenicity impracticable by this method.

When setting the maximum test concentration, it is important to test into the milligram-per-plate range where possible (Gatehouse et al., 1990), as some mutagens are only detectable when tested at high concentrations. However, for nontoxic, soluble mutagens an upper limit of $5\,mg\,plate^{-1}$ is recommended (DeSerres and Shelby, 1979). For less soluble compounds at least one dose exhibiting precipitation should be included.

Forward Mutation Tests Forward mutation is an endpoint that may arise from various events, including base substitutions, frameshifts, DNA deletions, and so on, as mentioned earlier.

7.2.9 Eukaryotic Mutation Tests

Prokaryotic systems, as described, have proved to be quick, versatile, and in many cases surprisingly accurate in identifying potential genetic hazards to humans. However, there are intrinsic differences between eukaryotic and prokaryotic cells in the organization of the genome and the processing of the genetic information. Thus, there is a place for test systems based on mammalian cells for fundamental studies to understand the mutation process in higher cells and for the use of such tests for screening for genotoxic effects.

The early work of Muller showed the usefulness of the fruit fly *D. melanogaster* as a higher system for measuring germ line mutations in a whole animal. The *Drosophila* sex-linked recessive lethal test has yielded much useful information and in the 1970s was a popular system for screening chemicals for mutation, but this test failed to perform well in international collaborative trials to study the utility of such tests to detect carcinogens and popularity wanted. Another *Drosophila* test devised in the 1980s, the SMART assay (somatic mutation and recombination test) shows much promise and has revived the popularity of *Drosophila* for screening for genotoxic agent.

A number of test systems that use cultured mammalian cells from both established and primary lines now have a large database of tested chemicals in the literature, are relatively rapid, and are feasible to use for genetic toxicity screening. These are discussed in the next section.

7.2.10 In Vitro Tests for Detection of Mammalian Mutation

There have been a variety of in vitro mutation systems described in the literature, but only a small number have been defined adequately for quantitative studies (Cole et al., 1990). These are based on the detection of forward mutations in a similar manner to the systems described earlier for bacteria. A defined large number of cells are treated with the test agent and then, after a set interval, exposed to a selective toxic agent, so that only cells that have mutated can survive. As cultured mammalian cells are diploid (or near diploid), normally there are two copies of each gene. Recessive mutations can be missed if a normal copy is present on the homologous chromosome. As mutation frequencies for individual genes are normally very low, an impossibly large population of cells would need to be screened to detect cells in which both copies are inactivated by mutation. This problem is overcome by measuring mutation in genes on the X chromosome in male cells where only one copy of the gene will be present or using heterozygous genes where two copies of a gene may be present but one copy is already inactive through mutation or deletion.

Many genes are essential for the survival of the cell in culture, and thus mutations in such genes would be difficult to detect. However, use has been made of genes that are not essential for cell survival but allow the cell to

salvage nucleotides from the surrounding medium. This saves the cell energy, as it does not have to make these compounds from simpler precursors by energy-expensive catabolism. These enzymes are located at the cell membrane. If the cell is supplied with toxic nucleotides, the "normal" unmutated cells will transport these into the cell and kill the cell. However, if the cells have lost the enzyme as a result of mutation (or chromosomal deletion, rearrangement, etc.), then they will not be able to "salvage" the exogenous toxic nucleotides and will survive. The surviving mutant cells can be detected by the formation of colonies on tissue culture plates or, in some cases, in the wells of microtiter plates.

One factor to take into account with these tests is that of expression time. Although a gene may be inactivated by mutation, the mRNA existing before the mutational event may decay only slowly, so that active enzyme may be present for some time after exposure to the mutagen. Thus, the cells have to be left for a period before challenging with the toxic nucleotide: This is the expression time and differs between systems.

Chinese Hamster Lines Chinese hamster cell lines have given much valuable data over the past 15 years, but their use for screening is limited by lack of sensitivity, as only a relatively small target cell population can be used, owing to metabolic cooperation (see Cole et al., 1990); however, they are still in use, so a brief description follows.

Chinese hamster ovary (CHO) and V79 lines have high plating efficiencies and short generation times (less than 24 h). These properties make the lines useful for mutagenicity experiments. Both cell lines have grossly rearranged chromosomal complements, which has an unknown effect on their responsiveness to mutagens (Tweats and Gatehouse, 1988). There is some evidence that Chinese hamster lines are undergoing genetic drift in different culture collections (Kirkland and Garner, 1987).

V79 System The Chinese hamster V79 line was established in 1958 (Ford and Yerganian, 1958). Publication of the use of the line for mutation studies (by measuring resistance to purine analogues due to mutation of the *hgprt* locus) occurred 10 years later (Chu and Malling, 1968). The V79 line was derived from a male Chinese hamster; hence, V79 cells possess only a single X chromosome.

V79 cells grow as a cell sheet or monolayer on glass or plastic surfaces. If large numbers of cells are treated with a mutagen, when plated out, cells in close contact can link via intracellular bridges. These allow the transfer of cellular components between cells such as mRNA. Thus, if a cell carries a mutation in the *hgprt* gene resulting in the inactivation of the relevant mRNA, it can receive viable mRNA or intact enzyme from adjacent nonmutated cells. Therefore, when the mutated cell is challenged with a toxic purine, it is lost, owing to the presence of active enzyme derived from the imported mRNA. This phenomenon is termed *metabolic cooperation* and severely limited the

sensitivity of lines such as V79 for mutagen detection. This drawback can be overcome to an extent by carrying out the detection of mutant clones in semi-solid agar (see, e.g., Oberly et al., 1987) or by using the "respreading technique" (Fox, 1981).

The preferred expression time for *hgprt* mutants is six to eight days, although care needs to be taken when testing chemicals well into the toxic range, where the "expression time" needs to be extended to allow recovery.

Preliminary Cytotoxicity Testing An essential first step is to carry out a preliminary study to evaluate the toxicity of the test material to the indicator cells under the conditions of the main mutagenicity test. When selecting dose levels, the solubility of the test compound, the resulting pH of the media, and the osmolality of the test solutions all need to be considered. The latter two parameters have been known to induce false-positive effects in in vitro mammalian tests (Brusick, 1986). The experimental procedure is carried out as follows:

1. Seek T75 plastic tissue culture flasks with a minimum of 2.5×10^6 cells in 120 mL of Eagle's medium containing 20 mM L-glutamine: 0.88 g L^{-1} sodium bicarbonate, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 50 μg mL^{-1} streptomycin sulfate, 50 IU mL^{-1} benzylpenicillin, and 7.5% of fetal bovine serum. Incubate the flasks for 18–24 h at 37 °C in a CO_2 incubator to establish monolayer cultures.
2. Prepare treatment medium containing various concentrations of test compound—for example, 19.7 mL of Eagle's medium (without serum) plus 300 μL of stock concentration of compound in a preferred solvent (e.g., water, ethanol, DMSO, etc.). The final concentration of solvent other than water should not exceed 1% v/v. Normally a range of 0–5000 μg mL^{-1} (final concentration) is covered. For a sparingly soluble compound, the highest concentration will be the lowest at which visible precipitation occurs. Similarly, if a compound has a marked effect on osmolality, concentrations should not be used that exceed 500 milliosmoles (mOsm) per kilogram. In addition, a pH range of 6.5–7.5 should be maintained.
3. Rinse each cell monolayer with a minimum of 20 mL phosphate-buffered saline (PBS) and then carefully add 20 mL of treatment medium. Incubate the flasks for 3 h at 37 °C in a CO_2 incubator.
4. After treatment, carefully discard the medium from each flask and wash each monolayer twice with PBS. Care needs to be taken to safely dispose of contaminated solutions.
5. To each flask add 10 mL of trypsin solution (0.025% trypsin in PBS). Once the cells have rounded up, neutralize the trypsin by adding 10 mL of complete medium. A cell suspension is obtained by vigorous pipetting to break up cell clumps.

6. Count the trypsinized cell suspension and dilute in complete media before assessing for survival. For each treatment set up five Petri dishes containing 200 cells per dish.
7. Incubate at 37 °C in a CO_2 incubator for 7–10 days.
8. Remove the medium and fix and stain the colonies using 5% Giemsa in buffered formalin. Once the colonies are stained, remove the Giemsa and count the colonies.

The method can be repeated including 20% v/v S9 mix.

To calculate percentage survival, the following formula is used:

$$\frac{\text{Cell titer in treated culture}}{\text{Cell titer in control culture}} \times \frac{\text{mean no. of colonies on treated plates}}{\text{mean no. of colonies on control plates}} \times 100$$

The cloning efficiency (CE) of the control culture is calculated as

$$\text{CE} = \frac{\text{mean no. of colonies per plate}}{\text{no. of cells per plate (i.e., 200)}} \times 100$$

In the absence of precipitation or effects on pH or osmolality, the maximum concentration of the main mutagenicity study is a concentration that reduces survival to approximately 20% of the control value.

Procedure for the Chinese Hamster V79/Hgprt Assay The assay usually comprises three test concentrations, each in duplicate, and four vehicle control replicates. Suitable positive controls are ethylmethane sulfonate (–S9) and dimethyl benzanthracene (+S9). V79 cells with a low nominal passage number should be used from frozen stocks to help minimize genetic drift. The procedure described includes a reseeding step for mutation expression.

Steps 1–5 are the same as the cytotoxicity assay. As before, tests can be carried out in the presence and in the absence of S9 mix:

6. Count the trypsinized cultures and assess a sample for survival as for the cytotoxicity assay. In addition, reseed an appropriate number of cells for estimation of mutation frequency at day 8 of the expression time. Transfer the cells to roller bottles (usually 490 cm^2) for this stage. Gas the bottles with pure CO_2, tighten the tops, and incubate the bottles at 37 °C on a roller machine (approximate speed 0.5–1.0 rev min^{-1}). Usually 10^6 viable cells are reseeded in 50 mL of Eagle's medium containing serum, but more cells are required at the toxic dose levels.
7. Subculture the bottles as necessary throughout the expression period to maintain subconfluency. This involves retrypsinization and determining the cell titer for each treatment. For each culture a fresh roller bottle is reseeded with a minimum of 10^6 cells.

8. On day 8, trypsinize, count, and dilute each culture again so that a sample cell population can be assessed for cloning efficiency and a second sample can be assessed for the induction of 6TG-resistant cells.
9. Dilute the cell suspension in complete medium and add 2×10^5 cells per Petri dish (10 Petri dishes per treatment). Add 6-thioguanine to the medium to a final concentration of $10\,\mu g\,mL^{-1}$.
10. Incubate the Petri dishes for 7–10 days and then remove the medium. Fix and stain the colonies are as previously. Then count the colonies (>50 cells per clone).

Mutation frequency in each culture is calculated as

$$\frac{\text{Mean no. colonies on thioguanine plates}}{1000 \times \text{mean no. colonies on survival plates}}$$

Data Analysis (Arlett et al., 1989) A weighted analysis of variance is performed on the mutation frequencies, as the variation in the number of mutations per plate usually increases as the mean increases. Each dose of test compound is compared with the corresponding vehicle control by means of a one-sided Dunnett's test and, in addition, the mutation frequencies are examined to see whether there is a linear relationship with dose.

The criterion employed for a positive response in this assay is a reproducible statistically significant increase in mutation frequency (weighted mean for duplicate treated cultures) over the concurrent vehicle control value (weighted mean for four independent control cultures). Ideally, the response should show evidence of a dose–response relationship. When a small isolated significant increase in mutation frequency is observed in only one of the two duplicate experiments, then a third test should be carried out. If the third test shows no significant effects, the initial increase is likely to be a chance result. In cases where an apparent treated-related increase is thought to be a result of unusually low variability or a low control frequency, comparison with the laboratory historical control frequency may be justified.

CHO/Hgprt System The CHO cells have 21 or 22 chromosomes with one intact X chromosome and a large acrocentric marker chromosome (Natarajan and Obe, 1982). The use of these cells in mammalian mutation experiments was first reported by Hsie et al. (1975) and was refined into a quantitative assay for mutagenicity testing by O'Neill. The performance of this system has been reviewed by the U.S. Environmental Protection Agency (EPA) Gene-Tox Program. The experimental procedure for this assay is similar to the V79/Hgprt system already described, and for more detailed descriptions the reader is referred to Li et al. (1987).

Mouse Lymphoma L5178Y TK$^{+/-}$ Assay Whereas the Chinese hamster cell systems are regarded as relatively insensitive, the mouse lymphoma L5178Y

$TK^{+/-}$ test is undoubtedly more sensitive. Unfortunately, there are persistent doubts regarding its specificity—that is, the ability to distinguish between carcinogens and noncarcinogens (see, e.g., Tennant et al., 1987). However, a great advantage is the ability of these cells to grow in suspension culture in which intracellular bridges do not occur. Thus, the problems of metabolic cooperation are avoided, which allows a large number of cells to be treated for optimum statistical analysis of results.

A candid historical overview of the development of the mouse lymphoma $TK^{(+/-}$ mutagenicity assay is given by its originator, Clive (1987). Initially methodologies were developed for producing the three TK genotypes ($TK^{+/+}$ and $TK^{-/-}$ homozygotes and the $TK^{+/-}$ heterozygotes (Clive et al., 1972). This first heterozygote was lost; however, it was recognized that subsequent heterozygotes produced distinctly bimodal distributions of mutant colony sizes, owing to differences in growth rate. These were interpreted in terms of single-gene (large-colony mutants) and viable chromosomal mutations (small-colony mutants). A period of diversification of the mouse lymphoma assay followed with controversy over the significance of small-colony mutants (Amacher et al., 1980).

Following this, a series of cytogenetic studies confirmed the cytogenetic interpretation for small-colony mutants (see, e.g., Hozier et al., 1982). Molecular studies showed that most mutations resulting in small-colony mutants involve large-scale deletions (Evans et al., 1986). A current theory states that, for many compounds, deletion mutants are induced by binding of the compound to complexes between topoisomerase II and DNA (Clive, 1989). Topoisomerases are enzymes that control supercoiling via breakage and reunion of DNA strands; it is the latter strep that is disrupted, which leads to chromosome damage and deletions. Further molecular studies (Applegate et al., 1990) have shown that a wide variety of genetic events can result in the formation of $TK^{+/-}$ genotype from the heterozygote, including recombinations and mitotic nondisjunction.

The $TK^{+/-}$ line was originally isolated as a spontaneously arising revertant clone from a UV-induced $TK^{-/-}$ clone. The parental $TK^{+/+}$ cell and the heterozygote were then the only TK-competent mouse lymphoma cells that could be maintained in THMG medium ($3\,\mu g\,mL^{-1}$ thymidine, $5\,\mu g\,mL^{-1}$ hypoxanthine, $0.1\,\mu g\,mL^{-1}$ methotrexate, and $7.5\,\mu g\,mL^{-1}$ glycine) (Clive, 1987). Thus, like most established lines, these cells are remote from wild-type cells. The karyotype of the $TK^{+/-}$–3.7.2C line has a modal chromosome number of 40, like wild type, but has a variety of chromosomal rearrangements and centromeric heteromorphisms (Blazak et al., 1986).

Two main protocols have been devised for carrying out mutation assays with mouse lymphoma L5178Y cells—that is, plating the cells in soft agar or a fluctuation test approach. It is the latter that is described in the following section, based on Cole et al. (1986). The reader is referred to Clive et al. (1987) for a full description of the soft-agar method.

Preliminary Cytotoxicity Assay The cells are maintained in RPMI 1640 medium containing 2.0 mM glutamine, 20 mM HEPES, 200 $\mu g\,mL^{-1}$ sodium pyruvate, 50 $IU\,mL^{-1}$ benzylpenicillin, 50 $\mu g\,mL^{-1}$ streptomycin sulfate, and 10% donor horse serum (heat inactivated for 30 min at 56 °C). This medium is designated CM10. Conditioned medium is CM10 in which cells have grown exponentially for at least one day. Treatment medium contains 3% horse serum and 30% conditioned media (CM3). Medium without serum is known as incomplete medium (ICM). If treatment time exceeds 3 h, treatment is carried out in CM10.

The method is as follows:

1. The cell titer of an exponentially growing culture of cells in CM10 is determined with a Coulter counter. The cell suspension is centrifuged at 70 *g* for 5 min and the supernatant is reduced such that 3 mL contains approximately 5×10^6 cells (3 h treatment) or 2×10^6 (treatment >3 h).

2a. For tests in the absence of S9 mix, treatment groups are prepared by mixing 3 mL of solution of test compound and 6.9 mL of ICM (3 h treatment) or 6.9 mL of CM10 (treatment >3 h).

2b. Tests in the presence of S9 mix are carried out in the same way, except the treatment medium contains 10% v/v S9 mix at the expense of ICM—that is, 3 mL cell suspension, 5.9 mL ICM, 1 mL S9 mix, and 0.1 mL test compound solution per vehicle. The composition of the S9 mix is as described earlier. It is prepared immediately before required and kept on ice until it is added to the test system. For the vehicle controls, if an organic solvent is used, it should not exceed 1% v/v.

3. After the treatment period, cells are spun down at 70 *g* for 5 min and the supernatant is transferred for assessment of pH and osmolality. The cell pellet is washed twice in PBS and then resuspended in 10 mL CM10. (All contaminated material and waste should be disposed of safely.)

4. The cell titer of each culture is counted and a sample diluted in CM10 for assessment of posttreatment survival. For this two 96-well microtiter plates are charged with 200 µL of a diluted cell suspension using a multichannel pipette such that each well contains on average one cell.

5. Plates are incubated for seven to eight days at 37 °C and 5% CO_2 in 95 ± 3% relative humidity.

6. The plates are removed from the incubator and 20 µL of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] at 5 $mg\,mL^{-1}$ (in PBS) is added to each well with a multichannel pipette. The plates are left to stand for 1–4 h and are then scored for the presence of colonies with a Titertek mirror-box, which allows direct viewing of the bottom surface of the plates.

7. Cytotoxicity can also be determined posttreatment as follows: T25 flasks are set up after treatment containing 0.75×10^5 cells mL^{-1} in 5 mL

CM10. Flasks are incubated with loose lids at 37 °C with 5% CO_2 in 95 ± 3% relative humidity. Two days later the cell titer of each culture is determined with a Coulter counter.

8. Following this procedure, various calculations are carried out to aid selection of dose levels for the main mutation assay.

   (a) *Cloning Efficiency* In microtiter assays calculations are based on the Poisson distribution:

$$P(o) = \frac{\text{no. of wells without colony}}{\text{total no. of cells}}$$

   (b) *Relative Survival* Relative survival (S) is calculated as

$$S = \frac{\text{CE of treated group}}{\text{CE of control group}}$$

   (c) *Growth* Growth in suspension (SG) is calculated as

$$\text{SG} = \frac{\text{cell count after 3 days}}{0.75 \times 10^5}$$

   Relative suspension growth (RSG) is calculated as

$$\text{RSG} = \frac{\text{SG of treated group}}{\text{SG of control group}} \times 100\%$$

Selection of Dose Levels The highest test concentration is selected from one of the following options, whichever is lowest:

- A concentration which reduces survival to about 10–20% of the control value.
- A concentration which reduces RSG to 10–20% of the control value.
- The lowest concentration at which visible precipitation occurs.
- The highest concentration which does not increase the osmolality of the medium to greater than 400 mmol kg^{-1} or 100 mmol above the value for the solvent control.
- The highest concentration that does not alter the pH of the treatment medium beyond the range 6.8–7.5.
- If none of these conditions are met, 5 mg mL^{-1} should be used.

Lower test concentrations are selected as fractions of the highest concentration, usually including one dose which causes 20–70% survival and one dose which causes >70% survival.

Main Mutation Assay The assay normally comprises three test concentrations, a positive control, and a vehicle control. All treatment groups are set up in duplicate. The expression time is two days, unless there are indications that the test agent inhibits cell proliferation, where an additional or possibly alternative expression time should be employed.

Stock cultures are established from frozen ampoules of cells that have been treated with thymidine, hypoxanthine, methotrexate, and glycine for 24 h, which purges the culture of preexisting $TK^{-/-}$ mutants. This cell stock is used for a maximum of two months.

Treatment is normally carried out in 50-mL centrifuge tubes on a roller machine. During the expression time the cells are grown in T75 plastic tissue culture flasks. For estimation of cloning efficiency and mutant induction, cells are plated out in 96-well microtiter plates. Flasks and microtiter plates are incubated at 37 °C in a CO_2 incubator as in the cytotoxicity assays.

Cell titers are determined by diluting the cell suspension in Isoton and counting an appropriate volume (usually 0.5 mL) with a Coulter counter. Two counts are made per suspension.

The experimental procedure is carried out as follows:

1. On the day of treatment stock solutions for the positive control and the various concentrations of test compound (selected as per the previous selection) are prepared.
2. Treatment is carried out in 30% conditioned media. The serum concentration is 3% (3 h treatment) or 10% (treated >3 h).
3. Cell suspensions of exponentially growing cells are prepared as in the cytotoxicity assay, except that 6 mL of media required for each treatment culture contains 10^7 cells (3 h treatment) or 3×10^6 cells (>3 h treatment). The number of cells per treatment may be increased if marked cytotoxicity is expected to allow enough cells to survive (e.g., if 20% survival or less is expected, 2×10^7 cells may be treated).
4. For tests in the absence of S9 mix, 6 mL of cell suspension, 0.2 mL test compound/vehicle, and 13.8 mL ICM (3 h treatment) or 13.8 mL CM10 (treatment >7 h) are mixed in the presence of S9 mix and 0.2 mL of test compound per vehicle is prepared.
5. After treatment the cells are centrifuged at 70 *g* for 5 min, supernatant is discarded, and the cell pellet is resuspended in PBS (pH 7). This washing procedure is repeated twice, and finally the cell pellet is resuspended in CM10.
6. Each culture is counted so that a sample of cells can be assessed for posttreatment survival, and the remaining cell population is assessed for estimation of mutation frequency.
7. For survival estimation, cells are placed into 96-well microtiter trays at a cell density of one cell per well as per the cytotoxicity assay.

8. For mutation estimation, the cells are diluted to a cell density of 2×10^5 cells mL^{-1} with CM10 in tissue culture flasks and the culture is incubated at 37 °C in a CO_2 incubator. On day 1 each culture is counted and diluted with fresh medium to a cell density of 2×10^5 cells mL^{-1} in a maximum of 100 mL of medium.
9. On day 2 each culture is counted again and an aliquot of cells taken so that: (a) A sample of the cell population can be assessed for cloning efficiency. Plates are incubated at 37 °C in a CO_2 incubator for seven days. (b) A sample of the cell population can be assessed for the induction of TFT (tetrafluorotoluene)-resistant cells (mutants). For this 2×10^3 cells are plated per well in 200 µL CM10 containing 4 µg mL^{-1} TFT. TFT and TFT-containing cultures must not be exposed to bright light, as the material is light sensitive. The plates are incubated for 10–12 days at 37 °C in a CO_2 incubator.
10. At the end of incubation 20 µL MTT is added to each well. The plates are left to develop for 1–4 h at 37 °C and then scored for colony-bearing wells. Colonies are scored by eye and are classified as small or large.

The calculation for cloning efficiency is made as for the cytotoxicity assay.

Relative total growth (RTG) is a cytotoxicity parameter which considers growth in suspension during the expression time and the cloning efficiency of the end of the expression time as follows:

$$\text{SG} = \frac{24\,\text{h cell count}}{2 \times 10^4} \times \frac{48\,\text{h cell count}}{2 \times 10^5}$$

$$\text{RTG} = \frac{\text{SG treated culture}}{\text{SG control culture}} \times \frac{\text{CE of treated culture}}{\text{CE of control culture}}$$

Mutation frequency (MF) is calculated as

$$\text{MF} = \frac{\text{InPo for mutation plates}}{\text{no. of cells per well} \times \text{CE}/100}$$

In Vivo Genotoxicity Tests for Assessment of Primary DNA Lesions Primary DNA lesions are detected with so-called indicator tests. These tests do not directly measure consequences of DNA interaction (i.e., mutation) but do detect effects related to the process of mutagenesis, such as DNA damage, recombination, and repair. Results from indicator tests can provide additional useful information in the context of extended genotoxicity testing. However, primary DNA lesions may be repaired error free and do not necessarily result in formation of mutations. The most commonly utilized assays in pharmaceutical development are the phosphorus-postlabeling assay

TABLE 7.8 Genotoxicity Tests Recommended by ICH

Genotoxicity Test	Mutation	Cell Type	Method
Test for gene mutation in bacteria	Gene	Bacterial	In vitro
In vitro cytogenetic assay using mouse lymphomas tk cells	Chromosome	Mammalian	In vitro
In vivo test for chromosomal damage using rodent hematopoietic cells	Gene	Mammalian	In vivo

and the comet assay. A comparison of different aspects of the methods described in the text is depicted in Table 7.8. Basic aspects regarding optimal study design for in vivo micronucleus assays are largely applicable to the design of supplemental in vivo assays. Specific or unique aspects on study protocols are described more extensively where appropriate.

Comet Assay The in vivo comet assay (single-cell gel electrophoresis) is increasingly being used as a supplement genotoxicity test for drug candidates (Hartman et al., 1993; Brendler-Schwaab et al., 2005) There are general review articles on the comet assay (Tice, 2000; Sheit and Hartman, 2005) and a general guideline for test conductance has been published as a result of the International Workshop on Genotoxicity Test Procedures (IWGTP).

More specific recommendations with the goal of gaining more formal regulatory acceptance of the comet assay were published following the fourth International Comet Assay Workshop (Hartman et al., 2007). An updated position paper on specific aspects of tests conditions and data interpretation was prepared following the IWGTP in 2005 (Burlinson et al., 2006). See Table 7.2.

Principle of Method The basic principle of the comet assay is the migration of DNA in an agarose matrix under electrophoretic conditions. When viewed through the microscope, a cell has the appearance of a comet, with a head (the nuclear region) and a tail containing DNA fragments or strands migrating in the direction of the anode. Among the various versions of the comet assay, the alkaline (pH of the unwinding and electrophoresis buffer at least 13) method enables detection of the broadest spectrum of DNA damage and is therefore urgently recommended (in the first instance) for regulatory purposes (Tice et al., 2000). The alkaline version detects DNA damage such as strand breaks, alkali-labile sites (ALS), and single-strand breaks associated with incomplete excision repair. Under certain conditions, the assay can also detect DNA–DNA and DNA–protein crosslinking, which (in the absence of other kinds of DNA lesions) appears as a relative decrease in DNA migration compared to concurrent controls. In contrast to other DNA alterations, crosslinks may stabilize chromosomal DNA and inhibit DNA migration (Merks et al., 1998). Thus, reduced DNA migration in comparison to the negative control (which

should show some degree of DNA migration) may indicate the induction of crosslinks, which are relevant lesions with regard to mutagenesis and should be further investigated. Increased DNA migration indicated the induction of DNA strand breaks and/or ALS. Furthermore, enhanced activity of excision repair may result in increased DNA migration. DNA excision repair can influence comet assay effects in a complex way (Speit and Hartmann, 1995). While DNA repair generally reduces DNA migration by eliminating DNA lesions, ongoing excision repair may increase DNA migration to incision-related DNA strand breaks. Thus, the contribution of excision repair to the DNA effects seen in the comet assay depends on the types of induced primary DNA damage and the time point of analysis (Collins et al., 1993). Test procedure aspects regarding test animals, test substance, use of concurrent negative and positive control animals, as well as dose selection for the design of a cytogenetic assay, as described in detail previously, are largely applicable to the design of an in vivo comet assay. In addition, more specific details can be found in an earlier publication. A single treatment or repeated treatments (generally at 24-h intervals) are equally acceptable. In both experimental designs, the study is acceptable as long as a positive effect has been demonstrated or, for a negative result, as long as an appropriate level of animal or tissue toxicity has been demonstrated or the limit dose with appropriate level tissue exposure has been used. For repeated treatment schedules, dosing must be continued until the day of sampling. On a daily basis, test substances may be administrated as a split dose (i.e., two treatments separated by no more than a few hours) to facilitate administering a large volume of material. The test may be performed in two ways. If animals are treated with the test substances once, then tissue/organ samples are obtained at 2–6 and 16–26 h after dosing. The shorter sampling time is considered sufficient to detect rapidly absorbed as well as unstable or direct-acting compounds. In contrast, the late sampling time is intended to detect compounds that are more slowly absorbed, disturbed, and metabolized. When a positive response is identified at one sampling time, data from the other sample time need not be collected. Alternatively, if multiple treatments at 24-h intervals are used, tissue/organ samples need be collected only once. The sampling time should be 2–6 h after the last administration of the test substance. Alternative sampling times may be used when justified on the basis of toxicokinetic data.

Selection of Tissues and Cell Preparation In principle, any tissue of the experimental animal, provided that a high-quality single-cell/nucleus suspension can be obtained, can be used for a comet assay. Selection of the tissue(s) to be evaluated should be based, wherever possible, on data from absorption, distribution, metabolism, excretion studies, and/or other toxicological information. A tissue should not be evaluated unless there is evidence of or support for exposure of the tissue to the test substance and/or its metabolite (s). In the absence of such information and unless scientifically justified, two tissues should be examined. Recommended tissues are liver, which is the major organ

for the metabolism of absorbed compounds, and a site of first-contact tissue—for example, gastrointestinal for orally administered substances, respiratory tract for substances administrated via inhalation, or skin for dermally applied substances. Which tissue is evaluated first is at the discretion of the investigator and both tissues need not be evaluated if a positive response is obtained in the first tissue evaluated.

Single-cell suspension can be obtained from solid tissue by mincing briefly with a pair fine scissors (Tice et al., 1991), incubation with digestive enzymes such as collagenase trypsin (Brendler Schwaab et al., 1994), or pushing the tissue sample through a mesh membrane. Cell nuclei can also be obtained by homogenization. During mincing or homogenization, ethylenediaminetetraacetic acid (EDTA) can be added to the processing solution to chelate calcium/magnesium and prevent endonuclease activation. In addition, radical scavengers (e.g., DMSO) can be added to prevent oxidant-induced DNA damage. Any cell dissociation method is acceptable as long as it can be demonstrated that the process is not associated with inappropriate background levels of DNA damage.

Cytotoxicity: Potential Confounding Factor A general issure with DNA strand break assays such as the comet is that indirect mechanisms related to cytoxicity may lead to enhanced strand break formation. However, since DNA damage in the comet assay is assessed on the level of individual cells, dead or dying cells may be identified on a microscopic slide by their specific image. Necrotic or apoptotic cells can result in comets with small or nonexistent head and large, diffuse tails as observed in vitro upon treatment with cytotoxic, nongenotoxic articles. However, such microscopic images are not uniquely diagnostic for apoptosis or necrosis since they may also be detected after treatment with high doses of radiation or high concentrations of strong mutagens. For the in vivo comet assay, only limited data are available to establish whether cytotoxicity results in increased DNA migration in tissues of experimental animals. Despite necrosis or apoptosis in target organs of rodents such as kidneys, testes, liver, or duodenum, no elevated DNA migration was observed. However, enhanced DNA migration was seen in homogenized liver tissue of mice dosed with carbon tetrachloride when histopathological examination showed evidence of necrosis in the liver. Therefore, to avoid potential false-positive effects resulting from cytotoxicity, recommendations regarding a concurrent assessment of target organ toxicity have been made, including dye viability assays, histopathology, and a neutral diffusion assay (Tice et al., 2000; Hartmann et al., 2001).

Biological Significance of Lesions Detected DNA lesions leading to effects in the comet assay can be strand breaks which may be relevant to the formation of chromosome aberrations or DNA modifications such as abasic (AP) sites with relevance to the induction of gene mutations. However, primary lesions detected by the comet assay may also be correctly repaired without resulting

in permanent genetic alterations. Neither the magnitude of DNA migration in the comet assay nor the shape of the comet can reveal the types of DNA damage causing the effect other biological significance, that is, their mutagenic potential. Therefore, conclusions regarding the mutagenicity of a test compound cannot be made solely on the basis of comet assay effects. There are a few limitations of the comet assay with regard to its application and interpretation of test results. For example, short-lived primary DNA lesions such as single-strand breaks, which may undergo rapid DNA repair, could be missed when using inadequate sampling times. However, an appropriate study design including only early preparation time points (i.e., at 3–6 h) is considered sufficient to ensure that these lesions are captured—in particular at higher dose levels, where DNA repair may be significantly delayed or even overwhelmed. In any case, it should be kept in mind that a negative comet result can be considered a strong indicator for the absence of a mutagenic potential.

Advantages The advantages of this assay for use in genotoxicity testing of drug candidates include applicability to various tissues and/or special cell types, sensitivity for detecting low levels of DNA damage, the requirement for small numbers of cells per sample, the general ease of test performance, the short time needed to complete a study, and relatively low cost. The comet assay can be applied to any tissue in the given in vivo model, provided that a single cell/nuclei suspension can be obtained. Therefore, the comet assay has potential advantages over other in vivo genotoxicity test methods, which are reliably applicable to rapidly proliferating cells only or have been validated preferentially in a single tissue only. The comet assay may detect a broader spectrum of primary DNA lesions, including single-strand breaks and oxidative-base damage, which may not be detected in the UDS (unscheduled DNA synthesis) test because they are not repaired by nucleotide excision repair. The advantages of the comet assay over the alkaline elution test include the detection of DNA damage on a single-cell level and the requirement for only small numbers of cells per sample. In contrast, when using the alkaline elution assay, large quantities of cells are necessary for the determination of genotoxic effects, and, therefore, only a limited number of organs/tissues can be evaluated using this technique. In particular, this seems important for investigation of suspected tissue-specific genotoxic activity, which includes "site-of-contact" genotoxicity (cases of high local versus low systemic exposure).

Limitations Experimental variability is an important issue and shoud be kept to a minimum to ensure reliable interpretation and comparability of the data obtained with other in vivo comet experiments. Experimental variability may result from shortcomings with regard to number of doses tested, number of animals per dose, number of slides per animal, number of cells analyzed, lack of sufficient DNA migration in cells of concurrent controls, and deviation from minimum time for treatment of slides with alkaline buffer. Considering

these discrepancies, data of comprehensive study as well as other study reports not in agreement with current recommendations should be interpreted with caution. This point was highlighted recently in a position paper on the use and status of the in vivo comet assay in genotoxicity testing which critically assessed published data produced under test conditions not fully in agreement with the minimal requirements for an acceptable test. For example, it was noted that positive comet assay data were published for compounds that have been assessed before to be neither genotoxic nor carcinogenic, such as food additives. Such isolated positive comet assay results should be critically evaluated in light of current recommendations to exclude methodological shortcomings and potential artifacts. In cases where negative carcinogenicity data are already available and the in vivo comet assay result represents an isolated positive finding in the context of existing genotoxicity data, the biological significance of the effect seen in the comet assay should be assessed with caution.

Data Analysis Data from the fluctuation test described above are analyzed by an appropriate statistical method as described in Robinson et al. (1989). Data from plate assays are analyzed as described in Arlett et al. (1989) for treat and plate tests.

Status of Mammalian Mutation Tests At present the only practical assays for screening new chemical entities for mammalian mutation are the mammalian cell assays described above. The protocols are well defined, and mutant selection and counting procedures are simple and easily quantified. In general, the genetic endpoints are understood and relevant to deleterious genetic events in humans. For these reasons the assays are still regarded as valuable in safety evaluation (Li et al., 1991). It is, however, recognized that there are still unknown factors and molecular events that influence test results. This can be illustrated by the conclusions of the third UKEMS (United Kingdom Environmental Mutagen Society) collaborative trial, which focused on tests with cultured mammalian cells. The following points were made:

- The number of cells to be cultured during expression imposes a severe limitation in the use of surface-attached cells.
- A careful determination of toxicity is important.
- S9 levels may need to be varied.
- The aromatic amine benzidine is mutagenic only at the TK locus in L5178Y $TK^{+/-}$ cells. The most disturbing finding was that benzidine (detectable without metabolism by S9 mix) did not produce detectable DNA adducts (as shown by ^{32}P-postlabelling) in L5178Y cells. Thus, the mechanism for mutagenesis in L5178Y cells benzidine remains to be elucidated (Arlett and Cole, 1990).

7.2.11 In Vivo Mammalian Mutation Tests

Mammalian mutation studies of chemicals in the whole animal have provided fundamental information on mutation parameters in germ cells such as dose response, dose fractionation, and sensitivity of various stages in gametogenesis, just as is known for ionizing radiation (Russell, 1989). This has led to estimations of the possible impact chemical mutagens may have on heritable malformation, inborn errors of metabolism, and so on. Today germ cell studies are still required when estimating the heritable damage a mutagen may inflict on exposed human populations.

The existing tests tend to be cumbersome and are not used for routine genetic toxicology screening, and thus only brief descriptions will follow. Reviews of existing data, particularly by Holden (Holden, 1982; Adler and Ashby, 1989), have indicated that most, if not all, germ cell mutagens also induce DNA damage in somatic cells, as detected by well-established assays such as the rodent micronucleus test. The converse is not true—that is, some mutagens/clastogens can induce somatic cell damage but do not induce germ cell changes, which probably reflects the special protection afforded to the germ cells, such as that provided by the blood–testis barrier. In other words, it appears that germ cell mutagens are a subset of somatic cell mutagens.

In vivo mammalian mutation tests are not restricted to germ cell tests. The mouse spot test described below first is, again, a test used for studying radiation-induced mutation but also has been used for screening chemicals for in vivo mutagenic potential. This test has had several proponents but compared with in vivo chromosomal assays is not widely used.

Mouse-Specific Locus Test The mouse somatic spot test is a type of specific locus test. The classical specific locus test was developed independently by Russell at Oak Ridge in the late 1940s (Russell, 1951, 1989) and Carter in Edinburgh (Carter et al., 1956). The test consists of treatment of parental mice homozygous for a wild-type set of marker loci. The targets for mutation are the germ cells in the gonads of the treated mice. These are mated with a tester stock that is homozygous recessive at the marker loci. The F_1 offspring that result are normally heterozygous at the marker loci and thus express the wild-type phenotype. In the event of a mutation from the wild-type allele at any of these loci, the F_1 offspring express the recessive phenotype.

The test marker strain (T) developed by Russell uses seven recessive loci: *a* (nonagouti), *b* (brown), c^{ch} (chinchilla), *d* (dilute), *p* (pink-eyed dilution), *s* (piebald), and *se* (short ear). As for the mouse spot test, these genes control coat pigmentation, intensity or pattern, and, for the *se* gene, the size of the external ear.

As the occurrence of mutation is rare even after mutagen treatment, the specific locus test is the ultimate study of mutation, requiring many thousands

of offspring to be scored plus significant resources of time, space, and animal husbandry. Because of these constraints, it is often difficult to define a negative result, as insufficient animals are scored or all stages of spermatogenesis are not covered. Of the 25 compounds tested in the assay, as reviewed by Ehling et al. (1986), 17 were regarded as "inconclusive" and 8 positive. The scale studies can reach is illustrated by the test of ethylene oxide described by Russell et al. (1984), where exposures of 101,000 and 150,000 ppm were used over 16–23 weeks. A total of 71,387 offspring were examined. The spermatogonial stem cell mutation rate in the treated animals did not differ significantly from the historical control frequency!

With regard to the design of the test, mice are mated when seven to eight weeks old. By this age all germ cell stages are present. The test compound is normally administered by the IP route to maximize the likelihood of germ cell exposure. The preferred dose is just below the toxic level so long as fertility is not compromised. One lower dose should also be included.

In males spermatogonia are most at risk, but it is also desirable that later stages also be exposed. Thus, the mice are mated immediately after treatment to two to four females. This is continued each week for seven weeks. Then the first group has completed its rearing of the first set of offspring and is remated. This cycle can be continued for the lifetime of the males. Tests can also be carried out by dosing females, when treatment is carried out for three weeks to cover all stages of oogenesis.

The offspring are examined immediately after birth for identification of malformations (dominant visibles) and then at weaning for the specific locus mutations. Presumptive mutant mice are checked by further crosses to confirm their status (Searle, 1984).

Comparison of mutation frequencies is made with the historical database. For definition of a positive result the same principles are recommended as for the mouse spot test (Selby and Olson, 1981). A minimum size of 18,000 offspring per group is recommended by those authors for definition of a negative result.

7.3 IN VITRO CYTOGENETIC ASSAYS

The in vitro cytogenetic assay is a short-term mutagenicity test for detecting chromosomal damage in cultured mammalian cells.

Cultured cells have a limited ability metabolically to activate some potential clastogens. This can be overcome by adding an exogenous metabolic activation system such as S9 mix to the cells (Ames et al., 1975; Madle and Obe, 1980; Natarajan and Obe, 1982; Maron and Ames, 1983).

Observations are made in metaphase cells arrested with a spindle inhibitor such as colchicine or colcemid to accumulate cells in a metaphase-like stage of mitosis (c-metaphase) before hypotonic treatment to enlarge cells and fixation with alcohol–acetic acid solution. Cells are then dispersed onto

microscope slides and stained and slides are randomized, coded, and analyzed for chromosome aberrations with high-power light microscopy. Details of the procedure are given in Dean and Danford (1984) and Preston et al. (1981, 1987). The UKEMS guidelines (Scott et al., 1990) recommend that all tests be repeated regardless of the outcome of the first test and that, if a negative or equivocal result is obtained in the first test, the repeat test should include an additional sampling time. In the earlier version of the guidelines (Scott et al., 1983) a single sampling at approximately 1.5 normal cycle times (–24 h for a 1.5-cell cycle) from the beginning of treatment was recommended, provided that a range of concentrations was used which induced marginal to substantial reductions in mitotic index, usually an indicator of mitotic delay. However, Ishidate (1988a) reported a number of chemicals which gave negative responses with a fixation time of 24 h but which were positive at 48 h. This was when a Chinese hamster fibroblast line (CHO) with a doubling time of 15 h was used. It would appear, therefore, that there are chemicals which can induce extensive mitotic delay at clastogenic doses and may be clastogenic only when cells have passed through more than one cell cycle since treatment (Thust et al., 1980). A repeat test should include an additional sample at approximately 24 h later, but it may only be necessary to score cells from the highest dose at this later fixation time. When the first test gives a clearly positive result, the repeat test need only utilize the same fixation time. The use of other sampling times is in agreement with other guidelines (European Community EEC Directive—OECD, 1983; American Society for Testing and Materials—Preston et al., 1987; Japanese guidelines—Japan Ministry of Health and Welfare (JMHW), 1984; Joint Directives, 1987; Ishidate, 1988b).

7.3.1 Cell Types

Established cell lines, cell strains, or primary cell cultures may be used. The most often used are Chinese hamster cell lines and human peripheral blood lymphocytes. The merits of these two cell lines have been reported (Ishidate and Harnois, 1987; Kirkland and Garner, 1987). The cell system must be validated and consistently sensitive to known clastogens.

7.3.2 Chinese Hamster Cell Lines

Chinese hamster ovary cells in which there has been an extensive rearrangement of chromosome material and the chromosome number may not be constant from cell to cell are frequently used. Polyploidy, endoreduplication, and high spontaneous chromosome aberration frequencies can sometimes be found in these established cell lines, but careful cell culture techniques should minimize such effects. Cells should be treated in exponential growth when cells are in all stages of the cell cycle.

7.3.3 Human Peripheral Blood Lymphocytes

Blood should be taken from healthy donors not known to be suffering from viral infections or receiving medication. Staff handling blood should be immunized against hepatitis B and regular donors should be shown to be hepatitis B antigen negative. Donors and staff should be aware of AIDS implications, and blood and cultures should be handled at containment level 2 (Advisory Committee on Dangerous Pathogens, 1984).

Peripheral blood cultures are stimulated to divide by the addition of a T-cell mitogen such as phytohemagglutinin (PHA) to the culture medium. Mitotic activity is at a maximum at about three days but begins at about 40 h after PHA stimulation and the chromosome constitution remains diploid during short-term culture (Evans and O'Riordan, 1975). Treatments should commence at about 44 h after culture initiation. This is when cells are actively proliferating and cells are in all stages of the cell cycle. They should be sampled about 20 h later. In a repeat study the second sample time should be about 92 h after culture initiation. Morimoto et al. (1983) report that the cycle time for lymphocytes averages about 12–14 h except for the first cycle.

Female donors can give higher yields of chromosome damage (Anderson et al., 1989).

7.3.4 Positive and Negative Controls

When the solvent is not the culture medium or water, the solvent, liver enzyme activation mixture and solvent, and untreated controls are used as negative controls.

Since cultured cells are normally treated in their usual growth medium, the solubility of the test material in the medium should be ascertained before testing. As pointed out earlier, extremes of pH can be clastogenic (Cifone et al., 1987), so the effect of the test material on pH should also be determined, but buffers can be utilized.

Various organic solvents are used, such as DMSO, dimethylformamide, ethanol, and acetone. The volume added must not be toxic to cells. Greater than 10% v/v water can be toxic because of nutrient dilution and osmolality changes.

A known clastogen should always be included as a positive control. When metabolic activation is used, a positive-control chemical known to require metabolic activation should also be used to ensure that the system is functioning properly. Without metabolic activation, a direct-acting positive-control chemical should be used. A structurally related positive control can also be used. Appropriate safety precautions must be taken in handling clastogens [International Agency for Research on Cancer (IARC), 1979; Medical Research Council (MRC), 1981].

Positive-control chemicals should be used to produce relatively low frequencies of aberrations so that the sensitivity of the assay for detecting weak clastogens can be established (Preston et al., 1987).

Aberration yields in negative and positive controls should be used to provide a historical database.

7.3.5 Treatment of Cells

When an exogenous activation system is employed, short treatments (about 2 h) are usually necessary because S9 mix is often cytotoxic when used for extended lengths of time. However, cells may be treated with chemicals either continuously up to harvest time or for a short time followed by washing and addition of fresh medium to allow cell cycle progression. Continuous treatment avoids centrifugation steps required with washing of cells and optimizes the endogenous metabolic capacity of the lymphocytes.

When metabolic activation is used, S9 mix should not exceed 1–10% of the culture medium by volume. It has been shown that the S9 mix is clastogenic in CHO cells and mouse lymphoma cells (Cifone et al., 1987; Kirkland et al., 1989) but not in human lymphocytes, where blood components can inactivate active oxygen species which could cause chromosome damage. When S9 mix from animals treated with other enzyme-inducing agents such as phenobarbitone/β-naphthoflavone is used, clastogenesis may be minimized (Kirkland et al., 1989).

Prior to testing, it is necessary to determine the cytotoxicity of the test material in order to select a suitable dose range for the chromosome assay both with and without metabolic activation. The range most commonly used determines the effect of the agent on the mitotic index (MI), that is, the percentage of cells in mitoses at the time of cell harvest. The highest dose should inhibit mitotic activity by approximately 50% (EEC Annex V) or 75% (UKEMS: Scott et al., 1990) or exhibit some other indication of cytotoxicity. If the reduction in MI is too great, insufficient cells can be found for chromosome analysis. Cytotoxicity can also be assessed by making cell counts in the chromosome aberration test when using cell lines. In the lymphocyte assay total white cell counts can be used in addition to MI. A dose which induces 50–75% toxicity in these assays should be accompanied by a suitable reduction in mitotic index.

If the test material is not toxic, it is recommended that it be tested up to 5 mg mL^{-1}. The UKEMS recommends that chemicals be tested up to their maximum solubility in the treatment medium and not just their maximum solubility in stock solutions.

For highly soluble nontoxic agents, concentrations above 10 mM may produce substantial increases in the osmolality of the culture medium, which could be clastogenic by causing ionic imbalance within the cells (Ishidate et al., 1984; Brusick, 1987a). At concentrations exceeding 10 mM the osmolality

of the treatment media should be measured, and if the increase exceeds 50 mmol kg^{-1}, clastogenicity resulting from high osmolality should be suspected and, according to the UKEMS, is unlikely to be of relevance to human risk. The UKEMS also does not recommend the testing of chemicals at concentrations exceeding their solubility limits as suspensions or precipitate.

A minimum of three doses of the test material should be used—the highest chosen as described above, the lowest on the borderline of toxicity, and an intermediate one. Up to six doses can be managed satisfactorily, and this ensures the detection of any dose response and that a toxic range is covered. MIs are as required for the preliminary study (at lease 1000 cells per culture). It is also useful to score endoreduplication and polyploidy for historical data. Cells from only three doses need to be analyzed.

The range of doses used at the repeat fixation time can be those which induce a suitable degree of mitotic inhibition at the earlier fixation time, but if the highest dose reduces the MI to an unacceptably low level at the second sampling time, the next highest dose should be chosen for screening.

A complete assay requires the test material to be investigated at a minimum of three doses together with a positive (untreated) and solvent-only control that can be omitted if tissue culture medium is used as a solvent. When two fixation times are used in repeat tests, the positive control is necessary at only one time but the negative or solvent control is necessary at both times.

Duplicates of each test group and quadruplicates of solvent or negative controls should be set up. The sensitivity of the assay is improved with larger numbers scored in the negative controls (Richardson et al., 1989).

7.3.6 Scoring Procedures

Prior to scoring, slides should be coded, randomized, and then scored "blind." Metaphase analysis should only be carried out by an experienced observer. Metaphase cells should be sought under low-power magnification and those with well-spread (i.e., non-overlapping), clearly defined nonfuzzy chromosomes examined under high power with oil immersion. It is acceptable to analyze cells with total chromosome numbers or that have lost one or two chromosomes during processing. In human lymphocytes ($2n - 46$) 44 or more centromeres and in CHO cells ($2n - 22$; range 21–24) 20 or more centromeres can be scored. Chromosome numbers can be recorded for each cell to give an indication of aneuploidy. Only cells with increases in numbers (above 46 in human lymphocytes and 24 in CHO cells) should be considered in this category, since decreases can occur through processing.

Recording microscope coordinates of cells is necessary and allows verification of abnormal cells. A photographic record is also useful of cells with aberrations. Two hundred cells (100 from each of two replicates) should be scored per treatment group. When ambiguous results are obtained, there may be further "blind" reading of these samples.

7.3.7 Data Recording

The classification and nomenclature of the International System for Human Cytogenetic Nomenclature (ISCN, 1985) as applied to acquired chromosome aberrations are recommended. Score sheets giving the slide code, microscope scorer's name, date, cell number, number of chromosomes, and aberration types should be used. These should include chromatid and chromosome gaps, deletions, exchanges, and others. A space for the vernier reading for comments and a diagram of the aberration should be available.

From the score sheets, frequencies of various aberrations should be calculated and each aberration should be counted only once. To consider a break as one event and an exchange as two events is not acceptable, since unfounded assumptions are made about mechanisms involved (Revell, 1974).

7.3.8 Presentation of Results

The test material, test cells used, method of treatment, harvesting of cells, cytotoxicity assay, and so on, should be clearly stated as well as the statistical methods used. Richardson et al. (1989) recommend that comparison be made between the frequencies in control cells and those at each dose level using Fisher's exact test.

In cytogenetic assays the absence of a clear positive dose–response relationship at a particular time frequently arises. This is because a single common sampling time may be used for all doses of a test compound. Chromosome aberration yields can vary markedly with posttreatment sampling time of an asynchronous population, and increasing doses of clastogens can induce increasing degrees of mitotic delay (Scott et al., 1990). Additional fixation times should clarify the relationship between dose and aberration yield.

Gaps are by tradition excluded from quantification of chromosome aberration yields. Some gaps have been shown to be real discontinuities in DNA (e.g., Heddle and Bodycote, 1970). Where chromosome aberration yields are on the borderline of statistical significance above control values, the inclusion of gaps could be useful. Further details on this approach may be found in the UKEMS guidelines (Scott et al., 1990).

Since chromosome exchanges are relatively rare events, greater biological significance should be attached to their presence than to gaps and breaks.

Chemicals which are clastogenic in vitro at low doses are more likely to be clastogenic in vivo than those where clastogenicity is detected only at high concentrations (Ishidate et al., 1988). Negative results in well-conducted in vitro tests are a good indication of a lack of potential for in vivo clastogenesis, since almost all in vivo clastogens have given positive results in vitro when adequately tested (Thompson, 1986; Ishidate et al., 1988).

7.4 IN VIVO CYTOGENETICS ASSAYS

Damage induced in whole animals can be detected in in vivo chromosome assays in either somatic or germinal cells by examination of metaphases or the formation of micronuclei. The micronucleus test can also detect whole chromosome loss or aneuploidy in the absence of clastogenic activity and is considered comparable in sensitivity to chromosome analysis (Tsuchimoto and Matter, 1979).

Rats and mice are generally used for in vivo studies, with the mouse being employed for bone marrow micronucleus analysis and the rat for metaphase analysis, but both can be used for either. Mice are cheaper and easier to handle than rats, and only a qualitative difference in response has been found between the species (Albanese et al., 1988). Chinese hamsters are also widely used for metaphase analysis because of their low diploid chromosome number of 22. However, there are few other historical toxicological data for this species.

7.4.1 Somatic Cell Assays

Metaphase Analysis Metaphase analysis can be performed in any tissue with actively dividing cells, but bone marrow is the tissue most often examined. Cells are treated with a test compound and are arrested in metaphase by the administration of colcemid or colchicine at various sampling times after treatment. Preparations are examined for structural chromosome damage. Because the bone marrow has a good blood supply, the cells should be exposed to the test compound or its metabolites in the peripheral blood supply, and the cells are sensitive to S-dependent and S-independent mutagens (Topham et al., 1983).

Peripheral blood cells can be stimulated to divide even though the target cell is relatively insensitive (Newton and Lilly, 1986). It is necessary to stimulate them with a mitogen since the number of lymphocytes which are dividing at any one time is very low. Cells are in G_0 when exposure is taking place, so they may not be sensitive to cell cycle stage specific mutagens and any damage might be repaired before sampling.

Micronuclei The assessment of micronuclei is considered simpler than the assessment of metaphase analysis (Collaborative Study Group, 1986, 1988). This assay is most often carried out in bone marrow cells, where polychromatic erythrocytes are examined. Damage is induced in the immature erythroblast and results in a micronucleus outside the main nucleus, which is easily detected after staining as a chromatid-containing body. When the erythroblast matures, the micronucleus, whose formation results from chromosome loss during cell division or from chromosome breakage forming centric and acentric fragments, is not extruded with the nucleus. Micronuclei can also be detected in peripheral blood cells (MacGregor et al., 1980). In addition, they can be detected in liver

(Tates et al., 1980; Braithwaite and Ashby, 1988) after partial hepatectomy or stimulation with 4-acetylaminofluorene or they can be detected in any proliferating cells.

7.4.2 Germ Cell Assays

The study of chromosome damage is highly relevant to the assessment of heritable cytogenetic damage. Many compounds which cause somatic cell damage have not produced germ cell damage (Holden, 1982) and, so far, all germ mutagens have also produced somatic damage.

Germ cell data, however, are needed for genetic risk estimation, and testing can be performed in male or female germ cells. The former are most often used, owing to their systemic effects in females. Testing in the male is performed in mitotically proliferating premeiotic spermatogonia, but chromosomal errors in such cells can result in cell death or prevent the cell from passing through meiosis. Damage produced in postmeiotic cells, the spermatids, or sperm are more likely to be transmitted to the F_1 progeny (Albanese, 1987). In females it is during early fetal development of the ovary that oocyte stage is the most commonly tested in the adult female. To test other stages during the first or second meiotic divisions demands the use of oocytes undergoing ovulation, which occurs naturally or is hormone stimulated. It is thus more difficult technically to test female germ cells.

7.4.3 Heritable Chromosome Assays

Damage may be analyzed in the heritable translocation test, which involves the examination in male F_1 animals if diakinesis metaphase 1 spermatocytes for multivalent association fall within the acceptable range for the laboratory for a substance to be considered positive or negative under the conditions of the study.

7.4.4 Germ Cell Cytogenetic Assays

Either mouse or rat can be used, but the mouse is generally the preferred species. Normally such assays are not conducted for routine screening purposes.

Spermatogonial metaphases can be prepared by the air-drying technique of Evans et al. (1964) for the first and second meiotic metaphase (MI and MII) in the male mouse. This method is not so suitable for rat and hamster. The numbers of spermatogonial metaphases can be boosted if, prior to hypotonic treatment, the testicular tubules are dispersed in trypsin solution (0.25%). At least one month between treatment and sample should be allowed to pass in the mouse to allow treated cells to reach meiosis. Brook and Chandley (1986) established that 11 days and 4 h was required for spermatogonial cells to reach preleptotene and 8 days and 10 h to reach zygotene. It takes 4 h for cells to

move from MI to MII, but test compounds can alter this rate. A search for multivalent formation can be made at MI for the structural rearrangements induced in spermatogonia. Cawood and Breckon (1983) examined the synaptonemal complex at pachytene using electron microscopy. Errors of segregation should be searched for at the first meiotic division in the male mouse, MII cells showing 19 (hypoploid) and 21 (hyperploid) chromosomes (Brook and Chandley, 1986). Hansmann and El-Nahass (1979), Brook (1982), and Brook and Chandley (1985) describe assays in the female mouse and procedures used for inducing ovulation by hormones and treatment of specific stages of meiosis.

7.5 SISTER CHROMATID EXCHANGE ASSAYS

SCEs are reciprocal exchanges between sister chromatids. They result in a change in morphology of the chromosome, but breakage and reunion are involved although the exact mechanism is unclear. They are thought to occur at homologous loci.

In 1958 Taylor demonstrated SCEs using autoradiographic techniques to detect the disposition or labeled DNA following incorporation of [^{3}H]-thymidine. 5-Bromo-2′-deoxyuridine (drdU) has now replaced [^{3}H]-thymidine and various staining methods have been used to show the differential incorporation of BrdU between sister chromatids: fluorescent—Hoechst 33258 (Latt, 1973), combined fluorescent and Giemsa (Perry and Wolff, 1974), and Giemsa (Korenberg and Freedlender, 1974). The fluorescent plus Giemsa procedure is recommended in view of the fact that stained slides can be stored and microscope analysis is simpler.

So that SCEs can be seen at metaphase, cells must pass through S phase (Kato, 1973; 1974; Wolff and Perry, 1974). SCEs appear to occur at the replication point, since SCE induction is maximal at the beginning of DNA synthesis but drops to zero at the end of the S phase (Latt and Loveday, 1978).

For SCE analysis in vitro, any cell type that is replicating or can be stimulated to divide is suitable. The incorporation of BrdU into cells in vivo allows the examination of a variety of tissues (Latt et al., 1980). Edwards et al. (1993) suggest that it is necessary to standardize protocols measuring SCE since different responses can be obtained depending on the extent of simultaneous exposure of test compound and BrdU.

7.5.1 Relevance of SCE in Terms of Genotoxicity

SCEs do not appear to be related to other cytogenetic events, since potent clastogens such as bleomycin and ionizing radiation induce low levels of SCE (Perry and Evans, 1975). The mechanisms involved in chromosome aberrations and SCE formation are dissimilar (e.g., Galloway and Wolff, 1979). There is not evidence that SCEs are in themselves lethal events, since there is little relationship to cytotoxicity (e.g., Bowden et al., 1979). It was suggested by

Wolff (1977a,b) that they relate more to mutational events due to a compatibility with cell survival. However, there are examples of agents that induce significant SCE increases in the absence of mutation (Bradley et al., 1979) as well as the converse (Connell, 1979; Connell and Medcalf, 1982).

The SCE assay is particularly sensitive for alkylating agents and base analogues, agents causing single-strand breaks in DNA, and compounds acting through DNA binding (Latt et al., 1981). The most potent SCE inducers are S-phase dependent. Painter (1980) reports that agents such as X-irradiation, which inhibits replicon initiation, are poor SCE inducers, whereas mitomycin C, which inhibits replication fork progression, is a potent SCE inducer.

7.5.2 Experimental Design

Established cell lines, primarily cell cultures of rodents, may be used. Detailed information on in vitro and in vivo assays may be obtained in reviews of SCE methods by Latt et al. (1977, 1981), Perry and Thomson (1984), and Perry et al. (1984). The in vitro methods will be briefly explored here.

Either monolayer or suspension cultures or human lymphocytes can be employed. Human fibroblasts are less suitable because of their long cell cycle duration.

The concentration of organic solvents for the test compound should not exceed 0.8% v/v, as higher concentrations could lead to slight elevations in the SCE level (Perry et al., 1984).

For monolayer cultures, the cultures are set up the day before BrdU treatment so that the cells will be in exponential growth before the addition of BrdU or the test compound. After BrdU addition the cells are allowed to undergo the equivalent of two cell cycles before cell harvest. A spindle inhibitor such as colchicine or colcemid is introduced for the final 1–2 h of culture to arrest cells in metaphase, after which the cells are harvested and chromosome preparations are made by routine cytogenetic techniques.

In the absence of metabolic activation, BrdU and the test agent can be added simultaneously and left for the duration of BrdU labeling. Shorter treatments should be used in the presence of metabolic activation or to avoid synergistic effects with BrdU, when cells can be pulse treated for, for example, 1 h before BrdU addition (see Edwards et al., 1993).

Peripheral blood cultures are established in medium containing BrdU and PHA. Colcemid is added 1–2 h before harvest and the cells are harvested between 60 and 70 h post-PHA-stimulation. Cell harvest and slide preparations are conducted according to routine cytogenetic methods.

Heparinized blood samples may be stored at 4 °C for up to 48 h without affecting the SCE response (Lambert et al., 1982). If the test agent is known to react with serum or red blood cells, the mononuclear lymphocytes may be isolated by use of a Ficoll/Hypaque gradient (Boyum, 1968).

If metabolic activation is not required, treatment is best conducted over the whole of the final 24 h of culture, or if metabolic activation is required, a pulse exposure may be employed to treat cultures at the first S phase at around 24–30 h or at 48 h for an asynchronous population.

Exposure of cells to fluorescent light during the culture period leads to photolysis of BrdU-containing DNA and a concomitant increase in SCE frequency (Wolff and Perry, 1974). Consequently, SCE cultures should be kept in the dark and manipulated under subdued light conditions such as yellow safe light. Furthermore, media used in SCE assays should be stored in the dark, since certain media components produce reactive SCE-inducing intermediates on exposure to fluorescent light (Monticone and Schneider, 1979).

Coded and randomized slides should be read. All experiments should be repeated at least once (Perry et al., 1984) with higher and lower concentrations of S9 mix if a negative response is achieved. Even for an apparently unambiguous positive response with a greater than twofold increase in SCEs over the background level at the highest dose and with at least two consecutive dose levels with an increased SCE response, a repeat study is necessary to show a consistent response.

The quality of differential staining will determine the ease and accuracy of SCE scoring, and, to eliminate variation, results from different observers should occasionally be compared. Furthermore, to avoid observer bias, scorers should have slides from different treatment groups equally distributed among them, as with all cytogenetic studies.

Issues in Assay Interpretation and Relevance The reason for consideration of a significant revision to S2 (such as is now under consideration) ties into issues and considerations as to perceived unacceptable error rates in test performance and difficulties in interpreting the relevance of findings. Supplement in vivo genotoxicity studies are used to (1) follow up on positive finding in one or more tests of the standard genotoxicity battery, (2) elucidate a potential contribution of genotoxicity to the induction of preneoplastic and/or neoplastic changes detected in long-term tests in rodents, and (3) elucidate mechanisms of micronucleus formation to differentiate clastogenic from aneuogenic effects since aneugenicity is well accepted to result from mechanisms of action for which thresholds exist, demonstrating that micronucleus formation as a result of chromosome loss should allow an acceptable level of human exposure to be defined. No matter the trigger for conducting supplemental in vivo genotoxicity testing, it is critical that the approach utilized—for example, the endpoint and target tissue assessed—is scientifically valuable such that the results will aid in interpreting the relevance of the initial finding of concern. Ultimately, the goal of supplemental genotoxicity testing is to determine if a genotoxic risk is posed to patients under the intended condition of treatment.

Follow-Up Testing of Drug Candidates Positive in Standard Genotoxicity Test Battery It has been reported that approximately 30–50% of pharmaceuticals produce positive genotoxicity results in vitro (Kirkland and Muller, 2000). In contrast, results from bone marrow cytogenetic assays are frequently negative, even for those compounds that produce positive results in vitro. This discrepancy may result from a number of major differences that exist when testing in cultured cells versus intact animals. For example, differing metabolic pathways can exist in vitro and in vivo, metabolic inactivation can occur in the intact animal, parent compound or active metabolite may not reach the target cell in vivo, rapid detoxification and elimination may occur, or plasma levels in vivo may not be comparable to concentrations that generate positive responses in the in vitro assay, which is often accompanied by high levels of cytotoxicity. It is also worth nothing that positive results generated in vitro may be secondary to effects, such as cytotoxicity, which may never be achieved under in vivo exposure conditions. Data from in vivo experiments are therefore essential before definitive conclusions are drawn regarding the potential mutagenic hazard to humans from chemicals that produce positive results in one or more in vitro tests.

Follow-Up Testing of Tumorigenic Drug Candidates Negative in Standard Genotoxicity Test Battery In carcinogenicity testing of pharmaceutical drug candidates of tumorigenic response in rodents, the ICH guidance S2B currently stipulates that such tumorigenicity is not clearly based on a nongenotoxic mechanism. Typically, supplemental in vivo genotoxicity tests should be performed with cells of the respective tumor target organ to distinguish between genotoxic and nongenotoxic mechanisms of tumor induction.

Endpoints Assessed in Supplemental Assays Commonly applied test systems are described that are used as supplemental genotoxicity assays. These assays differ with respect to the endpoints assessed:

1. Induction of primary DNA lesions, that is, measurement of exposure, uptake, and reactivity to DNA via the comet assay or P-postlabeling assay
2. Measurement of the repair of DNA lesion using the in-scheduled DNA synthesis (UDS) test
3. Measurement of induction of genetic drug transgenic animal assays for point mutations or the mouse spot test

The comet assay is the most commonly applied of these.

The issue of how to assess the relevance of a finding of genotoxicity in a candidate drug is a complex one. If the judgment is that such a finding is not relevant to human risks, there are two approaches to assessing and defend-

ing such a finding: the WOE (weight of evidence) or the MOA (made of action).

In the case of impurities in a marketed or candidate drug, the approach is more straightforward. One must reduce the level to or below the TTC (toxicological threshold of concern), the level at which no patient would receive more than 1.5 μg day^{-1} (EMEA, 2004).

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8

Repeat-Dose Toxicity Studies

In the broadest sense, subchronic and chronic studies for pharmaceutical products can incorporate any of the routes used to administer a therapeutic agent, use any of a number of animal models, and conform to a broad range of experimental designs. They can be two weeks long (what used to be called "subacute" studies because they were conducted at dose levels below those employed for single-dose or acute studies) or last up to a year. Another name for these studies is repeat-dose studies (Ballantyne, 2000; Wilson et al., 2001; Gad, 2008a)—that is, those studies whereby animals have a therapeutic agent administered to them on a regular and repeated basis by one or more routes over a period of one year or less. There is great flexibility and variability in the design of such studies.

This chapter seeks to provide a firm grasp of the objectives for repeat-dose studies, the regulatory requirements governing them, the key factors in their design and conduct, and the interpretation of their results.

8.1 OBJECTIVES

As with any scientific study or experiment (but especially for those in safety assessment), the essential first step is to define and understand the reason(s) for the conduct of the study—that is, its objectives. There are three major (scientific) reasons for conducting subchronic and chronic studies, but a basic

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characteristic of all but a few subchronic studies needs to be understood. The subchronic study is (as are most other studies in whole-animal toxicology) a broad screen. It is not focused on a specific endpoint; rather, it is a broad exploration of the cumulative biological effects of the administered agent over a range of doses. In fact, it is so broad an exploration that it can be called a "shotgun" study.

The objectives of the typical subchronic and chronic studies fall into three categories. The first is to broadly define the toxicity (and, if one is wise, the pharmacology and hyperpharmacology) of repeated doses of a potential therapeutic agent in an animal model (Traina, 1983). This definition is both qualitative (what are the target organs and the nature of the effects seen) and quantitative (at what dose levels, or, more importantly, at what plasma and tissue levels, are effects definitely seen and not seen).

The second objective (and the one that in the pharmaceutical industry laboratory usually compels both timing and compromising of design and execution) is to provide support for the initiation and/or continued conduct of clinical trials in humans (O'Grady and Linet, 1990; Smith, 1992). As such, subchronic studies should provide not only adequate clearance (therapeutic margin) of initial dose levels and duration of dosing but also guidance for any special measures to be made or precautions to be taken in initial clinical trials. Setting inadequate dose levels (either too low or too high) may lead to the failure of a study. A successful study must both define a safe, or "clean," dose level (one that is as high as possible, to allow as much flexibility as possible in the conduct of clinical studies) and demonstrate and/or characterize signs of toxicity at some higher dose. The duration-of-dosing issue is driven by a compromise between meeting regulatorily established guidelines (as set out in Table 8.1) and the economic pressure to initiate clinical trials as soon as possible.

The third objective is one of looking forward to later studies. The subchronic study must provide sufficient information to allow a prudent setting of doses for later, longer studies (including, ultimately, carcinogenicity studies). At the same time, the subchronic study must also provide guidance for the other (than dose) design features of longer term studies (such as what parameters to measure and when to measure them, how many animals to use, and how long to conduct the study).

TABLE 8.1 Duration of Treatment Supported by Preclinical Studies

Animal Study Length	Generally Allowed Human Dosing
2 Weeks	Up to 3 doses
1 Month	10 Days
3 Months	1 Month
1 Year (rodent); 9 months (dog)—(U.S.)/6 months (European Economic Community, Japan)	Unlimited

Source: ICH, 2000.

These objectives are addressed by the usual subchronic study. Some subchronic studies, however, are unusual in being conceived, designed, and executed to address specific questions raised (or left unanswered) by previous preclinical or early clinical studies. Such a special purpose is addressed separately.

Chronic studies (those that last six or nine months or a year) may also be conducted for the above purposes but are primarily done to fulfill registration requirements for drugs that are intended for continuous long-term (lifetime) use or frequent intermittent use.

8.2 REGULATORY CONSIDERATIONS

Much of what is done (and how it is done) in repeat-dose studies is a response to a number of regulations. Three of these have very broad impact. These are the good laboratory practiced (GLP) requirements, Animal Welfare Act requirements, and regulatory requirements that actually govern study design.

8.2.1 Good Laboratory Practices

Since 1978, the design and conduct of preclinical safety assessment studies for pharmaceuticals in the United States (and, indeed, internationally) have been governed and significantly influenced by GLPs. Strictly speaking, these regulations cover qualifications of staff and facilities, training, record-keeping, documentation, and actions required to ensure compliance with and effectiveness of these steps. Though the initial regulations were from the U.S. Food and Drug Administration (FDA, 1983), they have always extended to cover studies performed overseas (FDA, 1988a). Most other countries have adopted similar regulations. A discussion of these regulations is beyond the scope of the current chapter, but several aspects are central to this effort. Each technique or methodology to be employed in a study (such as animal identification, weighing and examination, blood collection, and data recording) must be adequately described in a standard operating procedure (SOP) before the study begins. Those who are to perform such procedures must be trained in them beforehand. The actual design of the study, including start date and how it is to be ended and analyzed, plus the principal scientists involved (particularly the study director) must be specified in a protocol that is signed before the study commences. Any changes to these features must be documented in amendments once the study has begun. It is a good idea that the pathologist who is to later perform or oversee histopathology be designated before the start of the study and that the design be a team effort involving the best efforts of the toxicologist, pathologist, and (usually for subchronic studies) the drug metabolism scientist.

8.2.2 Animal Welfare Act

Gone are the days when the pharmaceutical scientist could conduct whatever procedures or studies that were desired using experimental animals. The Animal Welfare Act (APHIS, 1989, amended every five years since) (and its analogues in other countries) rightfully requires careful consideration of animal usage to ensure that research and testing use as few animals as possible in as humane a manner as possible. As a start, all protocols must be reviewed by an institutional animal care and use committee. Such review takes time but should not serve to hinder good science. When designing a study or developing a new procedure or technique, the following points should be kept in mind:

1. Will the number of animals used be sufficient to provide the required data yet not constitute excessive use? (It ultimately does not reduce animal use to utilize too few animals to begin with and then have to repeat the study.)
2. Are the procedures employed the least invasive and traumatic available? This practice not only is required by regulations but also is sound scientific practice, since any induced stress will produce a range of responses in test animals that can mask or confound the chemically induced effects.

8.2.3 Regulatory Requirements for Study Design

The first consideration in the construction of a study is a clear statement of its objectives, which are almost always headed by meeting regulatory requirements to support drug development and registration. Accordingly, the relevant regulatory requirements must be analyzed, which is complicated by the fact that new drugs are no longer developed for registration and sale in a single-market country. The expense is too great, and the potential for broad international sales too appealing. While each major country has its own requirements as to study designs and studies required (with most of the smaller countries adhering to the regulations of one of the major players), harmonization has done much to smooth these differences (Adler and Zbinden, 1988). Meeting these regulatory requirements is particularly challenging for several reasons. First, the only official delineation of general requirements in the Untied States is dated (FDA, 1971), and recently special cases have arisen (anti-HIV agents, biotechnologically derived agents, therapeutic agents for neonates and the very elderly, etc.) that try the utility of these requirements. These needs have led to a stream of points to consider which seek to update requirements. Second, the term "guidelines" means different things in different countries (in the United States it means "requirements," in Japan "suggestions").

Agents intended to treat or arrest the progress of rapidly spreading life-threatening diseases (such as AIDS) are subject to less stringent safety assessment requirements prior to initial clinical evaluations than are other drugs. However, even though approval (if clinical efficacy is established) for

marketing can be granted with preclinical testing still under way, all applicable safety assessments (as with any other class of drugs) must still be completed (FDA, 1988b).

Drugs intended for use in either the elderly or the very young have special additional requirements for safety evaluation, in recognition of the special characteristics and potential sensitivities of these populations. For the elderly, these requirements call for special consideration of renal and hepatic effects [Center for Drug Evaluation and Research (CDER), 1989]. Likewise, drugs intended for the young require special studies to be performed in neonates and juvenile animals (usually of two or four weeks duration in rats).

In the last five to six years, a number of potentially important drugs have been produced by recombinant DNA technology. These biomacromolecules, which are primarily endogenously occurring proteins, present a variety of special considerations and concerns, including the following:

- Because they are endogenously occurring molecules, assessing their pharmacokinetics and metabolism presents special problems.
- Is the externally commercially produced molecule biologically equivalent to the naturally occurring one?
- As proteins, are they immunogenic or do they provoke neutralizing antibodies that will limit their usefulness?
- Because they are available only in very small quantities, the use of traditional protocols (such as those that use ever-increasing doses until an adverse effect is achieved) is impractical.
- Agents with such specific activity in humans may not be appropriately evaluated in rodents or other model species.

Each of these points must be addressed in any safety-testing plan (Weissinger, 1989). The requirements set out in this chapter are designed to do this (for repeat-dose testing).

8.3 STUDY DESIGN AND CONDUCT

8.3.1 Animals

In all but a few rare cases, for pharmaceutical safety assessment, separate studies in at least two species are required. Regulations require that both species be mammalian, and one of these must be a nonrodent; practice and economics dictate that the other species will be a rodent. With extremely rare exception, the rodent species employed is the rat (though the mouse also sees significant use). There is considerably more variability in the nonrodent species, with a range of factors determining whether the dog (most common choice), a primate species (typically the rhesus or cynomolgus, though some others are used in particular cases), the pig (particularly in Europe), or some other animal

(e.g., the ferret) is selected. The factors that should and do govern species selection are presented in detail in Gad (2008b). The use of multiple species is a regulatory requirement arising from experience and the belief (going back to 1944, at least) that it will provide a better chance of detecting the full range of biological responses (adverse and otherwise) to the new molecular entity being evaluated. This belief has come under fire in recent years (Zbinden, 1993) but is unlikely to be changed soon. Along the same lines, unless an agent is to be used by only one sex or the other of humans, equal numbers of both sexes of an animal species are utilized in the studies, with the sexes being treated as unrelated for purposes of statistical analysis. Also, except in rare cases, the animals used are young, healthy adults in the logarithmic phase of their growth curve. (The FDA specifies that rodents be less than six weeks of age at the initiation of dosing.)

Numbers of animals to be used in each dose group of a study are presented in Table 8.2. Though the usual practice is to use three different dose groups and at least one equal-sized control group, this number is not fixed and should be viewed as a minimum (see the section on study design later in this chapter). Use of more groups allows for a reduction in the risk of not clearly defining effects and establishing the highest possible safe dose at a modest increase in cost. There must be as many control animals as there are in the largest size test group to optimize statistical power.

Animals are assigned to groups (test and control) by one or another form of statistical randomization. Prior to assignment, animals are evaluated for some period of time after being received in house (usually at least one week for rodents and two for nonrodents) to ensure that they are healthy and have no discernible abnormalities. The randomization is never pure; it is always "blocked" in some form or another (by initial body weight, at least) so that each group is not (statistically) significantly different from the others in terms of the blocked parameters.

Proper facilities and care for test animals are not only a matter of regulatory compliance (and a legal requirement) but also essential for a scientifically sound and valid study. Husbandry requires clean cages of sufficient size and continuous availability of clean water and food (unless the protocol requires some restriction on their availability). Environmental conditions (temperature, humidity, and light–dark cycle) must be kept within specified limits. All

TABLE 8.2 Numbers of Animals for Chronic and Subchronic Study per Test Group

Study Length	Rats per Sex	Dogs per Sex	Primates per Sex
2–4 Weeks	5–10	3–4	3
3 Months[a]	20	6	5
6 Months	30	8	5
1 Year	50	10	10

[a]Starting with 13-week studies, one should consider adding animals (particularly to the high-dose group) to allow evaluation of reversal (or progression) of effects.

of these must in turn be detailed in the protocols of studies. The limits for these conditions are set forth in relevant National Institutes of Health and U.S. Department of Agriculture publications.

8.3.2 Routes and Setting Doses

Route (how an agent is administered to a test animal) and dose (how much of and how frequently an agent is administered) are inseparable in safety assessment studies and really cannot be defined independently. The selection of both begins with an understanding of the intended use of the drug in humans. The ideal case is to have the test material administered by the same route, at the same frequency [once a day, three times a day (t.i.d.), etc.], and for the same intervals (e.g., continuously if the drug is an intravenously infused agent) as the drug's eventual use in people. Practical considerations such as the limitations of animal models (i.e., there are some things you cannot get a rat to do), limitations on technical support,[1] and the like and regulatory requirements (discussed below as part of dose setting) frequently act or interact to preclude this straightforward approach.

Almost 30 routes exist for administration of drugs to patients, but only a handful of these are commonly used in preclinical safety studies (Gad, 1994). The most common deviation from what is to be done in clinical trials is the use of parenteral (injected) routes such as intravenous and subcutaneous deliveries. Such injections are loosely characterized as bolus (all at once or over a very short period, such as 5 min) and infusion (over a protracted period of hours, days, or even months). The term continuous infusion implies a steady rate over a protracted period, requiring some form of setup such as an implanted venous catheter or infusion port.

It is rare that the raw drug itself is suitable (in terms of stability, local tissue tolerance, and optimum systemic absorption and distribution) for direct use as a dosage form. Either it must be taken into a solution or suspension in a suitable carrier or a more complex formulation (a prototype of the commercial form) must be developed. Gad (2007) should be consulted for a more complete discussion of dose formulation for animals or humans. One formulation or more must be developed (preferably the same one for both animals and humans) based on the specific requirements of preclinical dosage formulation. For many therapeutic agents, limitations on volumes that can be administered and concentrations of active ingredient that can be achieved impact heavily on dose setting.

Setting of doses for longer term toxicity studies is one of the most difficult tasks in study design. The doses administered must include one that is devoid of any adverse effect (preferably of any effect) and yet still high enough to "clear" the projected clinical dose by the traditional or regulatory safety

[1] Many antiviral agents, particularly some anti-HIV agents, have rather short plasma half-lives, which requires frequent oral administration of the agent. Thirteen-week studies have been conducted with t.i.d. dosing of rats and monkeys, requiring around-the-clock shift work for technical staff of the laboratory.

factors (10 times for rodents, 5 times for nonrodents). At the same time, if feasible, at least one of the doses should characterize the toxicity profile associated with the agent (for some biotechnologically derived agents, particularly those derived from endogenous human molecules, it may only be possible to demonstrate biological effects in appropriate disease models and impossible to demonstrate toxicity). Because of limitations on availability of protodrugs, it is generally undesirable to go too high to achieve this second (toxicity) objective.

Traditionally, studies include three or more dose groups to fulfill these two objectives. Based on earlier results (generally, single-dose or two-week studies), doses are selected. It is, by the way, generally an excellent idea to observe the "decade rule" in extrapolation of results from shorter to longer studies; that is, do not try to project doses for more than an order-of-magnitude-longer study (thus the traditional progression from single-dose to 14-day to 90-day studies). Also, one should not allow the traditional use of three dose groups plus a control to limit designs. If there is a great deal of uncertainty, it is much cheaper in every way to use four or five dose groups in a single study than to have to repeat the entire study. Finally, remember that different doses may be appropriate for the different sexes.

It should also be kept in mind that formulating materials may have effects of their own, and a "vehicle" control group may be required in addition to a negative control group. Additionally the limitations on volumes of dose administration should be kept in mind as presented in Table 8.3.

8.3.3 Parameters to Measure

As was stated earlier, subchronic studies are shotgun in nature; that is, they are designed to look at a very broad range of endpoints with the intention of screening as broadly as possible the indications of toxicity. Meaningful findings

TABLE 8.3 Guidance on Volumes of Administration

	Routes and Volumes (mL kg^{-1} Administration Site)					
Species	Oral	SC	IP	IM	IV Bolus	IV (Slowing)
Mouse	10 (50)	10 (40)	20 (80)	0.5[a] (0.1)[a]	5	(25)
Rat	10 (40)	5 (10)	10 (20)	0.1[a] (0.2)[a]	5	(20)
Rabbit	10 (15)	1 (2)	5 (20)	0.25 (0.5)	2	(10)
Dog	5 (15)	1 (2)	1 (20)	0.25 (0.5)	2.5	(10)
Macaque	5 (15)	2 (5)	– (10)	0.25 (0.05)	2	—
Marmoset	10 (15)	2 (5)	– (20)	0.25 (0.5)	2.5	(10)
Minipig	10 (15)	1 (2)	1 (20)	0.25 (0.5)	2.5	(5)

Notes: —, data not available; SC, subcutaneous; IP, intraperitoneal; IM, intramuscular. For nonaqueous injectates consideration must be given to time of absorption before redosing. No more than two IM sites should be used per day. Subcutaneous sites should be limited to two to three per day. Figures in parentheses are total per day with multiple administrations.

[a]Milliliters.

Sources: Adapted from ECVAM, 2001.

are rarely limited to a single endpoint—rather, what typically emerges is a pattern of findings. This broad search for components of the toxicity profile is not just a response to regulatory guidelines intended to identify potentially unsafe drugs. An understanding of all the indicators of biological effect can also frequently help one to understand the relevance of findings, to establish some as unrepresentative of a risk to humans, and even to identify new therapeutic uses of an agent.

Parameters of interest in the repeat-dose study can be considered as sets of measures, each with its own history, rationale, and requirements. It is critical to remember, however, that the strength of the study design as a scientific evaluation lies in the relationships and patterns of effects that are seen in looking at each of these measures (or groups) not simply as independent findings but rather as integrated profiles of biological effects.

Body Weight Body weight (and the associated calculated parameter of body weight gain) is a nonspecific, broad screen for adverse systemic toxicity (Ellaben and Hart, 1998). Animals are initially assigned to groups based on a randomization scheme that includes having each group vary insignificantly from one another in terms of body weight. Weights are measured prior to the initial dose, then typically 1, 3, 5, 7, 11, and 14 days thereafter. The frequency of measurement of weights goes down as the study proceeds—after 2 weeks, weighing is typically weekly through 6 weeks, then every other week through 3 months, and monthly thereafter. Because the animals used in these studies are young adults in the early log phase of their growth, decreases in the rate of gain relative to control animals is a very sensitive (albeit nonspecific) indicator of systemic toxicity.

Food Consumption Food consumption is typically measured with one or two uses in mind. First, it may be explanatory in the interpretation of reductions (either absolute or relative) in body weight. In cases where administration of the test compound is via diet, it is essential to be able to adjust dietary content so as to accurately maintain dose levels. Additionally, the actual parameter itself is a broad and nonspecific indicator of systemic c toxicity. Food consumption is usually measured over a period of several days, first weekly and then on a once-a-month basis. Water consumption, which is also sometimes measured, is similar in interpretation and use. Additionally, particularly in nonrodents, loss of appetite is an early indicator of adverse effects in animals.

Clinical Signs Clinical signs are generally vastly underrated in value, probably because insufficient attention is paid to care in their collection. Two separate levels of data collection are actually involved here. The first is the morbidity and mortality observation, which is made twice a day. This generally consists of a simple cageside visual assessment of each animal to determine if it is still alive, and, if so, whether it appears in good (or at least stable) health. Historically, this regulatory required observation was intended to ensure that tissues

from intoxicated animals were not lost for meaningful histopathological evaluation due to autolysis (Arnold et al., 1990).

The second level of clinical observation is the detailed hands-on examination analogous to the human physical examination. It is usually performed against a checklist [see Gad (2007) for an example], and evaluation is of the incidence of observations of a particular type in a group of treated animals compared to controls. Observations range from being indicative of nonspecific systemic toxicity to fairly specific indicators of target organ toxicity. These more detailed observations are typically taken after the first week of a study and on a monthly basis thereafter.

Ophthalmological examinations are typically made immediately prior to initiation of a study (and thus serve to screen out animals with preexisting conditions) and toward the end of a study.

Particularly when the agent under investigation either targets or acts via a mechanism likely to have a primary effect on a certain organ for which functional measures are available, an extra set of measurements of functional performance should be considered. The organs or organ systems that are usually of particular concern are the kidneys, liver, cardiovascular, nervous, and immune. Special measures (such as creatinine clearance as a measure of renal function) are combined with other data already collected (organ weights, histopathology, clinical pathology, etc.) to provide a focused "special" investigation or evaluation of adverse effects on the target organ system of concern. In larger animals (dogs and primates) some of these measures [such as electrocardiograms (ECGs)] are made as a matter of course in all studies.

Clinical Chemistry and Pathology Clinical pathology covers a number of biochemical and morphological evaluations based on invasive and noninvasive sampling of fluids from animals that are made periodically during the course of a subchronic study. These evaluations are sometimes labeled as clinical (as opposed to anatomical) pathology determinations. Table 8.4 presents a summary of the parameters measured under the headings of clinical chemistry, hematology, and urinalysis using samples of blood and urine collected at predetermined intervals during the study. Conventionally, these intervals are typically at three points evenly spaced over the course of the study, with the first being one month after study initiation and the last being immediately prior to termination of the test animals. For a three-month study, this means that samples of blood and urine would be collected at one, two, and three months after study initiation (i.e., after the first day of dosing of the animals). There are some implications of these sampling plans that should be considered when the data are being interpreted. Many of the clinical chemistry (and some of the hematological) markers are really the result of organ system damage that may be transient in nature (see Table 8.5 for a summary of interpretations of clinical chemistry findings and Table 8.6 for a similar summary for hematological findings). The samples on which analysis is performed are from fixed points in time, which may miss transient changes (typically, increases) in some enzyme levels.

TABLE 8.4 Clinical Pathology Measures

Clinical Chemistry	Hematology	Urinalysis
Albumin	Erythrocyte count (RBC)	Chloride
Alkaline phosphatase (ALP)	Hemoglobin (HGB)	Bilirubin
Blood urea nitrogen (BUN)	Hematocrit (HCT)	Glucose
Calcium	Mean corpuscular hemoglobin (MCH)	Occult blood
Chloride	Mean corpuscular volume (MCV)	pH
Creatine	Platelet count	Phosphorus
Creatine phosphokinase (CPK)	Prothrombin time	Potassium
Direct bilirubin	Reticulocyte count	Protein
γ-Glutamyltransferase (GGT)	White cell count (WBC)	Sodium
Globulin	White cell differential count	Specific gravity
Glucose		Volume
Lactic dehydrogenase (LDH)		
Phosphorus		
Potassium		
Serum glutamic–oxaloacetic transaminase (SGOT)		
Serum glutamic–pyruvic transaminase (SGPT)		
Sodium		
Total bilirubin		
Total cholesterol		
Total protein		
Triglycerides		

There is now a concerted effort to improve predictive power of nonclinical safety assessment studies for major classes of issues that are "discovered" in clinical trials and marketed use of new drugs. In particular, there are efforts to identify biomarkers which can be measured in animal studies and have relevance and more similarity for clinical finding.

Particular focus has been on biomakers for the heart (see Braunwald, 2008), kidney, and liver. The kidney guidance is quite recent with European Medicines Agency (EMEA) and FDA announcements in late May 2008 that they have accepted seven biomarkers (Kim-1, albumin, total protein, Cystatin C, β_2-microglobulin, urinary clusterin, and urinary trefoil factor 3) for use in preclinical studies. The liver guidance is recent (EMEA, 2008), though this reflects an ongoing effort (see Kaplowitz, 2005) to try and reduce the occurrence of "idiosyncratic" liver toxicity in clinical trials. Table 8.7 presents a list of available clinical chemistry measures that are considered preclinical in the three major categories of hepatic toxicity.

It would seem likely that rather than these being adapted whole cloth into the standard design of repeat-dose studies, they are likely to be used in a more considered manner when there is cause for specific concern. This, however, must still be worded out.

Pharmacokinetics and Metabolism Pharmaceutical subchronic toxicity studies are always accompanied by a parallel determination of the

TABLE 8.5 Association of Changes in Biochemical Parameters with Actions at Particular Target Organs

	Organ System								
Parameter	Blood	Heart	Lung	Kidney	Liver	Bone	Intestine	Pancreas	Notes
Albumin				↓	↓				Produced by the liver. Very significant reductions indicate extensive liver damage.
ALP (alkaline phosphatase)					↑	↑	↑		Elevations usually associated with cholestasis. Bone ALP tends to be higher in young animals
ALT (alanine aminotransferase), also called SGPT (serum glutamic pyruvic transaminase)					↑				Evaluations usually associated with hepatic damage or disease. Prime clinical hepatic damage marker—Hy's law sets a boundary of a 3× increase of significant damage.
Bilirubin (total)	↑				↑				Usually elevated due to cholestasis either due to obstruction or hepatopathy.
BUN (blood urea nitrogen)				↑	↓				Estimates blood-filtering capacity of the kidneys. Does not become significantly elevated until kidney function is reduced 60–75%.
Calcium				↑					Can be life threatening and result in acute death.
Cholinesterase				↑	↓				Found in plasma, brain, and RBC.
CPK (creatinine phosphokinase)		↑							Most often elevated due to skeletal muscle damage but can also be produced by cardiac muscle damage. Can be more sensitive than histopathology.
Creatine				↑					Also estimates blood-filtering capacity of kidney as BUN does. More specific than BUN.

TABLE 8.5 ***Continued***

Parameter	Organ System								Notes
	Blood	Heart	Lung	Kidney	Liver	Bone	Intestine	Pancreas	
Glucose								↑	Alterations other than those associated with stress are uncommon and reflect an effect on the pancreatic islets or anorexia.
GGT (γ-glutamyltransferase)					↑				Elevated in cholestasis. This is microsomal enzyme and levels often increase in response to microsomal enzyme induction.
HBDH (hydroxybutyric dehydrogenase)		↑			↑				Most prominent in cardiac muscle tissue.
LDH (lactic dehydrogenase)		↑	↑	↑	↑				Increase usually due to skeletal muscle, cardiac muscle, and liver damage. Not very specific unless isozymes are evaluated.
Protein (total)				↑	↑				Absolute alterations are usually associated with decreased production (liver) or increased loss (kidney).
SGOT (serum glutamic oxaloacetic transaminase); also called AST (aspartate amino transferase)		↑		↑	↑			↑	Present in skeletal muscle and heart and most commonly associated with damage to these.
SDH (sorbitol dehydrogenase)					↑ or ↓				Liver enzyme which can be quite sensitive but is fairly unstable.QQSamples should be processed as soon as possible.

Source: Gad, 2007.

TABLE 8.6 Some Probable Conditions Affecting Hematological Changes

Parameter	Elevation	Depression
Red blood cells	1. Vascular shock 2. Excessive diuresis 3. Chronic hypoxia 4. Hyperadrenocorticism	1. Anemias a. Blood loss b. Hemolysis c. Low RBC production
Hematocrit	1. Increased RBC 2. Stress 3. Shock a. Trauma b. Surgery 4. Polycythemia	1. Anemias 2. Pregnancy 3. Excessive hydration
Hemoglobin	1. Polycythemia (increase in production of RBCs)	1. Anemias 2. Lead poisonings
Mean cell volume	1. Anemias 2. B_{12} deficiency	1. Iron deficiency
Mean corpuscular hemoglobin	1. Reticulocytosis	1. Iron deficiency
White blood cells	1. Bacterial infections 2. Bone marrow stimulation	1. Bone marrow depression 2. Cancer chemotherapy 3. Chemical intoxication 4. Splenic disorders
Platelets	—	1. Bone marrow depression 2. Immune disorder
Neutrophils	1. Acute bacterial infections 2. Tissue necrosis 3. Strenuous exercise 4. Convulsions 5. Tachycardia 6. Acute hemorrhage	1. Viral infections
Lymphocytes	1. Leukemia 2. Malnutrition 3. Viral infections	
Monocytes	1. Protozoal infections	
Eosinophils	1. Allergy 2. Irradiation 3. Pernicious anemia 4. Parasitism	
Basophils	1. Lead poisoning	

Source: Gad, 2007.

pharmacokinetics of the material of interest administered by the same route as that used in the safety study. This parallel determination consists of measuring plasma levels of the administered agent and its major metabolites either in animals that are part of the main study or in a separate set of animals (in parallel with the main study) that are dosed and evaluated to determine just these endpoints. The purpose of these determinations is both to allow a better interpretation of the findings of the study and to encourage the most accurate

TABLE 8.7 Clinical Chemistry Measures Considered Useful in Identifying Liver Toxicity

Parameters	Hepatocellular	Hepatobiliary	Mitochondrial
Alanine aminotransferase (ALT)	X		
Aspartate aminotransferase (AST)	X		
Sorbitol dehydrogenase (SDH)	X		
Glutamate dehydrogenase (GLDH)	X		X
Total bile acid (TBA)	X	X	
Alkaline phosphatase (ALP)		X	
γ-Glutamyltransferase (GGT)		X	
5′-nucleotidase (5-NT)		X	
Total bilirubin (TBILI)		X	
Potential ancillary markers			
Lactate			X
Lactate dehydrogenase (LHD)	X		
Ornithine carbamyltransferase	X		X
Unconjugated bilirubin (UBILI)	X		

possible extrapolation to humans. The first data of interest are the absorption, distribution, and elimination of the test material, but a number of other types of information can also be collected (Yacobi et al., 1989; Tse and Jaffe, 1991). For nonparenteral routes it is essential to demonstrate that systemic absorption and distribution of the test material did occur; otherwise, it is open to question whether the potential safety of the agent in humans has been adequately addressed (not to mention the implication for potential human therapeutic efficacy). A complication, however, is that there are limits as to how much blood may be collected from specific species at one time (see Table 8.8), particularly as samples must be drawn to allow evaluation of clinical chemistry, clinical pathology, and pharmacokinetics. This is even worse in the case of biologics, where adequate samples must also be drawn to allow the evaluation of antibody levels.

8.3.4 Other In-Life Endpoints for Evaluation

Ophthalmology Ophthalmological examination of all animals in a study (particularly nonrodents) should be performed both before study initiation and at the completion of the period at which the drug is administered. This should be performed by an experienced veterinary ophthalmologist.

Cardiovascular Function Particularly in light of recent concerns with drug-induced arrhythmias, careful consideration must be given to incorporating adequate evaluation of drug-induced alterations on cardiovascular function. This is usually achieved by measuring blood pressure, heart rate, and an ECG prestudy and periodically during the course of the study (usually at at least one intermediate period and at the end of the study) in the nonrodent species being employed.

TABLE 8.8 Total Blood Volumes and Recommended Maximum Blood Sample Volumes for Species of Given Body Weight

Species	Blood Volume (mL)	7.5% (mL)	10% (mL)	15% (mL)	20% (mL)
Mouse (25 g)	1.8	0.1	0.2	0.3	0.4
Rat (250 g)	16	1.2	1.6	2.4	3.2
Rabbit (4 kg)	224	17	22	34	45
Dog (10 kg)	850	64	85	127	170
Macaque (Rhesus) (5 kg)	280	21	28	42	56
Macaque (Cynomologus) (5 kg)	325	24	32	49	65
Marmoset (350 kg)	25	2.0	2.5	3.5	5
Mini pig (15 kg)	975	73	98	146	195

TABLE 8.9 FDA Draft Criteria for Neurotoxicity Screen as Component of Short-Term and Subchronic Studies

Histopathological examination of tissues representative of nervous system, including brain, spinal cord, and peripheral nervous system

Quantitative observations and manipulative test to detect neurological, behavioral, and physiological dysfunctions. These may include:

- General appearance
- Body posture
- Incidence and severity of seizure
- Incidence and severity of tremor, paralysis, and other dysfunction
- Level of motor activity and arousal
- Level of reactivity to stimuli
- Motor coordination
- Strength
- Gait
- Sensorimotor response to primary sensory stimuli
- Excessive lacrimation or salivation
- Pilorection
- Diarrhea
- Ptosis
- Other signs of neurotoxicity deemed appropriate

Neurotoxicology Table 8.9 presents the FDA's current draft criteria (FDA, 1993, 2000) for endpoints to be incorporated in studies as a screen for neurotoxicity. In practice, a functional observation battery is employed at several endpoints (usually one and three months into the study) to fill these requirements.

Immunotoxicology In response to concerns about potential effects of drugs on the immune system, the ICH and FDA (2006) have promulgated a guidance

TABLE 8.10 FDA Draft Recommendation for Type I Immunotoxicity Test That Can be Included in Repeated-Dose Toxicity Studies

Hematology	Clinical Chemistry
White blood cell counts	Total serum production
Differential white blood cell counts	Albumin
Lymphocytosis Lymphopenia	Albumin-to-globulin ratio
Eosinophilia	Serum transaminases
Histopathology	
Lymphoid tissues	
Spleen	
• Lymph nodes	
• Thymus	
• Peyer's patches in gut	
• Bone Marrow	
Cytology (if needed)[a]	
• Prevalence of activated macrophages	
• Tissue prevalence and location of lymphocytes	
• Evidence of B-cell germinal centers	
• Evidence of T-cell germinal centers	
Necrotic or proliferative changes in lymphoid tissues	

[a]More comprehensive cytological evaluation of the tissues would not be done unless there is evidence of potential immunotoxicity from the preceding evaluations.

calling for a basic set of potential indicators of immunotoxicity (Table 8.10) to be evaluated and considered in standard repeat-dose studies. Most of these endpoints are, it should be noted, already collected in traditional subchronic designs.

Pharmacokinetics All subchronic and chronic toxicity studies now incorporate (either in the study itself or in a parallel study) evaluation of the basic pharmacokinetics of a compound. This is discussed in detail in Chapter 15.

8.3.5 Histopathology

Histopathology is generally considered the single most significant portion of data to come out of a repeat-dose toxicity study. It actually consists of three related sets of data (gross pathology observations, organ weights, and microscopic pathology) that are collected during the termination of the study animals. At the end of the study, a number of tissues are collected during termination of all surviving animals (test and control). Organ weights and terminal body weights are recorded at study termination, so that absolute and relative (to body weight) values can be statistically evaluated.

These tissues, along with the organs for which weights are determined, are listed in Table 8.11. All tissues collected are typically processed for microscopic observation, but only those from the high-dose and control groups

TABLE 8.11 Tissues for Histopathology

Adrenals[a]	Mainstream Bronchi
Body and cervix	Major salivary glad
Brain, all three levels[a]	Mesenteric lymph nodes
Cervical lymph nodes	Ovaries and tubes
Cervical spinal cord	Pancreas
Duodenum	Pituitary
Esophagogastric junction	Prostate
Esophagus	Skeletal muscle from proximal hind limb
Eyes with optic nerves	Spleen[a]
Femur with marrow	Sternebra with marrow
Heart	Stomach
Ileum	Testes with epididymides[a]
Kidneys[a]	Thymus and mediastinal contents[a]
Large bowel	Thyroid with parathyroid[a]
Larynx with thyroid and parathyroid	Trachea
Liver[a]	Urinary bladder
Lungs[a]	Uterus including horns

[a]Organs to be weighed.

are necessarily evaluated microscopically. If a target organ is discovered in the high-dose group, then successively lower dose groups are examined until a "clean" (devoid of effect) level is discovered (Haschek and Rousseaup, 1991).

In theory, all microscopic evaluations should be performed blind (without the pathologist knowing from which dose group a particular animal came), but this is difficult to do in practice and such an approach frequently degrades the quality of the evaluation. Like all the other portions of data in the study, proper evaluation benefits from having access to all data that address the relevance, severity, timing, and potential mechanisms of a specific toxicity. Blind examination is best applied in peer review or consultations on specific findings.

In addition to the "standard" set of tissues specified in Table 8.8, observations during the course of the study or in other previous studies may dictate that additional tissues be collected or special examinations (e.g., special stains, polarized light or electron microscopy, immunocytochemistry, or quantitative morphometry) be undertaken to evaluate the relevance of or understand the mechanisms underlying certain observations.

Histopathology testing is a terminal procedure, and, therefore, sampling of any single animal is a one-time event (except in the case of a tissue collected by biopsy). Because it is a regulatory requirement that the tissues from a basic number of animals be examined at the stated end of the study, an assessment of effects at any other time course (most commonly, to investigate recovery from an effect found at study termination) requires that satellite groups of animals be incorporated into the study at startup. Such animals are randomly

assigned at the beginning of the study and are otherwise treated exactly the same as the equivalent treatment (or control) animals.

8.3.6 Study Designs

The traditional design for a repeat-dose toxicity study is very straightforward. The appropriate number of animals of each sex are assigned to each of the designated dose and control groups. Unfortunately, this basic design is taken by many to be dogma, even when it does not suit the purposes of the investigator. There are many possible variations to study design, but four basic factors should be considered: controls, the use of interval and satellite groups, balanced and unbalanced designs, and staggered starts.

Classically, a single control group of the same size as each of the dose groups is incorporated into each study. Some studies incorporate two control groups (each the same size as the experimental groups) to guard against having a statistically significant effect due to one control group being abnormal for one or more parameters (a much more likely event when laboratory animals were less genetically homogeneous than they are now). The belief is that a "significant" finding that differs from one (but not both) of the concurrent control groups and does not differ from historical control data can be considered as not biologically significant. This is, however, an indefensible approach. Historical controls have value, but it is the concurrent control group(s) in a study that is of concern.

Interval or satellite groups have been discussed at two earlier points in this chapter. They allow measurement of termination parameters at intervals other than at termination of the study. They are also useful when the manipulation involved in making a measurement (such as the collection of an extensive blood sample), while not terminal, may compromise (relative to other animals) the subject animals. Another common use of such groups is to evaluate recovery from some observed effect at study termination.

Usually, each of the groups in a study is the same size, with each of the sexes being equally represented. The result is called a balanced design, with statistical power for detection of effects optimized for each of the treatment groups. If one knows little about the dose–toxicity profile, this is an entirely sound and rational approach. However, there are situations when one may wish to utilize an unbalanced design—that is, to have one or more dose groups larger than the others. This is usually the case when either greater sensitivity is desired (typically in a low-dose group) or an unusual degree of attrition of test animals is expected (usually due to mortality in a high-dose group) or as a guard against a single animal's idiopathic response being sufficient to cause "statistical significance."

As it is the normal practice to have a balanced design, it is also traditional to initiate treatment of all animals at the same time. This may lead to problems at study termination, however. It is a very uncommon toxicology laboratory that can "bring a study down" on a single day. In fact, there are no laboratories

that can collect blood and perform necropsies in a single day on even the 48–80 dogs involved in a study, much less the 160–400+ rats in the rodent version. Starting all animals on a study the same day presents a number of less than desirable options. The first is to terminate as many animals as can be done each day, continuing to dose (and therefore further affect) the remaining test animals. Assuming that the animals are being terminated in a random, balanced manner, this means that the last animals terminated will have received from 3 to 10 additional days of treatment. At the least, this is likely to cause some variance inflation (and therefore both decrease the power of the study design and possibly confound interpretation). If the difference in the length of treatment of test animals is greater than 3% of the intended length of the study, one should consider alternative designs.

An alternative approach to study design that addresses this problem employs one of several forms of staggered starts. In these, distinct groups of animals have their dosing initiated at different times. The most meaningful form recognizes that the two sexes are in effect separate studies anyway (they are never compared statistically, with the treatment groups being compared only against the same-sex control group). Thus if the termination procedure for one sex takes three to five days, then one sex should be initiated on dosing one week and the other on the following week. This maximizes the benefits of common logistical support (such as dose formulation) and reduces the impact of differential length of dosing on study outcome.

A variation on this is to stagger the startup either of different dose groups or of the satellite and main study portions of dose groups. The former is to be avoided (it will completely confound study outcome), while the latter makes sense in some cases (pharmacokinetics and special measures) but not others (recovery and interval sacrifice).

8.4 STUDY INTERPRETATION AND REPORTING

For a successful repeat-dose study, the bottom line is the clear demonstration of a no-effect level, characterization of a toxicity profile (providing guidance for any clinical studies), enough information on pharmacokinetics and metabolism to scale dosages to human applications, and at least a basic understanding of the mechanisms involved in any identified pathogenesis. The report that is produced as a result of the study should clearly communicate these points—along with the study design and experimental procedures, summarized data, and their statistical analysis—and it should be GLP compliant, suitable for FDA submission format.

Interpretation of the results of a study should be truly scientific and integrative. It is elementary to have the report state only each statistically and biologically significant finding in an orderly manner. The meaning and significance of each in relation to other findings as well as the relevance to potential human effects must be evaluated and addressed.

The author of the report should ensure that it is accurate and complete but also that it clearly tells a story and concludes with the relevant (to clinical development) findings.

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9

Immunotoxicology in Drug Development

9.1 INTRODUCTION

Unlike many of the other areas covered in this volume, immunotoxicity evaluation of drugs has undergone fundamental changes since the first edition. Before, there was no specific guidance. Now, with the International Conference on Harmonisation (ICH) S8A in place and in force, there is both a requirement and a roadmap for immunotoxicity evaluations, though biologics are excluded from coverage.

This is in particular a concern as biologics have attained their therapeutic promise with their hyperpharmacology providing the largest portion of safety concern for them [from the "cytokine storm" of TGN-1412 to the unintended immune issues associated with most monoclonal antibodies (mAbs)]. With immune modulation of some form being the primary intended therapeutic effects and one-third of all new approved therapeutics being biologics, this is particularly a concern, while specifically not covered by ICH guidances, approaches and requirements for immunotoxicity evaluations of these moieties are addressed, however, in this chapter.

All three ICH regions have made strong efforts to harmonize the immunotoxicity risk assessment for investigational new drugs. These efforts culminated in the release of the ICH S8 guideline, which was adopted by the CHMP (Committee for Medicinal Products for Human Use) in October 2005 and came into force in the United States in April 2006, as well as the MHLW (Ministry of Health, Labor and Welfare, Japan) in April 2006. According to this current

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guideline, initial immunotoxicity assessment should be based on the evaluation of data already available from standard toxicity studies and other characteristics of the drug substance, such as pharmacological properties, the intended patient population, known drug class effects, clinical data for the drug, and its disposition. The need for additional immunotoxicity testing should be decided on the basis of a weight of evidence assessment, taking into account all available information. Testing should thus be a tiered approach, triggered and determined by concerns from the weight of evidence assessment.

Immunotoxicity is defined in the ICH S8 guideline as unintended immunosupression or enhancement. It must be noticed that drug-induced hypersensitivity and autoimmunity are not in the scope of ICH S8 (Pattels and Taylor, 2008). Surprisingly, the ICH S8 guideline excludes biological and biotechnology-derived products, probably the larger area of concern for pharmaceuticals. EMEA (2007) has promulgated specific guidance for immunogenicity evaluation of proteins.

The immune system is a highly complex system of organ systems, cells, and soluble factors distributed throughout the body and involved in a multitude of functions, including antigen presentation and recognition, amplification, and cell proliferation with subsequent differentiation and secretion of lymphokines and antibodies. In health, these are in a state of balance, and there are extensive mechanisms to maintain this balance. The resulting integrated system is responsible for defense against foreign pathogens and spontaneously occurring neoplasms and is readily triggered to response. To be effective, the immune system must be able to both recognize and destroy foreign antigens. To accomplish this, cellular and soluble components of diverse function and specificity circulate through blood and lymphatic vessels, thus allowing them to act at remote sites and tissues. For this system to function in balance and harmony requires regulation through cell-to-cell communications and precise recognition of self versus nonself. There are multiple opportunities for immunotoxicants to upset this balance by selectively disabling one or more of the cell types or alter membrane morphology and receptors. There are several undesired immune system responses that may occur upon repeated therapeutic administration of a pharmaceutical that may ultimately present barriers to its development, including:

- Down-modulation of the immune response (immunosuppression), which may result in an impaired ability to deal with neoplasia and infections. This is of particular concern if the therapeutic agent is intended to be used in patients with preexisting conditions such as cancer, severe infection, or immunodeficiency diseases.
- Up-modulation of the immune system (i.e., autoimmunity).
- Direct adverse immune responses to the agent itself in the form of hypersensitivity responses (anaphylaxis and delayed contact hypersensitivity).
- Direct immune responses to the agent that limit or nullify its efficacy (i.e., the development of neutralizing antibodies).

Iammune modulated responses to drugs ("drug allergies") are a major problem and a cause of discontinuance of use by patients who need access to the therapeutic benefits (Patterson et al., 1986), and there remains no adequate preclinical methodology for identifying/predicting these responses to orally administered small-molecule drugs (Hastings, 2001).

It is the intent of this chapter to provide an understanding of these adverse immunological effects, the types of preclinical tests that may be used to detect them, and approaches for testing and interpreting test results.

Immunotoxicology has evolved over the last 20 years as a specialty within toxicology that brings together knowledge from basic immunology, molecular biology, microbiology, pharmacology, and physiology. As a discipline, immunotoxicology involves the study of adverse effects that xenobiotics have on the immune system. As listed above, several different types of adverse immunological effects may occur, including immunosuppression, autoimmunity, and hypersensitivity. Although these effects are clearly distinct, they are not mutually exclusive. For example, immunosuppressive drugs that suppress suppressor-cell activity can also induce autoimmunity (Hutchings et al., 1985), and drugs that are immunoenhancing at low doses may be immunotoxic at high doses. Chemical xenobiotics may be in the form of natural or man-made environmental chemicals—pharmaceuticals and biologics that are pharmacologically, endocrinologically, or toxicologically active. Although, in general, xenobiotics are not endogenously produced, immunologically active biological response modifiers that naturally occur in the body should also be included, since many are not known to compromise immune function when administered in pharmacologically effective doses (Koller, 1987).

Although the types of immunological responses to various xenobiotics may be similar, the approach taken for screening potential immunological activity will vary depending on the application of the compound. Thus, this chapter will primarily focus on the immunotoxicology of pharmaceuticals. In contrast to potential environmental exposures, pharmaceuticals are developed with intentional but restricted human exposure and their biological effects are extensively studied in surveillance. Pharmaceuticals are developed to be biologically active, and, in some cases, intentionally immunomodulating or immunosuppressive. Many will react with biological macromolecules or require receptor binding in order to be pharmacologically active. By their nature, these interactions may result in toxicity to the cells of the immune system, may adversely alter the appearance of "self" to produce an autoimmune response, or may form a hapten, which may then elicit a hypersensitivity response. Because of the fast-expanding development of new drugs that can potentially impact the immune responsiveness of humans, immunotoxicity testing of new pharmaceutical products has become a growing concern.

Until recently, immunotoxicology in pharmaceutical safety assessment has been poorly addressed by both regulatory requirements/guidelines and existing practice. Notable exceptions are the testing requirements for delayed contact hypersensitivity for dermally administered agents and antigenicity/anaphylaxis testing for drugs to be registered in Japan. The most recently

announced regulatory expectation for parenterally administered protein or peptide agents produced by biotechnology is that the development of antibodies (neutralizing and otherwise) should be evaluated in at least one (preferably two) of the animal models used to assess general systemic toxicity.

Unanticipated immunotoxicity is infrequently observed with drugs that have been approved for marketing. With the exception of drugs that are intended to be immunomodulatory or immunosuppressive as part of their therapeutic mode of action, there is little evidence that drugs cause unintended functional immunosuppression in humans (Gleichmann et al., 1989). However, hypersensitivity (allergy) and autoimmunity are frequently observed and are serious consequences of some drug therapies (DeSwarte, 1986; Patterson et al., 1986; Vos et al., 1989; Choquet-Kastylevsky et al., 2001; Pieters, 2001; Luebke et al., 2007). An adverse immune response in the form of hypersensitivity is one of the most frequent safety causes for withdrawal of drugs that have already made it to the market (see Table 9.1) and accounts for approximately 15% of adverse reactions to xenobiotics (deWeck, 1983). In addition, adverse immune responses such as this (usually urticaria and frank rashes) are the chief "unexpected" finding in clinical studies. These findings are unexpected in that they are not predicted by preclinical studies because there is a lack of good preclinical models for predicting systemic hypersensitivity responses, especially to orally administered agents. As a consequence, the unexpected occurrence of hypersensitivity in the clinic may delay, or even

TABLE 9.1 Drugs Withdrawn from Market Due to Dose- and Time-Unrelated Toxicity Not Identified in Animal Experiments

Compound	Adverse Reaction	Year of Introduction	Years on Market
Aminopyrine	Agranulocytosis	ca. 1900	75
Phenacetin	Interstitial nephritis	ca. 1900	83
Dipyrone	Agranulocytosis	ca. 1930	47
Clioquinol	Subacute myelo-optic neuropathy	ca. 1930	51
Oxyphenisatin	Chronic active hepatitis	ca. 1955	23
Nialamide	Liver damage	1959	19
Phenoxypropazine	Liver damage	1961	5
Mebanazine	Liver damage	1963	3
Ibufenac	Hepatotoxicity	1966	2
Practolol	Oculo-mucocutaneous syndrome	1970	6
Alcolofenace	Hypersensitivity	1972	7
Azaribine	Thrombosis	1975	1
Ticrynafen	Nephropathy	1979	1
Benoxaprofen	Photosensitivity, hepatotoxicity	1980	2
Zomepirac	Urticaria, anaphylactic shock	1980	3
Zirnelidine	Hepatotoxicity	1982	2
Temafloxacin	Hepato- and renal toxicity	1990	2
Tronan	Hepato- and renal toxicity	1997	3
Renzalin	Hepatotoxicity	1996	4

Source: Adapted from Bakke et al. (1984) and updated.

preclude, further development and commercialization. Thus, a primary purpose for preclinical immunotoxicology testing is to help us detect these adverse effects earlier in development, before they are found in clinical trials.

9.2 REGULATORY POSITIONS

The pharmaceutical and medical device industries are increasingly concerned with whether preclinical testing of their products should include routine immunotoxicological screening or be done on an "as-needed basis," triggered by the toxicological profile of the xenobiotic established in routine preclinical safety testing (Bloom et al., 1987). Although the U.S. Food and Drug Administration (FDA) has not as yet officially released guidelines for immunotoxicity testing of pharmaceuticals, recent drug development efforts in the areas of biotechnology, prostaglandins, interleukins, and recombinant biological modifiers have elicited the expectation that the development of antibodies (neutralizing and otherwise) should be evaluated in at least one of the animal models used to assess general systemic toxicity. More to the point, draft guidelines have been released for devices [Center for Devices and Radiological Health (CDRH), 1997]. The other available guidance had been the draft guidelines in the revision of the "Redbook" (FDA, 1993). The ICH S8 guidance (2006) supersedes all of them (for pharmaceuticals) (Table 9.2).

The FDA had drafted a similar two-leveled approach (Hinton, 1992) for assessing immunotoxicity of food colors, additives, and drugs, and these are reflected in the S8 guidance. In all of these testing schemes, the initial tier generally includes a fundamental standard toxicity assessment with emphasis on histopathology of the major components of the immune system. Additional tiers are then added to more precisely evaluate the functionality of the components that appeared to be adversely affected in the first tier of tests. These test strategies are primarily geared toward the detection of small-molecule pharmaceutical-induced immunosuppression; thus the effectiveness of these test schemes for detecting immunostimulation has still not been determined (Spreafico, 1988). Table 9.3 presents items that should be considered in such an initial evaluation.

The FDA, ICH, and European Medicines Agency (EMEA) guidelines are not currently entirely aligned. The ultimate immune test would be to examine the effects of xenobiotics on the intact animal's response to challenge by viral, bacterial, or parasitic pathogens or neoplastic cells. The ability of the immune system to compensate or, conversely, its inability to compensate for loss or inhibition of its components is fully examined through host resistance mechanisms. This tiered test approach has been validated with 50 selected compounds, and results from these studies have shown that the use of only two or three immune tests are sufficient to predict known immunotoxic compounds in rodents with a >90% concordance (Luster et al., 1992a,b). Specifically the use of either a humoral response assay for plaque-forming colonies (PFC

TABLE 9.2 Comparison of Current ICH and Former European Union (EU) and U.S. Immunotoxicity

Parameter	ICH S8 (in Operation in All ICH Regions Since 2006)	FDA CDER (Still in Operation Since 2002)	EU CHMP (Still in Operation Since 2000)
Specific immunotoxicity guideline	Yes	Yes	No, included in guidance on repeat-dose toxicity.
Drug-induced hypersensitivity, immunogenicity, and autoimmunity excluded	Yes	No, these categories are included in the guideline.	Yes. (Note: Skin sensitizing potential addressed in CPMP Note for Guidance on Non-Clinical Local Tolerance Testing, 2001 [42].)
Screening study(ies) required	Yes, the initial screen for potential immunotoxicity involves standard toxicity studies (STSs) from short-term to chronic repeat-dose studies in rodents and nonrodents.	Yes, including all standard repeat-dose toxicology studies that have been performed.	Yes, screening required for all new active substances in at least one repeat-dose toxicity study (duration ideally should be 28 days). Rats or mice are species of choice.
Screening study(ies) immunotoxicity parameters	Changes in hematology, lymphoid organ weights, histopathology of immune system, and serum globulins and increased incidences of infections and tumors should be evaluated for signs of immunotoxic potential in the STSs.	Changes in hematology lymphoid organ weights, gross pathology and histopathology of immune system, and serum globulins and increased incidences of infections and tumors should be evaluated for signs of immunotoxic potential	Hematology, lymphoid organ weights, histopathology of lymphoid tissues, bone marrow, cellularity, distribution of lymphocyte subsets, and NK-cell activity (if latter two unavailable, primary antibody response to T-cell-dependent antigen).
Other factors to consider in evaluation of potential immunotoxicity and need for additional immunotoxicity studies	Pharmacological properties of drug; patient population; structural similarities to known immunomodulators; drug disposition; clinical data.	Patients population; known drug class effects [including structure-activity relationships (SARs)]; drug pharmacokinetics; clinical data. If drug intended for HIV, immune function studies required.	None specifically included in the guideline.
"Follow-on"/"additional" immunotoxicity studies	Additional studies may be required depending on the "weight of evidence review" of STSs and "other factors." Additional studies addressed in 3.2, 3.3, and Appendix of guideline.	Not specified.	Not specified.

TABLE 9.3 Typical Indicators of Immunotoxcity Which May Be Observed During Regulatory Repeat-Dose Toxicity Studies

Findings	Possible Indicator of
During In-Life Phase	
Increased frequencies of infectious disease	Immunosuppression
Increased frequencies of tumors in long-term studies in absence of genotoxicity or nongenotoxic indicators of tumorigenicity (e.g., endocrine)	Immunosuppression
Unexpected pathological symptoms or deaths shortly after administration	Hypersensitivity
Strong inflammatory reactions at site of administration	Hypersensitivity
Gross Necropsy	
Significant increase or decrease of size and weight of lymphatic organs	Unintended immunostimulation or immunosuppression
Hematology	
Changes in total or differential blood counts	Unintended immunostimulation or mmunosuppression
Anemia	Type II hypersensitivity
Altered frequencies of lymphocyte subsets (flow cytometry)[a]	Unintended immunostimulation or immunosuppression
Clinical Chemistry	
Altered total globulin levels or albumin: globulin ratio	Unintended immunostimulation or immunosuppression
Changes of immunoglobulin isotype levels[a]	Unintended immunostimulation or immunosuppression
Reduction of hemolytic complement activity[a]	Unintended immunostimulation type III hypersensitivity
Antinuclear or anticytoplasmic antibodies[a]	Unintended immunostimulation or autoimmunity
Histopathology	
Changes of cellularity and/or microanatomy of lymphatic organs	Unintended immunostimulation or immunosuppression
Vasculitis, glomerulonephritis	Type III hypersensitivity

[a]This parameter is normally not measured during standard toxicity studies but may be integrated when a focus is drawn on immunotoxicity assessment.

response) or determination of surface marker expression in combination with almost any other parameter significantly increased the ability to predict immunotoxicity when compared to the predictivity of any assay alone.

The FDA guidelines for immunotoxicity testing of food additives start with a type 1 battery of tests. Type 1 tests can be derived from the routine measurements and examinations performed in short-term and subchronic rodent toxicity studies, since they do not require any perturbation of the test animals (immunization or challenge with infectious agents). These measurements

include hematology and serum chemistry profiles, routine histopathological examinations of immune-associated organs and tissues, and organ and body weight measurements, including thymus and spleen. If a compound produces any primary indicators of immunotoxicity from these measurements, more definitive immunotoxicity tests, such as those indicated in the preceding paragraph, may be recommended on a case-by-case basis.

The following is a brief explanation of some of the indicators that may be used to trigger additional definitive testing and a description of some of the most commonly used assays to assess humoral, cell-mediated, or nonspecific immune dysfunction, which are common to most immunotoxicology test strategies.

9.2.1 CDER Guidance for Investigational New Drugs

The Center for Drug Evaluation and Research (CDER, 2001) promulgated draft guidance for pre-INDA (investigation new drug application) immunotoxicity clearly established the framework for the FDA's approach, which was finalized under ICH S8 (2006). It begins by characterizing five adverse-event categories:

- Immunosuppression
- Antigenicity
- Hypersensitivity
- Autoimmunity
- Adverse immunostimulation

Specific tests are proposed for each of these categories. The CDER notes that immune system effects in noclinical toxicology studies are often attributed and written off as due to stress (Ader and Cohen, 1993). Such effects are frequently reversible with repeat dosing and tend not to be dose related. It is also proposed that, when possible, dose extrapolations to those in clinical use be based on relative body area. Specific recommendations are made for when to conduct specific testing (as opposed to the broader general evaluations integrated into existing repeat-dose testing) (Figure 9.1) and for follow-up studies for exploring mechanisms (Figure 9.2).

9.3 OVERVIEW OF IMMUNE SYSTEM

A thorough review of the immune system is not the intent of this chapter, but a brief description of the important components of the system and their interactions is necessary for an understanding of how xenobiotics can affect immune function. A breakdown at any point in this intricate and dynamic system can lead to immunopathology.

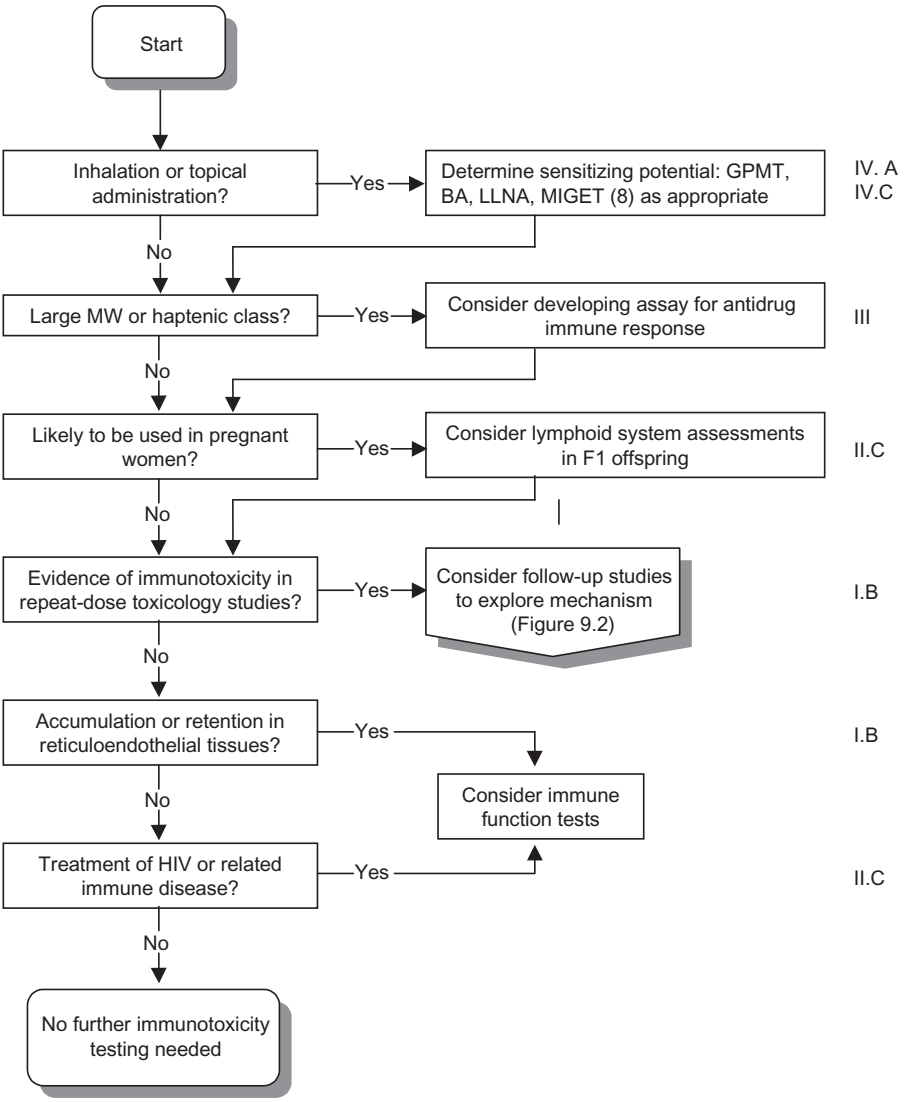

Figure 9.1 *CDER flowchart for determining when to conduct specific immunotoxicity testing. Annotations in right margin indicate location of text describing specific advice GPMT, guinea pig maximization test; BA, Buehler assay (Buehler patch test); LLNA, local lymph node assay; MIGET, mouse IgE test. (There is only a relatively small database available for assessing the usefulness of the MIGET for drug regulatory purposes.)*

The immune system is divided into two defense mechanisms: nonspecific, or innate, and specific, or adaptive, mechanisms that recognize and respond to foreign substances. Some of the important cellular components of nonspecific and specific immunity are described in Table 9.4. The nonspecific immune

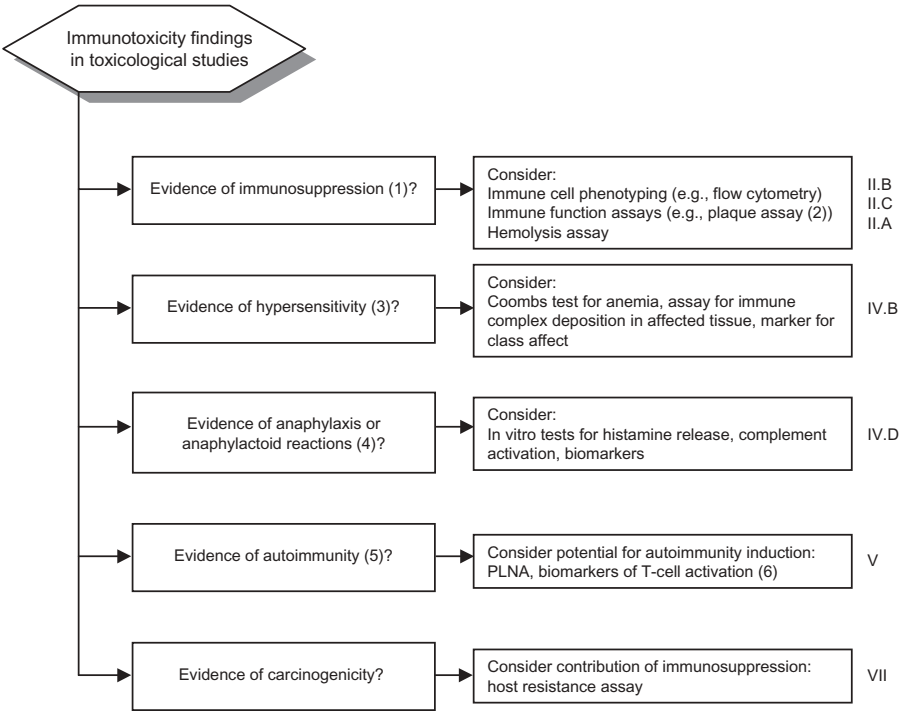

Figure 9.2 *Follow-up studies to consider for exploring mechanisms of immunotoxicity. Annotations in right margin indicate location of text describing specific advice. (1) Examples include myelosuppression, histopathology in immune-associated tissues, increased infection, tumors, decreased serum Ig, phenotypic changes in immune cells. (2) Other acceptable assays include drug effect on NK-cell function in vitro bastogenesis, cytotoxic T-cell function, cytokine production, DTH, host resistance to infections or implanted tumors. (3) Examples include anemia, leukopenia, thrombocytopenia, pnuemonitis, vasculitis, lupuslike reactions, glomerulonephritis. (4) Examples include cardiopulmonary distress, rashes, flushed skin, swelling of face or limbs. (5) Examples include vasculitis, lupuslike reactions, glomerulonephritis, hemolytic anemia. (6) There are no established assays that reliably assess potential for autoimmunity and acute systemic hypersensitivity. The popliteal lymph node assay (PLNA) has only a relatively small database available for assessing its usefulness for drug regulatory purposes.*

system is the first line of defense against infectious organisms. Its cellular components are the phagocytic cells such as the monocytes, macrophages, and polymorphic neutrophils (PMNs).

The innate immune system has been identified in immune defense in insects and alone in the phylogenetic sequence and serves the same purpose in all these organisms, including humans. The innate immune system encompasses all physical, chemical, and cellular barriers that protect the individual from microbial infections without the need to learn to discriminate self from nonself. The body protects itself from dangerous actions of the innate immune system by the lack of expression of molecular patterns of microorganisms and by the

TABLE 9.4 Cellular Components of Immune System and Their Functions

Cell Subpopulations	Markers[a]	Functions
	Nonspecific Immuity	
Granulocytes	—	Degranulate to release mediators
Neutrophils (blood)		
Basophils (blood)		
Eosinophils (blood)		
Mast cells (connective tissue)		
NK cells	—	Nonsensitized lymphocytes; directly kill target cells
Reticuloendothelial	CD14; HLA-DR	Antigen processing, presentation, and phagocytosis (humoral and some cell-mediated responses)
Macrophage (peritoneal, pleural, alveolar spaces)		
Histiocytes (tissues)		
Monocytes (blood)		
	Specific immunity	
Humoral immunity	CD19; CD23	Proliferate; form plasma cells
Activated B cells	—	Secrete antibody; terminally differentiated
Plasma cells	—	Secrete IgM antibodies (primary response)
Resting	—	Secrete IgG antibodies (secondary response)
Memory		
	Cell-Mediated Immunity	
T-Cell types	CD4; CD25	Assists in humoral immunity; required for antibody production
Helper (T_h)	CD8; CD25	Targets lysis
Cytotoxic (T_k)		Suppresses/regulates humoral and cell-mediated responses
Suppressor (T_s)		

[a]Activation surface markers detected by specific monoclonal antibodies; can be assayed with flow cytometry.

abundant expression of inhibitors. Most components of the innate immune system can work independently and in parallel to destroy microorganisms. Due to its redundancy, the innate immune system is rather robust regarding its actions.

The specific, or adaptive, immune system is present only in vertebrates and is characterized by memory, specificity, and the ability to distinguish self from nonself. The important cells of the adaptive immune system are the lymphocytes and antigen-presenting cells (APCs) that are part of nonspecific immunity. The lymphocytes, which originate from pluripotent stem cells located in the hematopoietic tissues of the liver (fetal) and bone marrow, are composed of two general cell types: T and B cells. The T cells differentiate in the thymus and are made up of three subsets: helper, suppressor, and cytotoxic. The B cells, which have the capacity to produce antibodies, differentiate in the bone

marrow or fetal liver. The various functions of the T cells include presenting antigen to B cells, helping B cells to make antibody, killing virally infected cells, regulating the level of the immune response, and stimulating cytotoxic activity of other cells such as macrophages (Male et al., 1987).

Activation of the immune system is thought to occur when APCs such as macrophages and dendritic cells take up antigen via F_c or complement receptors, process the antigen, and present it to T cells (see Figure 9.3). Macrophages release soluble mediators such as interleukin 1 (IL-1), which stimulate T cells to proliferate. Antigen-presenting cells must present antigen to T cells in

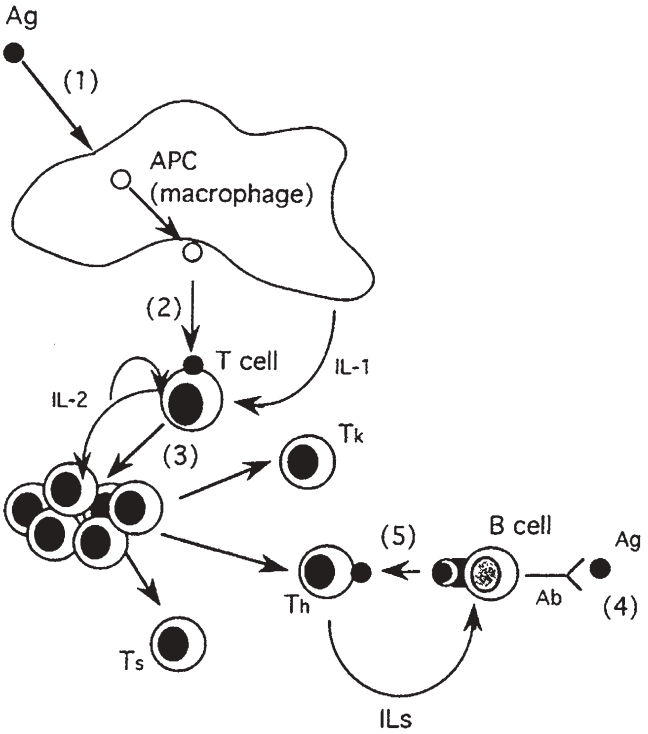

Figure 9.3 *Simplified schematic of immunoregulatory circuit that regulates activation of T cells and B cells involved in humoral (T-cell-dependent) and cell-mediated immunity. (1) Antigen (Ag) is processed by the APCs expressing class II MHC molecules. (2) Antigen plus class II MHC is then presented to antigen-specific T helper cells ($CD4^+$), which stimulates secretion of IL-2. (3) IL-2 in turn stimulates proliferation (clonal expansion) of T cells and differentiation into T suppressor (T_s), T killer (T_k), and T helper (T_h) effector cells. The expanded clone has a higher likelihood of finding the appropriate B cell that has the same antigen and class II molecules on its surface. (4) Next, the antigen binds to an antibody (Ab) on the surface of a specific B cell. (5) The B cell in turn processes the antigen and presents it (plus class II MHC) to the specific T_h cell. The T_h cell is then stimulated to secrete additional ILs that stimulate clonal expansion and differentiation of the antigen-specific B cell.*

conjunction with the class II major histocompatibility complex (MHC) proteins that are located on the surfaces of T cells. The receptor on the T cell is a complex of the Ti molecule that binds antigen, the MHC proteins, and the T3 molecular complex, which is often referred to as the CD3 complex. Upon stimulation, T cells proliferate, differentiate, and express interleukin 2 (IL-2) receptors. T cells also produce and secrete IL-2, which in turn acts on antigen-specific B cells, causing them to proliferate and differentiate into antibody-forming (plasma) cells.

The adaptive arm of the immune system, which depends on delicate tolerance mechanisms to shape the repertoire of the randomly produced antigen receptors, makes this part of the immune system susceptible to the induction of autoimmunity. In contrast to innate immune responses, induction of an adaptive immune response depends on a well-regulated temporal and spatial sequence of activation events, which requires the cooperation of several cell types and the migration of cells between different organs. As a result, activation of adaptive immune responses is a rather slow process, as compared with activation of innate defense mechanisms. All cellular interactions during the induction of an adaptive immune response are based on differentiated intercellular communication pathways involving a host of cytokines and surface receptors.

Antibodies circulate freely in the blood or lymph and are important in neutralizing foreign antigens. The various types of antibodies involved in humoral immunity and their functions are described in Table 9.5. There are multiple genes (polymorphisms) that encode diversity to the variable region

TABLE 9.5 Antibodies Involved in Humoral Immune Response

Antibodies	Serum Concentration		Characteristics/Functions
	$mg\,mL^{-1}$	%	
IgG	10–12	80%	Monomeric structure (γ-globulin); secreted from B cells during secondary response; binds complement; can cross placenta
IgM	1–2	5–10%	Pentameric structure; secreted from B cells during primary response; potent binder of complement; high levels indicative of SLE or rheumatoid arthritis; cannot cross placenta
IgA	3–4	10–15%	Dimeric or monomeric structures; found in seromucous secretions (breast milk); secreted by B cells associated with epithelial cells in gastrointestinal tract, lung, etc.
IgD	0.03	<1%	Monomer; extremely labile; functions not well known
IgE	<0.0001		Reaginic antibody involved in immediate hypersensitivity; antihelminthic; does not bind complement

Source: Adapted from Clark, 1983.

of the antibody. B cells are capable of generating further diversity to antibody specificity by a sequence of molecular events involving somatic mutations, chromosomal rearrangements during mitosis, and recombination of gene segments (Roitt et al., 1985).

The immune system is regulated in part by feedback inhibition involving complex interactions between the various growth and differentiation factors listed in Table 9.6. Since antigen initiates the signal for the immune response, elimination of antigen will decrease further stimulation (Male et al., 1987). T suppressor (T_s) cells also regulate the immune response and are thought to be important in the development of tolerance to self-antigens. In addition to the humoral immune system or the branch that is modulated by antibody, cell-mediated immunity and cytotoxic cell types play a major role in the defense against virally infected cells, tumor cells, and cells of foreign tissue transplants. Cytotoxic T_k (T killer) cells recognize antigen in association with class I molecules of the MHC, while natural killer (NK) cells are not MHC restricted. Cell killing results in a sequence of events following activation of the effector cell, lysosomal degranulation, and calcium influx into the targeted cell. The various types of cells involved in cell-mediated cytotoxicity and their mechanisms of action are outlined in Table 9.7.

9.4 IMMUNOTOXIC EFFECTS

The immune system is a highly integrated and regulated network of cell types that requires continual renewal to achieve balance and immunocompetence. The delicacy of this balance makes the immune system a natural target for cytotoxic drugs or their metabolites. Since renewal is dependent on the ability of cells to proliferate and differentiate, exposure to agents that arrest cell division can subsequently lead to reduced immune function or immunosuppression. This concept has been exploited in the development of therapeutic drugs intended to treat leukemias, autoimmune disease, and chronic inflammatory diseases and to prevent transplant rejection. However, some drugs may adversely modulate the immune system secondarily to their therapeutic effects.

Two broad categories of immunotoxicity have been defined on the basis of suppression or stimulation of normal immune function. Immunosuppression is a down-modulation of the immune system characterized by cell depletion, dysfunction, or dysregulation that may subsequently result in increased susceptibility to infection and tumors. By contrast, immunostimulation is an increased or exaggerated immune responsiveness that may be apparent in the form of a tissue-damaging allergic hypersensitivity response or pathological autoimmunity. However, as knowledge of the mechanisms involved in each of these conditions has expanded, the distinction between them has become less clear. Some agents can cause immunosuppression at one dose or duration of exposure and immunostimulation at others. For instance, the chemotherapeutic drug cyclophosphamide is in most cases immunosuppressive; however, it

TABLE 9.6 Growth and Differentiation Factors of Immune System

Factors	Cell of Origin	Primary Immune Functions
Interleukins[a]		
IL-1	Macrophage, B and T cells	Lymphocyte-activating factor; enhances activation of T and B cells, NK cells, and macrophages
IL-2	T cells (T_h)	T-cell growth factor; stimulates T-cell growth and effector differentiation; stimulates B-cell proliferation/differentiation
IL-3	T cells (T_h)	Mast- cell growth factor; stimulates proliferation/ differentiation of mast cells, neutrophils, and macrophages
IL-4	T cells (T_h), mast cells, B cells	B-cell growth factor; induces proliferation/ differentiation of B cells and secretion of IgA, IgG_1, and IgE; promotes T-cell growth; activates macrophages
IL-5	T cells (T_h)	Stimulates antibody secretion (IgA), proliferation of B cells, and eosinophil differentiation
IL-6	T cells, fibroblasts, monocytes	Stimulates growth/differentiation of B cells and secretion of IgG; promotes IL-2-induced growth of T cells
IL-7	Bone marrow stromal cells	Stimulates pre-B- and pre-T-cell growth/ differentiation; enhances thymocyte proliferation
IL-8	Monocytes, fibroblasts	Neutrophil chemotaxis
IL-9	T cells	Stimulates T cells and mast cells
IL-10	T cells	Stimulates mast cells and thymocytes; induction of class II MHC
Interferons (INFs)		
A-INF	Leukocytes, mast cells	Antiviral; increases NK-cell function, B-cell differentiation, potentiates macrophage production of IL-1
B-INF	Fibroblasts, epithelial cells	Antiviral; potentiates macrophage production of IL-1; increases NK-cell function
Γ-INF	T cells (T_h), cytotoxic T cells	Antiviral; activates macrophages; induces MHC class II expression on macrophages, epithelial, and endothelial cells
Tumor necrosis factors (TNFs)		
TNF-α	Macrophage, B and T cells	Catectin; promotes tumor cytotoxicity; activates macrophages and neutrophils; enhances IL-2 receptor expression on T cells; inhibits antibody secretion
TNF-β	T cells (T_h)	Lymphotoxin; promotes T-cell-mediated cytotoxicity
	NK cells	B-cell activation

	Stem Cells	Promotes Growth and Differentiation of
Colony stimulating factors (CSFs)		
Granulocyte CSF	Myeloid	Granulocytes and macrophages
Macrophage CSF	Myeloid	Macrophages and granulocytes
Granulocyte–macrophage CSF	Myeloid	Granulocytes, macrophages, eosinophils, mast cells, and pluripotent progenitor cells

[a]Includes lymphokines, monokines, and cytokines produced by T cells, macrophages, and other cells, respectively.

Source: Adapted from Golub and 2Green, 1991.

TABLE 9.7 Cells and Mechanisms Involved in Cell-Mediated Cytotoxicity

Cell Type	Mechanism of Cytotoxicity
T_k cells	T_k cells that are specifically sensitized to antigens on target cells interact directly with target cells to lyse them.
T_D	Cells involved in delayed hypersensitivity that act indirectly to kill target cells; T_D cells react with antigen and release cytokines that can kill target cells.
NK cells	Nonspecific T cells that react directly with target cells (tumor cells) without prior sensitization.
Null cells	Antibody-dependent cell-mediated cytotoxicity (ADCC) involving non-T/non-B cells (null cells) with F_c receptors specific for antibody-coated target cells.
Macrophages	Nonspecific, direct killing of target by phagocytosis; also involved in presenting antigen to specific T_k cells that can then mediate cytotoxicity as described above.

can also induce autoimmunity (Hutchings et al., 1985). Likewise, dimethylnitrosamine, a nitrosamine detected in some foods, has been shown to have both suppressing and enhancing effects on the immune system (Yoshida et al., 1989).

9.5 IMMUNOSUPPRESSION

The various cells of the immune system may differ in their sensitivity to a given xenobiotic. Thus, immunosuppression may be expressed as varying degrees of reduced activity of a single cell type of multiple populations of immunocytes. Several lymphoid organs such as the bone marrow, spleen, thymus, and lymph nodes may be affected simultaneously or the immunodeficiency may be isolated to a single tissue, such as the Peyer's patches of the intestines. The resulting deficiency may in turn lead to an array of clinical outcomes of varying ranges of severity. These outcomes include increased susceptibility to infections, increased severity or persistence of infections, or infections with unusual organisms (e.g., systemic fungal infections). Immunosuppression can be induced in a dose-related manner by a variety of therapeutic agents at dose levels lower than those required to produce overt clinical signs of general toxicity. In addition, immunosuppression can occur without regard to genetic predisposition given that a sufficient dose level and duration of exposure have been achieved.

Humoral immunity is characterized by the production of antigen-specific antibodies that enhance phagocytosis and destruction of microorganisms through opsonization. Thus, deficiencies of humoral immunity (B lymphocytes) may lead to reduced antibody titers and are typically associated with acute gram-positive bacterial infections (i.e., *Streptococcus*). Although chronic infection is usually associated with dysfunction of some aspect of cellular

immunity, chronic infections can also occur when facultative intracellular organisms such as *Listeria* or *Mycobacterium* evade antibodies and multiply within phagocytic cells.

Since cellular immunity results in the release of chemotactic lymphocytes that in turn enhance phagocytosis, a deficiency in cellular immunity may also result in chronic infections. Cellular immunity is mediated by T cells, macrophages, and NK cells involved in complex compensatory networks and secondary changes. Immunosuppressive agents may act directly by lethality to T cells or indirectly by blocking mitosis, lymphokine synthesis, lymphokine release, or membrane receptors to lymphokines. In addition, cellular immunity is involved in the production and release of interferon, a lymphokine that ultimately acts in blockage of viral replication (Table 9.6). Viruses are particularly susceptible to cytolysis by T cells since they often attach to the surface of infected cells. Thus, immunosuppression of any of the components of cellular immunity may result in an increase in protozoan, fungal, and viral infections as well as opportunistic bacterial infections.

Immune depression may result unintentionally as a side effect of cancer chemotherapy or intentionally from therapeutics administered to prevent graft rejection. In fact, both transplant patients administered immunosuppressive drugs and cancer patients treated with chemotherapeutic agents have been shown to be at high risk of developing secondary cancers, particularly of lymphoreticular etiology (Penn, 1977). Most of these drugs are alkylating or crosslinking agents that by their chemical nature are electrophilic and highly reactive with nucleophilic macromolecules (protein and nucleic acids). Nucleophilic sites are quite ubiquitous and include amino, hydroxyl, mercapto, and histidine functional groups. Thus, immunotoxic agents used in chemotherapy may induce secondary tumors through direct genotoxic mechanisms (i.e., DNA alkylation).

Reduced cellular immunity may result in increased malignancy and decreased viral resistance through indirect mechanisms as well by modulating immunosurveillance of aberrant cells. T lymphocytes, macrophage cells, and NK cells are all involved in immunosurveillance through cytolysis of virally infected cells or tumor cells, each by a different mechanism (Table 9.6) (Burnet, 1970). In addition to the common cell types described in Table 9.7, at least two other types of cytotoxic effector cells of T-cell origin have been identified, each of which has a unique lytic specificity phenotype and activity profile (Merluzzi, 1985). Of these, both lymphocyte-activated killer (LAK) and tumor-infiltrating lymphocyte (TIL) cells have been shown to lyse a variety of different tumor cells. However, TIL cells have 50–100 times more lytic activity than LAK cells. Most tumor cells express unique surface antigens that render them different from normal cells. Once detected as foreign, they are presented to the T helper cells in association with MHC molecules to form an antigen–MHC complex. This association elicits a genetic component to the immunospecificity reaction. T helper cells subsequently direct the antigen complex toward the cytotoxic T lymphocytes, which possess receptors for antigen–MHC complexes. These

cells can then proliferate, respond to specific viral antigens or antigens on the membranes of tumor cells, and destroy them (Yoshida et al., 1989).

In contrast, the macrophages and NK cells are involved in nonspecific immunosurveillance in that they do not require prior sensitization with a foreign antigen as a prerequisite for lysis and are not involved with MHC molecules. The enhancement of either NK-cell function or macrophage function has been shown to reduce metastasis of some types of tumors. Macrophage cells accumulate at the tumor site and have been shown to lyse a variety of transformed tumor cells (Volkman, 1984). NK cells are involved in the lysis of primary autochthonous tumor cells. Migration of NK cells to tumor sites has been well documented. Although not clearly defined, it appears that they can recognize certain proteinaceous structures on tumor cells and lyse them with cytolysin.

9.5.1 Immunosuppressive Drugs

Table 9.8 lists numerous types of drugs that are immunosuppressive and describes their immunotoxic effects. Several classes of drugs that characteristically depress the immune system are further discussed below.

Antimetabolites This class of drugs includes purine, pyrimidine, and folic acid analogues that have been successfully used to treat various carcinomas, autoimmune diseases, and dermatological disorders such as psoriasis. Because of their structural similarities to normal components of DNA and RNA synthesis, they are capable of competing with the normal macromolecules and alkylating biological nucleophiles.

Thioguanine and mercaptopurine are purine analogues structurally similar to guanine and hypoxanthine that have been used to treat malignancies. Azathioprine, an imidazolyl derivative of mercaptopurine, has been used as an immunosuppressive therapeutic in organ transplants and to treat severe refractory rheumatoid arthritis (Hunter et al., 1975) and autoimmune disorders, including pemphigus vulgaris and bullous pemphigoid. These drugs act as antimetabolites to block de novo purine synthesis through the erroneous incorporation of thioinosinic acid into the pathway in place of inosine. The antimetabolite can bind to the inosine receptor, which in turn will inhibit the synthesis of DNA, RNA, and protein and ultimately T-cell differentiation (Hadden et al., 1984). For example, both thioguanine and mercaptopurine can act as substrates for the hypoxanthine guanine phosphoribosyl transferase (HGPRT) enzyme to produce T-IMP (thioinosine monophosphate) and T-GMP (thioguanine monophosphate), respectively. Thioinosine monophosphate is a poor substrate for guanylyl kinase, which would normally catalyze the conversion of GMP to guanosine diphosphate (GDP) (Calabresi and Chabner, 1990). Thus T-IMP can accumulate in the cell and inhibit several vital metabolic reactions. At high doses, these drugs can suppress the entire immune system. However, at clinical dosages, only the T-cell response is affected,

TABLE 9.8 Immunosuppressive Drugs and Their Effects

Drugs	Biological Activity and Indications	Immunotoxic Effects
	Hormones and Antagonists	
Corticosteroids (prednisone)	Anti-inflammatory; SLE; leukemias; rheumatoid arthritis; breast cancer	Depresses T- and B-cell function; reduces lymphokines; alters macrophage function; increases infections
Diethylstilbestrol	Synthetic estrogen; cancer chemotherapy	Depletes or functionally impairs T cells; enhances macrophage suppressor cell; increases infections and tumorigenesis
Estradiol	Synthetic estrogen; dysmenorrhea; osteoporosis	Decreases T_h cells and IL-2 synthesis; increases T_s cell function, infections, and tumorigenesis
	Antibiotics	
Cephalosporins	β-Lactam antimicrobial	Granulocytopenia; cytopenia
Chloramphenol	Wide-spectrum antimicrobial	Pancytopenia, leukopenia (idiosyncratic)
Penicillins	β-Lactam antimicrobial	Granulocytopenia; cytopenia
Rifampin	Macrocyclic antibiotic	Suppresses T-cell function
Tetracyclines	Antimicrobial	Decreased migration of granulocytes
	Chemotherapeutics and Immunomodulators	
Arabinoside (AraA, AraC)	Antimetabolites; antivirals; leukemias; lymphomas	Leukopenia; thrombocytopenia
Azathioprine	Antimetabolite; leukemia; arthritis; transplant rejection	Inhibits protein synthesis; bone marrow suppression
Busulfan	Alkylating agent; chronic granulocytic leukemia	Leukopenia; myelosuppressive; granulocytopenia
Carmutin, Lomustin (BCNU, CCNU)	Alkylating agents; Hodgkin's disease; lymphomas	Delayed hematopoietic depression; leukopenia; thrombocytopenia
Chlorambucil	Alkylating agent; leukemia; lymphomas; vasculitis	Bone marrow suppression; myelosuppressive
Cyclophosphamide (cytotoxin)	Alkylating agent; cancer chemotherapy; transplant rejection; rheumatoid arthritis	Decreased T_s cells, B cells, and NK cells
Cyclosporin A	Transplant rejections	Depresses T cells; inhibits IL-2 production
Interferon	Immunomodulator; antiviral, hairy cell leukemia	Bone marrow suppression; granulocytopenia; leukopenia

TABLE 9.8 ***Continued***

Drugs	Biological Activity and Indications	Immunotoxic Effects
Melphalan (L-PAM)	Alkylating agent; breast and ovarian cancer	Leukopenia; bone marrow suppression; granulocytopenia; pancytopenia
6-Mercaptopurine	Antimetabolite; acute leukemias; arthritis	Decreased T-cell function; bone marrow suppression
Methotrexate	Folic acid analogue; cancer chemotherapy, arthritis	Inhibits proliferation; T-cell suppression; granulocytopenia; lymphocytopenia
Penostatin	Adenosine analog; T-cell leukemia	Inhibits adenosine deaminase; suppresses T and B cells
Zidovudine (AZT)	Antiviral (HIV)	Decreases T_h cells and granulocytes
	Miscellaneous	
Colchicine	Antimitotic; gout; anti-inflammatory	Inhibits migration of granulocytes; leukopenia; agranulocytosis
Diphenylhydantoin (phenytoin)	Antiepileptic	Leukocytopenia; neutrapenia
Indomethacin (indocin)	Nonsteroidal anti-inflammatory; analgesic; antipyretic	Neutrapenia
Procainamide	Antiarrhythmic	Agranulocytosis; leukopenia (rare)
Sulfasalazine	Antimicrobial anti-inflammatory; ulcerative colitis/inflammatory bowel diseases	Suppresses NK cells; impaired lymphocyte function

Source: Adapted primarily from Brunton et al., 2006.

without an apparent decrease in T-cell numbers (Spreafico and Anaclerio, 1977).

Pentostatin (2′-deoxycoformycin) is an adenosine analogue that is a potent inhibitor of adenosine deaminase. Pentostatin is particularly useful for treating T-cell leukemia since malignant T cells have higher levels of adenosine deaminase than most cells. Similar to individuals that are genetically deficient in adenosine deaminase, treatment with pentostatin produces immunosuppression of both T and B lymphocytes, with minimal effect on other tissues. As a result, severe opportunistic infections are often associated with its clinical use.

5-Fluorouracil (5-FU), adenosine arabinoside (AraA), and cytosine arabinoside (AraC) are pyrimidine analogues to uracil, adenine, and cytosine, respectively. 5-FU is used primarily to treat cancer of the breasts and gastrointestinal tract as well as severe recalcitrant psoriasis (Alper et al., 1985). AraC is predominantly indicated for the treatment of acute leukemia and non-Hodgkin's lymphomas. Although high-dose therapy with AraC has a good

likelihood of producing complete remission, it is often accompanied by severe leukopenia, thrombocytopenia, and anemia (Barnett et al., 1985). Likewise, myelosuppression is the major toxicity associated with bolus-dose regimens of 5-FU.

Glucocorticosteroids Corticosteroids are commonly used to reduce inflammation, to treat autoimmune diseases such as systemic lupus erythematosus (SLE), and as a prophylactic measure to prevent transplant rejection. The adrenocorticosteroid prednisone is often coadministered with other immunosuppressives such as cyclosporine and azathioprine (Elion and Hitchings, 1975). Glucocorticosteroids act pharmacologically by modulating the rate of protein synthesis. The molecule reacts with specific receptors to form a complex that crosses into the nucleus of the cell and regulates transcription of specific messenger RNA (mRNA). The corticosteroid complex releases inhibition of transcription, thus enhancing protein synthesis (Hollenberg et al., 1987). This may lead to the initiation of de novo synthesis of the phospholipase A2–inhibiting protein lipocortin, which blocks the synthesis of arachidonic acid and its prostaglandin and leukotriene metabolites (Haynes and Murad, 1985; Wallner et al., 1986). Glucocorticosteroids induce immunosuppression and anti-inflammation as a result of the inhibition of specific leukocyte functions such as lymphokine activity. Glucocorticoids can also inhibit recruitment of leukocytes and macrophages into the site of inflammation. In addition, amplification of cell-mediated immunity can be suppressed by inhibiting the interaction of IL-2 with its T-cell receptors. However, the immunosuppression is reversible and immune function recovers once therapy has ceased.

Cyclosporine Cyclosporin A (cyclosporine) is an 11-amino-acid cyclic peptide residue of fungal origin isolated from the fermentation products of *Trichoderma polysporum* and *Cylindrocarpon lucidum.* In addition to having a very narrow range of antibiotic activity, it was also found to inhibit proliferation of lymphocytes, which made it unsuitable as an antibiotic. Cyclosporine inhibits the early cellular response of helper T cells to antigens (Kay and Benzie, 1984) primarily by inhibiting production of IL-2 (Elliot et al., 1984), and at higher doses it may inhibit expression of IL-2 receptors (Herold et al., 1986). Cyclosporine does not prevent the stimulation of helper-T-cell clonal expansion by IL-2, only its activation. Since it is not myelosuppressive at therapeutic dosages, the incidence of secondary infection is lower than that induced by other classes of immunosuppressives. Thus, cyclosporine is ideal as an immunosuppressive agent to prevent transplant rejection and graft-host disease (Kahan and Bach, 1988). Cyclosporine has also been used as an antihelminthic and as an anti-inflammatory agent to treat rheumatoid arthritis and other autoimmune-type diseases.

Nitrogen Mustards Nitrogen mustards characteristically consist of a bis(2-chloroethyl) group bonded to nitrogen. These molecules are highly reactive

bifunctional alkylating agents that have been successfully used in cancer chemotherapy. Included in this group are mechlorethamine, L-phenylalanine mustard (melphalan), chlorambucil, ifosfamide, and cyclophosphamide. The cytotoxic effects of each on the bone marrow and lymphoid organs are similar; however, their pharmacokinetic and toxic profiles can vary on the basis of the substituted side group. For example, the side group may consist of a simple methyl group, as is the case of mechlorethamine, or substituted phenyl groups, in the cases of melphalan and chlorambucil.

Cyclophosphamide, which contains a cyclic phosphamide group bonded to the nitrogen mustard, is representative of this class. The parent compound itself is not active in vitro unless treated in conjunction with an exogenous P450 microsomal enzyme system (Colvin, 1982) such as rat liver S9 homogenate, which metabolizes it to a highly reactive alkylating agent (4-hydroxycyclophosphamide). Thus, in vivo, cyclophosphamide is not toxic until it is metabolically activated in the liver. Cyclophosphamide has been the most widely used nitrogen mustard where it has been effective as a cancer chemotherapeutic and to treat autoimmune-type diseases including SLE, multiple sclerosis, and rheumatoid arthritis (Calabresi and Parks, 1985). Treatment with cyclophosphamide suppresses all classes of lymphoid cells, which may result in reduced lymphocyte function as well as lymphopenia and neutropenia (Webb and Winkelstein, 1982). Thus, it has also been administered as a large single dose prior to bone marrow transplants to suppress cellular immunity and subsequently inhibit rejection (Shand, 1979).

Estrogens β-Estradiol (Luster et al., 1984; Pung et al., 1984) and therapeutics with estrogenic activity, such as diethylstilbestrol (DES), have also been shown to be immunosuppressive (Luster et al., 1985). Estrogens have been shown to increase T-suppressor-cell activity in splenocytes, decrease numbers of T helper cells, inhibit IL-2 synthesis, and modulate production of immunoregulatory factors (Luster et al., 1987). These effects have been particularly characterized in studies with DES, a nonsteroidal synthetic estrogen used widely in the treatment of prostate and breast cancers, as well as administered to pregnant women as a "morning after" contraceptive. Decreased mitogenicity of human peripheral blood lymphocytes has been observed in men treated with DES for prostate cancer and women exposed in utero (Haukass et al., 1982; Ways et al., 1987). In mice, thymic involution and atrophy with depletion of the cortical lymphocytes have been observed histologically. Function is also modulated, as evident by depressed mixed-lymphocyte responses, mitogenicity, and T-cell release of IL-2 (Pung et al., 1985). Dean et al. (1980) speculated that DES treatment selectively depletes or functionally impairs T cells and/or the induction of suppressor macrophages, resulting in immunosuppression. Macrophage suppressor cell activity is enhanced (Luster et al., 1980) and PMN cells accumulate following bacterial challenge. Although macrophage functions of phagocytosis and tumor growth inhibition are potentiated, defects in macrophage migration and decreased bactericidal activity contribute to

decreased host resistance with resulting increased susceptibility to bacterial infections.

Heavy Metals Some heavy metals such as gold and platinum are used pharmacologically as immunomodulators to treat rheumatoid arthritis and as antineoplastic drugs, respectively. Most heavy metals inhibit mitogenicity, antibody responses, and host resistance to bacterial or viral challenge and tumor growth. Platinum has been shown to suppress humoral immunity, lymphocyte proliferation, and macrophage function (Lawrence, 1985). Clinically, mild to moderate myelosuppression may also be evident with transient leukopenia and thrombocytopenia.

Likewise, injectable gold salts such as gold sodium thiomalate affect a variety of immune responses in humans (Bloom et al., 1987). Severe thrombocytopenia occurs in 1% of patients as a result of an immunological disturbance that accelerates the degradation of platelets. Leukopenia, agranulocytosis, and fatal aplastic anemia may also occur. Although better tolerated than parenteral preparations, administered orally the organic gold compound auranofin is also immunosuppressive. In a dog study, auranofin was shown to produce thrombocytopenia similar to that described in humans administered parenteral preparations (Bloom et al., 1985b). Long-term toxicity studies with these compounds in dogs show evidence of immune-modulating activity, possible drug-induced immunotoxicity, and treatment-related changes in immune function (e.g., lymphocyte activation).

Antibiotics β-Lactam-containing antibiotics such as the cephalosporins may also induce significant immunosuppressive effects (Caspritz and Hadden, 1987) in a small percentage of human patients. Adverse effects including anemia, neutropenia, thrombocytopenia, and bone marrow depression were observed in dogs administered high doses of cefonicid for six months (Bloom et al., 1985a). A similar syndrome has been characterized in cefazedone-treated dogs expressing an agglutinating red cell antibody. Further studies with this drug indicated that both cytopenia (Bloom et al., 1985a) and suppression of bone marrow stem cell activity appear to be antibody mediated (Deldar et al., 1985).

9.6 IMMUNOSTIMULATION

A variety of drugs as well as environmental chemicals have been shown to have immunostimulatory or sensitizing effects on the immune system and these effects are well documented in humans exposed to drugs (DeSwarte, 1986). The drug or metabolite can act as a hapten and covalently bind to a protein or other cellular constituent of the host to appear foreign and become antigenic. Haptens are low-molecular-weight substances that are not in themselves immunogenic but will induce an immune response if conjugated with

nucleophilic groups on proteins or other macromolecular carriers. In both allergy and autoimmunity, the immune system is stimulated or sensitized by the drug conjugate to produce specific pathological responses. An allergic hypersensitivity reaction may vary from one which results in an immediate anaphylactic response to one which produces a delayed hypersensitivity reaction or immune complex reaction. Allergic hypersensitivity reactions result in a heightened sensitivity to nonself-antigens, whereas autoimmunity results in an altered response to self-antigens. Unlike immunosuppression, which nonspecifically affects all individuals in a dose-related manner, both allergy and autoimmunity have a genetic component that creates susceptibility in those individuals with a genetic predisposition. Susceptible individuals, once sensitized, can respond to even minute quantities of the antigen. Several examples of drugs that can stimulate the immune system are presented in Table 9.9.

9.6.1 Hypersensitivity (or Allergenicity)

The four types of hypersensitivity reactions as classified by Coombs and Gell (1975) are outlined in Table 9.10. The first three types are immediate antibody-mediated reactions, whereas the fourth type is a cellular-mediated delayed-type response that may require one to two days to occur after a secondary exposure. Type I reactions are characterized by an anaphylaxis response to a variety of compounds, including proteinaceous materials and pharmaceuticals such as penicillin. Various target organs may be involved depending on the route of exposure. For example, the gastrointestinal tract is usually involved with food allergies, the respiratory system with inhaled allergens, the skin with dermal exposure, and smooth muscle vasculature with systemic exposure. The type of response elicited often depends on the site of exposure and includes dermatitis and urticaria (dermal), rhinitis and asthma (inhalation), increased gastrointestinal emptying (ingestion), and systemic anaphylactic shock (parenteral) (Steele et al., 1989).

Type I Hypersensitivity During an initial exposure, immunoglobulin E (IgE) antibodies are produced and bind to the cell surface of mast cells and basophils. Upon subsequent exposures to the antigen, reaginic IgE antibodies bound to the surface of target cells at the F_c region (mast cells and basophils) become crosslinked (at the F_{ab} regions) by the antigen. Crosslinking causes distortion of the cell surface and IgE molecule, which in turn activates a series of enzymatic reactions, ultimately leading to degranulation of the mast cells and basophils. These granules contain a variety of pharmacological substances (Table 9.11), such as histamines, serotonins, prostaglandins, bradykinins, and leukotrienes [slow-reacting substance of anaphylaxis (SRS-A) and ecdysone receptor (ECR)-A]. Upon subsequent challenge exposures, these factors are responsible for eliciting an allergic reaction through vasodilation and increased vascular permeability. The nasal passages contain both mast cells and plasma cells that secrete IgE antibodies. Allergic responses localized in the nasal mucosa result

TABLE 9.9 Drugs That Produce Immunostimulation

Drug	Type of Response
	Hypersensitivity
Antibiotics	
Cephalosporins	Anaphylaxis, urticaria, rash, granulocytopenia
Chloramphenicol	Rash, dermatitis, urticaria
Neomycin	Dermal exposure—rash, dermatitis
Sulfathiazole	Rash, dermatitis, urticaria
Spiramycin	Rash, dermatitis, urticaria
Quinolones	Photosensitivity
Tetracyclines	Photosensitivity, anaphylaxis, asthma, dermatitis
Others	
Allopurinol	Rash, urticaria, fever, eosinophilia
Avridine	DTH; increases NK cells, T cells, IL-1, and IL-2
Isoprinosine	DTH; increases T lymphocytes
Indomethacin	Rash, urticaria, asthma, granulocytopenia
Quinidine	Fever, anaphylaxis, asthma
Salicylates	Rash, urticaria
	Autoimmunity
Amiodarone	Thyroiditis
Captopril	Autoimmune hemolytic anemia, pemphigus, granulocytopenia
Chlorpromazine	Granulocytopenia
Halothane	Autoimmune chronic active hepatitis
Hydralizine	Autoimmune hemolytic anemia, drug-induced SLE, myasthenia gravis, pemphigus, glomerulonephritis, Goodpasture's disease
Methyldopa	Autoimmune hemolytic anemia, leukopenia, drug-induced SLE,
Nitrofurantoin	pemphigus
D-Penicillamine	Peripheral neuritis
	Autoimmunity, drug-induced SLE, myasthenia gravis, pemphigus, glomerulonephritis, Goodpasture's disease
Propranolol	Autoimmunity
Procainamide	Autoimmunity, drug-induced SLE, rash, vasculitis, myalgias
Pyrithioxine	Pemphigus
	Hypersensitivity and Autoimmunity
Antibiotics	
Isoniazid	Rash, dermatitis, vasculitis, arthritis, drug-induced SLE
Penicillins	Anaphylaxis, dermatitis; vasculitis, serum sickness, hemolytic
Sulfonamides	anemia
	Dermatitis, photosensitivity, pemphigus, hemolytic anemia, serum sickness, drug-induced SLE
Others	
Acetazolamide	Rash, fever, autoimmunity
Lithium	Dermatitis, autoimmune thyroiditis, vasculitis
Thiazides	Hypersensitivity, photosensitivity, autoimmunity (diabetes)
Phenytoin	Rash, drug-induced SLE, hepatitis

TABLE 9.10 Types of Hypersensitivity Responses

Type and Designation	Agents: Clinical Manifestations	Components	Effects	Mechanism
I: Immediate (reaginic)	Food additives (GI allergies; anaphylactic) Penicillin: urticaria and dermatitis	Mast cells; IgE	Anaphylaxis, asthma, urticaria, rhinitis, dermatitis	IgE binds to mast cells to stimulate release of humoral factors
II: Cytotoxic	Cephalosporine: hemolytic anemia Quinidine: thrombocytopenia	IgG, IgM	Hemolytic anemia, Goodpasture's disease	IgG and IgM bind to cells (e.g., RBCs), fix complement (opsonization), then lyse cells
III: Immune complex (arthus)	Methicillin: chronic glomerulonephritis	Antigen–antibody complexes (Ag–Ab)	SLE, rheumatoid arthritis, glomerular nephritis, serum sickness, vasculitis	Ag–Ab complexes deposit in tissues and may fix complement
IV: Delayed hypersensitivity	Penicillin: contact dermatitis	T_D cells; macrophages	Contact dermatitis, tuberculosis	Sensitized T cells induce a delayed-hypersensitivity response upon challenge

Source: Based on classification system of Coombs and Gell, 1975.

TABLE 9.11 Proteins and Soluble Mediators Involved in Hypersensitivity

Factor	Origin	Characteristics/Functions
Histamine	Mast cells, basophils	Contraction of smooth muscle; increases vascular permeability
Serotonin	Mast cells, basophils	Contraction of smooth muscle; leukotriene
SRS-A	Lung tissue	(Slow-reacting substance of anaphylaxis); Contraction of smooth muscle; acidic polypeptide
ECF-A	Mast cells	(Eosinophilic chemotactic factor of anaphylaxis); attracts eosinophils; small peptide
Prostaglandins	Various tissues	Modifies release of histamine and serotonin from mast cells and basophils

Source: Extracted and modified from Clark, 1983.

in dilation of the local blood vessels, tissue swelling, mucous secretion, and sneezing. Reactions localized in the respiratory tract, also rich in mast cells and IgE, result in an allergic asthma response. This condition is triggered by the release of histamine and SRS-A, which induce constriction of the bronchi and alveoli, pulmonary edema, and mucous secretions that block the bronchi and alveoli, together resulting in severe difficulty in breathing. In the case of a challenge dose of a drug administered systemically, the reactive patient may have difficulty breathing within minutes of exposure and may experience convulsions, vomiting, and low blood pressure. The effects of anaphylactic shock and respiratory distress, if severe, may ultimately result in death.

Antibiotics containing β-lactam structures, such as penicillin and cephalosporins, are the most commonly occurring inducers of anaphylactic shock and drug hypersensitivity in general. Other hypersensitivity reactions may include urticarial rash, fever, bronchospasm, serum sickness, and vasculitis with reported incidences of all types varying from 0.7 to 10% (Idsøe et al., 1968) and the incidence of anaphylactoid reactions varying from 0.04 to 0.2%. When the β-lactam ring is opened during metabolism, the penicilloyl moiety can form covalent conjugates with nucleophilic sites on proteins. The penicilloyl conjugates can then act as haptens to form the determinants for antibody induction. Although most patients that have received penicillin produce antibodies against the metabolite benzylpenicilloyl, only a fraction experience allergic reaction (Garratty and Petz, 1975), which suggests a genetic component to susceptibility.

Type II Hypersensitivity Type II cytolytic reactions are mediated by IgG and IgM antibodies that can fix complement, opsonize particles, or induce antibody-dependent cellular cytolysis reactions. Erythrocytes, lymphocytes, and platelets of the circulatory system are the major target cells that interact with the cytolytic antibodies causing depletion of these cells. Hemolytic anemia (penicillin, methyldopa), leukopenia, thrombocytopenia (quinidine), and/or granulocytopenia (sulfonamide) may result. Type II reactions involving the

lungs and kidneys occur through the development of antibodies (autoantibodies) to the basement membranes in the alveoli or glomeruli, respectively. Prolonged damage may result in Goodpasture's disease, an autoimmune disease characterized by pulmonary hemorrhage and glomerulonephritis. Several other autoimmune-type diseases have been associated with extended treatments with D-penicillamine and other pharmaceuticals. Various types of autoimmune responses and examples of drug-induced autoimmunity are discussed in further detail later in this section.

Type III Hypersensitivity Type III reactions (arthus) are characterized as immediate hypersensitivity reactions initiated by antigen–antibody complexes that form freely in the plasma instead of at the cell surface. Regardless of whether the antigens are self or foreign, complexes mediated by IgG can form and settle into the tissue compartments of the host. These complexes can then fix complement and release C3a and C5a fragments that are chemotactic for phagocytic cells. Polymorphonuclear leukocytes are then attracted to the site, where they phagocytize the complexes and release hydrolytic enzymes into the tissues. Additional damage can be caused by binding to and activating platelets and basophils, which, in the end, results in localized necrosis, hemorrhage, and increased permeability of local blood vessels. These reactions commonly target the kidney, resulting in glomerulonephritis through the deposition of the complexes in the glomeruli.

Some antibiotics (β-lactam) have been reported to produce glomerular nephritis in humans that has been attributed to circulating immune complexes. These complexes have also been observed in preclinical toxicology studies with baboons treated with a β-lactam antibiotic prior to the appearance of any biochemical or clinical changes (Descotes and Mazue, 1987). In addition, immunoglobulin complexes have been observed in rats treated with gold and autologus immune complex nephritis has been observed in guinea pigs (Ueda et al., 1980). Similar evidence of immunomediated nephrotoxicity has been reported in rheumatoid arthritis patients administered long-term treatments with gold compounds; proteinuria has been observed in approximately 10% of these patients.

Other target organs such as the skin with lupus, the joints with rheumatoid arthritis, and the lungs with pneumonitis may be affected. The deposition of antigen–antibody complexes through the circulatory system results in a syndrome referred to as serum sickness, which was quite prevalent prior to 1940 (Clark, 1983), when serum therapy for diphtheria was commonly used. Serum sickness occurs when the serum itself becomes antigenic as a side effect from passive immunization with heterologous antiserum produced from various sources of farm animals. The antitoxin for diphtheria was produced in a horse and administered to humans as multiple injections of passive antibody. As a consequence, these people often became sensitized to the horse serum and developed a severe form of arthritis and glomerulonephritis caused by deposition of antigen–antibody complexes. Clinical symptoms of serum sickness

present as urticarial skin eruptions, arthralgia or arthritis, lymphadenopathy, and fever. Drugs such as sulfonamides, penicillin, and iodides can induce a similar type of reaction. Although uncommon today, transplant patients receiving immunosuppressive therapy with heterologous antilymphocyte serum or globulins may also exhibit serum sickness.

Type IV Delayed-Type Hypersensitivity (DTH) Delayed-type hypersensitivity reactions are T-cell mediated with no involvement of antibodies. However, these reactions are controlled through accessory cells, suppressor T cells, and monokine-secreting macrophages, which regulate the proliferation and differentiation of T cells. The most frequent form of DTH manifests itself as contact dermatitis. The drug or metabolite binds to a protein in the skin or the Langerhans cell membrane (class II MHC molecules) where it is recognized as an antigen and triggers cell proliferation. After a sufficient period of time for migration of the antigen and clonal expansion (latency period), a subsequent exposure will elicit a dermatitis reaction. A 24–48-h delay often occurs between the time of exposure and onset of symptoms to allow time for infiltration of lymphocytes to the site of exposure. The T cells ($CD4^+$) that react with the antigen are activated and release lymphokines that are chemotactic for monocytes and macrophages. Although these cells infiltrate to the site via the circulatory vessels, an intact lymphatic drainage system from the site is necessary since the reaction is initiated in drainage lymph nodes proximal to the site (Clark, 1983). The release (degranulation) of enzymes and histamines from the macrophages may then result in tissue damage. Clinical symptoms of local dermal reactions may include a rash (not limited to sites of exposure), itching, and/or burning sensations. Erythema is generally observed in the area around the site, which may become thickened and hard to the touch. In severe cases, necrosis may appear in the center of the site followed by desquamation during the healing process. The immune-enhancing drugs isoprinosine and avridine have been shown to induce a DTH reaction in rats (Exon et al., 1986).

A second form of DTH response is similar to that of contact dermatitis in that macrophages are the primary effector cells responsible for stimulating $CD4^+$ T cells; however, this response is not necessarily localized to the epidermis. A classical example of this type of response is demonstrated by the tuberculin diagnostic tests. To determine if an individual has been exposed to tuberculosis, a small amount of fluid from tubercle bacilli cultures is injected subcutaneously. The development of induration after 48 h at the site of injection is diagnostic of prior exposure.

Shock, similar to that of anaphylaxis, may occur as a third form of a delayed systemic hypersensitivity response. However, unlike anaphylaxis, IgE antibodies are not involved. This type of response may occur 5–8 h after systemic exposure and can result in fatality within 24 h following intravenous or intraperitoneal injection.

A fourth form of delayed hypersensitivity results in the formation of granulomas. If the antigen is allowed to persist unchecked, macrophages and

fibroblasts are recruited to the site to proliferate, produce collagen, and effectively "wall off" the antigen. A granuloma requires a minimum of one to two weeks to form.

9.6.2 Photosensitization

Regardless of the route of exposure, some haptens (photoantigens) that are absorbed locally into the skin or reach the skin through systemic absorption can be photoactivated by ultraviolet (UV) light between 320 and 400 nm. Once activated, the hapten can bind to the dermal receptors to initiate sensitization (photoallergy). Subsequent exposures to the hapten in the presence of UV light can result in a hypersensitivity response. Clinical symptoms of photoallergy may occur within minutes (immediate hypersensitivity) of exposure to sunlight or 24 h or more after exposure (DTH). Symptoms may range from acute urticarial reactions to eczematous or papular lesions. Although both phototoxic and photoallergic reactions require the compound to be exposed to sunlight in order to elicit a response, their mechanisms of action are quite different. Since photosensitization is an immune-mediated condition, repeated exposures with a latency period between the initial exposure and subsequent exposures is required, the response is not dose related (small amounts can produce a response once sensitized), and not all individuals exposed to the compound will necessarily respond (genetic component to susceptibility). Although both conditions can present similar symptoms (erythema), phototoxicity is limited mainly to erythema, whereas photoallergy can result in erythema, edema, and dermatitis as described above.

Several drug classes, including tetracycline, sulfonamide, and quinolone antibiotics, as well as chlorothiazide, chlorpromazine, and amiodarone hydrochloride, have been shown to be photoantigens. Photosensitivity may persist even after withdrawal of the drug, as has been observed with the antiarrhythmic drug amiodarone hydrochloride, since it is lipophilic and can be stored for extended periods in the body fat (Unkovic et al., 1984). In addition, it is quite common for cross-reactions to occur between structurally related drugs of the same class.

9.6.3 Autoimmunity

In autoimmunity, as with hypersensitivity, the immune system is stimulated by specific responses that are pathogenic, and both tend to have a genetic component that predisposes some individuals more than others. However, as is the case with hypersensitivity, the adverse immune response of drug-induced autoimmunity is not restricted to the drug itself but also involves a response to self-antigens.

Autoimmune responses directed against normal components of the body may consist of antibody-driven humoral responses and/or cell-mediated DTH responses. T cells can react directly against specific target organs, or B cells

can secrete autoantibodies that target "self." Autoimmunity may occur spontaneously as the result of a loss of regulatory controls that initiate or suppress normal immunity causing the immune system to produce lymphocytes reactive against its own cells and macromolecules such as DNA, RNA, or erythrocytes.

Although autoantibodies are often associated with autoimmune reactions, they are not necessarily indicative of autoimmunity (Russel, 1981). Antinuclear antibodies can occur normally with aging in some healthy women without autoimmune disease, and all individuals have B cells with the potential of reacting with self-antigens through Ig receptors (Dighiero et al., 1983). The presence of an antibody titer to a particular immunogen indicates that haptenization of serum albumin has occurred as part of a normal immune response. However, if cells are stimulated to proliferate and secrete autoantibodies directed against a specific cell or cellular component, a pathological response may result. The tissue damage associated with autoimmune disease is usually a consequence of type II or III hypersensitivity reactions that result in the deposition of antibody–antigen complexes.

Several diseases have been associated with the production of autoantibodies against various tissues. For example, an autoimmune form of hemolytic anemia can occur if the antibodies are directed against erythrocytes. Similarly, antibodies that react with acetylcholine receptors may cause myasthenia gravis, those directed against glomerular basement membranes may cause Goodpasture's syndrome, and those that target the liver may cause hepatitis. Other forms of organ-specific autoimmunity include autoimmune thyroiditis (as seen with amiodarone) and juvenile diabetes mellitus, which can result from autoantibodies directed against the tissue-specific antigens thyroglobulin and cytoplasmic components of pancreatic islet cells, respectively. In contrast, systemic autoimmune diseases may occur if the autoantibodies are directed against an antigen that is ubiquitous throughout the body, such as DNA or RNA. For example, SLE occurs as the result of autoimmunity to nuclear antigens that form immune complexes in the walls of blood vessels and basement membranes of tissues throughout the body.

The etiology of drug-induced autoimmunity is not well established and is confounded by factors such as age, sex, and nutritional state as well as genetic influences on pharmacological and immune susceptibility. Unlike idiopathic autoimmunity, which is progressive or characterized by an alternating series of relapses and remissions, drug-induced autoimmunity is thought to subside after the drug is discontinued. However, this is not certain since a major determining factor for diagnosis of a drug-related disorder is dependent on the observation of remission upon withdrawal of the drug (Bigazzi, 1988).

One possible mechanism for xenobiotic-induced autoimmunity involves xenobiotic binding to autologus molecules, which then appear foreign to the immunosurveillance system. If a self-antigen is chemically altered, a specific T helper (T_h) cell may see it as foreign and react to the altered antigenic determinant portion, allowing an autoreactive B cell to react to the unaltered

hapten. This interaction results in a carrier–hapten bridge between the specific T_h and autoreactive B cell, bringing them together for subsequent production of autoantibodies specific to the self-antigen that was chemically altered (Weigle, 1980). Conversely, a xenobiotic may alter B cells directly, including those that are autoreactive. Thus, the altered B cells may react to self-antigens independent from T_h-cell recognition and in a non-tissue-specific manner.

Another possible mechanism is that the xenobiotic may stimulate nonspecific mitogenicity of B cells. This could result in a polyclonal activation of B cells with subsequent production of autoantibodies. Alternatively, the xenobiotic may stimulate mitogenicity of T cells that recognize self, which in turn activate B-cell production of antibodies in response to "self" molecules. There is also evidence to suggest that anti-DNA autoantibodies may originate from somatic mutations in lymphocyte precursors with antibacterial or antiviral specificity. For example, a single amino acid substitution resulting from a mutation in a monoclonal antibody to polyphorylcholine was shown to result in a loss of the original specificity and an acquisition of DNA reactivity similar to that observed for anti-DNA antibodies in SLE (Talal, 1987).

The mechanisms of autoimmunity may also entail interaction with MHC structures determined by the human leukocyte antigen (HLA) alleles. Individuals carrying certain HLA alleles have been shown to be predisposed to certain autoimmune diseases, which may account in part for the genetic variability of autoimmunity. In addition, metabolites of a particular drug may vary between individuals to confound the development of drug-induced autoimmunity. Dendritic cells, such as the Langerhans cells of the skin and B lymphocytes that function to present antigens to T_h cells, express class II MHC structures. Although the exact involvement of these MHC structures is unknown, Gleichmann et al. (1989) have theorized that self-antigens rendered foreign by drugs such as D-penicillamine may be presented to T_h cells by MHC class II structures. An alternate hypothesis is that the drug or a metabolite may alter MHC class II structures on B cells, making them appear foreign to T_h cells.

A number of different drugs have been shown to induce autoimmunity in susceptible individuals. A syndrome similar to that of SLE was described in a patient administered sulfadiazine in 1945 by Hoffman (see Bigazzi, 1988). Sulfonamides were one of the first classes of drugs identified to induce an autoimmune response, while to date, over 40 other drugs have been associated with a similar syndrome.

Autoantibodies to red blood cells and autoimmune hemolytic anemia have been observed in patients treated with numerous drugs, including procainamide, chlorpropamide, captopril, cefalexin, penicillin, and methyldopa (Logue et al., 1970; Kleinman et al., 1984). Hydralazine- and procainamide-induced autoantibodies may also result in SLE. Approximately 20% of patients administered methyldopa for several weeks for the treatment of essential hypertension developed a dose-related titer and incidence of autoantibodies to erythrocytes, 1% of which presented with hemolytic anemia. Methyldopa does

not appear to act as a hapten but appears to act by modifying erythrocyte surface antigens. IgG autoantibodies then develop against the modified erythrocytes.

D-Penicillamine is used to treat patients with rheumatoid arthritis, to reduce excess cystine excretion in patients with cystinurias, and as a chelating agent for copper in patients with Wilson's disease. D-Penicillamine can cause multiple forms of autoimmunity, including SLE, myasthenia gravis, pemphigus, and autoimmune thyroiditis. This drug is thought to act as immunomodulator in patients by initiating or even potentiating anti-DNA antibody synthesis (Mach et al., 1986). The highly reactive thiol group may react with various receptors and biological macromolecules to induce autoantibodies. Long-term (many months) treatment has been shown to induce autoimmunity resulting in myasthenia gravis in 0.5% of patients (Bigazzi, 1988) and SLE in approximately 2% of patients as exhibited by varying degrees of joint pain, synovitis, myalgia, malaise, rash, nephritis, pleurisy, and neurological effects. In patients exhibiting myasthenia gravis, D-penicillamine may act to alter the acetylcholine receptors. Autoantibodies to acetylcholine receptors have been detected in these patients and have been shown to decrease gradually after drug withdrawal concomitant with reversibility of the clinical syndrome. However, myasthenia gravis may persist for long periods of time after D-penicillamine therapy has ceased.

Although rare, cases of renal lupus syndrome and pemphigus blisters have also been reported as a consequence of D-penicillamine-induced immune complexes (Ntoso et al., 1986; Bigazzi, 1988) as well as with other drugs. With renal lupus syndrome, secondary glomerulonephritis may result if granular IgG antibodies are produced and deposited on the basement membranes. In patients with pemphigus blisters, autoantibodies to the intercellular substance of the skin have been recovered from the sera, and dermal biopsies have demonstrated intracellular deposits or immunoglobulin deposits on the basement membranes. Pemphigus has also been observed in patients treated with sulfhydryl compounds such as captopril and pyrithioxine (Bigazzi, 1988).

Some metals that are used therapeutically have also been shown to induce autoimmune responses. Gold salts used to treat arthritis may induce formation of antiglomerular basement membrane antibodies, which may lead to glomerulonephritis similar to that seen in Goodpasture's disease (see type II hypersensitivity). Since gold is not observed at the site of the lesions (Druet et al., 1982), it has been hypothesized that the metal elicits an antiself response. Lithium, used to treat manic depression, is thought to induce autoantibodies against thyroglobulin, which in some patients results in hypothyroidism. In studies with rats, levels of antibodies to thyroglobulin were shown to increase significantly in lithium-treated rats compared to controls immediately after immunization with thyroglobulin; however, rats that were not immunized with thyroglobulin did not produce circulation antithyroglobulin antibodies upon receiving lithium, and there was no effect of lithium on lymphocytic infiltration of the thyroid in either group (Hassman et al., 1985).

Some drugs such as penicillin have been shown to induce autoimmunity as well as anaphylaxis (Gleichman et al., 1989). The carbonyl of the β-lactam ring of penicillin can form a covalent penicilloyl conjugate with nucleophilic sites on proteins, particularly the amino groups of lysine residues. This conjugate, which acts as the major immunogenic determinant, may become biotransformed to other isomeric forms of clinical relevance (Batchelor et al., 1965).

A genetic predisposition to drug-induced development of SLE has been shown to occur in some individuals treated with the drugs hydralazine, isoniazid, procainamide, and sulphamethazine. A polymorphism, which is known to exist for the genes responsible for expression of hepatic *N*-acetyl transferase enzymes, determines the rate of acetylation of these drugs to regulate the rate of drug inactivation. Individuals that are relatively slow acetylators of these drugs are more likely to develop antinuclear antibodies and are at a higher risk for developing SLE (Perry et al., 1970). Other predisposing factors, such as HLA phenotype (HLA-DR4 and/or C4 allele), may also play a genetic role in determining susceptibility to hydralazine-induced SLE (Spears and Batchelor, 1987).

In addition, silicone-containing medical devices, particularly breast prostheses, have been reported to cause serum-sickness-like reactions, scleroderma-like lesions, and an SLE-like disease termed human adjuvant disease (Kumagai et al., 1984; Guillaume et al., 1984). Some patients may also present with granulomas and autoantibodies. Human adjuvant disease is a connective tissue or autoimmune disease similar to that of adjuvant arthritis in rats and rheumatoid arthritis in humans. Autoimmune-disease-like symptoms usually develop two to five years after implantation in a small percentage of people that receive implants, which may indicate that there is a genetic predisposition similar to that for SLE. An early hypothesis is that the prosthesis or injected silicone plays an adjuvant role by enhancing the immune response through increased macrophage and T-cell helper function. There is currently controversy as to whether silicone, as a foreign body, induces a nonspecific inflammation reaction, a specific cell-mediated immunological reaction, or no reaction at all. However, there is strong support to indicate that silicone microparticles can act as haptens to produce a delayed hypersensitivity reaction in a genetically susceptible population of people. It should be noted that there are currently no known drug-induced type I autoimmunities.

9.7 EVALUATION OF IMMUNE SYSTEM

The ICH/FDA CDER guidelines for immunotoxicity testing of small-molecule pharmaceuticals (ICH S8) start with evaluation of parameters evaluated in repeat-dose (typically 28-day) systemic toxicity studies. There tests can generally be derived from the routine measurements and examinations performed in short-term and subchronic rodent and nonrodent toxicity

studies, since they do not require any perturbation of the test animals (immunization or challenge with infectious agents). These measurements include hematology and serum chemistry profiles, routine histopathological examinations of immune-associated organs and tissues, and organ and body weight measurements including thymus and spleen. If a compound produces any primary indicators of immunotoxicity from these measurements, more definitive immunotoxicity tests, such as those indicated in the preceding paragraph, may be recommended on a case-by-case basis.

The following is a brief explanation of some of the indicators that may be used to trigger additional definitive testing and a description of some of the most commonly used assays to assess humoral, cell-mediated, or nonspecific immune dysfunction, which are common to most immunotoxicology test strategies.

9.7.1 Immunopathological Assessments

Various general toxicological and histopathological evaluations of the immune system can be made as part of routine preclinical safety testing to obtain a preliminary assessment of potential drug-related effects on the immune system. At necropsy, various immunological organs of the immune system such as thymus, spleen, and lymph nodes are typically observed for gross abnormalities and weighed in order to detect decreased or increased cellularity. Bone marrow and peripheral blood samples are also taken to evaluate abnormal types and/or frequencies of the various cellular components. Tables 9.12 and 9.13 summarize the observations and interpretations.

Organ and Body Weights Changes in absolute weight, organ-to-body weight ratios, and organ-to-brain weight ratios of tissues such as thymus and spleen are useful general indicators of potential immunotoxicity. However, these measures are nonspecific for immunotoxicity since they may also reflect general toxicity and effects on endocrine function that can indirectly affect the immune system.

9.7.2 Humoral Immune Response and Possible Entry Points for Immunotoxic Actions

Hematology Hemacytometers or electronic cell counters can be used to assess the numbers of lymphocytes, neutrophils, monocytes, basophils, and eosinophils in the peripheral blood, while changes in relative ratios of the various cell types can be assessed by microscopic differential evaluation. Similar evaluations can be performed with bone marrow aspirates, where changes may reflect immunotoxicity to the pluripotent stem cells and newly developing lymphoid precursor cells. Potential hematological indicators of immunotoxicity include altered white blood cell counts or differential ratios, lymphocytosis, lymphopenia, or eosinophilia. Changes in any of these

TABLE 9.12 Examples of Antemortem and Postmorten Findings That May Include Potential Immunotoxicity If Treatment Related

Parameter	Possible Observation (Cause)	Possible State of Immune Competence
	Antemortem	
Mortality	Increased (infection)	Depressed
Body weight	Decreased (infection)	Depressed
Clinical signs	Rales, nasal discharge (respiratory infection)	Depressed
	Swollen cervical area (sialodacryoadenitis virus)	Depressed
Physical examinations	Enlarged tonsils (infection)	Depressed
Hematology	Leukopenia/lymphopenia	Depressed
	Leukocytosis (infection/cancer)	Enhanced/depressed
	Thrombocytopenia	Hypersensitivity
	Neutropenia	Hypersensitivity
Protein electrophoresis	Hypogammaglobulinemia	Depressed
	Hypergammaglobulinemia (ongoing immune response or infection)	Enhanced/activated
	Postmortem	
Organ weights		
Thymus	Decreased	Depressed
Histopathology		
Adrenal glands	Cortical hypertrophy (stress)	Depressed (secondary)
Bone marrow	Hypoplasia	Depressed
Kidney	Amyloidosis	Autoimmunity
	Glomerulonephritis (immune complex)	
Lung	Pneumonitis (infection)	Depressed
Lymph node	Atrophy	Depressed
Spleen	Hypertrophy/hyperplasia	Enhanced/activated
	Depletion of follicles	Depressed B cells
	Hypocellularity of periarteriolar sheath	Depressed T cells
	Active germinal centers	Enhanced/activated
Thymus	Atrophy	Depressed
Thyroid	Inflammation	Autoimmunity

parameters can be followed up with more sophisticated flow cytometric analyses or immunostaining techniques that are useful for phenotyping the various types of lymphocytes (B cell, T cell) and the T-cell subsets ($CD4^+$ and $CD8^+$) on the basis of unique surface markers. Decreases or increases in the percentages of any of the cell populations relative to controls or in the ratios of B cells to T cells or $CD4^+$ to $CD8^+$ cells may be indicators of immunotoxicity.

Clinical Chemistry Nonspecific clinical chemistry indicators of potential immune dysfunction include changes in serum protein levels in conjunction

with changes in the albumin-to-globulin (A/G) ratio. Immunoelectrophoretic analysis of serum proteins can then be performed to quantify the relative percentages of albumin and the α-, β-, and γ-globulin fractions. To perform these assays, a drop of serum (antigen) is placed into a well cut in a gel, then the gel is subjected to electrophoresis so that each molecule in the serum moves in the electric field according to its charge. This separation is then exposed to specific antiserum, which is placed in a trough cut parallel to the direction in which the components have moved. By passive diffusion, the antibody reaches the electrophoretically separated antigen and reacts to form Ag–Ab complexes. The γ-globulin fractions can be separated and further quantified for the relative proportions of IgG, IgM, IgA, and IgE using similar techniques.

Serum concentrations of immunoglobulin classes and subclasses can also be measured using various techniques such as radioimmunoassays (RIAs) or enzyme-linked immunosorbent assays (ELISAs). In the ELISA, antigens specific for each class of immunoglobulin can be adsorbed onto the surfaces for microtiter plates. To determine the quantity of each antibody in a test sample, an aliquot of antiserum is allowed to react with the adsorbed antigens. Unreacted molecules are rinsed off and an enzyme-linked anti-Ig is then added to each well. Next, substrate is added and the amount of color that develops is quantified using a spectrophotometric device. The amount of antibody can then be extrapolated from standard curves since the amount of color is proportional to the amount of enzyme-linked antibody that reacts. Variations in levels of a given antibody may indicate the decreased ability of B cells of decreased numbers of B cells producing that antibody. In addition, serum autoantibodies to DNA, mitochondria, and parietal cells can be used to assess autoimmunity. Serum cytokines (IL-1, IL-2, and γ-interferon) can also be evaluated using immunochemical assays to evaluate macrophage, lymphocyte, and lymphokine activity; prostaglandin E_2 can also be measured to evaluate macrophage function.

CH50 determinations can be used to analyze the total serum complement and are useful for monitoring immune complex diseases (Sullivan, 1989); activation of complement (Table 9.13) in the presence of autoantibodies is indicative of immune complex diseases and autoimmunity. The various components of the complement system (C3, C4) can also be measured to assess the integrity of the system. For instance, low serum concentrations of C3 and C4 with a concomitant decrease in CH50 may indicate activation of complement, while a low C4 alone is a sensitive indicator of reduced activation of the complement system. Since C3 is used as an alternate complement pathway, it usually measures high. Therefore, a low C3 with a normal C4 may indicate an alternate pathway of activation.

Histopathology Histopathological abnormalities can be found in lymphoid tissues during gross and routine microscopic evaluations of the spleen, lymph nodes, thymus, bone marrow, and gut-associated lymphoid tissues such as Peyer's patches and mesenteric lymph nodes. Microscopic evaluations should

TABLE 9.13 Immune System Components in Organ Sites

Organ	Antigen-Presenting Cell	B Cell	CD4* T Cell	Possible Immunotoxic Entry Point	Outcome
Bone marrow	Differentiation from pluripotent stem cells	Differentiation from pluripotent stem cells; rearrangement of antigen receptors, central repertoire selection, homing, antibody production	Differentiation from pluripotent stem cells; rearrangement of antigen receptors	Inhibition of cell proliferation, interference with signal transduction, genotoxicity, interference with gene recombination, interference with DNA repair, interference with signal transduction, interference with apoptosis, interference with signal transduction, inhibition of protein synthesis	Leukopenia, malignancies, lymphopenia, selective lymphopenia, autoimmunity, impaired adaptive immune response
Thymus	—	—	Proliferation and positive/negative repertoire selection	Interference with signal transduction, interference with apoptosis	Selective lymphopenia, autoimmunity
Peripheral tissue	Antigen uptake and processing, maturation	—	—	Inhibition of cell motility, interference with phagolysosomal degradation, interference with intracellular transport, inhibition of cell motility, interference with signal transduction	Lack of antigen presentation, impaired adaptive immune response, lack of antigen presentation, impaired adaptive immune response
Lymphatic organ	Antigen presentation	Antigen uptake and presentation, cross-comunication with T cell, activation, proliferation, differentiation	Antigen recognition (antigen-presenting cell) and activation, proliferation, differentiation, cross-comunication with B cell	Inhibition of cell motility, interference with phagolysosomal degradation or intracellular transport, interference with signal transduction, inhibition of cytokine release, interference with signal transduction, inhibition of cell proliferation, interference with signal transduction	Impaired adaptive immune response, impaired adaptive immune response, autoimmunity, allergy, impaired adaptive immune response

Adapted from Haley et al., 2005.

include descriptive qualitative changes such as types of cells, densities of cell populations, proliferation in known T- and B-cell areas (e.g., germinal centers), relative numbers of follicles and germinal centers (immune activation), and the appearance of atrophy or necrosis. In addition, unusual findings such as granulomas and scattered, focal mononuclear cell infiltrates in nonlymphoid tissues may be observed as indicators of chronic hypersensitivity or autoimmunity. A complete histopathological evaluation should also include a quantitative assessment of cellularity through direct counts of each cell type in the various lymphoid tissues. In addition, changes in cellularity of the spleen can be more precisely quantitated from routine H&E (hematoxylin and eosin) sections using morphometric analysis of the germinal centers (B cells) and periarteriolar lymphocyte sheath (T cells). Similar morphometric measurements can be made of the relative areas of the cortex and medulla of the thymus. If changes in cellularity are apparent from routinely stained histopathology sections, special immunostaining (immunoperoxidase or immunofluorescence) of B cells in the spleen and lymph nodes using polyclonal antibodies to IgG, or immunostaining of the T cells and their subsets in the spleen using mono- or polyclonal antibodies to their specific surface markers, can be used to further characterize changes in cellularity.

Numerous physiological and environmental factors such as age, stress, nutritional deficiency, and infections may affect the immune system (Sullivan, 1989). Thus, adverse findings in animal studies may reflect these indirect immunotoxic effects rather than the direct immunotoxic potential of a chemical or drug. Indirect immunotoxic effects may be assessed through histopathological evaluations of endocrine organs such as the adrenals and pituitary.

It is also well known that the functional reserves of the immune system can allow biologically significant, immunotoxic insults to occur without the appearance of morphological changes. In addition, there is some built-in redundancy in the system in that several mechanisms may produce the same outcome. For instance, cytotoxic T cells may alone be sufficient to protect the organism against a bacterial infection; however, the body will also produce antibodies for future protection. Thus, if one mechanism is insufficient to fight off infection, the second mechanism can serve as a backup. Because of this functional reserve, adverse effects may remain subclinical until the organism is subjected to undue stress or subsequent challenge (Bloom et al., 1987). Therefore, routine immunopathological assessments as part of standard preclinical toxicity tests may not be sufficient to detect all immunotoxins. Although changes detected in routine toxicological and pathological evaluations are nonspecific and of undetermined biological significance to the test animal, they can be invaluable as flags for triggering additional testing.

9.7.3 Humoral Immunity

As described previously, the humoral immune response results in the proliferation, activation, and subsequent production of antibodies by B cells

following antigenic exposure and stimulation. The functionality and interplay between the three primary types of immune cells (macrophage, B cells, and T cells) required to elicit a humoral response can be assessed through various in vitro assays using cells from the peripheral blood or lymphoid tissues. (Burleson et al., 1995).

Antibody Plaque-Forming Cell (PFC) Assay The number of B cells producing antibody (PFC) to a T-dependent antigen such as sheep red blood cells (SRBCs) can be assessed in vitro following in vivo exposure to the test article and antigen (ex vivo tests). The PFC response to a T-dependent antigen is included as a tier I test by the National Toxicology Program (NTP) since it appears to be the most commonly affected functional parameter of exposure to immunosuppressants. However, this test is designated as a type II test in the FDA Redbook since it requires an in vivo immunization of the animals with antigen and thus cannot be evaluated as part of an initial toxicity screen.

Although this assay requires that B cells be fully competent in secreting antibodies, T cells and macrophage cells are also essential for the proper functioning of humoral immunity. However, this assay is nonspecific in that it cannot determine which cell type(s) is responsible for dysfunction. Macrophage cells are needed to process antigen and produce IL-1. T cells are needed for several functions including antigen recognition of surface membrane proteins and B-cell maturation through the production of various lymphokines that stimulate growth and differentiation. SRBCs are most commonly used as the T-dependent antigen, although T-cell-independent antigens may also be useful to rule out T helper dysfunction as a cause of immunodysfunction.

The PFC assay has evolved from methodology originally developed as a hemolytic plaque assay (or Jerne plaque assay) by Nils Jerne to quantitate the number of antibody-forming cells in a cell suspension plated with red blood cells (RBCs) onto agar plates (Jerne and Nordin, 1963). In its present form, animals are treated in vivo with the test compound, immunized with approximately 5×10^8 SRBCs administered intravenously within two to three days posttreatment, and then sacrificed four days (IgM) or six days (IgG) later. Antibody-producing spleen cell suspensions are then mixed in vitro with SRBCs, placed onto covered slides, and incubated for a few hours in the presence of complement. During incubation, antibody diffuses from the anti-SRBC-producing cells and forms Ag–Ab complexes on the surfaces of nearby SRBCs. In the presence of complement, the Ag–Ab complexes cause lysis of the SRBCs, resulting in the formation of small, clear plaques on the slide. Plaques are then counted and expressed as PFCs/10^6 spleen cells. A dose-related reduction in PFCs is indicative of immunosuppression.

B-Cell Lymphoproliferation Response The NTP has classified this assay as a tier I test since mitogenesis can be performed easily in tandem with other tests to provide an assessment of the proliferative capacity of the cells (Luster et al., 1988). Since this assay is performed ex vivo with peripheral blood (or

spleen) and is well characterized for use in various animal species, it has also been included as an expanded type I test in the revised Redbook.

The proliferation of peripheral blood or splenic B cells following stimulation with lipopolysaccharide (LPS) or other mitogens (pokeweed mitogen extract) is another measurement of humoral immunity. LPS (a bacterial lipopolysaccharide) is a B-cell-specific mitogen that stimulates polyclonal proliferation (mitosis) as part of the natural sequence of antigen recognition, activation, and clonal expansion. The mitogen interacts not with just one particular antigen-specific clone but with all cells bearing the carbohydrate surface marker for which it is specific. Since mitogens are both polyclonal and polyfunctional, they can stimulate a wider spectrum of antigenic determinants than antigens, which can only stimulate a low number (10^{-6}) of specific cells.

In this assay, lymphocytes from animals are treated in vivo and cultured in vitro in microtiter plates in the presence of tritiated [^{3}H]thymidine (or uridine) using a range of at least three concentrations of mitogen to optimize the response. Lymphocytes can be obtained aseptically from peripheral blood or from single-cell suspensions of spleen cells that are prepared by pushing the tissue through sterile gauze or 60-mesh wire screens. A decrease in DNA synthesis (incorporation of ^{3}H) as compared to the unexposed cells of control animals may indicate that the B cells were unable to respond to antigenic stimulation. Alternative methodology employs 18–20 h incubation with ^{125}I-labeled iododeoxyuridine ([^{125}I]IudR) and fluorodeoxyuridine (FudR) (White et al., 1985). After incubation, the cells are collected onto filter disks and then counted with a gamma counter.

Assays such as this that use polyclonal mitogens for activation may not be as sensitive as specific antigen-driven systems (Luster et al., 1988). In addition, suppression of the mitogen response does not always correlate with the PFC response. Since mitogenesis represents only a small aspect of B-cell function and maturation, this endpoint is not sensitive to early events that may affect activation or later events that may affect differentiation of B cells into antibody-secreting cells (Klaus and Hawrylowicz, 1984).

9.7.4 Cell-Mediated Immunity

T-Cell Lymphoproliferation Response This assay is analogous to the B-cell lymphoproliferative response assay described above. Thus, this assay is also classified as a tier I test by the NTP and as an expanded type I test in the revised draft of the Redbook.

T cells from the peripheral blood or spleen undergo blastogenesis and proliferation in response to specific antigens that evoke a cell-mediated immune response. T-cell proliferation is assessed using T-cell-specific mitogens such as the plant lectins, concanavalin A (Con A), and phytohemagglutinin (PHA) or T-cell-specific antigens (i.e., tuberin, *Listeria*). Uptake of ^{3}H as an indicator of DNA synthesis is used as described above for evaluating B-cell

proliferation. T-cell mitogens stimulate not only the synthesis of DNA but also the expression of cell-specific function. For instance, Con A can trigger the expression of T helper, suppressor, and cytotoxic effector cells, and either mitogen may induce the expression (or reexpression of memory cells) of differentiated function (Clark, 1983). Since cell populations responsive to Con A are thought to be relatively immature compared to those that are stimulated with PHA, the parallel usage of both mitogens may be useful for distinguishing the affected subset (Tabo and Paul, 1973). A secondary response to T-cell antigens such as purified protein derivative of tuberculin (PPD) or tetanus toxoid can also be assessed.

Mixed-Lymphocyte Response (MLR) Assay This assay has been shown to be sensitive for the detection of chemical-induced immunosuppression and is a recommended tier I assay by the NTP (Luster et al., 1988). In addition, it has been shown to be predictive of host response to transplantation and of general immunocompetence (Harmon et al., 1982).

The MLR assay assesses the ability of T cells to recognize foreign antigens on allogenic lymphocytes and, thus, is an indirect measure of the cell-mediated ability to recognize graft or tumor cells as foreign. Responder lymphocytes from animals treated in vivo with the test compound are mixed with allogenic stimulator lymphocytes that have been treated in vitro with mitomycin C or irradiated to render them unable to respond (Bach and Voynow, 1966). Both cell types are cultured in vitro for three to five days, then incubated with ^{3}H for an additional 6 h. Once the radiolabel is incorporated into the DNA of the responding cells, the DNA is extracted and the amount of radioactive label is measured to quantitate proliferation of the responder cells of drug-treated animals compared to those of the controls.

Cytotoxic T-Lymphocyte (CTL)–Mediated Assay This assay is similar to the MLR assay and can be performed in parallel or as a tier II follow-up to the MLR assay.

The CTL assay ascertains the ability of cytotoxic T cells to lyse an allogenic target cell or the specific target cell type with which they were immunized. In general, the cytolytic response of activated effector cells is assessed by measuring the amount of radioactivity (^{51}Cr) that is released from the target cell. When performed in conjunction with the MLR assay, lymphoid cells of the two strains are cultured together in vitro as described above; however, ^{51}Cr is added to the culture after four to five days (instead of ^{3}H). Both responder and target cells are labeled with the ^{51}Cr, which is taken up rapidly by the cells through passive diffusion but is released slowly as long as the cell membrane is intact. Furthermore, since chromium is reduced from Cr^{6+} to Cr^{3+}, and since Cr^{3+} enters the cells at a much slower rate than Cr^{6+}, the ^{51}Cr released from the damaged target cells is not significantly reincorporated into undamaged cells (Clark, 1983), which would reduce the sensitivity of the assay. Thus, the amount of chromium released into the medium and recovered in the super-

natant of the mixture of the cells is directly proportionate to the extent of lysis of the target cells by the sensitized responder cells.

In a capillary tube assay developed in 1962 by George and Vaughan, the inhibition of migration of macrophage cells can be used to access normal T-cell function (see Clark, 1983). T cells are obtained from the peripheral blood of animals treated in vivo with a test article and injected with antigen (e.g., tuberculin). These T cells are functioning normally and should release migration inhibition factor (MIF). As a consequence, the macrophages, which generally show a propensity for migration upon stimulation with the antigen, should show a MIF-induced reduction in migratory behavior.

Delayed-Type Hypersensitivity Response The DTH response assay is considered to be a comprehensive tier II assay for cell-mediated immunity by the NTP.

To express a DTH inflammatory response, the immune system must be capable of recognizing and processing antigen, blastogenesis and proliferation of T cells, migration of memory T cells to the challenge site of exposure to antigen, and subsequent production of inflammatory mediators and lymphokines that elicit the inflammatory response. Thus, by measuring a DTH response to an antigen, these assays assess the functional status of both the afferent (antigen recognition and processing) and efferent (lymphokine production) arms of cellular immunity. Various antigens have been used for assessing DTH, including keyhole limpet hemocyanin (KLH), oxazolone, dinitrochlorobenzene, and SRBCs (Vos, 1977; Godfrey and Gell, 1978; Luster et al., 1988).

In one such assay described by White et al. (1985), mice previously treated with the test article are sensitized to SRBCs by inoculation of SRBCs into the hind footpad and four days later are challenged in the same footpad. Seventeen hours following challenge, they are injected intravenously with ^{125}I-labeled human serum albumin (HSA), then sacrificed 2 h later. Both hind feet are then radioassayed in a gamma counter (the second foot serves as a control for background infiltration of the label). With a normal functioning cell-mediated response, ^{125}I-labeled HSA will extravasate into the edematous area produced by the DTH response (Paranjpe and Boone, 1972). In general, a decrease in the extravasation of ^{125}I-labeled HSA is indicative of immunosuppression of the efferent arm of the cell-mediated immune system.

To assay specific functionality of the afferent arm of the DTH response, proliferation of the popliteal lymph node cells to SRBCs can also be measured (White et al., 1985). As described above, mice treated with the test article are sensitized to SRBCs by inoculation of SRBCs into the hind footpad. However, 1.5 h later they are challenged intraperitoneally with FUdR and 2 h later they are administered [^{125}I]IUdR intravenously (instead of ^{125}I-labeled HSA). Mice are sacrificed 24 h after challenge and both popliteal lymph nodes are removed and counted in a gamma counter.

Similar assays for DTH have been traditionally performed with the antigen *Mycobacterium tuberculosis*, which preferentially elicits a cell-mediated

response. In this assay a small amount of antigen contained in the supernatant fluid from the medium in which the pathogen was grown is injected into the footpad. Upon challenge, a visible and palpable lump should appear by 48 h. The amount of swelling is then measured and compared with the footpad that did not receive the challenge. Alternatively, methods used by the NTP employ a modified ^{125}I-labeled uridine (UdR) technique to measure the monocyte influx at the challenge site (ear) injected with KLH antigen. This assay has been shown to correlate well with decreased resistance to infectious disease (Luster et al., 1988). However, one should note that regardless of which technique is used, anti-inflammatory drugs may produce false-positive results in this type of assay.

9.7.5 Nonspecific Immunity Function Assay

Natural Killer Cell Assays This assay is a tier I test for nonspecific immunity in the NTP testing scheme (Luster et al., 1988) and is proposed as an additional type I test in the draft Redbook.

Natural killer cells, like cytotoxic T cells, have the ability to attack and destroy tumor cells or virus-infected cells. However, unlike T cells, they are not antigen specific, do not have unique, conally distributed receptors, and do not undergo clonal selection. In in vitro or ex vivo tests, target cells (e.g., YAC-1 tumor cells) are radiolabeled in vitro or in vivo with ^{51}Cr and incubated in vitro with effector NK cells from the spleens of animals that had been treated with a xenobiotic. This assay can be run in microtiter plates over a range of various ratios of effector/target cells. Cytotoxic activity is then measured by the amount of radioactivity released from the damaged tumor cells, as was previously described for cytotoxic T cells. This assay can also be performed in vivo, where YAC-1 cells labeled with [^{125}I]IUdR are injected directly into mice and NK-cell activity is correlated with its level of radioactivity (Riccardi et al., 1979). Immunotoxicity observed as reduced NK-cell activity is correlated with increased tumorigenesis and infectivity.

Macrophage Function Several assays are available to measure various aspects of macrophage function, including quantitation of resident peritoneal cells, antigen presentation, cytokine production, phagocytosis, intracellular production of oxygen free radicals (used to kill foreign bodies), and direct tumor-killing potential. Techniques for quantitation of peritoneal cells and functional assays for phagocytic ability are classified as comprehensive tier II tests by the NTP and as additional type I tests in the draft Redbook.

Macrophage cells and other PMNs contribute to the first-line defense of nonspecific immunity through their ability to phagocytize foreign materials, including pathogens, tumor cells, and fibers (e.g., silica, asbestos). Xenobiotics can affect macrophage function by direct toxicity to macrophages or by modulating their ability to become activated. Differential counts of resident perito-

neal cells can be made as a rapid, preliminary assessment of macrophage function for xenobiotics that are not administered parenterally.

Numerous in vitro assays can be employed to assess common functions of macrophages and PMNs, including adherence to glass, migration inhibition, phagocytosis, respiratory activity (chemiluminescent assays or nitroblue tetrazolium), and target cell killing. In one such assay, the chemotactic response to soluble attractants is evaluated using a Boyden chamber with two compartments that are separated by a filter. Macrophage cells or PMNs from treated animals are placed in one side and a chemotactic agent in the other. Chemotaxis is then quantified by counting the number of cells that pass through the filter. In another assay, the ability of the macrophages to phagocytize foreign materials can be evaluated by adding fluorescent latex beads to cultures containing macrophage cells, then determining the proportion of cells that have phagocytized the beads using a fluorescent microscope or by flow cytometry (Duke et al., 1985). Similar functions can be evaluated by incubating the cells with known amounts of bacteria. The cells are then removed by filtration or centrifugation, the remaining fluid is plated onto bacterial nutrient agar, and, after a few days of incubation, the bacterial colonies are counted. Furthermore, the efficiency of the cells to kill the bacteria once phagocytized can be assayed by lysing the cells and plating the lysate onto bacterial agar.

Various in vivo assessments of macrophage function have also been used. For example, peritoneal exudate cell (PEC) recruitment can be assessed using eliciting agents such as *Corynebacterium parvum*, MVE-2, or thioglycolate (Dean et al., 1984). In one such assay (White et al., 1985), mice are injected intraperitoneally with thioglycolate and sacrificed five days later, and the peritoneal cavity is flushed with culture medium. The cell suspension is then counted, the cell concentration is adjusted to a known density ($2 \times 10^5\,mL^{-1}$), and the cells are cultured for 1 h in 24-well culture dishes. Adherent cells are then washed with medium and aliquots of ^{51}Cr-labeled SRBCs that were opsonized with mouse IgG are added to each well and incubated for various times. This same system can be used to assess adherence and chemotaxis of the PECs (Laskin et al., 1981). Phagocytosis can also be evaluated in vivo by measuring the clearance of injected particles from the circulation and the accumulation of the particles in lymphatic tissues such as the spleen.

Mast Cell/Basophil Function The function of mast cells and basophils to degranulate can be evaluated using a passive cutaneous anaphylaxis test (Cromwell et al., 1986). Serum containing specific anaphylactic (IgE) antibodies from donor animals previously exposed to a known antigen is first administered by intradermal (or subcutaneous) injection into unexposed host animals. After a sufficient latency period to allow binding of the donor IgE to the host tissue mast cells, the animals are administered a second intravenous injection of the antigen. The anaphylactic antibodies present in the serum will stimulate normally functioning mast cells to degranulate (release histamines) and produce a marked inflammatory response. Using similar in vitro assays

with mast cells and basophils, the quantities of histamines that are released from the cells can be measured directly in the culture medium.

9.7.6 Host Resistance Assays

Host resistance assays can be used to assess the overall immunocompetence of the humoral or cell-mediated immune systems of the test animal (host) to fend off infection with pathogenic microbes or to resist tumorigenesis and metastasis. These assays are performed entirely in vivo and are dependent on all of the various components of the immune system to be functioning properly. Thus, these assays may be considered to be more biologically relevant than in vitro tests that only assess the function of cells from one source and of one type. Since these assays require that the animal be inoculated with a pathogen or exogenous tumor cell, they cannot be performed as part of a general preclinical toxicity assessment and are thus classified as type II tests in the revised Redbook. These assays are also included as tier II tests by the NTP.

Several host resistance assays have been developed using various infectious agents, including bacteria (*Listeria monocytogenes*, *Streptococcus*, and *Escherichia coli*), viruses (influenza, cytomegalovirus, and herpes), yeast (*Candida albicans*), and parasites (*Trichinella spiralis* and *Plasmodium berghei*). These assays have been described in the NTP guidelines (Luster et al., 1988). In general, animals previously treated with a xenobiotic are injected with the pathogen at a target dose that is estimated to kill 10–30% of control animals (LD10–30). After a period of time, the animals receive a challenge dose at a much higher concentration (LD60–80) and by a different route to determine if animals are resistant to reinfection. Although these assays are similar in their mechanisms of resistance to different pathogens, they have been shown to differ with regard to varying degrees of susceptibility by the same drug (Morahan et al., 1979). Thus, for screening purposes, it is recommended that at least two tests be used (Descotes and Mazue, 1987). Although these tests are relatively easy to perform, those involving the use of pathogens require special handling, containment, and decontamination procedures to prevent infection to humans and spread throughout the animal colony.

Similar host resistance assays are used to evaluate the immunosurveillance of spontaneous tumors, which is assessed as the capacity of the organism to reject grafted syngeneic tumors. Various animal-bearing tumor models (Pastan et al., 1986) and host resistance models have been used to assess immunotoxicity. Several of the host resistance assays utilize cultured tumor cell lines such as PYB6 sarcoma and B16F10 melanoma cells that are used with C57/BL/6 mice or the MADB106 lung tumor cell lines that are used with Fischer 344 rats. For example, the PYB6 sarcoma model uses death as an endpoint. In this assay, syngeneic mice are injected with the PYB6 sarcoma cells and death due to tumor is recorded daily. In another routinely used assay, animals that have been treated with a xenobiotic are injected with either B16F10 melanoma cells

or Lewis lung carcinoma cells; then approximately 20 days later they are sacrificed and pulmonary tumors are measured and counted.

9.7.7 T-Cell-Dependent Antibody Response (TDAR)

The TDAR should be performed using a recognized T-cell-dependent antigen like SRBCs, BSA, or KLH that results in a robust antibody response. For the SRBC assay, IgM measurement is considered the most appropriate endpoint, whereas IgG measurement is considered to be most appropriate for BSA or KLH. Antibody can be measured by using an ELISA or other immunoassay method. One advantage of ELISA over the traditional PFC assay (Ladics, 2005) is that samples can be collected serially during the study, if necessary. Since immunization is likely to have effects on the hematology, clinical chemistry, and histology of lymphatic organs, TDAR studies should always be performed as separate studies, or at least in satellite groups of repeat-dose toxicity studies.

Brief details of the assay are as follows:

Treatment

1. Use a suitable SPF mouse strain like BALB/c or C57BL/6 × C3H F1 (B6C3F1).
2. Allocate 120 animals in six groups of 10 males and 10 females each. The group sizes may be reduced to 5 males and 5 female animals per group when a substantial immunosuppressive effect can be expected. The larger group size should be chosen when immunosuppressives are excluded, since otherwise the statistical power of the assay might be insufficient to prove a lack of immunosuppression.
3. Allow acclimatization for 7 days before sampling of pretest serum (day –7) from the test groups, and allow an additional 7 days of rest before first dosing. Pretest serum of recovery groups may be taken on day 35, which is 1 week postdosing.
4. Administer the test substance and vehicle daily over a period of 28 days (day 1 to day 28) to all animals using an appropriate route of administration.
5. Use a low, intermediate, and high dose level, whereby the high dose level should be above the no-observable-effect level (NOEL) and below a dose level that causes stress, if possible. The intermediate (or low) dose level should ideally represent the intended clinical dose level.
6. Immunize all mice of the test groups on day 14 and mice of the recovery groups on day 42 by intraperitoneal injection of 100 µg KLH per mouse without the use of adjuvant.
7. Sample immune serum from all animals of the test groups on day 29 and from all of the recovery groups on day 57.
8. Store serum at –20 °C until ELISA testing.

9.7.8 Hypersensitivity

Type I Hypersensitivity Although here are acceptable systems for evaluating type I (immediate) reactions following systemic exposure, there are no reliable animal models for predicting type I reactions following dermal applications or oral administrations of drug. Repeated exposure of a xenobiotic is required to produce a type I response. A drug in the form of a hapten must covalently bind to macromolecules (proteins, nucleic acids) before it can initiate a primary antibody response. Once sensitized, even the smallest exposure to the xenobiotic can elicit a rapid, intensive IgE antibody-mediated inflammatory response. With the exception of antivirals and chemotherapeutic drugs, most drugs should not be reactive with biological nucleophiles since these drugs are usually screened out as mutagens or carcinogens in preclinical safety studies. However, type I hypersensitivity is a particular problem with biotechnology products themselves (e.g., insulin, growth hormones, interleukins), trace impurities from the producing organisms (e.g., *E. coli* proteins, mycelium), or the vehicles used to form emulsions (Matori et al., 1985).

The production of neutralizing antibodies to recombinant DNA protein products or their contaminants may be assayed using ELISAs or RIAs. A suitable animal model used to evaluate the potential for a type I response to protein hydrolysates is detailed in the U.S. Pharmacopeia. This test is very sensitive for testing proteins administered by the parenteral route but is of little value for low-molecular-weight drugs and those that are administered orally (Descotes and Mazue, 1987). Active systemic anaphylaxis can be assessed in guinea pigs following systemic exposure to the test compound. For dermal exposures, however, rabbits or guinea pigs must be exposed to the test article by intradermal injections and then evaluated for their ability to mount a systemic anaphylactic response. The passive cutaneous anaphylaxis test (as described above for mast cells) can also be used to assess a potential anaphylactic response to a test compound. The serum containing potential anaphylactic (IgE) antibodies from donor animals previously exposed to the test compound is first administered by intradermal (or subcutaneous) injection into unexposed host animals. After a latency period, the animals are administered an intravenous injection of the test compound together with a dye. If anaphylactic antibodies are present in the serum, the subsequent exposure to the test compound will cause a release of vasoactive amines (degranulation of mast cells), ultimately resulting in the migration of the dye to the sites of the intradermal serum injections.

Types II and III Hypersensitivity No simple animal modes are currently available to assess type II (antibody-mediated cytotoxicity) hypersensitivity reactions. IgE antibodies and immune complexes in the sera of exposed animals can be assayed using ELISA or RIA techniques that require the use of specific antibodies to the drug.

Type III (immune-complex-related disease) reactions have been demonstrated by the presence of proteinuria and immune complex deposits in the kidneys of the Brown-Norway, Lewis, and PVG/C rat strains. However, susceptibility to the deposition and the subsequent lesions (glomerulonephritis) are often variable and dependent on the strain (Bigazzi, 1985). For example, despite the appearance of clinical signs and proteinuria, after two months administration of mercuric chloride, detectable levels of circulating antinuclear autoantibodies can no longer be observed in the Brown-Norway strain (Bellon et al., 1982). By contrast, in PVG/C rats administered mercuric chloride, immune complex deposition and antinuclear autoantibodies are present for longer periods of time; however, proteinurea is not observed (Weening et al., 1978).

Type IV Hypersensitivity There are several well-established preclinical models for assessing type IV (delayed-type) hypersensitivity reactions following dermal exposure, but not for predicting this response after systemic exposure.

Type IV hypersensitivity responses are elicited by T lymphocytes and are controlled by accessory cells and suppressor T cells. Macrophages are also involved in that they secrete several monokines, which results in proliferation and differentiation of T cells. Thus, there are numerous points along this intricate pathway in which drugs may modulate the final response. To achieve a type IV response, an initial high-dose exposure or repeated lower dose exposures are applied to the skin; the antigen is carried from the skin by Langerhans cells and presented to cells in the thymus to initiate T-cell proliferation and sensitization. Once sensitized, a second "challenge" dose will elicit an inflammatory response. Thus, before sensitivity can be assessed, each of the models used to evaluate dermal hypersensitivity requires as a minimum:

- Initial induction exposure
- Latency period for expression
- Challenge exposure

A preliminary test for acute irritancy is also required to ensure that the initial dose is sufficient to stimulate sensitization and that the challenge dose is sufficient to ensure expression of the response without producing irritation, which would confound the response. To confirm suspected sensitization or determine a threshold dose, each assay may also include a second challenge dose one to two weeks after the first challenge, at the same or lower concentrations. To increase penetration of the test article, various methods of abrasion (e.g., tape stripping) and occlusive coverings may also be used.

Several systems are used routinely to test compounds for dermal hypersensitivity. The two most commonly used, the modified Buehler test and the guinea pig maximization test (GPMT), are briefly reviewed. More detailed methodology and a description of alternative test systems can be found in Gad

and Chengelis (1998). Although either rabbits or guinea pigs are sensitive test species, guinea pigs have traditionally been the animal of choice. Guinea pig models of skin sensitization have been (and remain) widely used and have been valuable in assessing human risk (Andersen and Maibach, 1985).

Modified Buehler Buehler (1964) developed the first test system to use an occlusive patch to maximize dermal exposure and to increase the test sensitivity (Buehler, 1964). Although this assay is still insensitive for some xenobiotics that may not sufficiently traverse the epidermis, it is particularly useful for compounds that are either highly irritating by intradermal injection or cannot be dissolved or suspended in a form that is conducive to injection. Other advantages are that the test produces few false positives, rarely overpredicts the potency of sensitizers, and is less likely to produce limiting system toxicity or ulceration at the induction sites. Figure 9.4 shows the test design in its current [Organisation for Economic Co-operation and Development (OECD)] form. The assay is no longer accepted in Europe due to a belief that it has an unacceptable rate of false-negative outcomes and is appropriate only for true topical (dermal) exposures.

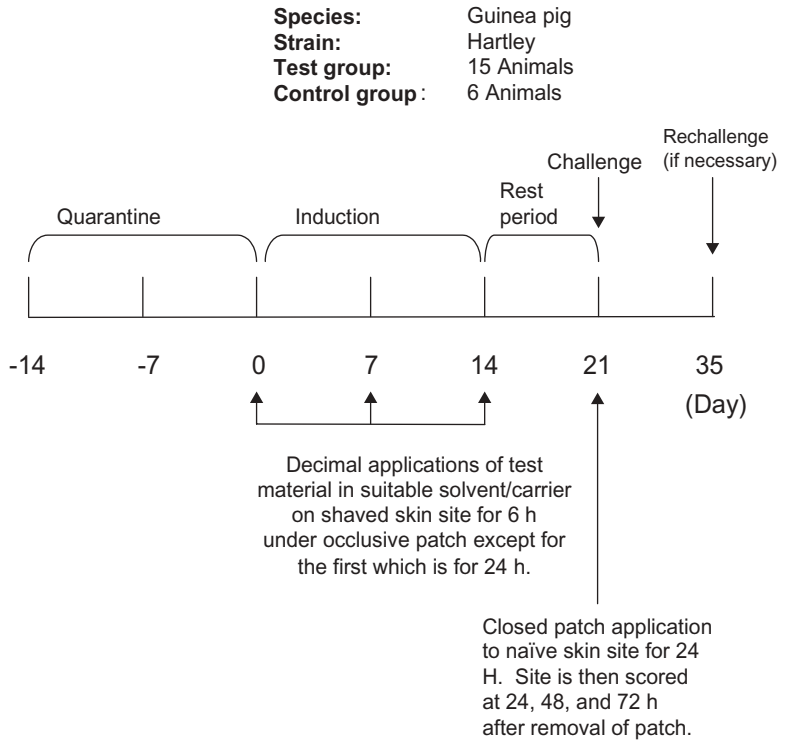

Figure 9.4 *Line chart for modified Buehler test for delayed contact dermal sensitization in guinea pig.*

During the induction phase, the test compound is applied to a cotton patch (1 in. × 1 in.) or placed in a Hill Top–style occlusive chamber. The patch is then placed onto a shaven area of epidermis on the left flank of a guinea pig and secured firmly in place for 24 h, after which time the patch is removed and the area is observed and scored for irritation (i.e., edema, erythema). A fresh patch is then reapplied for 6 h every other day during the induction period for a total of 10 treatments while continuing to score the application site at 24 and 48 h from the start of each treatment. Two weeks after the last induction exposure, the animals receive a challenge exposure for 24 h in the form of a patch applied to a shaven area of epidermis on the other flank (opposite the one used for induction). The challenge dose should be the highest concentration that does not produce dermal irritation after a single, 24-h exposure. The challenge site is observed for evidence of inflammation 24, 48, and 72 h after the patch is removed. Both the intensity and duration of a response to the test article compared to that of the vehicle are used to determine the potential and severity of sensitization.

Guinea Pig Maximization Test This assay, as developed by Magnusson and Kligman (1969), differs from the Buehler test in that the compound is administered by intradermal injection during the first stage of induction and coadministered with an adjuvant (Freund's complete adjuvant) during the induction phase to further stimulate the immune system. This test system is more sensitive (fewer false negatives) than the Buehler test; however, it may overpredict the potency for many sensitizers. Figures 9.5 and 9.6 illustrate the study design.

Prior to induction, a 4 × 6-cm area of fur is clipped from the shoulder region of each guinea pig. On day 0, three pairs of intradermal injections are made along opposite sides of the dorsal midline of the animal. The first pair (closest to the head) is administered as the test substance in the vehicle, the second pair is administered proximal to the first pair and consists only of Freund's

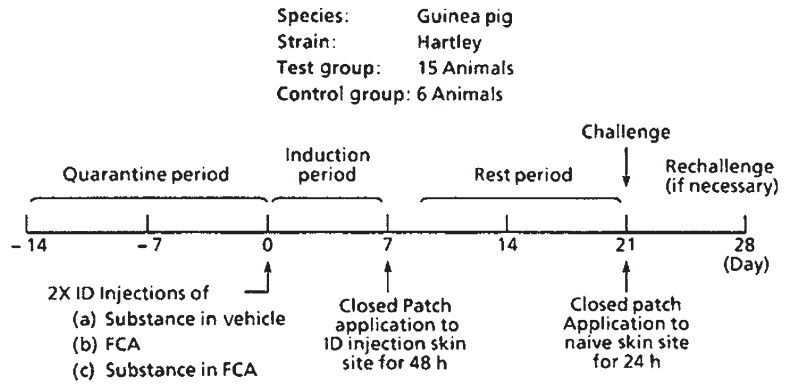

Figure 9.5 *Line chart for guinea pig maximization test for dermal sensitization.*

Outline of guinea pig maximization test

State	Induction		Challenge	Rechallenge
Day	0	7	21	28
Test group (15)	A. 0.1 mL substance ID B. 0.1 mL FCA ID C. 0.1 mL substance + FCA ID	Closed patch-48H application of substance	Closed patch-24H substance vehicle	Closed patch-24H vehicle
Test group (15)	A. 0.1 mL substance ID B. 0.1 mL FCA ID C. 0.1 mL vehicle + FCA ID	Closed patch-48H application of vehicle	Closed patch-24H substance vehicle	Closed patch-24H substance

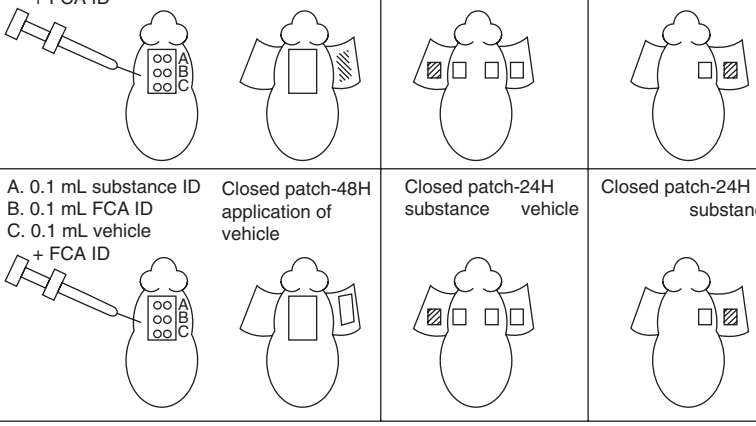

Figure 9.6 *Illustrative figures for injection and patching of animals in GPMT.*

complete adjuvant (FCA), and the third pair (spaced most posteriorly) is administered as the test substance in FCA. Seven days later (day 7), a mild to moderately irritating dose of the test article is spread onto a 1 × 2-in. filter paper patch, secured, and occluded for 48 h on the epidermal site that received the initial injections. On day 21, an area of fur on each flank is shaved and a 1 × 1-in. patch containing a nonirritating concentration of the test article is applied to one flank and a patch containing vehicle alone is applied to the other flank. The patches are secured and occluded for 24 h, and the challenge sites are scored for inflammation 24 and 48 h after removal of the patches. The incidence of animals that respond as well as the intensity and duration of a response to the test article are used to determine the potential and severity of sensitization.

9.7.9 Local Lymph Node Assay

This method has developed out of the work of Ian Kimber and associates (Kimber et al., 1986, 1991; Kimber and Weisenberger, 1989). It has the advantage over the other methods discussed in this chapter in that it provides an objective and quantifiable endpoint. The method is based on the fact that dermal sensitization requires the elicitation of an immune response. This immune response requires proliferation of a lymphocyte subpopulation. The local lymph node assay (LLNA) relies on the detection of increased DNA

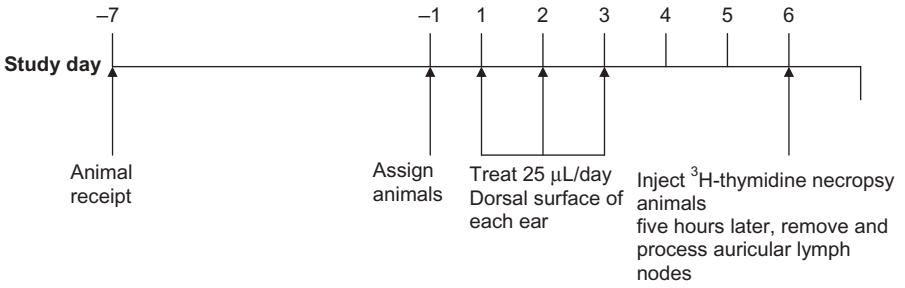

Figure 9.7 *Mouse LLNA.*

synthesis via tritiated thymidine incorporation. Sensitization is measured as a function of lymph node cell proliferative responses induced in a draining lymph node following repeated topical exposure of the test animal to the test article. Unlike the other tests discussed in this chapter, this assay looks only at the induction phase, as there is neither a challenge (elicitation) phase nor a sufficient period of evaluation for development of the underlying clonal expansion in response. Additionally, acute clinical formulations cannot be evaluated in this test system—meaning the all-important question of whether there is actually the potential for the drug to react with the immune system in clinical use remains unaddressed. Rather, in the case of a positive outcome, such interaction is presumed to occur.

The typical test (illustrated in Figure 9.7) is performed using mice—normally female CBA mice 6–10 weeks of age. Female BALB/c and ICR mice have also been used. After animal receipt, they are typically acclimated to standard laboratory husbandry conditions for 7–10 days. The usual protocol will consist of at least two groups (vehicle control and test article treated) of five mice each. They are treated on the dorsal surface of both ears with 25 µL (on each ear) of test article solution for three consecutive days. Twenty-four to 48 h after the last test article exposure, the animals are given a bolus (0.25-mL) dose of [^{3}H]thymidine (20 µCi with a specific activity of 5.0–7.0 Ci/mmol) in phosphate-buffered saline (PBS) via a tail vein. Five hours after the injection, the animals are euthanized by CO_2 asphyxiation and the auricular lymph nodes removed.

After removal, the lymph nodes can either be pooled by group or processed individually. Single-cell suspensions are prepared by gentle mechanical disaggregation through a nylon (100-µm) mesh. Cells are washed twice by centrifugation in an excess of PBS. After the final supernatant wash is removed, the cells are precipitated with cold 5% trichloroacetic acid (TCA) and kept at 4 °C for 12–18 h. The precipitate is then pelleted by centrifugation and resuspended in 1 mL 5% TCA, and the amount of radioactivity is determined by liquid scintillation counting using established techniques for tritium.

The data are reduced to the stimulation index (SI):

$$\text{SI} = \frac{H(\text{dpm})\text{treated group}}{H(\text{dpm})\text{control group}}$$

An SI of 3 or greater is considered a positive response, that is, the data support the hypothesis that the test material is a sensitizer.

The test article concentration is normally the highest nonirritating concentration. Several concentrations could be tested at the same time should one wish to establish a dose–response curve for induction. The test is easiest to perform if the vehicle is a standard nonirritating organic, such as acetone, ethanol, or dimethylformamide, or a solvent–olive oil blend. Until a laboratory develops its own historical control base, it is also preferable to include a positive control group. Either 0.25% dinitricholorobenzene or 0.05% oxazalone is recommended as a positive control. If the vehicle for the positive control is different than the vehicle for the test material, then two vehicle control groups may be necessary.

This method has been extensively validated in two international laboratory exercises (Basketter et al., 1991; Loveless et al., 1996). In the earlier work (Basketter et al., 1991), there was good correlation between the results obtained with guinea pig tests and those obtained with the LLNA. In the 1996 report, for example, five laboratories correctly identified dinitrochlorobenzene and oxazalone as sensitizers and the fact that *p*-aminobenzoic acid was not (Loveless et al., 1996). Arts and colleagues (1996) demonstrated that rats could be used as well as mice. Interestingly, they validated their assay (for both rats and mice) using BrDU uptake and immunohistochemical staining (rather than [^{3}H] thymidine) to quantitate lymph node cell proliferation.

This method is relatively quick and inexpensive because it uses relatively few mice (which are much less expensive than guinea pigs) and takes considerably less time than traditional guinea pig assays (Basketter and Scholes, 1991). It has an advantage over other methods in that it does not depend on an arguably subjective scoring system and produces a quantifiable endpoint. It does require a radiochemistry laboratory and license. Unless one already has an appropriately equipped laboratory used for other purposes (most likely metabolism studies), setting one up for the sole purpose of running the LLNA does not make economic sense. The standard version of the test has been adopted by the OECD (OECD429), ICCVAM (Interagency Coordination Committee on the Validation of Alternative Methods), European Medicines Agency (EMEA), and FDA (see Figure 9.8) but also has been shown to have a modest false-positive rate (misidentifying strong irritants as sensitizers).

Indeed, it has become clear that certain classes of structures (e.g., surfactants, fatty acids, fatty alcohols, siloxanes, and polyols) yield high incidences of false-positive outcomes. Though initially thought to be associated with (and due to) strong irritant responses, this is now clearly not the case (Kreiling et al., 2008).

The other possibility, of course, is clinical evaluation using the human repeat insult path test (HRIPT). This five-week hazard test is rarely taken due to cost,

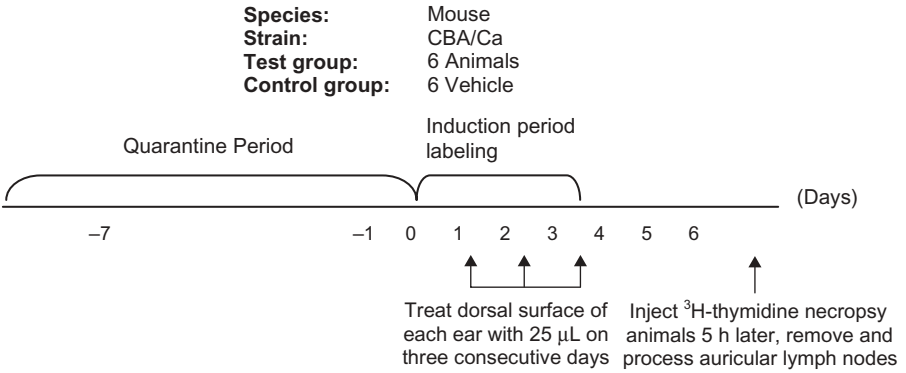

Figure 9.8 *Mouse LLNA (ICVAM protocol) Modification using flow cytometry instead of radiolabeling is preferable.*

though actual clinical formulations are effectively evaluated clinically in the normal course of clinical development of a drug.

9.7.10 Photosensitization

Some compounds can act as photoantigens that require exposure to UV light to become photoactive haptens. The physiochemical characteristics of compounds can sometimes reveal them as potentially photoactive, particularly if they are photounstable to light in the UV range. There are several in vivo tests that are used for determining photosensitization. The two assays described here are similar to those previously described for DTH with the primary exception that the dermal test sites are exposed to a light source during the induction and challenge phases. Like the DTH assays, these assays may also include a second challenge dose or the use of various methods of abrasion and occlusion to increase dermal penetration of the test article. The methods outlined below are more thoroughly described in Gad and Chengelis (1998).

Harber and Shalita Method This method (Harber and Shalita, 1975) is similar to the Buehler test in that the compound is applied topically to guinea pigs without the use of adjuvants; however, the test site is not occluded during exposure. During the induction phase, the compound is applied on alternate days during a 12-day period for a total of six applications. Thirty minutes after each application, the test sites are exposed to a sunlamp for 30 min and then to a black light for 30 min. The challenge dose is applied 21 days after the last induction exposure. Thirty minutes after application, the challenge site is shielded with a 3-mm-thick piece of glass, while the site is exposed to black light for an additional 30 min. The glass filters out erythrogenic (causing redness) radiation of less than 320 nm that may confound scoring the reaction. The challenge sites are observed and scored for inflammatory reactions 24 h later.

Armstrong Method This test (Ichikawa et al., 1981) resembles the guinea pig maximization test in that the FCA is injected intradermally at the test sites; however, covered Hill Top chambers are used to apply and occlude the test article at the test site, as was described for the Buehler test. During induction, four intradermal injections of FCA are administered at the test site; the test article is applied to the Hill Top chamber, which is then applied over the test site and occluded. After 4 h, the patches are removed and the test site is exposed to UV-A light (320–400 nm) for 30 min. Five additional applications of the test article (without FCA) with subsequent exposure to light are made on alternate days throughout the 11-day induction period. Nine to 13 days after the last induction exposure, the animals are challenged for 2 h with a nonirritating concentration of the test article on an occluded Hill Top chamber. The patches are then removed, and the sites are exposed to the UV-A light. Each site is graded for inflammation 24 and 48 h after challenge.

Despite its extreme clinical importance, the evaluation of small-molecule pharmaceuticals for allergenic potential is extremely unreliable in nonclinical toxicology studies. First, a drug that has been proved to be immunogenic in a laboratory animal species may not necessarily be immunogenic in humans, and vice versa. Second, the subtle factors that determine whether an individual responds to an antigen with an IgG or an IgE response can hardly be extrapolated from one species to another. Thus, an immune response that manifests as an allergic response in a laboratory animal may not necessarily manifest as an allergic response in humans, and vice versa.

With regard to antibody-mediated hypersensitivity, three methods have been used extensively to assess the induction of drug-specific (type I) anaphylactic reactions:

- Passive cutaneous anaphylaxis (PCA) assay
- Active cutaneous anaphylaxis (ACA) assay
- Active systemic anaphylaxis (ASA) assay

All three assays are normally conducted in guinea pigs, which is the only rodent species that actually develops symptoms of severe anaphylactic reactions and even fatal allergic shock. However, the usefulness of these assays for the safety assessment of drugs is considered limited. Since IgE as well as IgG antibodies can cause anaphylactic reactions in guinea pigs, a positive result in any of the three assays can only be weighted as proof of immunogenicity, but not allergenicity of a drug. The PCA, ACA, and ASA assays are therefore not requested or recommended for the routine evaluation of allergenicity of investigational new drugs by any regulatory agency.

The situation is as unsatisfactory for the prediction of a type II and III allergenic potential of drugs. Although there are examples of drugs that are associated with type II and III hypersensitivity reactions, there are no standard nonclinical methods for predicting these effects. Manifestations of both kinds of immunopathies are often indistinguishable from direct, non-immune-

mediated drug toxicity. Thus, in some instances of hemolytic anemia, vasculitis, or glomerulonephritis, which may be observed during standard toxicity studies, follow-up studies should be considered to determine if antibody-mediated immune mechanisms are involved.

Since all available nonclinical assays to assess the antibody-based allergenic potential of drugs have a limited predictivity for the human situation, detection of drug immunogenicity should already be considered to be a potential safety alert. Whether proven immunogenicity in a nonclinical test does in fact lead to allergic implications in patients can only be convincingly demonstrated (or excluded) in clinical trials or even later still during postmarketing surveillance of the approved drug.

The most robust and predictive procedures available for assessment of allergenicity are those measuring the skin-sensitizing potential of topically administered drug substances. In these cases, a drug has to permeate the keratinized skin, bind to MHC molecules of dermal APCs, and stimulate $CD4^+$ T cells for proliferation and T_H1 differentiation. Any drug that is able to induce the above sequence of events will inevitably induce an inflammatory reaction, a so-called DTH reaction, after subsequent challenge exposure to the skin. Thus, in this special situation of dermal sensitization, a proof of immunogenicity is also a proof of allergenicity.

When a drug is intended for topical administration (dermal, ocular, vaginal, rectal), the skin-sensitizing potential of the drug should be determined using an appropriate assay based on sensitization and challenge as part of nonclinical safety evaluation. The most common methods for evaluating the dermal sensitizing potential of drugs have been the Buehler assay (BA) and the GPMT. Both in vivo guinea pig–based methods are reliable and have demonstrated a high correlation with known human skin sensitizers. Techniques using mice, like the mouse ear swelling test, which uses an induction and challenge pattern similar to the traditional guinea pig tests, or the murine LLNA, correlate well with traditional guinea pig tests. Especially the LLNA, which is designed to detect lymphoproliferation in draining lymph nodes of the exposition area instead of inflammation following challenge, gives quantitative results. Furthermore, the assay is now accepted by most regulatory agencies with regard to reduction, refinement, and replacement of animal experimentation.

Pseudoallergic (anaphylactoid) reactions, which are independent of antigen-specific immune responses, result from direct drug-mediated histamine release or complement activation. Anaphylactoid reactions can be differentiated from true IgE-mediated anaphylaxis by in vitro testing of drug-induced histamine release from mast cell lines or by the detection of activated complement products in serum of animals showing signs of anaphylaxis.

9.8 APPROACHES TO COMPOUND EVALUATION

As outlined above, there are numerous assays available to assess the various endpoints that are relevant to immunotoxicity. Early in the development

process, a new compound should be evaluated with regard to various factors that may flag it as a potential immunotoxin, including chemical, structural, or physiochemical properties (e.g., photoallergin) and therapeutic class (i.e., immunomodulators, anti-inflammatory drugs, and antimetabolites). Compounds from therapeutic or structural classes that are known to be potential immunotoxins or immunomodulators should be evaluated for the effects in question on a case-by-case basis. With the exception of immunomodulators, protein products, and products of biotechnology, the majority of pharmaceuticals can be assessed for most forms of immunotoxicity during routine preclinical toxicity tests. In general, a well-conducted preclinical toxicity study can detect most serious immunotoxins in the form of altered clinical, hematological, or histological endpoints. For example, possible effects on humoral immunity may be indicated from clinical observation of gastrointestinal or respiratory pathology, changes in serum total protein and globulin, and histological changes in lymphoid cellularity. Likewise, effects on the cell-mediated response may be observed as increases in infections and tumor incidences and by changes in the T-cell compartments of lymphoid tissues. In the case of immunosuppressive drugs such as cyclophosphamide and cyclosporin A, the immune effects seen in rodents are similar to those observed in the clinic (Dean et al., 1987).

If perturbations are observed in any hematological or histopathological indicators of immunotoxicity, it is then prudent to follow up these findings with one or more of the following:

- Use of special immunochemical and cytological assays that can be performed retrospectively on samples taken from the animals in question.
- Use of more specific in vitro assays to further assess effects on the pertinent target system and potential mechanism of activity.
- Use of more specific in vitro and ex vivo assays to determine toxicological significance.
- Inclusion of additional nonroutine parameters for immunotoxicity assessment in subsequent (longer term) toxicity assays. This can also include additional satellite groups for functional tests that may require coadministration of adjuvants, pathogens, or tumor cells.

9.8.1 Use of In Vivo Tests

In vivo tests are more relevant indicators than are in vitro tests of immunotoxicity since the dynamic interactions between the various immunocomponents as well as the pertinent pharmacokinetic (absorption, distribution, plasma concentrations) and metabolic factors are taken into consideration. However, it is important to select the appropriate animal model and to design the protocol such that it will accurately reflect drug (or relevant metabolite) exposure to humans. For example, one should consider species variability

when selecting the animal model since biological diversity may further obscure the ability to accurately predict human toxicity.

Species Selection When possible, the species selected should demonstrate similar pharmacology and toxicity profiles to those anticipated in the clinic. Thus, the test animals should metabolize the drug and express the same target organ responses and toxic effects as humans. Although the rat and dog are the most common species used in preclinical safety tests, they are not as well characterized and validated as the mouse for assessing effects on immune function. For most immunosuppressive drugs, rodent data on target organ toxicities and comparability of immunosuppressive doses have been reflective of what was later observed in the clinic. Immunosuppressive effects and the doses that produced them have been shown to be similar in the various species that are typically used in preclinical safety tests (Dean and Thurmond, 1987). An exception has been seen with glucocorticosteroids, which are lympholytic in rodents but not in primates (Claman, 1972; Haynes and Murad, 1985). Although some compounds may show different pharmacokinetics and pharmacological effects in rodents than in humans, rodents still appear to be the most appropriate animals for assessing immunotoxicity of non-species-specific compounds (Dean and Thurmond, 1987).

The appropriate animal model is also important when performing follow-up testing or additional mechanistic tests to further investigate findings observed as part of the routine preclinical safety tests. When possible, these studies should employ the same animals or animal model in which the change was initially observed for several reasons, as outlined by Bloom et al. (1987), including:

- The incidence of adverse effect may be low and not easy to reproduce.
- Another species may not be genetically susceptible to the toxic effect.
- The biological significance of the change is well defined in that model.
- If the change follows long-term exposure to the drug, reproducing the effect in another model may be costly and impractical.

Route and Treatment Regimen When possible, it is important to administer the compound by the route and treatment regimen most appropriate for demonstrating the specific response and/or reflecting the intended clinical route of administration. It is also necessary for the compound to be in the same dosage form (i.e., salt form, excipients, solubilizers) that will be used clinically. With the exception of tests for contact hypersensitivity, most of the in vivo tests can be carried out with a minimum of three dose levels, which are needed to assess a dose–response relationship. Dose levels should range from the proposed clinical dose, or one that approximates the no-effect level, to a maximum-tolerated (or limit) dose that is lower than the LD_{10} but that produces some evidence of general toxicity (e.g., reduced body weight). A wide

dose interval may be necessary to detect immune changes that show a nonlinear dose response. Proper dose selection is crucial for a meaningful interpretation of test results since severe stress and malnutrition may produce indirect immunotoxic effects that would confound a clear interpretation of the data.

For compounds such as antibiotics, with a relatively short duration of therapeutic exposure, a short treatment period of one to two weeks in the animal model is generally appropriate. Longer treatments may not be suitable for these drugs since animals can adapt to toxic doses or develop a tolerance by inducing enzymes that increase metabolism of the drug. However, for compounds with intended chronic or prolonged usage, animals should be treated at least daily for at least a month to assess the cumulative effects of the drug.

9.8.2 Use of In Vitro Tests

In vitro tests are useful as sensitive follow-up tests to determine potential effects or mechanisms of effects on specific cell types at the cellular and molecular levels. In addition, most are relatively simple to perform and ex vivo tests can be performed in conjunction with preclinical in vivo tests. There are several advantages to using in vitro tests:

- Specific cell types of humoral components of the immune system can be isolated and studied.
- Cells can be stimulated with various mitogens to assess their proliferative functions in vitro.
- For mechanistic studies, cells and their secretory products can be systematically studied in isolation and in various combinations to assess their interactions and cell-to-cell communications.

However, for general preclinical assessments and screening purposes, in vitro tests should be well validated and used cautiously for several reasons:

- They may over- or underestimate an effect or give contradictory results compared to in vivo tests.
- Most immunotoxic responses express a clear dose–response relationship that can be used for human risk assessment. However, it is more difficult to extrapolate in vitro concentrations than in vivo animal doses (plasma concentrations) to the clinical dose.
- It is difficult to simulate in vitro the interaction of all of the various cell types and modulators of immune function that make up the in vivo system.
- Cells can be harvested from a variety of sources and each source may have a different sensitivity since they may be at various stages of maturation or activation.

9.8.3 Assessment of Immunotoxicity and Immunogenicity/ Allergenicity of Biotechnology-Derived Drugs

This is an area outside of current ICH S8 guidance and yet of clinical importance to the safety of current new therapeutics. The decision on a suitable species for preclinical immunotoxicity assessment of biotechnology-derived drugs must be made on a case-by-case basis. In any case, the limits of predictivity should be clearly stated in the rationales for choosing a certain assay protocol. The biological activity together with species and/or tissue specificity of many biotechnology-derived pharmaceuticals (e.g., recombinant cytokines, therapeutic antibodies) often preclude standard toxicity testing designs in commonly used species (e.g., rats and dogs). The same holds for immunotoxicity testing. The design of immunotoxicity testing programs for biotechnology-derived drugs should include the use of relevant species. A relevant species is one in which the test material is pharmacologically active due to expression of the receptor or an epitote (in the case of monoclonal antibodies). A variety of techniques (e.g., immunochemical or functional in vitro tests) can be used to identify a relevant species. In some cases nonhuman primates may be the only suitable species available. When no relevant species exists, the use of transgenic mice expressing the human receptor or epitope may be accepted by regulatory agencies. The information gained from use of a transgenic mouse model expressing the human receptor is optimized when the interaction of the product and the humanized receptor has similar physiological consequences to those expected in humans.

In other cases, the use of the homologous animal protein instead of the human counterpart may be considered. While useful information may also be gained from the use of homologous proteins, it should be noted that the production process, range of impurities/contaminants, pharmacokinetics, and exact pharmacological mechanism(s) may differ between the homologous form and the product intended for clinical use. Thus, from a formalistic point of view, the test item used in such protocols is not identical to the drug substance to be assessed. Results from such studies can therefore only be weighed as "supportive data." In such situations, it is highly recommended to discuss the testing strategy with the responsible regulatory agency for scientific advice. Where it is not possible to use transgenic animal models of homologous proteins, it may be advisable to assess certain aspects of potential immunotoxicity in vitro using human material like peripheral blood mononuclear cells (PBMCs), monocyte-derived macrophages, or long-term cultivated cell lines of hematopoietic origin.

Most biotechnology-derived pharmaceuticals intended for human use are per se immunogenic in animals. The induction of antibody formation in animals is therefore not predictive of a potential for antibody formation in humans. In this regard, the results of, for example, a guinea pig anaphylaxis test, which is usually positive for xenogenic protein products, is not predictive for reactions in humans. Such studies are therefore considered of little value

for the routine evaluation of these types of products. It must be kept in mind that even humanized proteins may be immunogenic in humans. In most cases, reliable information on immunogenicity of biotechnology-derived drugs can therefore only be obtained during clinical studies. However, immunogenicity studies in animals using biotechnology-derived drugs may yield valuable information when comparing the immunogenic potential of a test compound with a biosimilar reference compound or between different production batches.

Even if immunogenicity assessment of biotechnology-derived pharmaceuticals has limited predictivity for the human situation, measurement of antibodies associated with the administration of biotechnology-derived drugs should always be included in the design of a repeat-dose toxicity study (Wierda et al., 2001; Vohr, 2005). Antibody responses should be characterized with regard to titer, number of responding animals, and neutralizing or nonneutralizing antibodies. Furthermore, the detection and quantization of antibodies should be correlated with any pharmacological and/or toxicological changes. Specifically, the effects of antibody formation on pharmacokinetic/pharmacodynamic parameters, incidence and/or severity of adverse effects, complement activation, or the emergence of new toxic effects should be considered when interpreting the data. Attention should also be paid to the evaluation of possible pathological changes related to immune complex formation and deposition.

9.8.4 Suggested Approaches to Evaluation of Results

Several rodent toxicity studies have shown impaired host resistance to infectious agents or tumor cells at exposure levels of drugs that did not cause overt signs of toxicity (Vos, 1977; Dean et al., 1982). One serious limitation to the incorporation of specific immunotoxicological evaluations into general use in safety assessment for pharmaceuticals is a lack of clarity in how to evaluate and use such findings. This problem is true for all new diagnostic techniques in medicine and for all the new and more sensitive tools designed to evaluate specific target organ toxicities. Ultimately, as we have more experience and a reliable database that allows us to correlate laboratory findings with clinical experience, the required course of action will become clearer. However, some general suggestions and guidance can be offered:

- First, it is generally agreed that adverse effects observed above a certain level of severity should be given the same importance as any other life-threatening events when assessing biological significance. These effects are so severe that they are detected as part of the routine evaluations made in safety assessment studies. Such findings may include death, severe weight loss, early appearance of tumors, and the like. Findings such as significantly increased mortalities in a host resistance assay would also fit into this category.
- Second, there are specific endpoint assays for which an adverse outcome clearly dictates the action to be taken. These endpoints include either

immediate or delayed hypersensitivity reactions because once the individual is sensitized, a dose–response relationship may not apply.

- Third, as with most toxicological effects, toxic effects to the immune system are dependent upon dose to the target site. The dose–response curve can be used to determine no-effect and low-effect levels for immunotoxicity. These levels can then be compared to the therapeutic levels to assess whether there is an adequate margin of safety for humans.

If we consider both the specific immunotoxicity assays surveyed earlier in the chapter and the arrays of endpoints evaluated in traditional toxicology studies, which may be indicative of an immune system effect, these guidelines leave many potential questions unanswered. As additional data on individual endpoints indicative of immune system responses are collected, the pharmaceutical toxicologist is challenged with various issues regarding assay interpretation and relevance to proposed (or future) clinical trials. For example, what do significant but non-life-threatening decreases in antibody response, lymphocyte numbers, macrophage functions, or host resistance in an animal mean about the clinical use of a drug in a patient? The intended patient population is clearly relevant here—if the disease is one in which the immune system is already challenged or incorrectly modulated, any immune system effect other than an intended one should be avoided. There are several additional considerations and questions that should be answered when evaluating the biological and clinical significance of a statistically significant immune response:

1. *Is there a dose response?* The dose response should be evaluated as a dose-related trend in both incidence and severity of the response. If there is a dose-related response, is the lowest dose (preferably plasma level) at which the effect is seen near or below the target clinical dose (plasma level), and is there an adequate therapeutic margin of safety?
2. *Does the finding stand alone?* Is a change observed in only one parameter, or are there correlated findings that suggest a generalized, biologically significant effect? For example, are there changes in lymph node and spleen weights and morphological changes in these tissues to accompany changes in lymphocyte numbers?
3. *Is the effect a measure of function or a single endpoint measurement?* Functional measures such as host resistance of phagocytosis involve multiple cells and immunocomponents and, therefore, are considered to be more biologically relevant than a significant change in a single endpoint measurement (e.g., T-cell number).
4. *Is the effect reversible?* Reversibility of a response is dependent on the drug itself, exposure levels/duration, and factors related to the test animal (metabolic capability, genetic susceptibility, etc.). Most effects produced by immunosuppressive drugs have been shown to be reversible after cessation of therapy, such as those produced during cancer chemother-

apy. However, if a tumor develops before the immune system is restored, the effect is not reversible, as is the case of secondary tumors related to chemotherapy.

5. *Is there sufficient systematic toxicity data available at levels that demonstrate adequate exposure?* If a study was designed such that there was insufficient exposure or duration of exposure to potential lymphoid target tissues, the test protocol may not be adequate to demonstrate an adverse effect.

In general, a well-conducted long-term study in two species with no indication of immunotoxicity, based on the considerations outlined above, should be adequate to evaluate the potential for drug-induced immunotoxicity. If the results from these studies do not produce evidence if immune-specific toxicity after examination of standard and/or additional hematological, serum chemical, and histopathological parameters, then additional testing should not be indicated. However, if there are structure–activity considerations that may indicate a potential for concern, of if significant abnormalities are observed that cannot be clearly attributed to other toxicities, then it is important to perform additional tests to fully assess the biological significance of the findings.

9.9 PROBLEMS AND FUTURE DIRECTIONS

There are some very pressing problems for immunotoxicology, particularly in the context of pharmaceuticals and biological therapeutics and the assessment of their safety. Unlike industrial chemicals, environmental agents, or agricultural chemicals, pharmaceutical products are intended for human exposure, are usually systemically absorbed, and have intentional biological effects on humans—some of which are intentionally immunomodulating (interleukins, growth factors) or immunotoxic (cyclosporin, cyclophosphamide).

Data Interpretation The first major issue was presented and explored in the preceding section. This is how to evaluate and utilize the entire range of data that current immunotoxicological methodologies provide to determine the potential for immunotoxicity and how to interpret the biological significance of minor findings.

Appropriate Animal Models As previously addressed, most routine preclinical toxicology tests are performed with rats and dogs; therefore, toxicity, pharmacokinetic, and pharmacology data are most abundant for these species. However, most immunological parameters are best characterized and validated with mice. In addition, the NTP test battery was developed for the mouse, and some of these assays cannot be readily transferred to the rat. Over the last few years, several laboratories have begun adapting tests to both the rat and the dog (Bloom et al., 1987; Thiem et al., 1988); however, efforts need

to continue along these lines to further our understanding of the immune responses in these species and how they correlate with other animal models and humans.

Indirect Immunotoxic Effects A problem related to data interpretation is how to distinguish secondary effects that may indirectly result in immunotoxicity from the primary effects of immunotoxicity in preclinical toxicity studies. Various factors may produce pathology similar to that of an immunotoxin, including:

- Stress in a chronically ill animal as related to general toxicity, such as lung or liver damage, can result in immune suppression.
- Malnutrition in animals with drug-induced anorexia or malabsorption can trigger immune suppression.
- Infections and/or parasites may also modulate immune parameters.

These indirect factors must be systematically ruled out, and additional mechanistic studies may be necessary to address this problem. The potential for some indirect effects may be assessed through histopathological evaluation of endocrine organs such as the adrenals and pituitary.

Hypersensitivity Tests Probably the largest immunotoxicity concern in clinical studies is unexpected hypersensitivity reactions. While the available guinea-pig- and mouse-based tests for delayed contact hypersensitivity resulting from dermal exposure are generally good predictors, there are currently no well-validated models for either immediate or (dermal) delayed hypersensitivity responses resulting from either oral ingestion or parenteral administration. Yet these two situations are the largest single cause for discontinuing clinical trials.

One assay that may hold some promise for delayed hypersensitivity is an adoptive transfer-popliteal lymph node assay (Klinkhammer et al., 1988). This assay, based on the techniques previously described for the popliteal lymph node assay, allows assessment of hypersensitivity following systemic exposure of the drug. Donor mice are first injected with drug for five consecutive days. After a four-week latency period, potentially sensitized T cells obtained from the spleen are injected into the footpad of a syngenic mouse together with a subcutaneous challenge dose of the drug. Two to five days after the cell transfer, the popliteal lymph nodes are measured and observed for evidence of a response (enlargement). Once this assay is validated, it should allow for a more relevant assessment of hypersensitivity for drugs that are administered systemically (Gleichmann et al., 1989).

Autoimmunity Traditional methods for assessing immunotoxicity as part of routine preclinical toxicity tests are primarily geared toward the detection of immunosuppressive effects. Although it is possible to incorporate clinical

methods for detecting immune complexes and autoantibodies into the preclinical test protocols, the significance of adverse findings is ambiguous. Since these effects have a genetic component to their expression, the relevance of findings in animals is of questionable significance, particularly since these findings in the clinic do not always correlate with pathological effects.

Functional Reserve Capacity As previously discussed, the immune system has a tremendous reserve capacity that offers several levels of protection and backups to the primary response. As a consequence, this functional reserve can allow biologically significant, immunotoxic insults to occur without the appearance of morphological changes. Furthermore, adverse effects may remain subclinical until the organism is subjected to undue stress or subsequent challenge. Thus, there is some concern that routine immunopathological assessments by themselves may not be sufficiently sensitive to detect all immunotoxins, particularly when testing is conducted in a relatively pathogen-free, stress-free laboratory environment.

Significance of Minor Perturbations Although the immune system has a well-developed reserve capacity, some of these systems may act synergistically rather than independently. For instance, a macrophage can recognize and kill bacteria coated with antibodies more effectively than can either the macrophage or antibodies alone. Thus, even minor deficiencies and impairments may have some impact on the organism's ability to fend off infection or tumors, particularly if the organism is very young, old, ill, stressed, genetically predisposed to certain cancers, or otherwise immunocompromised. These considerations lead to some additional questions that must be addressed:

- What level of immunosuppression will predispose healthy or immunocompromised individuals to increased risk of infections or tumors?
- Will slight disturbances or immunosuppression lead to a prolonged recovery from viral or bacterial infections?
- Will slight up-modulation for extended periods result in autoimmune diseases or increased susceptibility to allergy?
- Are individuals that are slightly immunosuppressed at higher risk of developing AIDS after exposure to HIV?

Biotechnology Products Immunotherapeutics such as interferons and interleukins hold tremendous promise for those diseases where malfunctioning of the immune system is not the root of pathogenesis. Likewise, many of the new approaches to therapy of yet untreatable diseases are aimed at modulating the body's own immune system. Many of the new therapeutics coming from biotechnology are proteins of human origin. As such, they can evoke antibody responses in nonhuman species that are not indicative of what will be seen in patients. Meaningful evaluations must allow the toxicologist to

discriminate between those responses that are relevant to clinical development/utilization and those that are not.

In summary, it is the role of preclinical immunotoxicology testing to allow us to identify potential immune hazards early in development, before they are found in the clinic, and to provide us with a mechanistic understanding for the basis of these effects so that we may direct the development of alternative agents and/or treatment regimens to avoid them. The challenge for the toxicologist is to determine the appropriate course of action for evaluating each unique drug and to differentiate the desired therapeutic effects from the undesired and potentially adverse effects.

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10

Nonrodent Animal Studies

10.1 INTRODUCTION

Most safety assessment studies are conducted in rodents (rats, mice, and hamsters) or their close "cousins" rabbits and guinea pigs. Outside of the pharmaceutical, medical device, and veterinary product industries, it has become rare for the practicing toxicologist to have close familiarity with the nonrodent animal species addressed in this chapter. Indeed, it is unlikely that a toxicologist has received any significant academic experience or training with these species. Additionally, use of these species in the European Union (EU) even for therapeutics has become very difficult at best. However, the proper use of nonrodent species is essential in the evaluation of potential new therapeutic entities, on both scientific and regulatory grounds. Indeed, there are numerous studies showing significantly better concordance between humans and nonrodents than humans and rodents for detection of adverse responses to pharmaceuticals (Olson et al., 2000). This is tacitly recognized in regulatory practice for in those new product development plans where a single species is deemed appropriate and sufficient (medical devices, protein therapeutic, and 505(b)(2) approval candidates), the single species is overwhelming a nonrodent.

In addition to rodent studies, regulatory guidelines for pharmaceuticals require that repeated-dose safety studies of up to nine months (in the United States, six months elsewhere) in duration be conducted in a nonrodent species. The most commonly used nonrodent species is the dog followed by an NHP

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(nonhuman primate) and then the pig. Another nonrodent model used to a limited extent in systemic safety evaluation is the ferret. The major objectives of this chapter are (1) to discuss differences in rodent and nonrodent experimental design, (2) to examine the feasibility of using the dog, NHP, pig, and ferret in safety assessment testing, and (3) to identify the advantages and limitations associated with each species.

10.2 COMPARISON BETWEEN RODENT AND NONRODENT EXPERIMENTAL DESIGN: NUMBER OF ANIMALS

One of the main differences in experimental design between rodent and nonrodent safety studies is the number of animals used (Table 10.1). In general, approximately nine times as many rodents are used in toxicity studies as compared to nonrodents. This difference is reflected in the 2005 estimates of overall usage of animals in the United States (10 million animals total) as published by the National Research Council (NRC, 1998), which showed that only about 10% of the animals used in general research for that year were NHPs (2%), pigs (1%), or dogs (7%). The smaller number of nonrodents used is related in part to the higher costs associated with their purchase, housing, and maintenance and in part to their limited use in other areas of research.

10.3 DIFFERENCES IN STUDY ACTIVITIES

10.3.1 Blood Collection

In rodent studies, large numbers of satellite animals (often close to the number used in the main study phase) are usually needed for pharmacokinetic blood sampling, whereas with most nonrodent species adequate blood samples can be collected from the main study animals without compromising their health status (Diehl et al., 2001).

TABLE 10.1 Comparison of Rodent and Nonrodent Experimental Design

	Total Numbers of Animals in Study (No./Group/Sex)			
Duration of Study	Rat	Dog	Pig	Monkey
4 weeks	360 (20)[a]	40 (4)	40 (4)	32 (4)
13 weeks	280 (20)[a,b]	48 (6)[b]	48 (6)[b]	48 (6)[b]
52 weeks/9 months	360 (10)[a,b]	64 (8)[b]	64 (8)[b]	48 (6)[b]

[a]Includes satellite animals for pharmacokinetic evaluation.
[b]Number of animals/group includes several animals.

10.3.2 Dosing

The most appropriate route of oral administration is probably capsule dosing for dogs and gavage for monkeys and ferrets. Nasal gastrogavage is also occasionally used for dogs and primates.

10.3.3 Handling of Animals

Once rodents are acclimated to handling, they are generally relatively easy to work with. In contrast, some nonrodent species, such as NHPs and pigs, are often difficult to handle because of their size, strength, emotionality, and aggressiveness. This can make the conduct of routine study activities [such as dosing, blood collection, and recording electrocardiograms (ECGs)] relatively time consuming as well as stressful to the animals.

10.3.4 Behavioral Evaluation

Behavioral assessment of nonrodents is generally more difficult than evaluation of rodents because of their larger size, difficulties associated with handling and manipulation, and greater awareness of and reactivity to the experimenter. Such factors can confound detection and/or interpretation of more subtle test compound–related behavioral changes. However, there is now a published and validated functional observations battery (FOB) for neurological exams of dogs and it is widely used (Gad et al., 2003).

10.4 NONRODENT MODELS

This section is devoted to the definition and comparison of the four nonrodent animal models (dog, ferret, pig, and NHP) in terms of experimental procedures, environmental and dietary requirements, as well as advantages and disadvantages of use in safety assessment testing.

10.4.1 Dog

Environmental and Dietary Requirements Typical housing for laboratory dogs consists of stainless steel or fiberglass cages (of dimensions appropriate to the dog's size) or indoor pens (typical dimensions are 3 ft, 8 in. wide, 8 ft high, and 10 ft long). Two important aspects of the laboratory dog's environment are the need for exercise and socialization. Recent amendments to the U.S. Department of Agriculture's (USDA's) Animal Welfare Act require that an exercise and socalization program be established for dogs maintained in a laboratory environment. Difficulty often arises in establishing a program that will be truly beneficial to the animals. One important consideration is whether dogs should be group or individually exercised. Studies have demonstrated that dogs exercised alone tend to spend most of their time walking or inves-

tigating the area rather than jumping or running (Campbell et al., 1988), which suggests that group exercise is more beneficial.

The need for a certain degree of socialization is also important, in terms of both dog–dog and dog–human contact. If at all possible, dogs should share a cage or pen with another animal. One study approach undertaken by some laboratories has been to allow dogs of the same sex and treatment group to have daily contact with each other, usually from early evening to early morning. If study dictates do not make this approach feasible, efforts should be made to ensure the animals are housed in such a way that they have visual, auditory, and olfactory access to each other.

Recommended dry-bulb temperatures and relative humidity ranges for dogs are 64.4–84.2 °F (16–27 °C) and 30–70%, respectively [U.S. Department of Health and Human Services (DHHS), 1985]. Increases in temperature and high humidity are of particular concern because of the dog's limited capacity to dissipate heat (primarily through panting and, to a lesser extent, through radiation and conduction). Dogs would likely not survive exposure for extended periods of time to environments where the temperature is in excess of 40 °C and 40% relative humidity (Norris et al., 1968).

While the dog is a carnivore, it is able to adapt to an omnivorous diet. Requirements for dietary sources of energy, amino acids, glucose precursors, fatty acids, minerals, vitamins, and water have been established based on recommendations by the NRC (1985). Adult beagles maintained in a laboratory environment function well with one feeding of standard laboratory chow per day. In safety assessment testing, however, some compounds may induce serious dietary deficiencies through induced loss of appetite, malabsorption, or vomiting, and, in these cases, it may be advisable to provide a dietary supplement.

The dog's requirement for water appears to be self-regulated and depends on factors such as the type of feed consumed, ambient temperature, amount of exercise, and physiological state; therefore, in most cases, dogs should have free access to water.

Common Study Protocols The dog is the most commonly used nonrodent species in safety assessment testing (i.e., acute, subchronic, and chronic studies and cardiovascular safety pharmacology studies). The exception to this is its use in developmental toxicity and reproductive studies. For developmental toxicity studies, the dog does not appear to be as sensitive an indicator of teratogens as other nonrodent species such as the monkey (Earl et al., 1973) or the ferret (Gulamhusein et al., 1980), and, for reproductive studies, the dog is not the species of choice because fertility testing is difficult to conduct (due to prolonged anestrus and the inability to predict the onset of proestrus) and there is no reliable procedure for induction of estrus or ovulation.

Examples of experimental designs and suggested timing of various study activities for 4- and 13-week dog studies are shown in Tables 10.2 and 10.3, respectively. Beagles are generally in the age range of 6–9 months at study

TABLE 10.2 Four-Week Dog or Primate Toxicity Study

Experimental Design

Four to five groups (including a control group)—4/sex/group main groups, plus extra in high-dose and control groups to evaluate recovery
Repeated daily dosing for 28 or 29 days
Necropsy starting on day 29

Study Activities

Daily observations: pretreatment and twice daily during study period
Physical examinations: pretreatment and after dosing during weeks 2 and 4
ECG: pretreatment and after dosing during weeks 2 and 4
Ophthalmic examinations: pretreatment and during week 4
Body weight: pretreatment, weekly, and prior to scheduled necropsy
Feed consumption: pretreatment and weekly
Clinical lab: twice before first dosing day, before dosing on day 2, during week 2, and prior to scheduled necropsy
Urine collection: pretreatment and during weeks 2 and 4
Pharmacokinetic: blood collected at specified times after dosing on days 1 and 28

Source: Adapted from a table in *Animal Models in Toxicology* (Gad, 2006).

TABLE 10.3 Thirteen-Week Dog Toxicity Study

Experimental Design

Four groups (including control group)—6/sex/group (plus 3/sex extra in high-dose and control groups for assessing potential recovery)
Repeated daily dosing for 91–93 days
Necropsy of main group (4/sex/group) on week 14
Necropsy of reversal group (2/sex/group) on week 18

Study Activities

Daily observations: pretreatment, twice daily during treatment, and once daily during reversal
Physical examinations: pretreatment, after dosing during weeks 4, 8, and 13 of treatment, and during week 4 of reversal
ECG: pretreatment, after dosing during weeks 4, 8, and 13 of treatment, and during week 4 of reversal
Ophthalmic examinations: pretreatment, during weeks 6 and 13 of treatment and during week 4 of reversal
Body weight: pretreatment (3 times), weekly during treatment and reversal periods, and prior to scheduled necropsy
Feed consumption: pretreatment, weekly through first month, bimonthly during remainder of treatment period, and weekly during reversal
Clinical lab: pretreatment, during weeks 4 and 8 of treatment, prior to scheduled necropsy, and during weeks 1 and 4 of reversal
Urine collection: pretreatment, monthly during treatment, and during week 4 of reversal.
Pharmacokinetic samples: blood collected at specified times after dosing on day 1 and during weeks 6 and 12

Source: Adapted from a table in *Animal Models in Toxicology* (Gad, 2006).

start, and the number of animals per sex per treatment group (*N*) will depend on the duration of the study. For a 2–4-week study without a reversal phase, *N* will likely be 4, whereas for a 26-week or 1-year study, *N* will be larger (e.g., *N* may be 9 including 3 per sex per group for the reversal phase); it should be noted that it has become a U.S. Food and Drug Administration (FDA) expectation for a high-dose and control group to include animals to allow the assessment of recovery.

Dogs should be selected for study use on the basis of acceptable body weights, urinalysis, and clinical pathology findings as well as physical, ophthalmic, and electrocardiographic evaluations. To minimize familial effects, efforts should be made to ensure that no two littermates of the same sex are assigned to the same treatment group.

Because most, if not all, study-related activities are conducted in the same dogs, the stress induced by repeated manipulation of dogs for activities such as blood collection, ECG, and physical examinations needs to be taken into consideration. Efforts should be made wherever possible to separate study activities by several days.

General Study Activities

Dosing Techniques The most frequently used route of administration in dog safety assessment studies is oral. Dosing by capsule is usually the preferred oral route in the dog. Gavage is also used, but it is a more labor intensive technique, and there is always the possibility of gavage error or aspiration. Since dogs have a natural tendency to vomit, it is recommended that they be sham dosed with empty capsules or gavaged with a water solution for several days prior to starting a study so that they can become acclimated to the dosing procedure. If the test substance in irritating (and therefore proemetic), the use of enterically coated capsules should be considered. Attention should be paid, however, to the time course of emesis. If it follows dose administration by more than 4–6 h, the cause will not have been due to gastrointestinal (GI) tract irritation, as the material will have already moved from the stomach.

Next to oral dosing, the most common dosing route for dogs is intravenous. For bolus or limited infusion intravenous dosing, the femoral, cephalic, and saphenous veins are commonly used. For continuous infusion, the jugular is often the vein of choice, and the procedure will require surgical preparation either for a direct line catheterization or subcutaneous insertion of a vascular access port (a rigid, multipuncturable reservoir equipped with an indwelling catheter).

Other routes of administration used less commonly in dog safety studies are subcutaneous, intramuscular, intraperitoneal, rectal, and vaginal and dermal.

Clinical Observations and Physical Examinations Daily clinical observations in dog safety studies, usually conducted pretreatment (prior to cage

cleaning) and at a specified time(s) after dosing, consist of a home cage observation with notation of clinical signs indicative of poor health (such as salivation, weight loss, abnormal feces, and vomitus) or abnormal behavior (such as reduced activity or increased aggression).

Physical examinations are conducted less frequently and generally involve the evaluation of gait, mobility, demeanor, and reflexes (pupillary light, corneal patellar, wheelbarrowing, hopping, etc.) as well as an examination of the head (eyes, ears, mouth, teeth, gums, and tongue), body (palpation for signs of masses and nodal swellings), and urogenital and anal regions.

Feed Consumption Feed consumption is relatively easy to measure in the dog since dogs do not usually spill much of their feed. Generally, the full feed bowl is weighed at the beginning and the empty bowl at the end of the feeding period (usually a 4-h period). This is repeated over two or three consecutive days and the average daily feed consumption is calculated from the numbers.

Electrocardiograms The collection of recorded ECGs in test dogs has become increasingly of interest due to the concurrence to the QT prolonguative by drugs and International Conference on Harmonisation (ICH)/FDA/European Medicines Agency (EMEA) mandated evaluation studies. Traditionally, a 10-lead system consisting of bipolar leads (I, II, III), augmented unipolar leads (aVR, aVL, and aVF), and unipolar precordial leads [V10, CV6LL(V2), CV6LU(V4), CV5RL(rV2)] has been recommended for dogs in the conscious state (Detweiler et al., 1979; Detweiler, 1980). For toxicity studies, dog ECGs are usually recorded by technical personnel and read at a later time by a veterinary cardiologist. Depending on the length of the study and the pharmacological–toxicological profile of the test compound, ECGs may be recorded as frequently as every day or as infrequently as every three months. Dog ECGs can also be highly variable. Factors that can affect the quality of the tracing include the positioning of the electrodes, the positioning of the dog, and the degree of nervousness and excitability of the animal. Conditioning the dogs to the electrode clips and the recording position (usually sphinx or right-lateral recumbency) during the pretreatment period will help improve the quality of the recording.

The use of surgically implanted sensors to be able to remotely monitor electrophysiology, blood pressure, and blood gases has become very common for cardiovascular safety pharmacology studies. It should always be the case using such dogs that a concurrent evaluation of baseline and vehicle effects be performed prior to that of acute drug grounds.

Blood and Urine Collection As mentioned previously, serial blood samples can be fairly easily collected from the dog. The jugular vein is probably the most commonly used vein because of its size and accessibility. Other veins used less frequently are the cephalic, femoral, brachial, and saphenous.

Due to the difficulty in obtaining sufficient volumes of urine in dogs over short collection periods, urine is usually collected overnight (approximately a 16–17-h period) in stainless steel metabolism cages. It is recommended that a sample for urinalysis be taken early in the collection process and that all samples be collected in light-resistant containers to help avoid problems such as dissolution of urine casts, increased bacterial activity, and breakdown of bilirubin with exposure of the sample to light.

Advantages and Disadvantages Some of the advantages and disadvantages of using the dog in safety assessment studies are listed in Table 10.4. With respect to its medium size and even temperament, the beagle is certainly a desirable nonrodent model. The relative ease in handling beagles makes them suitable for activities such as serial collection of blood samples and recording of electrocardiograms.

Disadvantages include an often wide variation in size and body weight and a loud, penetrating bark. The large amount of space required to house dogs and the current emphasis on regular exercise may also be disadvantages. Test compound requirements are generally higher for the dog than for either the NHP or ferret when these are alternative nonrodents species used in safety testing. This may be a problem in the early period of drug development when compound availability is often limited. Other problems center around the dog's tendency to vomit, which can be a disadvantage when compounds are orally administered, and the fact that, unlike rodents, studies requiring large numbers of dogs need careful advance planning to ensure that sufficient numbers of animals of the appropriate age can be obtained in a timely manner.

10.4.2 Ferret

The ferret, *Mustela putorius furo*, is a small carnivore that has become an increasingly popular species in various areas or research, including anatomy,

TABLE 10.4 Use of Beagle in Safety Assessment Studies

Advantages
Medium size
Moderate length of hair coat
Adaptability to living in group housing
Ease of handling (e.g., dosing, blood collection, ECG)
Disadvantages
Variation in size and body weight
Loud, penetrating bark
Greater test compound requirements than smaller nonrodent species
Availability
Exercise and housing requirements

Source: Adapted from a table in *Animal Models in Toxicology* (Gad, 2006).

virology, bacteriology, physiology (GI, pulmonary, and cardiovascular), pharmacology, neurology, teratology, and, to some extent, toxicology. The reader is referred to the excellent review by Fox (1988) on the biology and diseases of the ferret and to the chapter in Gad (2006) or the recent paper by Gad (2000a) on the ferret as an animal model in toxicology. Since 1990, the literature has reported on work done by Pfizer, Hoffman LaRoche, Gilead, Bristol Myers Squibb, Merck, Yamanouchi, Proctor and Gamble, Abbot, and Glaxo Wellcome using ferrets in pharmaceutical development. The ferret is also the species of choice for respiratory virus (especially influenza) efficacy studies.

Environmental and Dietary Requirements For reasons of environmental control, ferrets used in safety assessment studies should be housed indoors. It has been suggested that an optimal temperature range for the ferret is 40–65 °F (4–18 °C), while relative humidity should be maintained in the range of 40–65% (Fox, 1988). The ferret does not tolerate heat well due to its lack of well-developed sweat glands; the primary method of regulating heat loss appears to be through panting (Moody et al., 1985).

Since ferrets are seasonal breeders, the female being monestrus and an induced ovulator, the breeding cycle can be controlled by varying the length of exposure to artificial light. For safety studies, it is desirable to prevent both estrus in females and increased sexual activity in males; thus it has been recommended that the light period be kept short (Fox, 1988). In this laboratory, a 9-h light–15-h dark cycle has been used successfully for this purpose.

Ferrets should be housed in well-ventilated rooms that provide at least 10–15 air changes per hour. Good ventilation is important since ferrets are susceptible to respiratory viral infections. Additionally, there is a need to dissipate the musky odor of the animals. While housing standards for ferrets are not specified in the NRC (2004) *Guide to the Care and Use of Laboratory Animals*, space requirements of $49 \times 46 \times 46$ cm have been defined by other groups (Wilson and Donnoghue, 1982). Stainless steel cat or rabbit cages equipped with a drop pan to catch feces and urine are a suitable form of primary housing for ferrets. Ferrets are more content when they have access to a small secluded nesting area within their cage in which they can sleep, such as hammocks. The use of paper to line the cage or drop pan is not recommended, since the ferrets are likely to eat it. For socialization purposes, ferrets should be housed as a group or have visual access to neighboring ferrets if housed individually.

Since ferrets eat only their caloric requirements, and since their GI transit time is short (3–5 h), it is recommended that they receive diet ad libitum. Only one of the available standardized ferret chows commercially available should be fed. The most important dietary variable is the quality of the protein, and ferrets appear to do best with a high percentage of animal protein in their diet (Morton and Morton, 1985). Feed consumption will be higher in the fall and

winter and lower in the spring and summer. Hairball laxative is essential during the spring and summer months when the animals experience considerable hair loss. Water should be available at all times.

Study Protocols Historically, the ferret was used more often in teratology (Hoar, 1984), reproductive (Hoar, 1984), and acute safety studies than in repeated-dose studies (4–52 weeks in duration). This has changed since 2000, however, and the use of the ferret in pivotal repeated-dose safety assessment testing (Thornton et al., 1979; Hart, 1986; Haggerty et al., 1989) has increased, particularly for vaccines.

An example of the experimental design for a 4-week pivotal study in ferrets is shown in Table 10.5. Young adult ferrets are usually in the age range of 9–11 months at study start, and there should be sufficient numbers of animals in each group for statistical confidence (generally in the range of six to eight animals per sex per group reflecting the high degree of intergroup variability in the species). For longer term studies, the number of animals per group would be increased to include reversal group animals (three to four animals per sex per dose group).

Assignment of ferrets to a study should be based on evaluation of pretreatment clinical signs and body weights as well as physical, electrocardiographic, and opthalmological findings.

For longer term studies, females should be spayed to avoid the development of aplastic anemia, which will occur if the animals go into heat and are not bred (Morton and Morton, 1985).

As with dogs, efforts should be made to separate study activities as much as possible to minimize the stress of multiple activities being performed in the same animals.

TABLE 10.5 Four-Week Ferret Toxicity Study

Experimental Design
Five groups (including control group)—7/sex/group
Repeated daily dosing for 28 or 29 days
Necropsy starting on day 29
Study Activities
Daily observations: pretreatment and twice daily during study period
Physical examinations: pretreatment and after dosing during weeks 1, 2, and 4
ECG: pretreatment and after dosing during weeks 2, and 4
Ophthalmic examinations: pretreatment and during week 4
Body weight: pretreatment, twice weekly, and prior to scheduled necropsy
Feed consumption: pretreatment and weekly during the study
Clinical lab: pretreatment, week 2, and prior to scheduled necropsy
Urine collection: pretreatment and during weeks 2 and 4
Pharmacokinetic samples: blood collected at specified times after dosing on days 1 and 28

General Study Activities

Dosing Techniques Oral dosing of ferrets is usually done by gavage. One method is to hold the ferret perpendicular to the floor and insert the appropriate size stainless steel gavage needle into the animal's mouth, back into the esophagus, and down toward the stomach. Confirmation of correct positioning of the tube can be determined by visual inspection of the aspirate. As with dogs, ferrets have a tendency to retch or vomit, and daily gavaging with a water solution for several days prior to starting a study (for adaptation purposes) is recommended.

Repeated daily intravenous dosing in the ferret is generally considered to be technically difficult and time consuming; the use of an indwelling catheter is recommended (Moody et al., 1985). There are, however, reports in the literature of subchronic intravenous dosing (three times weekly for three months) of the ferret via the caudal vein (Mclain et al., 1987).

Dosing techniques such as intramuscular, intradermal, subcutaneous, and intraperitoneal administration can be used for the ferret. Care needs to be taken, however, when administering lipophilic compounds by the subcutaneous or intradermal routes to avoid inadvertently injecting compounds into the ferret's thick layer of subcutaneous fat, which can result in poor absorption (Moody et al., 1985).

Clinical Observations and Examinations Daily clinical observations will usually begin the week prior to study start and continue twice daily (pre- and postdosing) throughout the study. Ferrets are observed in their home cage for signs of physical debilitation (such as abnormal feces or vomitus), behavioral abnormalities, hair loss, swelling of the vulva (females), and testicular prominence (males). A physical examination should periodically be made and should include measurement of rectal temperature; observation of general demeanor and activity; palpation of the head, thorax, and abdomen; examination of eyes, ears, and body orifices; and testing of the pupillary and patellar reflexes.

Feed Consumption Feed consumption can be measured over two to three consecutive days and the average daily intake calculated. A problem with measuring feed intake in ferrets is their tendency to dig through their feed bowl, which often results in an unacceptable amount of spillage. Use of a feed follower may help reduce the spillage.

Electrocardiograms Most electrocardiographic evaluation in the ferret has been previously done in the anesthetized animal, though sensors may be surgically implanted. This allows electrocardiograms to be recorded using the limb (I, II, and III) and augmented (aVR, aVL, and aVF) leads. It is quite possible to obtain fairly good quality ECGs in the conscious ferret using leads I, II, and III. The standard position used for recording ECGs in the conscious or anesthetized ferret is right-lateral incumbency. ECGs have also been measured in

the ferret using surface electrodes placed between two points on the chest, with the signals being led off to an amplifier by a long, flexible cable and recorded on magnetic tape for later analysis (Andrews et al., 1979). The advantage of such a system is that the animals are allowed to move freely during the recording.

Blood and Urine Collection About 5–10 mL of blood can be collected from adult ferrets using retroorbital blood collection techniques. Other methods of blood collection include cephalic and jugular veins and caudal tail venipuncture as well as bleeding via the ventral tail artery. Cardiac puncture is also used, but in the opinion of this and other (Hart, 1986) laboratories, the procedure is traumatic and can cause myocardial scarring. Blood collection from the tail can be difficult because the ferret tail is short and the tail veins and arteries cannot be seen. For all the above-mentioned collection techniques, some form of pharmacological or mechanical restraint is required. To facilitate serial blood collection, methodology has been developed for a tethered restraint system with an implanted indwelling venous jugular catheter, which does not interfere with the normal activities of the ferrets and allows blood sampling to occur from outside the cage (Jackson et al., 1988).

For urine collection, glass or plastic rat metabolism cages work well for short-term or overnight collection. Care needs to be taken to avoid contamination of the urine with feces.

Advantages and Disadvantages Two advantages to using the ferret are its cost and its size (Table 10.6). The cost of the ferret is approximately one-tenth that of the dog. The ferret's smaller size means that it is easier to maintain and more economically housed and fed than the dog (Hart, 1986). Smaller size also means that test compound requirements for the ferret will be considerably less than those for larger nonrodent species (e.g., on the order of one-tenth of that needed for the dog). Another advantage is that if exercise

TABLE 10.6 Use of Ferret in Safety Assessment Studies

Advantages
Small size
Significantly lower cost than most other nonrodents
Lower test material requirements (relative to larger nonrodents)
Adaptability to exercise program
Disadvantages
Pervading musky odor
Rodents' inherent fear of ferrets
Can be difficult to handle
Background disease profile with resulting "background noise" and increased variability in clinical and anatomical pathology

requirements are ever established for the ferret, it will be an easier species than the dog for which to design an acceptable exercise program.

Disadvantages associated with the ferret include its pervading musky odor and its background disease profile. While the scent glands can be removed, 90% of the animal's odor is derived from sebaceous secretions onto the skin. However, neutering the males and spaying the females, in addition to descenting, will markedly reduce the odor. Rats should be housed as far away from ferrets as possible because of their inherent fear of ferrets (triggered by olfactory stimulation), which can interrupt breeding cycles or disturb other physiological functions (Fox, 1988). In this laboratory's experience, ferrets, which are generally less docile than dogs, can be difficult to handle and prone to bite, especially when restrained for activities such as ophthalmic and ECG examinations. The lack of easily accessible veins for intravenous dosing and serial blood collection is also a disadvantage.

The major disadvantage in the use of the ferret in safety studies is the profile of diseases associated with the species and the resulting variability in background clinical and anatomical pathology. Pneumonitis and hepatic lymphoid accumulation, associated with chronic parvovirus infection, have been observed in ferrets in this laboratory (Haggerty et al., 1989). Submucosal lymphoid nodules of the intestines are also a common finding (Hart, 1986). Additionally, a relatively high incidence of electrocardiographic (atrial or ventricular premature depolarization, atrial and ventricular extrasystoles) and ophthalmological (optic nerve hypoplasia and cataracts) anomalies have been found in ferrets in this laboratory. While it may be possible to work with the animal suppliers to reduce the chances of receiving animals with background ECG or ocular abnormalities, at the present time there is no supplier of a disease- and viral-free ferret.

10.4.3 Pig

Background The use of pigs (*Sus scrufa*) in biomedical research is well established. In toxicology, whereas the use of pigs in the United States is largely limited to dermal studies, in Europe they have become very popular for pharmaceutical studies in place of dogs and primates. They have been extensively used for surgical (Swindle et al., 1988) and physiological (primarily cardiovascular, renal, and digestive) research (Khan, 1984; Clausing et al., 1986) for years. Until relatively recently, their use in toxicity testing was uncommon except in the testing of veterinary or herd management drugs intended for use in swine or in dermal toxicity and absorption studies. Because of their well-accepted physiological similarities to humans, minipigs are becoming increasingly attractive toxicological models (Table 10.7). In fact, they are already more frequently used in nutritional toxicology studies (Clausing et al., 1986). Among the more common experimental animals, pigs are the only ones whose use is on the increase (Khan, 1984). Their expense (both in procurement and maintenance) and their relatively large size have mitigated against their

TABLE 10.7 Minipig in Toxicity Testing

Due to many advantages, mini- and micropigs are real alternatives to use of nonrodents (dogs, ferrets, and primates)
Minnesota minipig introduced in 1949
Body weights at age 2 years
Yucatan minipig: 70–90 kg
Yucatan micropig 40–45 kg
Göttinger micropig: 35–40 kg
Use in general toxicity testing and reproduction, teratological and behavioral toxicity (aspects of public acceptance as a species for testing)

TABLE 10.8 Main Advantages of Minipig

Similarity to humans in
• Cardiovascular anatomy and physiology
Ventricular performance
Electrophysiology
Coronary artery distribution
• Human skin
Thickness and permeability
Pigmentation
Allergic reaction
Reaction to burning and distress
• Gastrointestinal system and digestion
• Renal system
• Immune system (FDA: “better than rodents”)
• P450 total enzyme activity (especially CYP2E1, CYP3A4)

use in more general toxicity testing. The development of minipigs has resulted in a strain of more manageable size. In addition, the increase in expense in the use of dogs as well as the perceived lay opposition to their uses make minipigs (in Europe and Israel) even more attractive as a nonrodent species for general toxicity studies. The dog is a far more common companion animal and many of the recent developments in animal care and use laws have made specific provisions about the care of dogs. Minipigs have been shown to be more sensitive to a wide variety of drugs and chemicals (e.g., carbaryl, methylmercury) than dogs (Khan, 1984). The FDA has kept its own breeding colony of minipigs since the early 1960s. In short, there are scientific, economic, and sociological reasons that make minipigs good toxicological models. The reader is referred to an excellent short review by Phillips and Tumbleson (1986) that puts the issue of minipigs in biomedical research into the context of modeling in general. Table 10.8 presents the advantages of the minipig.

Several breeds of miniature swine have been developed. In the United States, these include the Yucatan micro- and minipigs, the Handford, the Sinclair, the Pitman-Moore, and the Hormel. The Yucatan and the Sinclair tend to be the most commonly used, though the Göttinger (widely used in Europe) is seeing increasing use (Ellegaard et al., 1995). Panepinto and Phillips (1986) have discussed the characteristics, advantages, and disadvantages of the

Yucatan minipig in some detail. In Europe, the Göttinger minipig is extensively used. At sexual maturity (4–6 months) the typical minipig weighs 20–40 kg, as compared to 102 kg for the more common pig, 8–15 kg for the dog. Micropigs weigh about 14–20 kg at sexual maturity. The minipig and the dog have comparable life spans; for example, Peggins et al. (1984) reported that the average life span for miniature swine is 15–17 years. The average beagle dog may have a life span of 8–12 years. Most of this discussion will focus on the purpose-bred minipigs, primarily the Yucatan and the Sinclair.

The greatest area of use of the pig in pharmaceutical safety assessment is for dermal agents. It should be noted that there are differences in skin thicknesses in different species, and these differences are not well characterized.

Husbandry

Housing A general review of handling and husbandry is given by Panepinto (1986) and Swindle et al. (1988). Young weanling pigs can be kept for short periods of time (up to one month) in standard dog cages with the floor modified with narrow mesh to account for the smaller foot of the pig. After that, however, their rapid growth generally makes such caging inappropriate. Larger stainless steel cages would be extremely expensive. Standard dog runs could have enough floor space to be converted for pigs, but smooth flooring does not provide appropriate footing for pigs and needs to be covered with wood chip bedding (Swindle et al., 1988). Although pigs are very social, they do not have to be group housed, as discussed by Barnett and Hensworth (1986); individually housed swine show little evidence of a chronic stress response. Insufficient space, on the other hand, can cause chronic stress in pigs. Hunsaker et al. (1984) have described an inexpensive caging system for miniature swine that is appropriate for toxicology studies. The flooring and walls are constructed of 0.50-cm welded wire coated with polyvinyl chloride polymer. As described, the unit has sufficient room for two pigs separated by a partition. These units are relatively inexpensive and provide more than sufficient floor space (about 17 ft^2 per pig) to meet the recommendations for pigs.

Water and Feed Like all animals, pigs should be permitted free access to potable water, preferably from a municipal water supply intended for human consumption. Drinking water intended for pigs does not have to be filtered or deionized. Various diets have been described. Because of their size (i.e., high maintenance charges and test article demands), pigs have seldom been used for chronic studies where the possibility of waterborne environmental contaminants could influence a study.

For miniature swine, the consistent use of a certified chow from a major manufacturer is recommended (Swindle et al., 1988). Free access to feed is not recommended as pigs will eat to excess and grow quite large. Available feed should be restricted to approximately 4% of body weight per day to prevent the animals from becoming obese.

Restraint and Dosing In general, minipigs are docile and easily socialized and trained. Barnett and Hensworth (1986) recommended a socialization regimen of 2 min of gentle interaction (e.g., striking). Pigs, like most experimental animals, are rarely simply kept and fed but have to be occasionally restrained so samples can be taken and other measurements made. Restraint methods designed for commercial swine should not be used for laboratory swine. Panepinto (1986) have described a sling method that provides restraint with minimal stress. The most frequently mentioned dosing routes in the literature are dietary admix, dermal (topical), gavage, and intravenous injections. Generally, minipigs are restrained in a sling while beginning dosing by the active route such as gavage. If the experiment requires the implantation of, for example, an indwelling catheter, minipigs can be anesthetized with ketamine ($20\,mg\,kg^{-1}$ IM) as described by Swindle et al. (1988).

Clinical Laboratory Clinical chemical and hematological parameters for minipigs have been studied. Ranges for some of the more commonly examined parameters from Yucatan minipigs are summarized in Tables 10.9 and 10.10 (from Radin et al., 1986). Parsons and Wells (1986) have published similar data on the Yucatan minipig. Brechbuler et al. (1984), Oldigs (1986), Ellegaard et al. (1995), and Koch et al. (2001) have published on the Göttinger minipig. Middleton and co-workers have published extensive lists (organized by age and sex) on the hematological parameters (Burks et al., 1977) and serum electrolytes (Hutcheson et al., 1979) for the Sinclair minipig. In general, the clinical laboratory picture of the various strains are quite similar. No real

TABLE 10.9 Minipig Clinical Chemistry Parameters in Different Strains

Parameter	Yucatan	Göttinger
Glucose ($mmol\,L^{-1}$)	3.75 ± 0.64	5.98 ± 1.01
Urea ($mmol\,L^{-1}$)	7.84 ± 2.64	3.19 ± 1.15
Creatinine ($\mu mol\,L^{-1}$)	115 ± 16	52.2 ± 11.1
Total protein ($g\,L^{-1}$)	74 ± 9	54.0 ± 4.6
Albumin ($g\,L^{-1}$)	50 ± 6	26.2 ± 6.0
Bilirubin total ($\mu mol\,L^{-1}$)	3.42 ± 1.37	—
Triglycerides ($mg\,L^{-1}$)	267 ± 134	565 ± 250
Total cholesterol ($mmol\,L^{-1}$)	1.85 ± 0.38	1.65 ± 0.38
γ-Glutamyl transpeptidase ($U\,L^{-1}$)	61.6 ± 11.2	—
Alanine aminotransferase ($U\,L^{-1}$)	72.5 ± 13.6	—
Aspartate aminotrasnferase ($U\,L^{-1}$)	40.3 ± 5.9	—
Na^+ ($mmol\,L^{-1}$)	140.5 ± 4.2	142.3 ± 3.00
K^+ ($mmol\,L^{-1}$)	4.1 ± 0.3	3.94 ± 0.32
CL^- ($mmol\,L^{-1}$)	103.1 ± 4.3	101.3 ± 3.6
Ca^{2+} ($mmol\,L^{-1}$)	2.62 ± 0.18	2.58 ± 0.16
PO_4^{2-} ($mmol\,L^{-1}$)	2.41 ± 0.26	1.61 ± 0.30

Note: Data are mean ± SD. From Parsons and Wells, 1986; Brechbuler, Kaeslin, and Wyler, 1984; Oldigs, 1986.

TABLE 10.10 Minipig Hematological Parameters in Different Strains

Parameter	Yucatan	Göttinger
Red blood cells ($10^6\,mm^{-3}$)	7.61 ± 0.15	7.0 ± 0.80
Hemoglobin ($g\,dL^{-1}$)	14.87 ± 0.18	14.9 ± 1.20
Hematocrit (%)	44 ± 0.5	44.6 ± 4.1
Mean corpuscular volume (fL)	58.5 ± 0.8	64.4 ± 3.7
Mean corpuscular hemoglobin (pg)	19.8 ± 0.3	21.4 ± 1.3
Mean corpuscular hemoglobin concentration ($g\,dL^{-1}$)	33.9 ± 0.3	33.2 ± 0.8
White blood cells ($10^3\,mm^{-3}$)	12.73 ± 0.41	12.6 ± 3.0
Lymphocytes ($10^3\,mm^{-3}$)	7.25 ± 0.24	5.75 ± 1.52
Neutrophils (mm^{-3})	4.47 ± 0.24	5.27 ± 1.29
Eosinophils (mm^{-3})	534 ± 57	517 ± 31
Monocyte (mm^{-3})	422 ± 35	945 ± 71
Basophils (mm^{-3})	89 ± 15	63 ± 1.3
Platelets ($10^3\,mm^{-3}$)	—	441 ± 119

Source: From Burks et al. 1977 (12 months old, sexes pooled); Radin et al., 1986.

differences between sexes have been identified, but age can be very much a factor. For example, serum creatinine can be 33% higher in a three-month-old as compared to 18-month-old Sinclair minipigs (based on data reported by Burks et al., 1977). As with other species, health status, feed composition, feeding regimen, fasting state, season, time of day, and so on, can affect clinical laboratory results in the minipig. Toxicological experiments should not be run without concurrent controls.

Xenobiotic Metabolism Some critical parameters of hepatic microsomal drug metabolism in the minipig, common swine, and rats are given in Table 10.11. As most investigators tend to use younger minipigs, the values reported in this table are for young (less than four-year-old) minipigs. Relatively few papers have examined the mitochondrial mixed functional oxidase (MMFO) in a broad age range (10 months to 12 years) of Hanford minipigs. They identified definite age-related differences. The amounts of cytochrome P-450 (CYP), the MMFO activity with aniline and *p*-chloro-*N*-methylaniline, and glucoronosyl transferase activity were all significantly higher in middle-aged (5–8-year) versus young (less-than-4-year) minipigs. Freudenthal et al. (1976) examined Hanford minipigs in the age range of 2–8-months and obtained somewhat different CYP (approximately $0.95\,nmol\,mg^{-1}$) values than did Peggins et al. (1984) (approximately 0.50 nmol). The reported ranges for aniline hydroxylase (about $0.70\,nmol\,min^{-1}\,mg^{-1}$) and uridine 5′-diphospho (UDP)-glucoronosyl transferase (about $50\,nmol\,min^{-1}\,mg^{-1}$) were similar in the two papers. Hence, the available data on the MMFO of young Hanford minipigs are fairly consistent. CYP isoenzymes 1A, 2A, 2B, 2C, 2D, 2E, and 3A have all been well characterized in the pig (Gad, 2006).

The flavin adenosine dinucleotide (FAD) containing monooxygenase (FMFO) has traditionally been studied in hog liver obtained from slaughter-

TABLE 10.11 Comparison of Xenobiotic Metabolism Systems in Rat and Pig

Enzyme	Rat[a]	Minipig[b]	Common swine[a]
Cytochrome P-450[c]	0.59 ± 0.04	0.95 ± 0.02	0.30 ± 0.04
MMFO activity[d]			
Ethylmorphine	5.09 ± 0.34	8.53 ± 0.51	1.39 ± 0.16
Ethoxyresorufin	0.134 ± 0.022		0.88 ± 0.02
Epoxide hydrolase styrene oxide	8.36 ± 2.48	—	11.4 ± 1.67
UDP-glucoronosyl transferae			
1-Naphthol	6.43 ± 1.66	—	5.50 ± 0.89
4-Nitrophenol	4.51 ± 0.50	5.5 ± 1.5	9.38 ± 1.07
Glutathione *S*-transferase			
DNCB	2659 ± 168	—	2746 ± 499
DCNB	118 ± 8.8	—	2.44 ± 0.23
PAPS sulfotransferase			
2-Naphthol	0.785 ± 0.066	—	0.095 ± 0.025
Acetyltransferase			
p-Aminobenzoate	0.77 ± 0.23	—	0.621 ± 0.111

[a]From Mueller et al., 1980; Smith et al., 1984; Watkins and Klaassen, 1986.
[b]From Freundenthal et al., 1976; Peggins et al., Shipley, and Weiner, 1984.
[c]nmol/mg microsomal protein.
[d]All enzyme activities; nmol/min/mg (either microsomal or cytosolic) protein.

houses (Tynes and Hodgson, 1984). Interestingly, when FMFO activity is compared between species, substrate specificities are found to be generally very similar (Tynes and Hodgson, 1984). Rettie et al. (1990) isolated and studied the FMFO from Yucatan minipig liver. As with the enzyme studied from other species, the hepatic enzyme exists as a single isozymic species, is active with both dimethylanaline (*N*-oxide formation) and alkyl *p*-tolyl sulfides (sulfoxidation), and is enantioselective in metabolite formation. It would thus appear that the minipig does not differ appreciably from regular swine in the presence or activity of FMFO.

Perhaps some aspects of minipig xenobiotic metabolism can be inferred from studies in regular swine. For example, Rendic et al. (1984) demonstrated that cimetidine and ranitidine are excellent inhibitors of the porcine MMFO in vitro and is probably also inhibitory in microsomal preparations from minipigs (Van Ryzin and Trapold, 1980). Walker et al. (1978) reported on epoxide hydratase activity in various species, including the pig. Depending on the substrate, the pig had activities equivalent to or greater than that of the rat. This was confirmed by Smith et al. (1984) and Watkins and Klaassen (1986). The MMFO, epoxide hydrolase, UDP-glucoronosyl transferase, *N*-acetyl transferase, glutathione *S*-transferase, and sulfotransferase activities in regular swine may be used to help infer the expected activity in minipigs until more complete and specific information appears in the literature on minipigs.

There are relatively few papers that compare in vivo pharmacokinetic behavior of a specific chemical in the minipig versus another animal. Schneider et al. (1977) reported on the toxicology and pharmacokinetics of cyclotri-

methylenetrinitramine in the rat and minipig. Rats convulsed within the first several hours after receiving this chemical, whereas minipigs convulsed 12–14 h later. This is consistent with the observation that at 24 h postdosing (100 mg kg^{-1} PO), the plasma levels were 3.0 μg mL^{-1} in rats and 4.7 μg mL^{-1} in minipigs. Other differences in pharmacokinetics and metabolism between the two species were described. The latent period for convulsion development was more similar between minipigs and humans than between rats and humans. The implication in this paper is that the minipig is a more suitable model for the study of the toxicity and metabolism of the nitramines than rats.

Dermal Toxicity Although rabbits are commonly used for the assessment of primary (and even cumulative) dermal irritation, pigs are now generally considered to be better models for the more sophisticated study of dermal permeability and toxicity. As reviewed by Sambuco (1985), human and porcine skin are similar with regard to sparsity of the pelage, thickness and general morphology, epidermal cell turnover time, size, and orientation, and distribution of vessels in the skin. The particularly thin haircoat and lack of pigments of the Yucatan minipig makes it particularly ideal for dermal studies. The size of the animal also provides the additional practical advantage of abundant surface area for multiple site testing.

Sambuco (1985) has described the sunburn response of the Yucatan minipig to ultraviolet (UV) light, suggesting that this species would also make a good model in phototoxicity as well as photocontact dermatitis studies. Thirty 12-cm sites were demarcated, permitting the study of 15 different dermal dosages of UV radiation.

Mannisto and co-workers (1984) have published a series of articles on the dermal toxicity of the anthralins in the minipig. In one experiment, 24 sites per minipig were used to assess the acute dermal irritation of various concentrations to four different chemicals per site. The range of concentrations tested permitted them to calculate the median erythema concentration and median irritation concentration with relatively few animals. They were able to show clear differences between anthralin congeners (antipsoriatic drugs) with regard to irritation. When compared to other species (mouse and guinea pig) the response of the minipig was the most similar to humans in that in both species these chemicals are delayed irritants, and several days postexposure may pass before the maximal irritant response is presented.

Likewise, Hanhijarvi et al. (1985) studied the chronic, cumulative dermal effects of anthralin chemicals in minipigs. Using only 12 animals they were able, by having 32 sites per animal, to study the effects of two different chemicals (dithranol and butantrone, both anthralins) in three different formulations at three different concentrations each. The protocol also included observations for systemic toxicity, clinical laboratory measurements, plasma drug analyses, and gross and histopathological examinations.

In a third report (very similar to the second), Hanhijarvi et al. (1985) clearly demonstrated that the type of vehicle can greatly influence irritation in that

dithranol was clearly more irritating when applied in paraffin that when applied in a gel. They were also able to demonstrate that although dithranol was less irritating than butantrone acutely, the cumulative irritations (mean scores at the end of six months of six times per week applications) were quite similar (Mannisto et al., 1984). There was no evidence of systemic toxicity or of test article in plasma with either species.

Cardiovascular Toxicity In general, the published literature consistently maintains that the cardiovascular systems of swine and humans are very similar. For example, as reviewed by Lee (1986), swine, including minipigs, have a noticeable background incidence of atherosclerotic lesions and swine fed high-lipid diets will develop even more extensive atherosclerotic lesions. High-lipid diets will produce lesions similar to advanced atheromatous lesions seen in humans. Although few drugs or chemicals have been shown to cause atherosclerosis, this information has three general applications to toxicology and pharmacology. First, the feeding regimen of minipigs should be carefully controlled in general toxicity studies to minimize the incidence of arterial disease, especially in long-term studies. Second, the pathologist should be aware of the natural background of this disease when preparing a diagnosis. Third, the minipig could provide a convenient model for the study of atherosclerotic disease and the screening of potential therapies.

The minipig has been used to study cardiotoxicity, particulary with medical device and drug/device combination products. Van Vleet et al. (1984) reported that minipigs were the only species studied other than dogs to develop cardiac damage in response to large doses of minoxidil. In both pig and the dod, minoxidil cardiotoxicity is characterized by vascular damage (with hemorrhage in the arterial epicardium) and myocardial necrosis (mostly of the left ventricular papillary muscles). Interestingly, in the dog the atrial lesion is largely restricted to the right atrium, whereas in the pig it is restricted to the left atrium. These lesions can be produced in roughly 50% of the minipigs given $10\,mg\,kg^{-1}$ of minoxidil for two days and sacrificed 48 h after the last dose (Herman et al., 1988, 1989). Herman and colleagues have published extensive descriptions of minoxidil-induced lesions in minipigs in comparison to those produced in dogs (Herman et al., 1988, 1989). The right versus left arterial difference is believed to be due to differences in the anatomical pattern of coronary circulation between two species (Herman et al., 1988).

Minipigs are also sensitive to the cardiotoxic effect of doxorubicin. When given six intravenous injections of either 1.6 or $2.4\,mg\,kg^{-1}$ of doxorubicin at three-week intervals, minipigs develop cardiac lesions similar to those seen in dogs, rabbits, and other experimental animals (Herman et al., 1989). The lesion is characterized by cytoplasmic vacuolation and varying degrees of myofibrillar degeneration and loss. Thus, the minipig is sensitive to the cardiotoxic effect of two well-known and extensively studied chemicals. Therefore, it is a suitable nonrodent species for the general assessment of cardiotoxicity.

Advantages and Disadvantages There is one disadvantage to the use of minipigs: their size. Although minipigs are smaller than regular swine, at maturity they are generally larger than beagle dogs. Among the advantages are the facts that they are long-lived, cooperative animals with well-defined physiological and metabolic characteristics. As they are not either popular companion animals (like dogs) or physically resemble humans (like monkeys), minipigs are not specifically discussed in animal "welfare" laws like the other two species. Depending on their final form, new animal welfare regulations could make the space and maintenance costs for dogs and monkeys very prohibitive. This may make minipigs increasingly more attractive as a nonrodent species for general toxicity testing.

10.4.4 Nonhuman Primates

Nonhuman primates are often the nonrodent species of choice for safety assessment studies. There are over 500 species of NHPs that differ widely from each other in size and physical characteristics. Most of the monkeys used in experimental research belong to the suborder Anthropoidea and especially to the superfamilies of Ceboidea (marmoset, squirrel monkey) and Cercopitcoidea (macaque, papio species, rhesus). These have been popular because of (1) assumed better concordance of effects seen to those in humans and (2) smaller weights (and therefore reduced compound requirement). However, predominant factors leading to a decision whether or not to select primates as the nonrodent species for safety evaluation are summarized as follows (Hobson, 2000).

Primates Are Selected for Safety Studies Because

- They are the only species which exhibit the human response to the test article.
- Due to smaller body size they conserve rare or expensive test articles.
- They do not form neutralizing antibodies to the test article.
- They are physiologically more similar to humans.
- Regulatory agencies require their use.
- Prior development history dictates species choice.
- Known class effects have previously been seen in primates.

Primates May Not be Selected for Safety Studies Because

- Perceived expense
- Facility and logistic concerns
- Limited supplies
- Biosafety concerns
- Perceived animal rights or animal welfare concerns or pressures

- Tradition and prior development history
- Regulatory agency direction
- Data suggesting that other animal models are the "most sensitive species"

Tradition and cost are the two most frequently quoted reasons for selecting dogs as the second toxicology species instead of NHPs. Many pharmaceutical companies, especially those that primarily work with small molecules, have many years of background data in dogs and do not choose to venture into primate research without a compelling reason to do so. Secondary concerns often center on perceived biosafety or animal rights issues. Contrary to conventional wisdom, primate studies are often more cost efficient than studies in dogs. Although the purchase cost for primates is approximately twice that of dogs, many other factors suggest that the total cost of a primate safety study may be less than the cost for a similar-sized dog study. Husbandry costs are higher in dogs because of the USDA requirement for exercise. Approximately four-fold more building space is required for a dog study due to the larger cages needed. Perhaps most importantly, the smaller body size of macaques means that the requirement for expensive or scarce test articles in a primate study is approximately a third of that of a dog study.

COMPARISON OF COSTS FOR TYPICAL 90-DAY STUDIES CONDUCTED WITH NHPS OR DOG

Animal cost (assume 40 animals)	
Cost of dogs	$40 \times \$900 = \$36{,}000$
Cost of primates	$40 \times \$2200 = \$88{,}000$
Per diem	
Dogs	$90 \text{ days} \times \$11 \times 40 = \$39{,}600$
Primates	$90 \text{ days} \times \$7 \times 40 = \$25{,}200$
Test article (at $\$400\,mg^{-1}$)	
Dogs	$10\,kg \times 90 \text{ days} \times 40 \text{ animals} \times 100\,\mu g\,kg^{-1}\,day^{-1} = \$1{,}440{,}000$
Primates	$4\,kg \times 90 \text{ days} \times 40 \text{ animals} \times 100\,\mu g\,kg^{-1}\,day^{-1} = \$576{,}000$

Clearly, the amount and cost of the test article and the length of the study determine which species is most cost efficient.

Because NHPs are phylogenetically closer to humans than other species, there is less chance that they will recognize human protein, peptide, or antibody-based biopharmaceuticals as foreign. Thus, they are often selected for safety studies of these materials. Although highly conserved proteins may not be immunogenic in lower species, clearly the formation of neutralizing antibodies to less conserved proteins during a safety study can confound experimental results (Dean et al., 1990). The formation of neutralizing antibodies to human biopharmaceuticals almost never occurs in chimpanzees (which no longer may be used in pharmaceutical research) but occurs more

and more frequently as the primate phylogenetic tree is descended. It is generally believed that the NHP phylogenetic difference from humans is ranked as follows: great apes, baboons, other Old World primates (including macaques), and New World primates. Clearly, as proteins are modified, they can become immunogenic in all primate species, including humans.

The physiological similarity and phylogenetic proximity of NHPs to humans are often cited as a rationale for primate selection for safety studies, especially when mechanisms of toxicity or pharmacological action are expected to be closely related to potential physiological reactions in humans. Likewise, species selection is often based on the demonstration of pharmacological activity of the test article. Many biopharmaceuticals do not exhibit their intended activity in nonprimate species, whereas small molecules may have activity across all species.

Regulatory agencies sometimes suggest (read "dictate") use of primates for certain study designs or drug classes. These requirements are often a surprise to companies when they are first presented. Usually they are derived from confidential data that the regulatory agencies have previously reviewed. Often the regulatory bodies are privy to data that suggest that a class effect is seen in primates and not in other species or that primates are the most sensitive species. An example was regulatory agency encouragement to perform cardiovascular evaluations of oligonucleotide pharmaceutical candidates in primates (Black et al., 1993, 1994). This was based on background information that suggested that oligonucleotides induced complement activation and the attendant hemodynamic and cardiovascular changes in primates but not in other species (Galbraith et al., 1994).

Animal welfare and conservative issues have frequently led to decisions to avoid primate use. Through the mid-1980s many NHPs used in medical research came from wild populations. This led to strong conservationist concerns with the use of monkeys in research. Now, however, almost all NHPs used in research are purpose bred and the conservationist concern has abated. Although there is some animal rights pressure specifically directed against primate use, it is not as formidable as the well-financed and sophisticated efforts to prevent the use of cast-off dogs (pound or shelter dogs or dogs from other class B sources in research). As a consequence, a few pharmaceutical companies are considering switching to NHPs for their second toxicology species.

Environmental and Dietary Requirements For most NHP species, room temperatures should be maintained in the range of 75 ± 5 °F with a relative humidity of 40% or greater. These temperature and humidity ranges have been found to be beneficial to the prevention of pneumonia and bloody nose syndrome. Rooms in which monkeys are housed should have 10–15 air changes per hour and be kept under negative pressure in relation to other parts of the building. Where there is significant risk of airborne infection, it is necessary to contain infected animals in units designed to remove the air away from personnel (Mazue and Richez, 1982).

Physical comfort should be an important consideration when determining the appropriate housing for NHPs. For individually housed NHPs, the floor area and height of cages should be about $0.28\,cm^2 \times 76.20\,cm$ for animals in the weight range of 1–3 kg and $0.40\,cm^2 \times 76.20\,cm$ for monkeys weighing 3–10 kg (DHHS, 1985). Probably the most common form of commercially available housing is mobile stainless steel rack–mounted cages. Group housing of NHPs used in safety studies is likely to become more common in the future as a result of the USDA current animal welfare regulations, which require that NHPs have the opportunity for socialization.

Another requirement of the new animal welfare regulations is that any cage or pen in which NHPs are housed must also contain toys, food, or other objects that animals can manipulate as they would objects in their natural environment. From experience, laboratories have found that toys in themselves are not sufficient since the animals quickly lose interest. Effective enrichment materials include foraging boards (fur-covered objects under which food is buried) and puzzle feeders for more complex foraging.

In many laboratories, monkeys are often fed commercial pelleted chow ad libitum supplemented with fresh fruits and bread. Like the human and guinea pig, the monkey cannot synthesize vitamin C and, thus, has a dietary requirement for this vitamin. Powdered chow is an inefficient form for feeding NHPs because a high percentage of the diet is wasted. Also, dust associated with the chow can cause respiratory problems in some species (NRC, 1978). Even with pelleted or extruded food, monkeys will waste about 50% of the ration sorting through the pellets (Mazue and Richez, 1982). Monkeys should have ad libitum access to water, and it is important that the device (either a water bottle equipped with a sipper tube or an automatic watering system) be fixed securely to the cage to avoid detachment by the animal.

Common Study Protocols Group sizes and numbers of animals per group for NHP toxicology studies vary slightly from country to country and from company to company; however, with the movement for international harmonization there is trend toward less variation in study design. Selection of group size is a compromise among regulatory guidelines, cost, statistical power, and conservation of animals.

An example of a protocol for a four-week safety study in cynomolgus monkeys is shown in Table 10.2. Cynomolgus monkeys are generally in the age range of one to three years at study start. A two- or four-week study will usually have about four animals per sex per group, not including the mandatory extra animal for evaluating recovery. For the longer term studies, the number of animals per group will be larger in order to include reversal animals. As with dogs and ferrets, monkeys should be selected for study use based on acceptable pretreatment body weights, clinical laboratory profiles, and physical, ECG, and ophthalmic examinations.

One aspect of study design in NHPs that is not well understood is caused by the variability in the age at which monkeys undergo puberty. Although age

at the onset of puberty is highly variable within macaque species, there is a remarkable correlation between body weight and sexual maturity in macaques. *Rhesus* females undergo menarche at 3000 ± 200 g irrespective of age, whereas males tend to become sexually mature around 4500 g. This means that a "typical" study is initiated with sexually mature females and sexually immature males. This practice is debatable and is certainly not universally adopted. A few pharmaceutical companies require mature animals of both sexes. Because sexual maturation in males occurs many months later than in females, rearing costs are higher for males and animal numbers may be limited because the younger males may have already been sold with their female birth-year counterparts.

General Study Activities

Common Dosing Techniques Dosing routes and permissible volumes for NHPs vary between laboratories. The volume limitations from our laboratory are presented in Table 10.12.

Primates offer all of the possible dosing routes available in humans, but body size often limits dosing volumes. If volumes for subcutaneous or intramuscular injections exceed those suggested above, enzyme elevations [particularly alanine aminotransferase (ALT) and aspartate aminotransferase (AST)] are frequently observed (unpublished results). Continuous infusion techniques in alert animals are available in some laboratories either through use of programmable backpack pumps or jacket-and-tether systems (Perkin and Stejskal, 1994).

Probably the most common oral route is gavage. This procedure usually requires some degree of physical restraint of the animal (by one or more persons) while a stomach tube for dosing is inserted either orally or intranasally. Other oral dosing methods include buccal, capsule, or addition of the test compound to the drinking water. It is also possible to prepare a modified diet admixture consisting of test compound, diet meal, water, agar, and a jelling agent. This type of preparation will reduce both the feed spillage and the dust normally associated with powdered chow; however, it is susceptible to microbial growth and must be kept frozen or refrigerated (NRC, 1978).

Bolus intravenous, intramuscular, or subcutaneous injections can be administered by a single person by securing the animal's arm through the cage bars

TABLE 10.12 Permissible Dosing Volumes for Nonhuman Primates

Route	Maximum Permissible Dose[a]
Intravenous	Varies with duration of administration and character of test article
Subcutaneous	2 mL site^{-1} and 5 mL kg^{-1}
Intramuscular	0.25 mL site^{-1} and 0.5 mL kg^{-1}
Oral/nasogastric	5 mL kg^{-1}

[a]Values are given for single or infrequent administration. Smaller volumes are appropriate for repeated dosing.

(Mazue and Richez, 1982). For safety considerations, many investigators prefer to have the animal physically restrained by a second person before the injection is given. Arterial injections (via the femoral artery) as well as limited or continuous intravenous infusions (via catheterization of the femoral or jugular vein) are other less commonly used parenteral routes in the monkey.

Other routes of administration sometimes used in monkey safety assessment studies are intravaginal dosing, topical application, inhalation, and nasal administration.

Clinical Observations and Examinations As with other species, it is important to have a good understanding of the types of normative behaviors and clinical signs that can be seen in normal, untreated monkeys before attempting to make observations in drug-treated animals. Cage-side observations in the monkey should be conducted at least two times daily to monitor general health and behavior. The first observation should be made before cages are cleaned in the morning, and the floors of the cages should be critically examined for signs of blood, abnormal feces, or vomitus. Clinical signs to which investigators should pay particular attention include reduced activity and lethargy, excessive excitation, reduced feed consumption, vomiting, and abnormal feces. If at all possible, the same people should work on a study for its entirety. The behavior of more timorous monkeys can be affected by the presence of unfamiliar personnel, resulting in undesirable clinical signs such as a loss of appetite and lethargy (Evans et al., 1982). To circumvent these kinds of problems, isolated observation using a video camera system may be a preferable approach.

Physical examinations of monkeys are usually conducted no more than once a week and generally consist of the measurement of rectal temperature, observation of general demeanor, palpation of the head, thorax, and abdomen, examination of eyes, ears, and bodily orifices, as well as testing of the pupillary and patellar reflexes.

Feed Consumption As mentioned previously, monkeys tend to scatter their feed, which can make feed consumption difficult to measure. It may be possible to successfully monitor feed consumption in monkeys by using the larger chow biscuits and counting the number of biscuits (or fractions of biscuits) consumed over two consecutive 24-h periods.

Electrocardiograms and Cardiovascular Measurements The availability of excellent good laboratory practices (GLP)–validated telemetry systems has led to recent increases in the number of cardiovasular safety pharmacology studies conducted in primates. In addition, telemetry is now sometimes included as a design element in standard safety studies. Because of the ability to collect large amounts of high-quality data over an extended time, total numbers of animals can often be reduced by appropriate application of telemetry. Indeed, it is often difficult to avoid statistical and reporting problems

caused by the temptation to collect too much data using telemetry. Implanted transmitters can function continuously for up to a year without battery replacement while providing data such as blood pressure, heart rate, ECG, body temperature, and activity.

For safety assessment studies, it is preferable to record monkey ECGs in the conscious animal, which, if using standard ECG techniques, requires chairing the animal. Electrocardiographic leads used in this laboratory include II, aVL, and V10. To help reduce emotional tachycardia, it is recommended that there be pretreatment habituation (no more than 10–30 min at least twice before study start) to the chairing and attachment of the surface electrodes. Probably the best and least stressful approach to monitoring ECG activity in conscious monkeys is automatic monitoring using a biotelemetry system. With this system, a transmitter surgically implanted subcutaneously along the dorsal midline broadcasts a radio signal encoding the ECG to a receiver mounted on top of the animal's cage and a computer records the signal at 2-min intervals (Line et al., 1989).

Blood and Urine Collections For blood collection, the rhesus can be bled from the saphenous or femoral vein. For female rhesus monkeys, it may not be possible to use the saphenous vein because of the swelling of the sex skin (i.e., the edematous thickening and reddening of the skin over the external genital region, rump, and tail that often extend down the leg to the knee). For the cynomolgus and squirrel monkeys, the veins are very small, and the femoral vein is usually the one of choice. Depending on the species, 2 (marmoset) to 24 (cynomolgus) milliliters may be collected. However, experimental designs in primates are often constrained by the limitations in the amount of blood that can be safely and humanely obtained during the course of a study (Fuller et al., 1992). With increasing emphasis on obtaining toxicokinetic data during safety studies, these constraints have become more vexing. A guideline for maximum blood withdrawal is 10 mL kg^{-1} day^{-1} (Heiser, 1970). More blood can be collected, but hematocrits should be monitored (Schalm, 1975). These amounts do not approach maximum amounts allowable for humane considerations but do represent the maximum that can be collected without causing more than slight decreases in hematological parameters (notably hematocrit, hemoglobin, and red cell count). Currently used catheter material and vascular access ports long-term frequent blood collection without the catheter clotting and emboli problems experienced in the past. The new vascular access ports remain patent for over a year with routine maintenance. Vascular access ports are particularly useful in primates where frequent samples are required because blood sample collection through ports appears to be far less stressful than collection by needle stick. We have also found them useful when evaluating anticoagulant test articles because venipuncture is contraindicated. Sample quality is also superior with ported collections.

Urine collection in NHPs can be measured using either a metabolism cage or a collection pan (equipped with a screen to catch the feces), which is

inserted under the floor grid of the home cage. The advantage of the latter system is that the animals do not need to be removed from their home cage; however, care needs to be taken to avoid contamination of the urine with drinking water.

Advantages and Disadvantages Advantages of using NHPs in safety assessment studies include their phylogenetic proximity as well as their physiological, behavioral, and, often, metabolic similarities to humans (Table 10.13). An example is the similarity between the ovarian cycle of female monkeys and women (Mazue and Richez, 1982), which makes the monkey the ideal animal model for reproductive studies. Another advantage associated with most species of monkeys used in safety assessment studies is that they are much smaller than nonrodents such as the dog and thus, like the ferret, require less test compound.

The most significant disadvantage to working with monkeys is the serious spontaneous diseases they can carry that are transmissible, and often life threatening, to humans. An example of one such disease is herpesvirus simiae (B virus). B virus is widespread, especially among wild-caught and to some extent laboratory-bred rhesus monkeys, including cynomolgus monkeys. Human exposure to B virus occurs during handling of monkeys and monkey tissues (via contact with tears, blood, or saliva of infected animals) and is associated with a high incidence of human mortality (DiGiacomo and Shah, 1972). Other serious to very serious diseases that can be transmitted from monkey to human are Marburg disease, viral hepatitis, tuberculosis, and monkeypox.

10.5 STATISTICS IN LARGE-ANIMAL STUDIES

Large-animal toxicology studies, typically ranging from 14 days to generally a maximum of 52 weeks, pose different types of statistical problems and open up new possibilities in terms of statistical evaluations. Standard statistical

TABLE 10.13 Use of Nonhuman Primate in Safety Assessment Studies

Advantages
Small size of many species
Less test material needed than for other nonrodent species
Physiological, behavioral, and, often, metabolic similarities to humans
Disadvantages
Limited availability
Cost
Need to develop environmental enrichment program
High potential for spontaneous diseases

methods used for chronic toxicology studies, such as one-way designs, often do not provide any meaningful insights because of small sample sizes used in large-animal studies. The designs for such studies are, generally speaking, nonoptimal. As a consequence, an investigator must attempt to use optimal statistical methods to evaluate such studies. Fortunately, for many of the relevant parameters for such studies, there are fewer to no dropouts (if one is careful) and there are repeated measurements on the same parameters of interest, at both pre- and posttreatment intervals. Optimality of statistical methods for such studies is then achieved by making use of the longitudinal observations in the analysis. The optimality can be further enhanced by introducing sex as a factor in the evaluation of the data in many such studies.

Many of the standard assumptions in both parametric and distribution-free statistical methods cannot be meaningfully tested in large-animal studies because of extremely small sample sizes (which is dictated not necessarily by scientific doctrine but by economic and minimum regulatory requirements). Fortunately, by making use of solid biological as well as statistical judgments, we seem to have made many discoveries in terms of human safety and efficacy in large-animal toxicology.

Instead of conventional textbook-type layout, this discussion will try to focus on various issues in large-animal toxicology experiments with plausible examples. One word of caution before we get deeper into our discussion: As in most areas of applied statistics, there really is no gospel in what we will be discussing today. Many statisticians may have variations of the theme to be brought out here.

10.5.1 Reasons for Small Sample Sizes in Large-Animal Toxicology

The following are some of the main reasons for having only three to five dogs or monkeys per sex in a typical large-animal study:

1. These studies are very expensive. A typical full-fledged study may cost as much as $30,000–$500,000 (for 26 weeks).
2. There is tremendous pressure from animal rights groups to look for alternatives, rather than using dogs and monkeys for investigative purposes.
3. Regulatory agencies throughout the world recognize these two facts and recommend such small sample sizes as minimum requirements. As a consequence, the pharmaceutical and chemical industries are reluctant to expand the scopes of such studies.

10.5.2 Cross-Sectional or Longitudinal Analysis?

Many of the studies we deal with have various parameters, such as body weight, food consumption, clinical chemistry, and hematology, that are collected repeatedly at various pre- and posttreatment intervals. Unfortunately, many investigators in the field do not take advantage of this important design

feature of such studies. Instead, the literature is full of simple parametric or distribution-free one-way techniques such as Student's *t* test, Wilcoxon–Mann–Whitney rank test, and one-way analysis-of-variance (ANOVA) methods that are being widely used sometimes without satisfaction. The argument then is given that "although there is apparent biological effect (or lack of it), because of small sample sizes and poor statistics, no significant effects can be determined from these data," or something like that. If truth be known, the small-sample-size part of this arguments may be correct; however, no attempts were made to optimize the statistical methods above using the various pieces of the particular design. The repeated-sampling part of the design (repeated measures) is very important for such studies and therefore should be incorportated in the analysis of the data. After all, design of experiment and analysis of data are inseperable. There are advantages and disadvantages of such analyses (the advantages generally outweigh the disadvantages) as described below.

10.5.3 Repeated Measures

Advantages

1. Between-subject variations are excluded from the experimental and stochastic errors.
2. Only the within-subject variation is included in the mean-square error (MSE) term.
3. Each subject becomes its own control.
4. The number of subjects in an experiment are kept to a minimum.
5. Both type I (false-positive) and type II (false-negative) error rates are minimized, thereby increasing power of the test statistic to be employed while decreasing inconsistent significant effects.

Disadvantages

1. Order of the treatment may cause interference, which can be avoided by appropriate randomization.
2. There is the possibility of carry-over effects. This is more crucial in Latin square and other cross-over designs. Knowledge of pharmacokinetics and metabolism of a compound under study generally helps in avoiding this problem.
3. Exact permutation and distribution-free techniques are not as widely developed as in the cases of one-way methods.
4. Power and sample size computations are a little more difficult to compute than for one-way designs.
5. Generally computers are required for performing the analyses using specialized software (not a major issue in most societies nowadays).
6. The results are a little more difficult to interpret than their one-way counterparts.

10.5.4 Common Practices in Large-Animal Toxicology

Older (and some newer) literature in large-animal toxicology is full of two-sample, one-way parametric, and distribution-free techniques. Some of the newer works use repeated-measures and even multivariate techniques. The following is a brief exposé of various methods used in the field:

1. One-way analysis of variance/covariance/regression and preplanned and post hoc group comparisons.
2. Two-sample Student's *t* test, Wilcoxan–Mann–Whitney rank test, and so on
3. Graphical display of response over time (as two- or three-dimensional plots)
4. Univariate repeated-measures analysis of variance/covariance techniques
5. Multivariate analysis of variance/covariance (MANOVA/MANCOVA) techniques

Methods 1 and 2 above should not be preferred in global analyses. Graphical displays have tremendous values as exploratory data analysis (EDA) techniques with the type of data one encounters in these studies. For formal analyses, one could weigh univariate repeated and other factorial designs against their true multivariate counterparts.

10.5.5 Univariate (Repeated-Measures) Techniques

Advantages

1. Easier to compute
2. Less susceptible to violation of normality
3. Exact and distribution-free tests easier to compute
4. Require smaller sample sizes; there is more power
5. Very few test statistics to deal with: classical ANOVA *F*; Greenhouse–Geisser and Huynh–Feldt adjusted degrees of freedom (df), and ANOVA *F*
6. Biologically meaningful and easier to resolve contrasts and multiple comparison tests
7. Missing values easily handled

Disadvantages

1. Susceptible to heteroscedasticity (heterogeneity of variances)
2. Less fancy compared to multivariate techniques

10.5.6 Multivariate Techniques

Advantages

1. Less susceptible to heteroscedasticity
2. Handles multiple dependent variables
3. Real fancy compared to univariate ANOVA/ANCOVA techniques

Disadvantages

1. More susceptible to violation of normality
2. Less power than univariate ANOVA, particularly with small sample sizes
3. Contrasts and multiple comparisons difficult to construct
4. Missing values more difficult to handle
5. Computationally more difficult (a mute point nowadays with personal computers)
6. Too many test statistics to deal with, sometimes giving contradictory answers

10.5.7 Some Other Design Factors to be Considered in Analysis

Most of the toxicological studies are designed to evaluate efficacy and safety in both sexes. With small sample sizes, one can increase the power efficiency of the particular test statistic by including sex as a factor in a full factorial analysis (not combining the two sexes) where appropriate. The factorial analysis will reveal whether there is any need to separate the two sexes. The other design fact that should be weighed carefully is the presence of any concomitant variables or covariates. For example, most large-animal studies will involve collection of data both prior to the beginning of the experiments as well as after. Thus pretreatment values and other characteristic control variables (e.g., body weights) may be important covariates in the analysis of the data. There are both advantages and disadvantages in including covariates in the analysis:

Advantages

1. Increases precision of an analysis (indirect or statistical control of variability)
2. Correction of bias
3. Elimination of extraneous variation in the data

Disadvantages

1. Unequal intra- and intergroup covariate slopes—may actually introduce bias as a consequence.
2. Nonlinearity of covariate slopes—may have the same effect as above.
3. In some cases the covariates may be affected by treatment.

An example is shown in Table 10.14. A two-factor ANOVA for the covariate, as shown in Table 10.15, clearly indicates that the two sexes started with approximately the same means ($p = 0.5598$). Moreover, there were no differences between the group means in either sex, as indicated by the large tail probabilities for treatment ($p = 0.8823$) and sex–treatment interaction ($p = 0.6532$). These facts justify using sex as a factor in the analysis, as was done here.

There are various other ways of examining the variate in question in this case. Let us first examine a simple one-way ANOVA of the variate by sex as in Table 10.16. In neither of the two cases was there any indication of significant treatment differences at any reasonable level. Because the two sexes did not show any pretreatment differences based on the two-factor analysis of the covariate, let us combine the two sexes and analyze the data by one-way ANOVA as in Table 10.17. In this case, because of the increased sample sizes for combining the two sexes, there was an indication of some treatment differences ($p = 0.0454$). Unfortunately, this analysis assumes that because there was no pretreatment difference between the two sexes, that pattern will hold

TABLE 10.14 Example 1

	Control		Treatment 1		Treatment 2	
Sex	Covariate	Variate	Covariate	Variate	Covariate	Variate
Male	40	95	30	85	50	90
	35	80	40	100	40	85
	40	95	45	85	40	90
	50	105	40	90	30	80
	45	100	40	90	40	85
Raw mean	42.0	95.0	39.0	90.0	40.0	86.0
SD	5.7	9.4	5.8	6.1	7.1	4.2
Female	50	100	50	100	45	95
	30	95	30	90	30	85
	35	95	40	95	25	75
	45	110	45	90	50	105
	30	88	40	95	35	85
Raw mean	38.0	97.6	41.0	94.0	37.0	89.0
SD	9.1	8.1	7.4	4.2	10.4	11.4

TABLE 10.15 Two-Factor Analysis of Variance for Covariate

Source	Sum of Squares	DF	Mean Squares	*F*	Tail Probability
Mean	46,807.50000	1	46,807.50000	785.58	0.0000
Sex	20.83333	1	20.83333	0.35	0.5598
Treatment	15.00000	2	7.50000	0.13	0.8823
Sex × treatment	51.66667	2	25.83333	0.43	0.6532
Error	1,430.00000	24	59.58333		

TABLE 10.16 One-Way Analysis of Variance of Variable of Sex

Source	Sum of Squares	DF	Mean Squares	*F*	Tail Probability
		Males			
Mean	122,401.66667	1	122,401.66667	2576.88	0.0000
Treatment	203.33333	2	101.66667	2.14	0.1603
Error	570.00000	12	47.50000		
		Females			
Mean	131,227.26667	1	131,227.26667	1841.36	0.0000
Treatment	186.53333	2	93.26667	1.31	0.3061
Error	855.20000	12	71.26667		

TABLE 10.17 One-Way Analysis of Variance for Combined Sexes

Source	Sum of Squares	DF	Mean Squares	*F*	Tail Probability
Mean	253,552.13333	1	253,552.13333	4549.999	0.0000
Treatment	387.26667	2	193.63333	3.47	0.454
Error	1,504.60000	27	55.72593		

TABLE 10.18 Two-Factor Analysis of Variance with Sex as Factor

Source	Sum of Squares	DF	Mean Squares	*F*	Tail Probability
Mean	253,552.13333	1	253,552.13333	4269.75	0.0000
Sex	76.80000	1	76.80000	1.29	0.2667
Treatment	387.26667	2	193.63333	3.26	0.0559
Sex × treatment	2.60000	2	1.30000	0.02	0.9784
Error	125.20000	24	59.38333		

during the posttreatment period. That often may not be the case because of biological reasons.

The above analysis establishes that there was no significant sex difference, as indicated by the tail probabilities for sex ($p = 0.2667$) and sex–treatment interaction ($p = 0.9784$). There was also some indication that there may have been some treatment effect across the treatment groups in both sexes ($p = 0.0559$) (Table 10.18). Examination of the variate means indicated that both sexes seemed to have lower means than their respective controls. The picture was clouded by the fact that there was such a slightly lower tendency, though not very consistent, in the covariate means as well. Under this circumstance, it is more appropriate to take both the covariate and the variate into any optimal analysis. Table 10.19 shows an ANCOVA for the factorial model.

As the ANCOVA table indicates, there was definite significant treatment effect ($p = 0.0104$), but this effect was not sex specific because there was no significant sex–treatment interaction ($p = 0.7613$). Furthermore, there was a signficinat difference between the two sexes in terms of magnitude but not in

TABLE 10.19 Analysis of Covariance of Factorial Model

Source	Sum of Squares	DF	Mean Squares	*F*	Tail Probability
Mean	147.42310	1	147.42310	5.65	0.0262
Sex	292.81064	2	146.40532	5.61	0.0104
Treatment	14.41235	2	7.20617	0.28	0.7613
Sex × treatment	824.75245	1	824.75245	31.59	0.0000
Error	600.44755	23	26.10642		

	Adjusted Cell Means and Standard Errors		
	Control	Treatment 1	Treatment 2
	Males		
Mean	93.10140	90.37972	85.62028
Standard error	2.30985	2.28601	2.28601
	Females		
Mean	98.73916	92.86084	90.89860
Standard error	2.29398	2.29398	2.30985

the direction of the effect. These findings are apparent in the covariate-adjusted means in all groups in both sexes. The magnitude of the treatment effect became amplified by introducing the covariate in the model. As can be seen from the two ANOVA and ANCOVA tables above, despite the fact that the ANCOVA error term lost one degree of freedom (df = 23) as opposed to the ANOVA error term (df = 24), the former gains some edge over the latter because of increased precision. Precision in this context is defined as the ratio between the MSEs of ANOVA and ANCOVA. For this example:

$$\begin{aligned}\text{Precision} &= \frac{1/\text{MSE}_{\text{ANCOVA}}}{1/\text{MSE}_{\text{ANOVA}}} \\ &= \frac{\text{MSE}_{\text{ANOVA}}}{\text{MSE}_{\text{ANCOVA}}} \\ &= \frac{59.38333}{26.10642} \sim 2.3\end{aligned}$$

In other words, we have gained about 2.3-fold precision by ANCOVA over ANOVA in resolving treatment effect.

With the advent of powerful personal computers and the availability of sophisticated "do-it-all" statistical packages, there is a trend among nonstatisticians (even some statisticians) to accept the results from these packages without contemplating twice. Many of these packages have flexible features that allow one to perform different types of analyses with the same data set, inappropriately or appropriately sometimes. What popular statistical packages give is not necessarily correct statistics or they may not be correct under spe-

cific designs. Some programs, for example, BMDP's 2V (1992), have "intelligence" built into them whereby they can identify the design based on the data matrix. By correctly following the data matrix setup specified in the manual, one can simply press the button and get the appropriate analysis needed. On the other hand, incorrect specification of the data matrix will produce incorrect results (although some programs, such as 2V, will often give an error message or prompt to make sure one wants what one is asking for; some, such as SAS's PROC GLM, may not and give results that are not even remotely related to the design). In other words, one must know some statistics and must be well versed in the features of the particular package before using it. The one-time famous mathematician–statistician–composer–pianist–singer–producer–recording artist Tom Leher (1959), in one of his famous monologues, said, "Life is a sewer; what one gets out of it depends on what one puts into it." Statistical packages are exactly like that.

10.5.8 Missing Values

All investigators know that missing values are a nuisance. They also create statistical nightmares. Classical statistical techniques were not geared toward having missing values in experiments. Unfortunately, in real-life situations, it just happens. Animals may die or are censored for various reasons. There are various techniques of calculating missing values for specific designs (Miller, 1981) just like there are for extreme values or outliers [Statistical Analysis System (SAS), 1996]. In neither case is there any unique way of handling them that is completely agreed upon by statisticians. One should remember that every time a missing value is computed and used in statistical analyses, one loses a degree of freedom. In large-animal toxicology, with small sampling sizes, one must be very careful about dealing with missing values. In a repeated-measures analysis, if one observation is missing from an animal during one interval, classical techniques automatically will exclude observations from that animal for all remaining intervals. Newer techniques based on regression or imputation have been developed in recent years and have been implemented in popular packages such as BMDP (5V) or SAS (PROC MIXED). Within a single package, there may be various techniques based on assumptions on covariance structures (unstructured, compound symmetry, etc.) and statistical algorithms (maximum likelihood, restricted minimum likelihood, etc.). The results sometimes could be very different under the same assumptions and algorithms. As a result, given the same compound symmetry assumption and using the same restricted maximum-likelihood (REML) algorithm, two well-known programs give different quantitative results. These methods are still experimental in nature and should not be taken for granted. Actually, the BMDP manual clearly warns users about the nature of this method. Consequently, the best way to avoid confusion is to try to make sure that missing values do not occur in key parameters in *large-animal* studies (Thakur, 2000).

10.6 SUMMARY

While there are advantages and disadvantages associated with all three nonrodent species, the dog is probably the nonrodent species most frequently used in safety assessment studies. This is because dogs are relatively docile and even tempered, they are generally more easy to obtain and relatively less expensive than monkeys, they carry less serious diseases than the ferret and the monkey, and they have a more extensive historical database in safety studies. It should be noted, however, that if the technical and health problems associated with the ferret can be overcome, its small size in terms of compound requirements, cost, and housing may make it an ideal nonrodent species for future use in safety assessment studies.

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11

Developmental and Reproductive Toxicity Testing

11.1 INTRODUCTION

The goal of testing the developmental and reproductive toxicity of drug candidates in laboratory animals is to predict which agents would adversely affect the ability to achieve and maintain pregnancy and for normal development of offspring in humans and to allow evaluation of the potential risks to patients. This testing involves an extensive battery of studies based historically on guidelines promulgated by the U.S. Food and Drug Administration (FDA) in 1966 (see FDA, 1966, 1982, 1984; D'Aguanno, 1973) and subsequently modified by the International Conference on Harmonisation (ICH). These guidelines established three basic types of studies, segments I, II, and III, that are based on dosing during sequential phases of the reproductive cycle. These guidelines represented a dramatic increase in the extent and sophistication of testing expected of new drug candidates. The impetus for this intensified interest was the tragic epidemic of phocomelia and other congenital malformations caused in the early 1960s by the exposure of pregnant women to the sedative thalidomide. (For an excellent discussion of the history of the thalidomide tragedy, see pp. 228–249 in Schardein (1993)]. Table 11.1 presents the most recent guidelines.

The types of developmental and reproductive toxicity studies performed prior to 1993 and the methods used have been extensively documented (see Palmer, 1981; Christian, 1983; Heinrichs, 1985; Heywood and James, 1985;

Drug Safety Evaluation, Second Edition, by Shayne Cox Gad

TABLE 11.1 Current Regulatory Guidelines—ICH, FDA: Medical Agents

ICH(2000)	Detection of toxicity to reproduction for medicinal products (ICH S5)
ICH(2000)	Detection of toxicity to reproduction for medicinal products (ICH S5)
FDA	International Conference on Harmonisation: Guideline on detection of toxicity to reproduction for medicinal products. *Fed.* Reg., September 22, 1994, Vol. 59, No. 183
FDA	International Conference on Harmonisation: Guideline on detection of toxicity to reproduction for medicinal products; Addendum on Toxicity to make fertility. *Fed.* Reg., April 5, 1996, Vol. 61, No. 67

Persaud, 1985; Schardein, 1988; Tyl, 1988; Christian and Hoberman, 1989; and Manson and Kang, 1989; Khera, 1991). Since June 20, 1979, the FDA has required that these studies be conducted according to good laboratory practice (GLP) regulations (see FDA, 1978, 1987). The conduct of these studies had been complicated by the need to satisfy worldwide regulatory guidelines that varied from country to country. As a result, studies were conducted for regulatory purposes that, from a scientific viewpoint, were redundant, superfluous, and/or unnecessarily complex. This situation was changed in 1993 when the International Conference on Harmonisation of Technical Requirements for the Registration of Pharmaceuticals for Human Use standardized worldwide requirements in the guideline "Detection of Toxicity to Reproduction for Medicinal Products" (ICH, 1994, 2000a, b).

This chapter briefly describes the current standard study designs and then focuses on current issues in developmental and reproductive toxicity testing (also see Hood, 2006).

11.2 ICH STUDY DESIGNS

The ICH S5 A and B guideline allows for various combinations of studies. The studies conducted must include evaluation of the following components:

1. Male and female fertility and early embryonic development to implantation
2. Embryo–fetal development
3. Pre- and postnatal development, including maternal function

These components would normally be evaluated in a rodent species (preferably the rat), and, in addition, embryo–fetal development would be evaluated in a second species, typically the rabbit. The "most probable option" in the ICH guideline is the case where three rodent studies would be conducted that separately addressed each of the components listed above. These study designs are described below. Table 11.2 presents a comparison of ICH, FDA, European, and Japanese guidelines. The day of insemination or detection of evidence of mating is considered day 0 of gestation and the day of birth is

TABLE 11.2 Comparison of ICH Stages and Study Types with Similar Observations Made

ICH Stage	FDA Guidelines	Great Britain and EEC Guidelines	Japanese Guidelines	EPA OPPTS, OECD, and FDA Redbook Guidelines
A—Premating to conception: reproductive functions in adult animals, including development and maturation of gametes, mating behavior, and fertilization	Segment I	Segment I	Segment I	Multigeneration One generation
B—Conception to implantation: reproductive functions in adult female, preimplantation and implantation stages of conceptus	Segment I	Segment I	Segment I	Multigeneration One generation Developmental toxicity
C—Implantation to closure of the hard palate: adult female reproductive functions and development of embryo through major organ formation	Segments I, II	Segments I, II	Segment II	Multigeneration One generation Developmental toxicity Developmental neurotoxicity
D—Closure of the hard palate to the end of pregnancy: adult female reproductive function, fetal development, and growth and organ development and growth	Segments I, II, III	Segments I, II	Segment II	Multigeneration One generation Developmental toxicity Developmental neurotoxicity
E—Birth to weaning: adult female reproduction function, adaptation of neonate to extrauterine life, including preweaning development and growth (postnatal age optimally based on postcoital age)	Segments I, III, pediatric	Segments I, II, III	Segments II, III	Multigeneration One generation Developmental toxicity Developmental neurotoxicity
F—Weaning to sexual maturaity: (pediatric evaluation when treated) postweaning development and growth, adaptation to independent life and attainment of full sexual development	Pediatric	Segment I	Segments II, III	Multigeneration Developmental neurotoxicity Developmental immunotoxicity

Note: Bolded information indicates treatment interval.

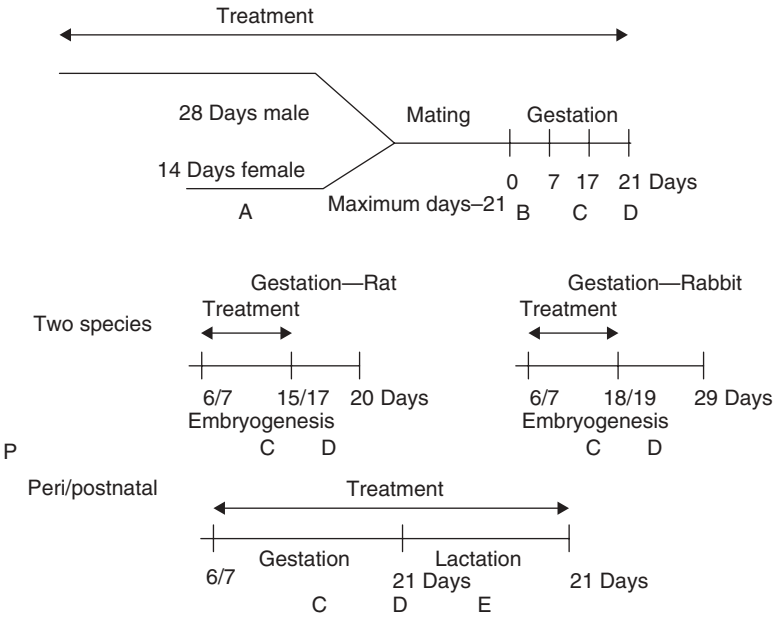

Figure 11.1 *Line charts for ICH stage study designs.*

considered postpartum and postnatal day 0. Figure 11.1 presents line charts for the ICH stage study designs.

11.2.1 Male and Female Fertility and Early Embryonic Development to Implantation

The purpose of this component is to assess the effects that result from treatment during maturation of gametes, during cohabitation, and, in females, during gestation up through the time of embryo implantation (typically last dose on day 6 of gestation). Assuming that the findings from a toxicity study of at least one month in duration do not contraindicate, the treatment period begins in males four weeks before male–female cohabitation and, in females, two weeks prior to cohabitation. A group size of 16–24 litters would generally be considered acceptable.

Minimal in-life observations include:

1. Clinical signs and mortality daily
2. Body weight twice weekly
3. Food consumption weekly
4. Vaginal cytology daily during cohabitation
5. Valuable target effects seen in previous toxicity studies

Females are sacrificed after the middle of the gestation period. Males are sacrificed at any time after the end of the cohabitation period, but it is generally advisable to retain the males until after the outcome of the first mating is known to ensure that a repeat cohabitation with untreated females will not be needed to determine if an observed effect on mating performance is a male effect. Males are treated until termination. Terminal examination of adults includes:

1. Necropsy
2. Preservation of organs with gross changes and sufficient control organs for comparison
3. Preservation of testes, epididymides, ovaries, and uteri
4. Sperm count and sperm viability
5. Count of corpora lutea and implantation sites
6. Count of live and dead conceptus

Among the study designs conducted before the ICH guidelines, the segment I fertility study conducted according to Japanese guidelines is most similar to this ICH study design. The major differences are the shortening of the treatment period of males prior to cohabitation from the duration of spermatogenesis (60–80 days) to 4 weeks and the addition of sperm evaluation. The justifications given for shortening the treatment period of males are:

1. Careful organ weight and histopathological evaluation of testes in general toxicity studies will detect most testicular toxins.
2. Fertility is an insensitive measure of testicular effects.
3. Compounds known to affect spermatogenesis generally exert their effects during the first four weeks of treatment.

Sperm counts can be performed with sperm from either the testis or the epididymis. Sperm motility is commonly being treated as a measure of sperm viability. The addition of sperm evaluation greatly increases the sensitivity of the study to detect effects on sperm maturation and the current study design will likely detect more male effects than previous designs even though the treatment period has been shortened.

11.2.2 Embryo–Fetal Development

The purpose of this component is to detect anatomical effects on the developing conceptus by treatment during the period of organogenesis from implantation to closure of the secondary palate. The study design is very similar to the historical segment II developmental toxicity study. A group size of 16–24 litters would generally be considered acceptable. The following is recommended:

	Rat	Rabbit	Mouse
Treatment period, gestational days	6–17	6–18	6–15
Group size, mated or inseminated	25	20	25

Minimal in-life observations include:

1. Clinical signs and mortality daily
2. Body weight twice weekly
3. Food consumption weekly
4. Valuable target effects seen in previous toxicity studies

Females are sacrificed at the end of the gestation period, about one day prior to parturition (day 20 or 21 for rats, day 28 or 29 for rabbits, and day 17 or 18 for mice). Terminal examinations include:

1. Necropsy
2. Preservation of organs with gross changes and sufficient control organs for comparison
3. Count of corpore lutea and live and dead implantations
4. Fetal body weight
5. External, visceral, and skeletal examination of fetuses
6. Gross evaluation of placenta

A minimum of 50% of fetuses are to be examined for visceral alterations and a minimum of 50% for skeletal abnormalities. When a fresh tissue microdissection technique is being used for the visceral examination of rabbit fetuses, all fetuses should be examined for both visceral and skeletal abnormalities.

Interpretation of results requires understanding and utilizing the following definitions:

Malformation Structural change that is likely to be permanent and detrimental to the survival or well-being of the fetus in the species/strain of animal being tested.

Alternation Change that is, in isolation, unlikely to be detrimental to the survival or well-being of the fetus in the species/strain of animal being tested.

Variant Observation occurring frequently in a particular strain of animal.

11.2.3 Adverse Effects

The following definitions should be referred to when considering whether an observed effect of treatment is adverse or not:

1. Treatment-related trend in incidence of specific or related malformations
2. Treatment-related increase in alterations, the cumulative effect of which is considered to be detrimental to the well-being of the fetus
3. Treatment-related increase in alterations, which are related in nature or derivation to treatment-related malformations evident on the study
4. Treatment-related marked change in the incidence of a group of alterations, which although their form is normal for a previous or future stage of development, that is, their occurrence suggests precocious or delayed development, their presence in a marked degree suggests some permanent change in the rate of development of the fetus and could be detrimental to its future development
5. Marked treatment-related increase in the occurrence of a specific alteration, in which the form is not predictive of the normal chronological order of development (e.g., bent scapula)

11.2.4 Pre- and Postnatal Development

The purpose of this component is to detect effects of treatment from implantation through lactation on the pregnant and lactating female and on the development of the conceptus and offspring through sexual maturity. The study design is similar to the previous segment III study design except that dosing begins on day 6 of gestation instead of day 15. A group size of 16–24 litters would generally be considered acceptable (with 25 mated females being recommended).

Minimal in-life observations for parental (F_0 generation) females include:

1. Clinical signs and mortality daily
2. Body weight twice weekly
3. Food consumption weekly
4. Valuable target effects seen in previous toxicity studies
5. Length of gestation
6. Parturition

Parental females are sacrificed after weaning of the F_1 generation. The age of sacrifice of the F_1 generation animals is not specified in the ICH guideline and varies among laboratories. Typically, they are sacrificed intermittently, with some laboratories reducing litter size on postnatal day 0, 3, or 4, on postnatal day 21 or at weaning, at male–female cohabitation to produce an F_2 generation, and the terminal sacrifice after production of the F_2 generation. Terminal examinations for maternal animals and offspring include:

1. Necropsy of all parental and F_1 adults
2. Preservation of organs with gross changes and sufficient control organs for comparison
3. Count of implantations

Additional observations of the F_1 generation include:

1. Abnormalities
2. Live and dead offspring at birth
3. Body weight at birth
4. Pre- and postnatal survival, growth, maturation, and fertility
5. Physical development, including vaginal opening and preputial separation
6. Sensory function, reflexes, motor activity, learning, and memory

11.2.5 Single- and Two-Study Designs for Rodents

Except for the embryo–fetal development component in rabbits, the components described above can be combined into fewer, larger studies instead of conducting each component separately. Acceptable alternatives include the "single-study design" and "two-study design." The choice may be made based on when study results are needed (how soon are females to be incorporated in clinical studies) and compound availability.

In the "single-study design," all of the above components are combined into one study. The dosing period, extending from before mating through lactation, is a combination of that for the fertility study together with that for the pre- and postnatal development study. Subgroups of animals are terminated at the end of gestation for fetal examination.

There are a variety of possible "two-study designs." One is to conduct the single study described above except that, instead of having subgroups for fetal examination, a separate embryo–fetal development study in rodents is conducted. Another two-study design consists of combining the embryo–fetal development study with the pre- and postnatal development study such that the two studies to be conducted would be (1) the fertility study and (2) the pre- and postnatal development study with subgroups terminated at the end of gestation for fetal examination. A third possible two-study design is to combine the fertility study with the embryo–fetal development study. In the first study, treatment would extend through the end of organogenesis and then, at termination at the end of gestation, there would be a complete fetal examination. The second study would be the pre- and postnatal development study.

For all the options described above, effects on male and female fertility can be evaluated separately by conducting separate studies in which only one sex is treated. The treatment periods are the same, but the treated animals are

cohabited with untreated animals of the opposite sex. In the male fertility study, the untreated females are terminated after the middle of gestation and terminal observations include embryo survival and possibly external examination of fetuses (if terminated at the end of gestation) (Tanimura, 1990). The advantage of conducting separate male and female studies is that, if there are effects, is it clear which sex was affected by treatment. Often when effects are seen in a combined male and female study, additional work is required to resolve which sex was affected. Either a second cohabitation of the treated males with untreated females is added or studies with only one sex treated must then be conducted.

With the possible exception of combining the female fertility component with the embryo–fetal development component, the combined-study approach is used often. The female fertility and embryo–fetal development components are needed to support clinical trials in women of childbearing potential in most countries and thus will be conducted early in the development of a drug. However, since the pre- and postnatal development component is not routinely required for clinical studies of women of childbearing potential and represents a large commitment of resources, it will not generally be conducted until late in the drug development process.

11.2.6 Preliminary Studies

According to the ICH guideline, "some minimal toxicity is to be expected to be induced in the high dose dams" in the reproductive toxicity studies. In some cases, particularly for the fertility and early embryonic development study, available information from general toxicity studies in the selected rodent species may be sufficient to allow the selection of dosage levels for a reproductive toxicity study with the goal of achieving minimal toxicity in high-dose dams. However, pregnant females sometimes respond differently to toxins than nonpregnant females, the duration of dosing for reproductive toxicity studies is different than for general toxicity studies, and toxicity may not have been achieved in the subacute toxicity studies. Thus, it is often necessary to conduct a range-finding study in pregnant rodents prior to the embryo–fetal development study. A range-finding study in rabbits is almost always required since only rarely are results available from other toxicity studies.

The range-finding study in pregnant animals (rodents or rabbits) is similar to the embryo–fetal development study discussed above except that there may be more dosage groups, group size is smaller (6–10 inseminated or mated females per group), and there is no need to examine fetuses for visceral or skeletal abnormalities. Evaluating litters from range-finding studies for resorption, fetal weight, and external abnormalities is valuable for providing an early indication of marked developmental toxicity. This is particularly important if conceptus survival at a particular dosage level would be inadequate to evaluate effects on development in the subsequent embryo–fetal development study. Once it has been determined during a range-finding study that a particu-

lar dosage level causes toxicity exceeding the minimal toxicity desired for the embryo–fetal development study, it is best to terminate that dosage group since continued treatment and evaluation unnecessarily expose animals to toxicity, any subsequent data collected are not useful for risk assessment (since it is known that excessive maternal toxicity itself causes developmental toxicity), and investment of resources is therefore unwarranted.

11.2.7 Toxicokinetics

The ICH guidelines do not require that toxicokinetic studies be conducted except that "at the time of study evaluation further information on kinetics in pregnant or lactating animals may be required according to the results obtained." In addition, the guidelines state that "it is preferable to have some information on kinetics before initiating reproduction studies." In practice, however, at least some degree of toxicokinetic sampling and analysis is performed to verify exposure and allow risk assessment.

The major toxicokinetic issue for reproductive toxicity studies is whether systemic exposure in the selected species and route is adequate relative to the systemic exposure with the clinical regimen. Often, this information is available for the selected rodent species from studies conducted independently from the reproductive toxicity studies. For rabbits, though, there is rarely toxicokinetic information available from other studies. Accordingly, it is advisable to conduct at least a crude evaluation of systemic exposure in the rabbit. It is best if theses data are available prior to the embryo–fetal development study so that, if the rabbit is found to have inadequate systemic exposure, an alternative species may be selected before the investment of resources in a large rabbit study. The collection of blood samples for toxicokinetic evaluations may be incorporated into the range-finding study in pregnant rabbits. However, rabbits stressed by multiple bleedings should not be retained for evaluation of developmental toxicity and satellite groups of toxicokinetic animals for bleeding only may be needed.

It would be ideal to have data from five to eight time points following the first and last doses to examine accumulation and other changes in kinetic parameters during pregnancy and, since physiology changes rapidly during gestation, to have data periodically during gestation as well. However, from a practical point of view, the question being asked (what is the approximate systemic exposure?) does not justify a comprehensive kinetic evaluation. When circumstances dictate that a toxicokinetic evaluation be performed, determining maternal plasma levels at a few postdosing intervals during a single 24-h period of gestation, preferably during the period when serious adverse effects are most likely to be induced (days 9–12 of gestation), will generally provide adequate information.

Only in special circumstances will the determination of embryo levels of drug add meaningfully to the assessment of human risk from a drug. In such studies, even if it is found that the embryo is not exposed, the lack of exposure

of the embryo would not necessarily indicate an invalid study or increased human risk since there may also be no exposure in human embryos. When embryo level studies are conducted, the selection of day(s) of gestation to harvest embryos is severely restricted by the sensitivity of the assay. Often, the earliest day that allows the collection of sufficient tissue for assay is gestational day 10 or 11.

11.2.8 Timing of Studies

The definition of which studies need to be performed in advance of clinical trials has not been addressed yet by the ICH process and is currently monitored by the regulatory agencies of individual countries and institutional review boards (IRBs). Embryo–fetal development studies in two species are almost universal prerequisites for clinical studies in women of childbearing potential. Some regulatory agencies also request that a fertility study in female rodents be conducted before clinical trials in women of childbearing potential. A fertility study in male rodents is required before clinical trials in men in Japan. Some pharmaceutical companies have internal guidelines that specify compliance with all the guidelines listed above, regardless of the location of the clinical trials.

The most conspicuous exception to the policy described above is the position of the FDA (1993). The FDA withdrew the restriction on the participation of women of childbearing potential in early clinical trials, citing "(1) exclusion of women from early trials is not medically necessary because the risk of fetal exposure can be minimized by patient behavior and laboratory testing, and (2) initial determinations about whether that risk is adequately addressed are properly left to patients, physicians, local IRBs and sponsors with appropriate review and guidance by FDA, as are all other aspects of the safety of proposed investigations." The policy of excluding women has been replaced by one that specifies that "the patients included in clinical trials should, in general, reflect the population that will receive the drug when it is marketed." In fact, inclusion of women at the earliest possible stages is frequently mandated. This led to the current situation where requirements as to when developmental and reproductive testing must be performed are very different between ICH regions, with FDA requirements generally not mandating any such studies until after phase I unless a potential risk/concern is indicated by a drug mechanism or finding in other studies (most commonly the initial repeat-dose toxicity studies).

To comply with FDA policy, at least for the conduct of clinical trials in the United States, pharmaceutical companies have a few choices. They can conduct the standard battery of reproductive studies prior to enrolling women of childbearing potential in early clinical trials. The possible negative impact would be a delay in the initiation of clinical trials. Alternatively, pharmaceutical companies can enroll women of childbearing potential in early clinical trials without having conducted any reproductive toxicity studies and accept

the additional risk resulting from exposure to untested drugs during inadvertent or undetected pregnancy. In either case, the incidence of pregnancy during clinical trials can be decreased by pregnancy testing and/or assurances of contraception.

11.3 METHODOLOGICAL ISSUES

11.3.1 Control of Bias

An important element to consider when designing developmental and reproductive toxicity studies is the control of bias. For example, animals should be assigned to groups randomly and preferably blocked by body weight. This can be accomplished by first ranking the animals in order of body weight and then, starting with the lightest or heaviest, assigning by rank to groups based on a list of sets of random permutations of numbers (e.g., 1, 2, 3, and 4 if there are four groups, where 1 represents the control group, 2 represents the low-dose group, etc.). Housing of treatment groups should also be unbiased. This can be done by a "Latin square" design where each block of four cages (if there are four groups) includes an animal from each group. It is often an acceptable compromise to have animals from different groups in alternating vertical columns with all the animals in a column from the same group. This provides equal vertical balancing for all groups. Historically, it has proven unwise to have groups segregated on separate racks.

The order of sacrifice on the day of cesarean sectioning should be balanced by group (again using random permutations) since fetuses continue to grow during the day and an unbalanced time of sacrifice would bias fetal weights, particularly for rodents. Alternatively, all animals can be killed at about the same time in the morning and the fetuses stored for examination later the same day. Fetal examinations should be conducted blind, that is, without knowledge of treatment group.

11.3.2 Diet

It is known that rodents require a diet relatively rich in protein and fats for successful reproduction (Zeman, 1967; Chow and Rider, 1973; Turner, 1973; Mulay et al., 1982). Consequently, rodents are fed high-protein, high-fat diets ad libitum for reproductive toxicity studies and also generally as a maintenance diet for all toxicity studies. Female rats fed in this manner begin to show decreases in fertility, litter size, and the incidence of normal estrus cycling at the age of six months (Matt et al., 1986, 1987). The disadvantage of this feeding practice is that the animals more quickly acquire age-related diseases and sexual dysfunction and die sooner than if they are fed a restricted amount of calories (for review, see Weindruch and Walford, 1988). In relatively short-term studies (such as standard ICH studies), this rapid aging does not present a

problem. However, for male breeding colonies or multigeneration studies with multiple litters per generation, it could be advantageous to restrict caloric intake, at least when the animals are not being bred. Restriction of food intake to achieve a 30% decrease in body weight gain compared to ad libitum–fed controls has no adverse effect on male rat reproduction (Chapin et al., 1991), although it does affect reproduction in mice (Gulati et al., 1991) and female rats (Chapin et al., 1991).

Dietary restriction is even more important for rabbits. Rabbits fed ad libitum fare very poorly. Some laboratories restrict New Zealand white rabbits to 150–180 g per day of a high- (at least 13.5%) fiber diet. However, even this regimen results in some rabbits going off feed late in gestation. It has been observed that by restricting New Zealand white rabbits to only 125 g of food per day nearly all control animals retain appetite throughout gestation and fewer of these animals abort (Clark et al., 1991). More uniform food consumption late in gestation is associated with greater uniformity in maternal body weight change and fetal weight. This decreased variability makes these measures more sensitive indicators of maternal and developmental toxicity. Thus, 125 g is the preferred daily ration for New Zealand white rabbits.

11.3.3 Clinical Pathology

Regulatory guidelines require that there be maternal toxicity at the highest dosage level in embryo–fetal developmental toxicity studies. It is important to avoid excessive toxicity in these studies since it is known that marked maternal toxicity can cause secondary developmental toxicity (see discussion in Section 11.4.3). This secondary developmental toxicity is irrelevant to the assessment of the developmental hazard of the test agent and thus simply confounds the interpretation of the data.

The traditional indicators of maternal toxicity in range-finding studies in pregnant animals (mortality, body weight, food consumption, and clinical signs) do not always provide a sensitive measure of toxicity. This insensitivity is a particular problem for rabbit studies since typically no other toxicity studies are conducted in rabbits and body weight change in rabbits is very variable (typically –100 to +400 g during gestation), making it a particularly insensitive indicator of toxicity.

Thus, it is desirable to improve the assessment of toxicity in range-finding studies in pregnant animals. Complete histopathological examination is not practical. However, it is often feasible to perform hematological and serum biochemical analyses that can significantly increase the changes of detecting significant toxicity and provide important information for selecting an appropriate highest dosage level for the embryo–fetal developmental toxicity study.

Based on more than 20 years of experience, body weight effects most often provided the basis for the selection of dosage levels in the segment II study. However, there have been cases where clinical pathology was or would have been useful to justify dosage selection. For example, the nonsteroidal anti-

inflammatory drug diflunisal caused a decrease in erythrocyte count from 6.0×10^6 to $2.9 \times 10^6\,mm^{-3}$ at a dosage level ($40\,mg\,kg^{-1}\,day^{-1}$) that caused only a 1% decrease in body weight in pregnant rabbits. The severe hemolytic anemia caused by this excessively high dosage level in turn caused secondary axial skeletal malformations in the fetuses (Clark et al., 1984). Also, the angiotensin-converting enzyme (ACE) inhibitor enalapril caused an increase in serum urea nitrogen from 16 to $46\,mg\,dL^{-1}$ (highest value 117) at a dosage level ($10\,mg\,kg^{-1}\,day^{-1}$) that had no apparent effect on body weight but caused a significant ($p < 0.05$) increase in resorptions (Minsker et al., 1990). Serum urea nitrogen concentration was used to select dosage levels for a subsequent ACE inhibitor, lisinopril. Likewise, the routine use of clinical pathology in range-finding studies has previously been proposed (Wise et al., 1988). The animals can be bled on the day after the last dose or sooner to detect transient effects or to allow an evaluation of the data prior to cesarean section.

11.3.4 Gravid Uterine Weights

Effects of treatment on maternal body weight gain are commonly evaluated as indicators of maternal toxicity. However, maternal body weight gain is influenced by fetal parameters such as live fetuses per litter and fetal body weight. Thus, effects indicative of developmental toxicity could contribute to decreased maternal body weight gain and confound the interpretation of maternal toxicity. In addition, other maternal but pregnancy-related parameters, such as volume of intrauterine fluid, could be affected by treatment and contribute to effects on overall body weight gain.

In an attempt to correct this complication, some laboratories weigh the gravid uterus at cesarean section and then subtract the weight of the gravid uterus from the body weight gain to obtain an adjusted weight gain that is more purely maternal. This adjustment is imprecise but not inappropriate for rats for which gravid uterine weight is correlated with and generally substantially less than maternal body weight change during gestation (e.g., see Figure 11.2 for which the correlation coefficient $r = 0.63$ and $p < 0.001$). However, the subtraction of gravid uterine weight from maternal weight gain is an overadjustment for rabbits. The maternal body weight gain of rabbits during gestation is generally less than the weight of the gravid uterus (see Figure 11.3). Moreover, gravid uterine weight is correlated with maternal body weight change in some but not all studies. For example, in the 53 untreated rabbits from the study shown in Figure 11.3, $r = 0.54$ and $p < 0.001$. However, in a study of 32 rabbits treated with a methylcellulose vehicle, $r = 0.21$ and $p = 0.25$. Thus, subtracting the gravid uterine weight from the maternal weight gain is not always appropriate. A preferred method for adjusting maternal body weight gain for possible developmental effects is to test and, if appropriate, use gravid uterine weight as a covariate (J. Antonello, personal communication, 1990). This method can be used for both rats and rabbits and for body weight change intervals in addition to those ending at study termination.

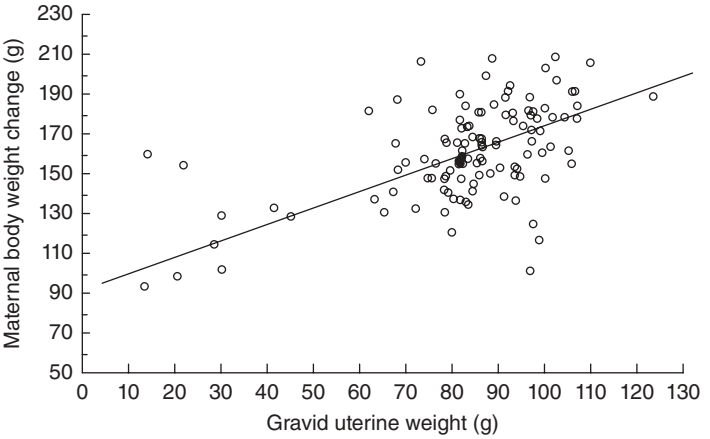

Figure 11.2 *Relationship between gravid uterine weight and maternal body weight change in control rats between days 0 and 20 of gestation. One hundred and twenty pregnant Sprague-Dawley [Crl:CD(SD)BR] rats were dosed orally with 0.5% aqueous methylcellulose on days 6–17 of gestation and cesarean sectioned on day 20 of gestation. The gravid uterus from each animal was removed and weighed.*

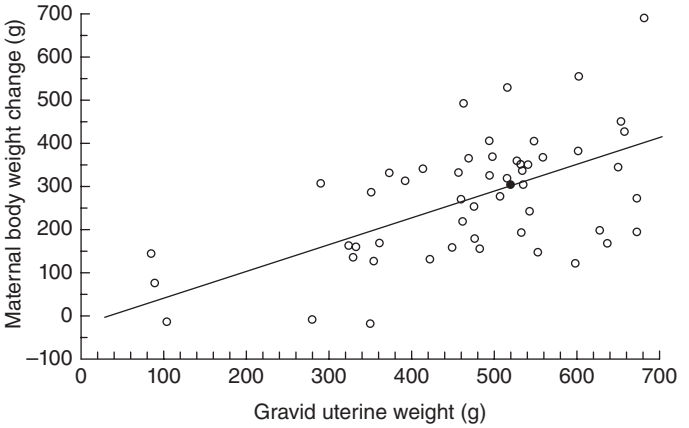

Figure 11.3 *Relationship between gravid uterine weights and maternal body weight change in untreated rabbits between days 0 and 28 of gestation. Fifty-three pregnant New Zealand white rabbits that had not been treated with control article or test agent were cesarean sectioned on day 28 of gestation. The gravid uterus from each animal was removed and weighed.*

Alternatively, to avoid weighing the uterus (or if the analysis is being performed retrospectively and uterine weights are unavailable) or if a more purely fetal adjustment is desired, one can use the sum of the live fetal weights within the litter (total live fetal weight) as the covariate instead of gravid uterine weight. As expected, total live fetal weight is very highly correlated with gravid uterine weight in control animals (r is 0.99 in control rats and 0.95

in control rabbits; J. Antonello, personal communication, 1990). Thus, in general, using either gravid uterine weight or total live fetal weight as the covariate will yield similar results. However, if treatment was to have an effect on gravid uterine weight that was not reflected in total live fetal weight (e.g., if the volume of amniotic, extracoelomic, or intrauterine fluid was affected), then total live fetal weight may not be highly correlated with gravid uterine weight and, hence, not interchangeable as a covariate. In that case, only weighing the gravid uterus would allow the detection of these effects not revealed by total live fetal weight.

11.3.5 Implant Counts and Determination of Pregnancy

Two observations suggest that the remnants of embryos that die soon after implantation are not apparent at gross examination of the uterus near term. First, embryos that were observed to be resorbing at laparotomy early in gestation left no readily visible trace near term (Staples, 1971). Second, occult implantation sites can be revealed near term by staining the uterus with ammonium sulfide or sodium hydroxide (Salewski, 1964; Yamada et al., 1988). It is not known if the uterine staining techniques reveal all implantation sites. It is clear, though, that when uterine staining techniques are not used, very early resorptions may not be included in what is termed the "resorption rate" but instead may contribute to the apparent "preimplantation loss" or, if no implantation sites were detected, the rate of "nonpregnant" animals.

In normal circumstances, probably very few implantation sites are not detected without staining. However, cases have occurred in which treatment effects were probably detected only as a result of uterine staining. For example, in one rabbit study with drug treatment starting on day 6 of the gestation, a drug-treated group had four litters that had implantation sites that were seen only after staining with ammonium sulfide, indicating very early drug-induced resorption. For critical studies in rabbits designed to determine early effects on resorption and abortion rates, it would be advantageous to measure plasma levels of progesterone on day 6 of gestation since low levels indicate nonpregnant animals (Adams et al., 1989, 1990).

11.3.6 Fetal Examinations

Many fetal anomalies, such as cleft palate, exencephaly, ectrodactyly, and missing vertebra, are discrete and distinct and therefore easy to recognize objectively. Some anatomical structures, though, occur along a continuous gradation of size and shape and are only considered anomalous if the deviation from the typical exceeds a somewhat arbitrarily selected threshold. These anomalies are observed in all examination types and include, for example, micrognathia, reduced gallbladder, enlarged heart, distended ureter, wavy rib, and incomplete ossification at many sites. In many cases, it cannot be said with certainty whether a specific degree of variation from normal would have

resulted in an adverse consequence to the animal and should therefore be considered abnormal. In the absence of certainty about outcome, the best approach is to uniformly apply a single criterion within a study (and preferably among studies) so that all treatment groups are examined consistently. The subjectivity (and hence fetus-to-fetus variability) of the examination can be minimized by having the criteria be as clear and objective as possible. For example, when examining for incompletely ossified thoracic centra or supraoccipitals, it can be required that the ossification pattern be absent (unossified), unilateral, or bipartite (which are objective observations) before recording as an observation. Subjective criteria such as being dumbbell or butterfly shaped would not be applied.

Examination of External Genitalia One aspect of external anatomy that is largely overlooked in the examination of offspring exposed in utero to test agents is the external genitalia, even though major malformations can occur in those structures. For example, hypospadias are malformations in the male in which the urethra opens on the underside of the penis or in the perineum. Hypospadias can occur in the male rat following in utero exposure to antiandrogens (e.g., Neumann et al., 1970), testosterone synthesis inhibitors (e.g., Bloch et al., 1971), or finasteride, a 5α-reductase inhibitor (Clark et al., 1990b). However, it is impractical to detect hypospadias in fetuses or young pups. Although the genital tubercle of the normal male rat fetus is grossly distinguishable from that of the normal female as early as day 21 of gestation (the female has a groove on the ventral side), the difference is very subtle and partial feminization of the male genital tubercle would be very difficult to ascertain. Routine histological examination is obviously too labor intensive to be considered. Hypospadias can readily be determined, though, by expressing and examining the penis of the adult. Thus, it is recommended that adult F_1 males be examined for hypospadias. If the timing of the separation of the balano–preputial membrane is being included in the pre- and postnatal development study as a developmental sign (see Korenbrot et al., 1977), the examination of the penis for hypospadias can be conducted at the same time.

The critical period for the induction of hypospadias by finasteride in rats is days 16–17 of gestation (Clark et al., 1990a). It is unlikely that other agents would have a much earlier critical period since testosterone synthesis, which is required for the development of the penile urethra, begins in the rat on day 15 of gestation (Habert and Picon, 1984). Thus, if treatment in the embryo–fetal development study terminates on day 15 of gestation (as is done in some laboratories), it is doubtful that hypospadias could be induced. However, hypospadias could be induced in the pre- and postnatal development study. Since the formation of the penile urethra in the rat is not completed until day 21 of gestation (Anderson and Clark, 1990), it could be argued that "major organogenesis" continues until that time.

One parameter that is readily and commonly measured as an indicator of effects on differentiation of the external genitalia in rodent fetuses is the sexu-

ally dimorphic distance between the anus and the genital tubercle (anogenital distance). However, it should not be assumed that anogenital distance is synonymous with hypospadias, since effects on anogenital distance are not necessarily predictive of hypospadias. Finasteride caused both hypospadias and decreased anogenital distance in male offspring but with very different dose–response relationships and only a slight tendency for animals with hypospadias to have a shorter anogenital distance (Clark et al., 1990b). Also, the effects on anogenital distance were largely reversible, whereas hypospadias were not. Another agent, triamcinolone acetonide, caused dramatic (reversible) decreases in anogenital distance in male rat fetuses on day 20 of gestation but did not affect the development of the genital tubercle and did not cause hypospadias (Wise et al., 1990b). Thus, decreased anogenital distance per se does not necessarily indicate a serious congenital anomaly.

When evaluating effects of treatment on fetal anogenital distance, it is obviously important to correct for effects on fetal weight. One approach is to calculate "relative" anogenital distance, the ratio between anogenital distance and another linear measure, for example, biparietal diameter (head width). The cube root of fetal weight simulates a linear measure (Wise et al., 1990b) and can also be used to normalize anogenital distance. Another approach is to compare the anogenital distance in a weight-reduced treatment group to that in a weight-matched control group at a younger age.

Visceral Fetal Examinations The examination of the abdominal and thoracic viscera of fetuses is performed either fresh without fixation ("Staples technique") or after Bouin's fixation by making freehand razor blade sections ("Wilson's technique"; Wilson, 1965). Both techniques have advantages. The fresh examination technique, which may require less training to become thoroughly proficient, provides a more easily interpreted view of heart anomalies. The examination must be performed on the day the dam is terminated, though, so having a large number of litters to examine in one day requires that a large team of workers be committed to the task.

With both techniques, the heads of one-half of the fetuses can be fixed in Bouin's fixative for subsequent freehand sectioning and examination. A common artifact induced by fixation in rabbit fetal heads is retinal folding.

Whether or not the kidneys are sliced transversely to examine the renal pelvis varies among laboratories. Hydronephrosis, delayed papillary development, and distended renal pelvis are most readily detected in this manner. However, it is not necessary to slice the kidneys to detect the urinary retention that can lead to distended renal pelvis and hydronephrosis. This point was demonstrated in a study in which 200,000 IU kg^{-1} day^{-1} of vitamin A administered orally on days 8–10 of gestation induced hydronephrosis and/or distended renal pelvis in 29 fetuses (R. Clark, personal communication). In all of these 29 fetuses (and two others), a distended ureter also occurred. Thus, a distended ureter may be a more sensitive indicator of urinary retention than a distended renal pelvis.

Skeletal Fetal Examination There is variability in the development of the fetal skeleton, including numbers of vertebrae and ribs, patterns of sternebral ossification, alignment of ribs with sternebrae, and alignment of ilia with lumbar and sacral vertebrae. There is also extensive plasticity in the development of the skeleton beyond the fetal stage. For example, it is known that markedly wavy ribs in fetuses can resolve so that the ribs in the adult are normal (Saegusa et al., 1980; Nishimura et al., 1982) and supernumerary ribs can be resorbed (Wickramaratne, 1988). This variability and plasticity complicates the classification of anomalies as true malformations as opposed to variations of normal. There is no unanimity on terminology, but, in general, a variation tends to be an alteration that occurs at relatively high spontaneous incidence (>1%), is often reversible, and has little or no adverse consequence for the animal.

When tabulating and interpreting fetal skeleton data, a distinction is made between alterations in the pattern of development and simple delays in development that are considered to be less serious. A delay in skeletal development is usually apparent as a delay in ossification, as evidenced by an increased incidence of specific, incompletely ossified sites or decreases in counts of ossified bones in specific regions (e.g., sacrocaudal vertebrae). These delays are normally associated with decreases in fetal weight and commonly occur at dosage levels of the test agent that also cause decreased maternal body weight gain.

When determining the criteria for recording skeletal alterations, particularly sites of incomplete ossification, it is legitimate to consider the resulting incidences. For example, including an unossified fifth sternebra in the criteria for recording incomplete sternebral ossification may increase the control incidence to a very high proportion (over 95%) of fetuses affected, which would then reduce the sensitivity for detecting treatment effects. The additional effort expended in recording the extra observations due to sternebra 5 would be wasted. In addition, recording high incidences of incomplete ossification at many sites is not worth the effort involved. The ossification at various sites is highly correlated, so recording at multiple sites is redundant. In some cases, the incidences can be reduced to reasonable levels (1 to 20% of control fetuses) and the criteria simultaneously being made more objective by requiring that the bone be entirely unossified before recording.

11.3.7 Developmental Signs

The postnatal evaluation of F_1 pups includes the observation of developmental signs in two or more pups per sex per litter. In general, the acquisition of these developmental landmarks, including anatomical changes (e.g., ear pinna detachment, incisor eruption, hair growth, and eye opening) and reflexes (negative geotaxis, surface righting, and free-fall righting), are highly correlated with body weight, but as indicators of developmental toxicity they are not as sensitive as body weight (Lochry et al., 1984; Lochry, 1987) and thus have

minimal value. Possible exceptions to this generality are the ontogeny of the auditory startle reflex and the markers of sexual maturation (vaginal patency, testes descent, and balano–preputial separation in males).

The examinations for developmental signs should be performed daily starting before and continuing until the criterion is achieved. The separation of the balano–preputial membrane of the penis (occurring at postnatal weeks 6–7; Korenbrot et al., 1977) is becoming the preferred landmark of sexual maturation in males. The timing of the testes descent is more variable and very dependent on the achievement criteria used. Another advantage of determining the time of the balano–preputial separation is that anomalies of the penis may be observed at the same time (as noted above).

11.3.8 Behavioral Tests

The trend within reproductive toxicology is to move from simple determinations of developmental landmarks and reflexes to more sophisticated and sensitive behavioral tests. This process was accelerated by the U.S. Environmental Protection Agency (EPA), which issued guidelines requiring a "developmental neurotoxicity" study of compounds that meet any of several broad criteria (EPA, 1991). The behavioral tests to be performed in this study are extensive and rigidly defined. As laboratories become equipped and trained to meet these guidelines, they are adding such tests to their evaluations of pharmaceuticals. The suggestions for routine testing made below are considered reasonable for pre- and postnatal development studies intended as routine screens. It is suggested that testing be conducted on one or two adults per sex per litter, keeping the range of actual ages as tight as possible.

Measurement of motor activity is commonly performed in the dark in cages or plastic boxes (open field) or residential mazes in which movement is quantitated by infrared detectors or by recording the interruption of light beams as the test subject moves through a horizontal grid of light beams. Possible parameters to evaluate include horizontal activity (light beams interrupted), number of movements, and time spent in the middle of the cage. The test period is selected to be long enough (normally 30–50 min) to allow the activity of the animals to decrease to an approximately constant level (asymptote). Testing of young pups (e.g., 13 days of age) is not recommended as their activity level is fairly constant during the test period and young unweaned pups should not be separated from their mothers for extended periods of time.

Another test paradigm for detecting treatment effects on brain functioning in F_1 offspring measures auditory startle habituation. In this test, the animal is placed in a chamber with a floor that detects movement. The animal is exposed to a sequence of 50–60 auditory stimuli, each at 110–120 decibels for 20–50 s and separated by 5–20 s. The gradual diminution of the animal's movement response is indicative of normal habituation.

There is not a consensus about the procedures to use to test for effects on learning and memory. The two most commonly used techniques are the water-

filled maze, which is preferred for measuring learning, and passive avoidance, which is preferred for measuring memory (see Buelke-Sam et al., 1985). Retention is tested in a repeat test conducted approximately one week later.

11.3.9 Detecting Effects on Male Reproduction

Male fertility studies with typical group sizes (15–30 males per group) are very insensitive for detecting effects on male fertility. If the control fertility rate is 80%, even a group size of 30 will only detect (at the 5% significance level) a 38% decrease in fertility 80% of the time and a 50% decrease 95% of the time (J. Antonello, personal communication, 1990). To detect slight effects on male fertility would require enormous group sizes. Mating each male with more than one female provides a more precise estimate of the reproductive capacity of each male but does not greatly increase statistical power. If multiple matings are to be done, it is recommended that the cohabitations with multiple females be sequential rather than concurrent.

It is difficult to detect effects on male fertility not only because of group size considerations, but also those mediated by decreased sperm production because of the normally huge excess of sperm included in a rat ejaculate. Sperm production can be decreased by up to 90% without effect on fertility (either pregnancy rate or litter size) in the rat. This is not the case for men, so the sperm excess in the rat represents a serious flaw in the rat model (see Working, 1988). To address this deficiency and improve the sensitivity of the model, it is advisable to determine the effects of the test agent on testes weights, testicular spermatid counts, and histopathology of the testes (preferably plastic sections) in the male fertility study and/or the 14-week toxicity study. In some cases, these parameters may be more predictive of possible effects on male fertility in humans than the fertility rate in rats.

11.4 DATA INTERPRETATION

11.4.1 Use of Statistical Analyses

Statistical analysis is a very useful tool for evaluating the effects of treatment on many developmental and reproductive toxicity parameters. For some parameters, such as maternal body weight changes, fetal weight, and horizontal activity in an open field, the comparison to the concurrent control is the primary consideration and, assuming adequate group size, the investigator relies heavily on the results of appropriate statistical analyses to interpret differences from control.

For other parameters, though, statistical analysis is just one of several considerations that include historical control data and other relevant information about the test agent and related test agents. For example, statistical analysis of a low incidence of an uncommon fetal malformation will usually not be significant ($p > 0.05$) even if treatment related, due to the low power for detect-

ing such effects with typical group sizes. In such cases, examination of the historical control data becomes paramount. If two fetuses with a particular malformation occur in separate litters only in a high-dose group, the finding is of much more concern if it is a very rare malformation than if recent historical control groups have had a few fetuses with that malformation.

Other known effects of the test agent or related agents also sometimes contribute to data interpretation. For example, a low incidence of a malformation may be considered treatment related if it is at the low end of a typical dose–response curve or if it is in a high-dose group and that malformation is an expected effect of the test agent. In general, though, a single occurrence of a fetal malformation in a treatment group (with none in control) is not cause for alarm, since this occurs in almost every study (together with occurrences of some malformations only in the control group).

Statistical methods exist to appropriately analyze most developmental and reproductive toxicity parameters. Exceptions to this are the "*r/m*" litter parameters in which, for each litter, there is a number affected divided by the number in the litter. These parameters include preimplantation loss (r = corpora lutea without implants, m = corpora lutea), resorption rate (r = resorptions, m = implants), and the family of alteration rates (r = affected fetuses, m = fetuses). There are two factors complicating the statistical analysis of these data that have heretofore been inadequately handled (Clark et al., 1989). One is that almost all of these parameters have a strong dependence on m. For example, both preimplantation loss (Figure 11.4) and resorption rate (Figure 11.5) are normally higher at both the low and high extremes of m. In contrast, supernumerary rib tends to occur at higher incidences in average-size litters (Figure 11.6). The second factor that complicates the statistical analysis of *r/m* data is that affected implants tend to occur in clusters within litters ("litter effects"); that is, the intralitter correlation is greater than the interlitter correlation. For example, the total number of litters affected with anasarca, missing vertebra, and supernumerary rib is much less than would be expected by change based on the number of affected fetuses (Table 11.3 and Figure 11.7).

These problems have been resolved for analysis of resorption rate (and preimplantation loss) in Sprague-Dawley rats using a three-step process (Soper and Clark, 1990). First, based on an analysis of data from 1379 control rat litters examined since 1978, a likelihood score was derived for each (r,m) couplet based on the incidence of that couplet given that value of m. These scores were approximately equal to r. Second, an analysis of 136 litters from groups with slight effects on resorption rate revealed that, at low-effect doses of embryocidal test agents, the increases in resorptions tended to occur as increased numbers of resorptions within affected litters rather than as an increased proportion of affected litters. To maximize the difference in scores between control and affected litters, the scores for controllike litters (r = 1, 2, 3) were downgraded from r (1, 2, and 3) to 0.4, 1, and 2.4, respectively. Third, to arrive at the final score for each litter, the modified r score for each litter

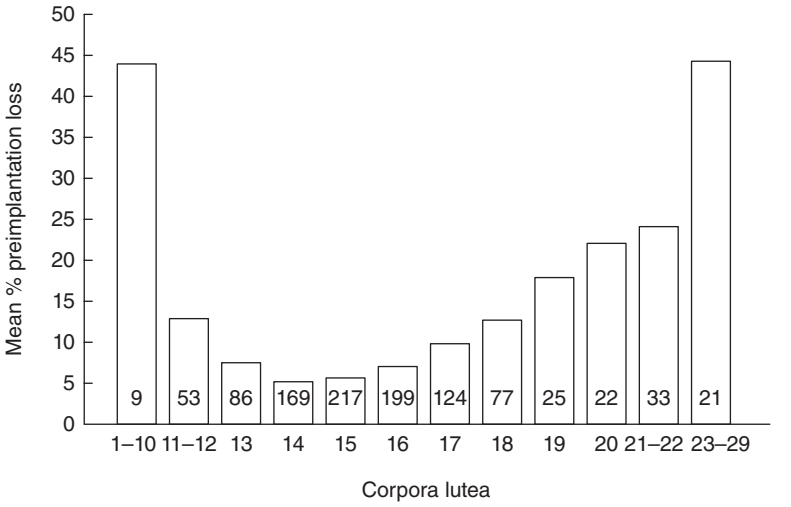

Figure 11.4 *Effect of litter size on mean percentage preimplantation loss in 1035 control rat litters. Between 1970 and 1988, 1035 control rats were cesarean sectioned on day 20 of gestation and the numbers of resorptions and implants were counted. Numbers within the bars indicate number of litters.*

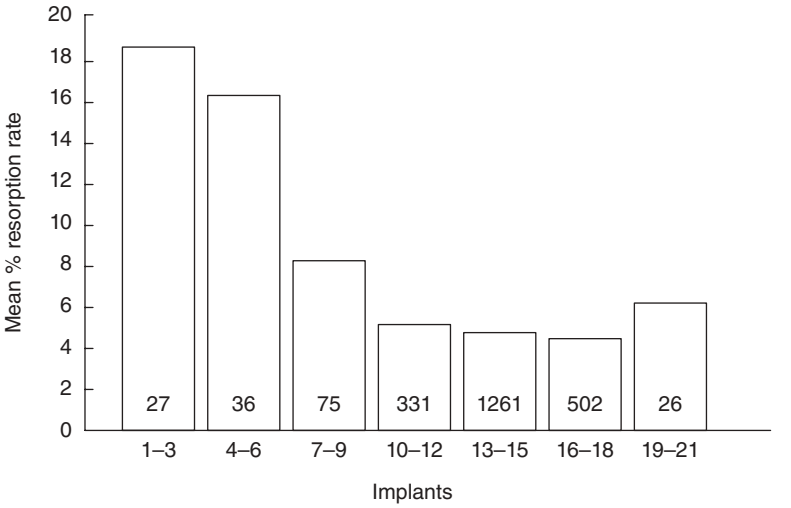

Figure 11.5 *Effect of litter size on mean percentage resorption rate in 2258 control rat litters. Between 1970 and 1988, 2258 control rats were cesarean sectioned on day 20 of gestation and the numbers of resorptions and implants were counted. Numbers within the bars indicate number of litters.*

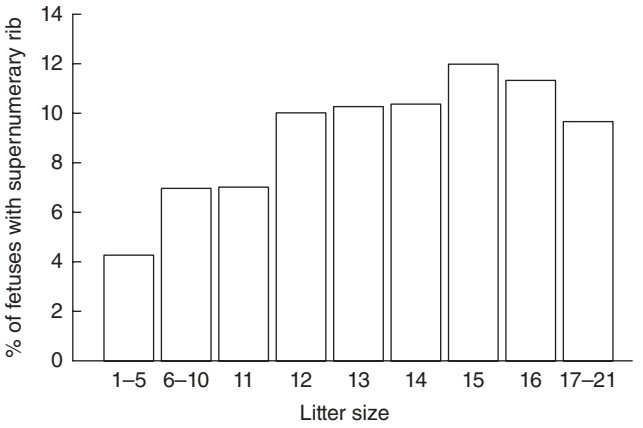

Figure 11.6 *Effect of litter size (live fetuses per litter) on incidence of supernumerary rib in 1379 control rat litters. Between 1978 and 1988, fetal skeletons from 1379 litters of control rats were stained with alizarin red and examined for supernumerary rib.*

TABLE 11.3 Pregnancy Categories

A: Adequately tested in humans, no risk (0.7% of approved drugs)
B/C/D: (Increasing levels of concern) (92.3% of approved drugs—66% in C)
X: Contraindicated for use in pregnancy (7.0% of approved drugs)
A: Animal studies and well-controlled studies in pregnant women failed to demonstrate a risk to the fetus.
B: Animal studies have failed to demonstrate risk to fetus; no adequate and well-controlled studies in pregnant women.
C: Animal studies showed adverse effect on fetus; no well-controlled human studies.
D: Positive evidence of human fetal risk based upon human data, but potential drug benefit outweighs risk.
X: Studies show fetal abnormalities in animals and humans; drug is contraindicated in pregnant women.

Note: Use in-pregnancy Ratings; FDA, 1979.

was divided by the expected control value for that value of m. This last step makes the litter score immune to spontaneous or treatment-related effects on m. The final "robust" scores have more power for detecting effects than various other measures (raw r/m, affected litters/litters, r, $\Sigma r/\Sigma n$, and the likelihood score) and has a lower false-positive rate with fluctuations in m.

A covariance analysis (Snedecor and Cochran, 1980) can be used to reduce variability in a parameter and thereby increase sensitivity. For example, much of the variability in fetal weight data is due to variable litter size and, for rats, litters being sacrificed at different times during the workday. The variability due to these sources can be reduced by using litter size and time of sacrifice as potential covariates. Similarly, litter size and length of gestation can be used as covariates for neonatal pup weights and body weight at the beginning of

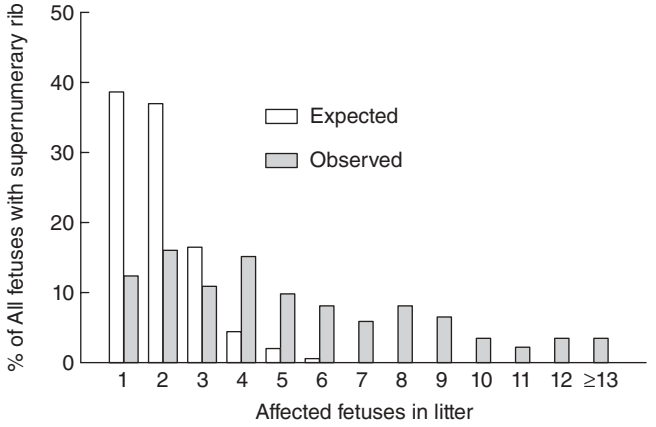

Figure 11.7 *Litter effect with supernumerary rib in 1379 control litters. Between 1978 and 1988, fetal skeletons from 1379 litters of control rats were stained with alizarin red and examined for supernumerary rib in addition to other anomalies. The calculation of the expected number of fetuses with supernumerary rib in each litter was based on the assumption that each fetus had an equally likely chance of having supernumerary rib independent of the incidence among littermates (K. Soper, personal communication, 1990).*

treatment can be used as a covariate for maternal body weight changes during the treatment period of an embryo–fetal development study.

11.4.2 Potential Hazard Categories of Developmental Toxins

It is generally agreed that an agent that causes developmental toxicity in laboratory animals at dosage levels that cause no maternal toxicity (i.e., "selective" developmental toxins) is potentially more hazardous to humans than an agent that causes developmental toxicity only at maternotoxic dosages ("nonselective" developmental toxins; e.g., see Johnson 1981; Schwetz, 1981; Fabro et al., 1982; Johnson, 1984; Johnson and Christian, 1984). This position is based on the supposition that pregnant women will avoid being exposed to toxic dosages of pharmaceuticals (which is usually but not always true). Developmental toxins can also be categorized as acting directly on the embryo or indirectly via an effect on the mother. All selective developmental toxins are presumably direct acting. Nonselective developmental toxins can either act directly or indirectly.

Direct-acting developmental toxins may be potentially more hazardous to humans than indirectly acting ones even if the direct developmental toxicity occurred only at maternotoxic dosages in the species of laboratory animals tested. When the developmental toxicity of an agent is secondary to maternal toxicity in all species tested, the dose–response curves for developmental and maternal toxicity in various species may be invariably linked and developmental toxicity would never occur at nonmaternotoxic dosages. However, when an agent acts directly on the embryo to cause developmental toxicity, the dose–

response curves may not be linked and, although they may be superimposed in the species of laboratory animals tested, they may not be superimposed in other species including humans. Thus, a direct-acting developmental toxin that is nonselective in one species may be selective in another species.

The ranking of potential developmental hazard in terms of selective, direct/nonselective, and indirect is more meaningful than the use of the terminology of specific/nonspecific and malformation/variation. However, when it cannot be determined if observed developmental toxicity is a direct or indirect effect, the alternative terminology becomes useful. A nonspecific effect (including in some cases decreased fetal weight, supernumerary rib, cleft palate in mice, and abortion in rabbits) is one that occurs commonly in response to high toxic dosages of a test agent. What makes a nonspecific effect generally less important than a specific effect is that nonspecific effects commonly occur only at maternally toxic dosages ("coeffective") and may be secondary to maternal toxicity—that is, indirect. However, when an apparently nonspecific adverse developmental effect is selective (direct), that is, it occurs at nonmaternotoxic dosages, it may nevertheless be indicative of a potential developmental hazard.

In general, an agent that induces a malformation (i.e., a teratogen) is considered to be more of a potential hazard than one that induces only a minor variation. Also, there has traditionally been more of a stigma associated with an agent that induces malformations than one that causes resorptions, even though embryo death is obviously a seriously adverse outcome. The point that makes the distinction among malformations, variations, or resorptions less important is that an agent that perturbs development to cause one effect in one species may cause a different effect in another species. Thus, any developmental toxic effect at nonmaternotoxic dosages should be considered carefully.

11.4.3 Associations between Developmental and Maternal Toxicity

The developmental toxicity of many pharmaceuticals occurs only at maternally toxic dosages (Khera, 1984, 1985; Schardein, 1987). Also, there are several compounds for which there is evidence that their developmental toxicity is secondary to their maternal toxicity. The decreased uterine blood flow associated with hydroxyurea treatment of pregnant rabbits may account for the embryotoxicity observed (Millicovsky et al., 1981). The teratogenicity of diphenylhydantoin in mice may be secondary to decreased maternal heart rate (Watkinson and Millicovsky, 1983) as supported by the amelioration of the teratogenicity by hyperoxia (Millicovsky and Johnston, 1981) and the dependence on maternal genotype in genetic crosses between sensitive and resistant strains (Johnston et al., 1979; Hansen and Hodes, 1983). The hemolytic anemia caused in pregnant rabbits by diflunisal was severe enough to explain the concomitant axial skeletal malformations (Clark et al., 1984). Acetazolamide-induced fetal malformations in mice are apparently related to maternal hypercapnia (Weaver and Scott, 1984a,b) and hypokalemia (Ellison and Maren,

1972). The increased resorption rate induced in rabbits by the antibiotic norfloxacin depends on exposure of the maternal gastrointestinal tract (Clark et al., 1991).

In addition, various treatments that simulate effects that can result from pharmaceutical treatment have been shown to cause developmental toxicity. Food deprivation can cause embryo–fetal toxicity and teratogenicity in mice (Szabo and Brent, 1975; Hemm et al., 1977) and rats (Ellington, 1980) and fetal death, decreased fetal weight, and abortions in rabbits (Matsuzawa et al., 1981; Clark et al., 1991). Treatments that result in maternal hypoxia, such as hypobaric exposure (Degenhardt and Kladetzky, 1955) and blood loss (Grote, 1969), have been shown to be teratogenic. Also, the results from testing with numerous agents suggest that supernumerary rib in mice is caused by maternal stress (Kavlock et al., 1985; Beyer and Chernoff, 1986).

Thus, in any case where developmental toxicity occurs at dosage levels with only moderate to severe maternal toxicity, the possibility of the developmental toxicity being secondary to the maternal toxicity can be considered. That is not to say, however, that it can be concluded that the developmental toxicity is secondary any time there is coincident maternal toxicity. To the contrary, it is usually very difficult to establish a causal relationship. Superficially similar types of maternal toxicity do not always cause the same pattern of developmental toxicity (Chernoff et al., 1990). This may be because the developmental toxicity is secondary to maternotoxicity, but, since typical developmental toxicity studies include only a very cursory evaluation of maternal toxicity, the developmental toxicity may be secondary to an aspect of maternotoxicity that is not even being measured.

To demonstrate that a developmental effect is secondary to a particular parameter of maternal toxicity, it is necessary but not sufficient to show that all mothers with developmental toxicity also had maternal toxicity and that the severity of the developmental effect was correlated with the maternal effect. An example of such a correlation is shown in Figure 11.8, in which a drug-induced effect on maternal body weight change in rabbits is correlated ($r = 0.45$, $p < 0.05$) with a drug-induced decrease in fetal weight. Other examples where this approach has been used to evaluate the relationship between maternal and developmental toxicity include (1) the negative correlation between resorption rate and maternal body weight change in norfloxacin-treated rabbits (Clark et al., 1991), supporting the contention that the developmental toxicity was secondary, and (2) the lack of correlation between embryotoxicity and maternal body weight change in pregnant mice treated with caffeine and L-phenylisopropyladenosine (Clark et al., 1987), suggesting no causal relationship between developmental and maternal toxicity may be required.

11.4.4 Assessment of Human Risk

Most test agents can be demonstrated to be developmentally toxic if tested under extreme conditions. This fact has been popularized as Karnofsky's law:

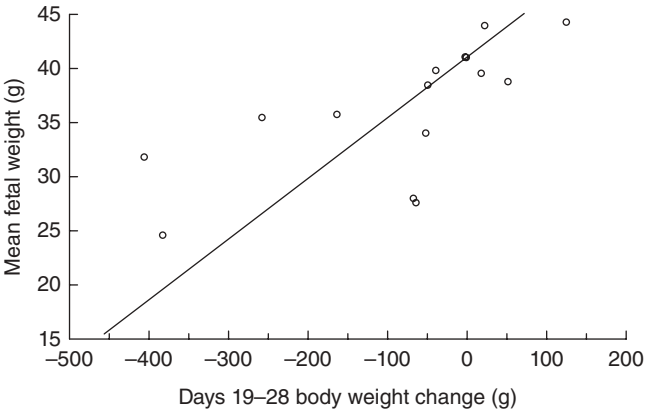

Figure 11.8 *Correlation between drug-induced effects on maternal body weight change and fetal weight in rabbits. The data were collected from the high-dosage group of a developmental toxicity study of a prospective drug candidate. The rabbits were dosed orally with the test agent from days 6–18 of gestation. On day 28 of gestation, the rabbits were cesarean sectioned and the live fetuses weighed.*

"Any drug administered at the proper dosage, at the proper stage of development, to embryos of the proper species … will be effective in causing disturbances in embryonic development" (Karnofsky, 1965, p. 185). In practice, about 37% of 3301 chemicals tested have been found to be teratogenic according to one tabulation (Schardein, 1993, p. viii; see also Shepard, 1998). Contributing to this high rate is the practice of testing maternotoxic doses (to satisfy regulatory guidelines) that in some cases result in developmental toxicity secondary to maternal toxicity. Despite the high rate of positives in animal tests, very few xenobiotics are known to cause developmental toxicity in humans as commonly used. Thus, simply the induction of developmental toxicity by a test agent in animals does not necessarily indicate that that test agent will be a developmental hazard to human conceptus under normal exposure conditions.

When a prospective drug under development for use in women of childbearing potential is determined to cause developmental toxicity in laboratory animals, the first question to be considered is whether that agent would cause developmental toxicity in humans at the anticipated therapeutic dosage level. This assessment and the related decision of whether to continue development of the drug candidate are currently based on the following:

1. The ratio between the estimated systemic exposure at the lowest effect level [or highest no-observed-effect level (NOEL)] and the estimated systemic exposure at the anticipated therapeutic dosage level (the "safety factor")
2. Whether the effect is selective, direct, and/or specific

3. The potential benefit to the patient population (compared to other available therapies)

The most common finding is that minor, nonselective, nonspecific developmental toxicity (e.g., decreased fetal weight) is observed at dosages at least 10-fold above the anticipated therapeutic dosage level. In this situation, development of the agent would normally proceed even if the "safety factor" were only 3–5. This is the case since (1) many pharmaceuticals cause maternal toxicity in laboratory animals at low multiples (e.g., 10) of the clinical exposure, (2) nonspecific developmental toxicity commonly accompanies maternal toxicity, and (3) pharmaceuticals fitting this pattern do not usually cause developmental effects as used clinically (which often includes the practice of not prescribing for women known to be pregnant).

In contrast, a drug candidate that selectively causes major malformations at a dosage threefold higher than the clinical dosage would likely not be developed to treat a non-life-threatening disease. However, it might be developed if the disease to be treated was particularly debilitating, no other effective therapy was available, and it was felt that the exposure of pregnant women could be largely avoided.

Until this year, once a new pharmaceutical is approved by the FDA, it is placed in one of five pregnancy categories (A, B, C, D, or X) based on the results of animal developmental toxicity studies and, when available (usually not), information from human usage experience (see Table 11.2 and Frankos, 1985). Note that the categorization does not depend on the safety factor for a developmental effect or whether the effect is major, selective, direct, or specific (although these factors may be considered when determining if a drug is to be approved). Most often, there are positive findings in animals and no experience in pregnant women and the drug is placed in pregnancy category C, indicating that it is to be used in pregnancy only if the potential benefit justifies the risk to the conceptus. Thus, it is left to the prescribing physician to regulate the exposure of pregnant women to the drug. If animals studies were negative and there is no information on effects on pregnant women, the agent is placed in pregnancy category B, indicating that the agent is to be used in pregnancy only if clearly needed. If developmental toxicity has been established in women (or, in some cases, if only strongly suspected), the agent is placed in category D or X. With category D, women may be prescribed the drug if the benefit outweighs the risk and the patient is informed of the potential hazard to the conceptus. Category X drugs are contraindicated in women who are or may become pregnant. Table 11.4 summarizes these categories and the proportions of drugs in each in 2000 [according to the *Physician's Desk Reference* (PDR)].

The FDA has adopted (2009) a change in these labeling requirements first proposed in May 2008. A new FDA pregnancy labeling guidance was proposed for labeling of drugs for reproductive and pregnancy risks. The proposed rule would remove the letter categories from the pregnancy section. The new format would have three sections:

TABLE 11.4 Pregnancy Categories[a]

	Outcome of Animal Studies		
Outcome of Human studies	+	–	Not Available
+	X or D	X or D	X or D
–	B	A	A or B
Not available	C_1	B	C_2

[a]A, B, C_2: Use during pregnancy only if clearly needed.
C_1: Use during pregnancy only if the potential benefit justifies the potential risk to the fetus.
D: If used during pregnancy, the patient should be apprised of the potential hazard to the fetus.
X: Contraindicated in women who are or may become pregnant.

1. *Fetal Risk Summary* This section would begin with a one-sentence risk conclusion that characterizes the likelihood that the drug increases the risk of four types of developmental abnormalities: structural anomalies, fetal and infant mortality, impaired physiological function, and alterations to growth. An example of a risk conclusion based on human data is: "human data do not indicate that Drug X increases the overall risk of structural anomalies." Many of the risk conclusions in the proposed rule are standardized statements that must be used. This would be followed by a summary of significant effects.
2. *Clinical Considerations* This component would address three main topics important when counseling with and prescribing for women who are pregnant, lactating, or of childbearing age.
3. *Data* This section would have a more detailed discussion of available data. Human data would appear before animal data.

The pregnancy section would also include information about whether there is a pregnancy registry for the drug. Pregnancy exposure registries collect and maintain data on the effects of approved drugs that are prescribed to and used by pregnant women. The lactation section of prescription drug labeling would provide information on use of the drug while breastfeeding and would use the same format as the pregnancy section.

Using the highest NOEL for determining a safety factor has the following flaws:

1. The determination of a true no-effect level (should one actually exist, which is debatable in some cases) is impossible given the statistical power associated with the group sizes typically used; thus, the reported NOEL is very dependent on the selected group size.
2. The NOEL depends greatly on the selection of dosage levels; unless the selected dosage is just below the threshold for detectable effects, the reported NOEL is an underestimate; thus, tightly spaced dosage levels favor the determination of a higher NOEL.

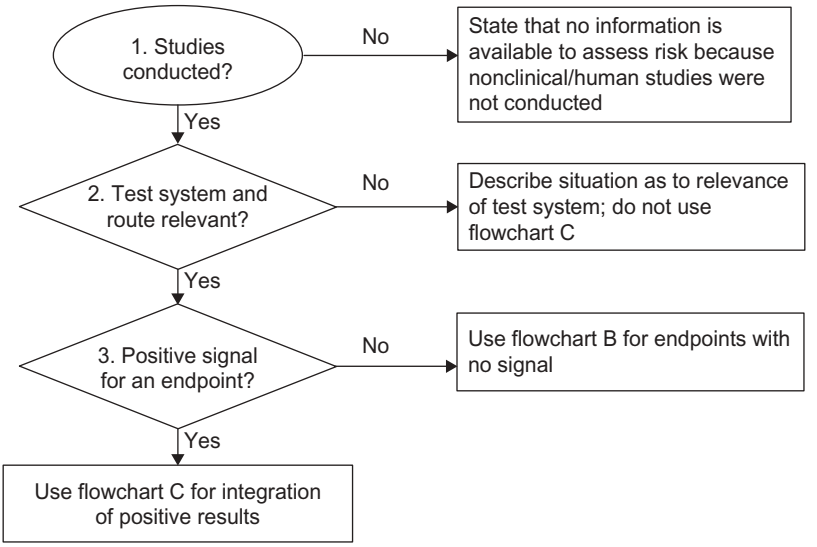

Figure 11.9 *Flowchart A. Overall decision tree for evaluation on reproduction/developmental toxicity risk from Wedge Document, 1999, distributed through www.FDA.gov (June 1999).*

Accordingly, the FDA has developed a sequential method of evaluating and dealing with reproductive and developmental analysis. This is called wedge analysis and is demonstrated in Figures 11.9–11.11.

11.5 IN VITRO TESTS FOR DEVELOPMENTAL TOXICITY

Many in vitro systems have been proposed as tests for developmental toxicity (for review, see Brown, 1984; Lin, 1987; In Vitro Teratology Task Force, 1987; and Gad, 2000). Various uses have been suggested for these in vitro tests, including the following:

1. A general prescreen to select likely developmental toxins for subsequent whole-animal studies
2. A prescreen to select among possible backups to a lead drug candidate that had been found to be developmentally toxic
3. To study mechanisms of developmental toxicity
4. To provide supplementary information about developmental toxicity in addition to that provided by whole-animal studies
5. To replace whole animals for evaluating developmental toxicity

Uses 1 and 5 above are very unlikely to be applicable to the pharmaceutical industry. One problem with in vitro systems for these purposes is that the

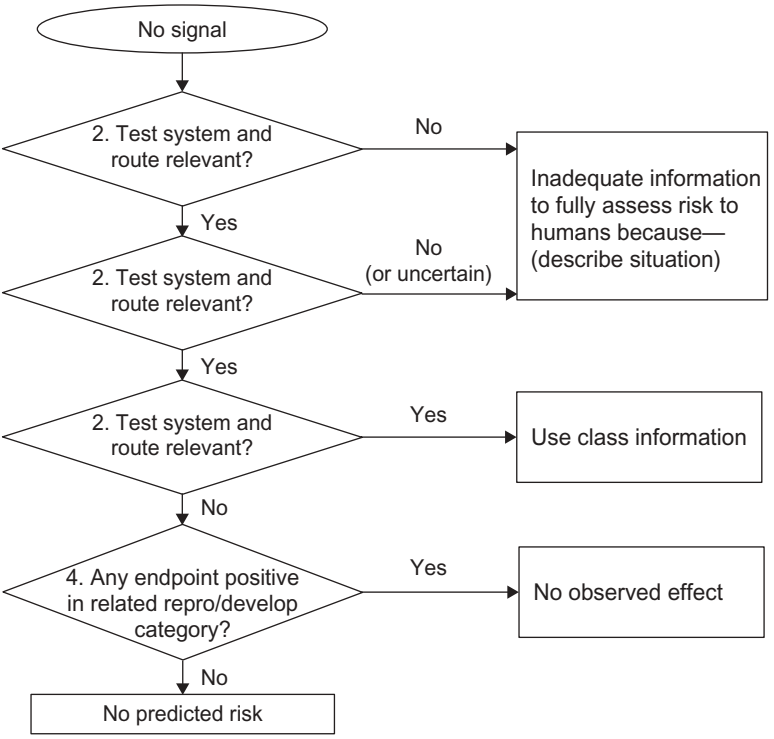

Figure 11.10 *Flowchart B. Decision tree for endpoints with no signal from Wedge Document, 1999, distributed through www.FDA.gov (June 1999).*

percentages of the agents that are positive are very high, for example, 69% of agents tested in the mouse ovarian tumor (MOT) cell attachment test and 72% of agents tested in the mouse limb bud (MLB) assay. High correlations between in vivo and in vitro results have been reported based on the limited number of validation work completed. But these correlations have compared an in vitro end point to teratogenicity in laboratory animals without regard to maternotoxicity. Thus, the question that these screens seem to be answering is: Can this agent be teratogenic or developmentally toxic in laboratory animals at any dosage level? However, as discussed above, it is not important for the purpose of safety assessment if an agent can be developmentally toxic in laboratory animals at high, maternotoxic dosages. The important question for prospective screens to answer is this: Is the agent a selective or direct developmental toxin? For these reasons, a promising drug candidate would not be dropped from development due to a positive result in a current in vitro test and a negative result would not preclude the need for whole-animal studies.

To relate a positive finding in an in vitro test to the in vivo situation, one must either compare the concentration that caused the positive developmental effect in vitro to the exposure level of the embryo in vivo or compare the in

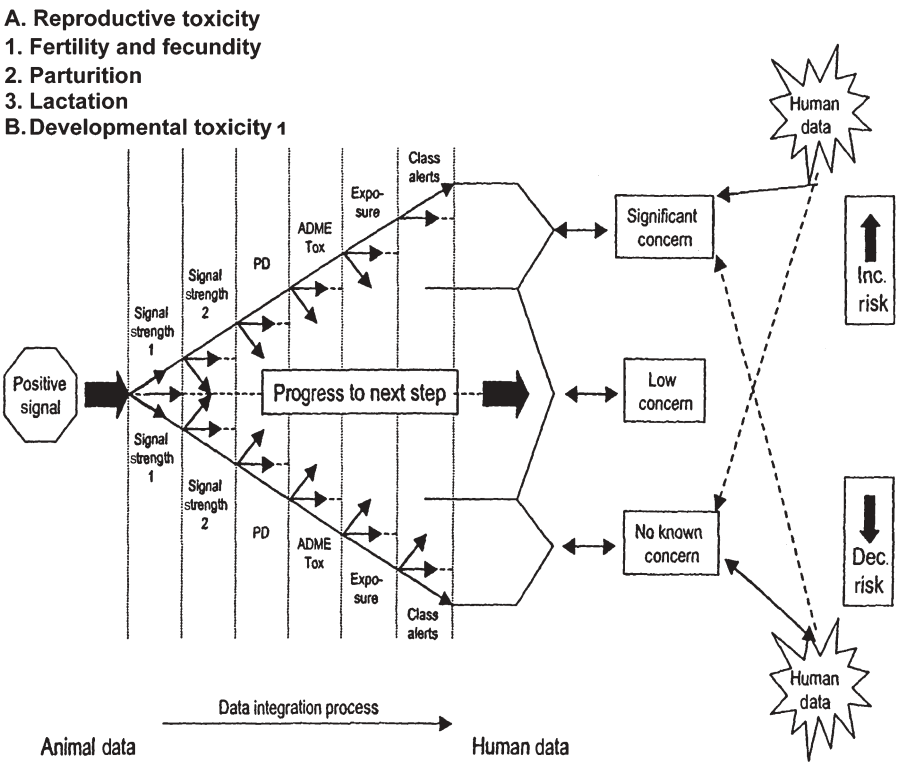

Figure 11.11 *Flowchart C. Integration of positive reproduction/ancillary study results from Wedge Document, 1999, distributed through www.FDA.gov (June 1999).*

vitro concentration for a developmental effect to the maternotoxicity that would be associated with exposure at that concentration in vivo. To do the necessary pharmacokinetic studies in vivo would defeat the purpose of using an in vitro test. It would be very desirable and may be possible, though, to have an endpoint in an in vitro test that would correlate with maternal toxicity.

Currently, only the *Hydra* system incorporates a measurement of "toxicity" to the adult to provide a comparison of the sensitivity of the "embryo" with that of the adult (Johnson et al., 1988). However, the *Hydra* screen has not been fully validated as being predictive of results in mammals and has fallen from favor. Thus, a major goal of research directed toward developing an in vitro teratogen screen should be to find a simple yet appropriate measure of toxicity unrelated to development. This would allow the comparison of the dose for a 50% effect (ED_{50}) of "developmental toxicity" as measured in vitro to an ED_{50} for "adult" toxicity in vitro. The validation of such a dual in vitro system would involve comparing the developmental selectivity in vitro to that in vivo for a large number of compounds. In a preliminary effort in this regard,

effects on cell division in the rat limb bud micromass assay were considered as a possible correlate of maternal toxicity (Wise et al., 1990a).

Another possible use of in vitro developmental toxicity tests would be to select the least developmentally toxic backup from among a group of structurally related compounds with similar pharmacological activity (use 2 above), for example, when a lead compound causes malformations in vivo and is also positive in a screen that is related to the type of malformation induced. However, even for this limited role for a developmental toxicity screen, it would probably also be desirable to have a measure of the comparative maternotoxicity of the various agents and/or information on the pharmacokinetics and distribution of the agents in vivo.

In vitro developmental toxicity systems have clearly been useful for studies of mechanisms of developmental effects (e.g., Daston et al., 1989)—use 3 above. It is unclear, though, whether in vitro developmental toxicity tests will provide useful information about developmental toxicity that is not derived from whole-animal studies (use 4). As is true for a possible use as a prescreen, the interpretation of a positive finding in an in vitro test will depend on knowing the exposure level in vivo. When this is known, the in vitro information could be helpful. The results of in vivo studies, though, would still likely be considered definitive for that species.

11.6 APPRAISAL OF CURRENT APPROACHES FOR DETERMINING DEVELOPMENTAL AND REPRODUCTIVE HAZARDS

The current system for testing new pharmaceuticals for developmental and reproductive toxicity has been largely intact since 1966. In that time, no thalidomide-like disasters have occurred. It cannot be proven, but there is a good chance that these two statements are linked, that is, that the testing system has prevented potent, selective, human teratogens from being marketed. Indeed, the development of many compounds has been terminated because of positive findings in standard developmental toxicity studies. We do not know for certain if any of these agents would have been developmental hazards in humans, but it seems very likely. Due to the limited information on developmental toxicity of chemical agents in humans and the obvious inability to conduct controlled human studies, the correlation between animal studies and human findings is uncertain and it is difficult to extrapolate precisely from animals to humans (see Frankos, 1985). However, the worst hazards—the few dozen selective developmental toxins that are known to be teratogens in humans—are generally also selective teratogens in animals. Thus, although the current battery of animal studies is not perfect, it appears to have been adequate and effective in performing the important task of preventing the widespread exposure of pregnant women to dangerous developmental toxins. In the few cases where new pharmaceuticals have been shown to cause malformations in humans, animal studies had been positive and provided an early warning to the potential problem.

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12

Carcinogenicity Studies

12.1 INTRODUCTION

In the experimental evaluation of substances for carcinogenesis based on experimental results of studies in a nonhuman species at some relatively high dose or exposure level, an attempt is made to predict the occurrence and level of tumorogenesis in humans at much lower levels. In this chapter we will examine the assumptions involved in this undertaking and review the aspects of design and interpretation of traditional long-term (lifetime) animal carcinogenicity studies as well as some alternative short-term models. It should be noted that these are required of the majority of marketed drugs but are only performed on the minority of drugs which reach a stage of development where a marketing application is likely and to occur within three or so years.

At least in a general way, we now understand what appear to be most of the mechanisms underlying chemical- and radiation-induced carcinogenesis. The most recent regulatory summary on identified carcinogens [National Institutes of Health (NIH), 2000] lists 44 agents classified as known to be human carcinogens. Several hundred other compounds are also described as having lesser degrees of proof. A review of these mechanisms is not germane to this chapter [readers are referred to Miller and Miller (1981) for a good short review], but it is now clear that cancer as seen in humans is the result of a multifocal set of causes.

Drug Safety Evaluation, Second Edition, by Shayne Cox Gad

Mechanisms and Theories of Chemical Carcinogenesis

1. Genetic (all due to some genotoxic event)
2. Epigenetic (no mutagenic event)
3. Oncogene activation
4. Two-step (induction/promotion)
5. Multistep (combination of above)

Looked at another way, the four major carcinogenic mechanisms are DNA damage, cell toxicity, cell proliferation, and oncogene activation. Any effective program to identify those drugs which have the potential to cause or increase the incidence of neoplasia in humans must effectively screen for these mechanisms (Kitchin, 1999; McGregor, 2000; Powell and Berry, 2000; Williams and Iatropoulos, 2001).

The single most important statistical consideration in the design of bioassays in the past was based on the point of view that what was being observed and evaluated was a simple quantal response (cancer occurred or it did not) and that a sufficient number of animals needed to be used to have reasonable expectations of detecting such an effect. Though the single fact of whether or not the simple incidence of neoplastic tumors is increased due to an agent of concern is of interest, a much more complex model must now be considered. The time to tumor, patterns of tumor incidence, effects on survival rate, and age of first tumor all must now be captured in a bioassay and included in an evaluation of the relevant risk to humans.

The task of interpreting the results of any of the animal-based bioassays must be considered from three different potential perspectives as to organ responsiveness:

I. Those organs with high animal and low human neoplasia rates
II. Those organs with high neoplasia rates in both animals and humans
III. Those organs with low animal but high human neoplasia rates

Note that not considered is the potential other case—where the neoplasia rates are low for both animals and humans. This is a very rare case and one for which our current bioassay designs probably lack sufficient power to be effective.

In group I, the use of animal cancer data obtained in the liver, kidney, forestomach, and thyroid gland are perceived by some as being hyperresponsive, too sensitive, and of limited value and utility in the animal cancer data obtained in group I organs. The liver is such a responsive and important organ in the interpretation of cancinogenesis data that the discussion of this subject area has been broken up into three chapters for human, rat, and mouse data. Peroxisome proliferation in the liner, particularly in mice, is an area of interpretive battle as in many cases the metabolism and mechanisms involved are not relevant to humans.

Group II organs (mammary gland, hematopoietic, urinary bladder, oral cavity, and skin) are less of an interpretive battleground than group I organs. For group II organs, all four major mechanisms of carcinogenesis (electrophile generation, oxidation of DNA, receptor–protein interactions, and cell proliferation) are known to be important. The high cancer rates for group B organs in both experimental animals and humans may at first give us a false sense of security about how well the experimental animal models are working. As we are better able to understand the probable carcinogenic mechanism(s) in the same organ in the three species, we may find that the important differences between the three species are more numerous than we suspect. This is particularly true for receptor-based and for cell-proliferation-based carcinogenic mechanisms.

Animal cancer data of group III organs are the opposite of group I organs. Group III organs have low animal cancer rates and high human cancer rates. In contrast to the continuing clamor and interpretive battleground associated with group A organs, there is little debate over group III organs. Few voices have questioned the adequacy of the present-day animal bioassay to protect the public health from possible cancer risks in these group III organs. Improved efforts must be made toward the development of cancer-predictive systems or short-term tests for cancer of the prostate gland, pancreas, colon/rectum, and cervix/uterus.

Carcinogenicity bioassays are the longest and most expensive of the extensive battery of toxicology studies required for the registration of pharmaceutical products in the United States and in other major countries. In addition, they are often the most controversial with respect to interpretation of their results. These studies are important because, as noted by the International Agency for Research on Cancer (IARC, 1987), "in the absence of adequate data on humans, it is biologically plausible and prudent to regard agents for which there is sufficient evidence of carcinogenicity in experimental animals as if they presented a carcinogenic risk to humans."

In this chapter, we consider the major factors involved in the design, conduct, analysis, and interpretation of carcinogenicity studies as they are performed in the pharmaceutical industry.

12.2 REGULATORY REQUIREMENTS AND TIMING

The prior U.S. Food and Drug Administration (FDA) guidance on the need for carcinogenicity testing of pharmaceuticals presented a dual criteria: that such studies were required to support registration of a drug that was to be administered for a period of three months or more (in Japan and Europe this was stated to be six months or more) and such testing had to be completed before filing for registration in such cases. International Conference on Harmonisation (ICH, 1996) guidelines now fix this triggering human exposure period at six months, excluding agents given infrequently through a lifetime

or for shorter periods of exposure unless there is reason for concern (such as positive findings in genotoxicity studies, structure–activity relationships suggesting such risk, evidence of preneoplastic lesions in repeat-dose studies, or previous demonstration of carcinogenic potential in the product class that is considered relevant to humans). Such studies are still only required to be completed before filing for registration. Most developers conduct carcinogenicity studies in parallel with phase III clinical studies.

Endogenous peptides, protein substances, and their analogues are generally not required to be evaluated for carcinogenicity. There are three conditions which call the need into question however:

- Products where there are significant differences in biological effects to the natural counterparts
- Products where modifications lead to significant changes in structure compared to the natural substance
- Products resulting in humans having a significant increase over the existing local or systemic concentration.

The ICH has also given guidance on design, dose selection, statistical analysis, and interpretation for such studies (1996, 1997a,b). The FDA has also offered guidance, the most recent form (FDA, 2001) in a 44-page document available online.

There has been extensive debate and consideration on the relevance and value of the traditional long-term rodent bioassays. The FDA looked at rat and mouse studies for 282 human pharmaceuticals, resulting in the conclusion that "sufficient evidence is now available for some *alternative in vivo carcinogenicity models to support their application as complimentary studies in combination with a single 2-year carcinogenicity study*" (italics added) to identify transspecies tumorigens (Contrera et al., 1997, pp. 130–131).

The Europeans, meanwhile, have focused on the need for better care in study design, conduct, and interpretation (Spindler et al., 2000), aiming to incorporate these in the revision of the CPMP (Center for Proprietary Medicinal Products) carcinogenicity guidelines (Weaver and Brunden, 1998).

12.3 SPECIES AND STRAIN

Two rodent species are routinely used for carcinogenicity testing in the pharmaceutical industry—the mouse and the rat. Sprague Dawley–derived rats are most commonly used in American pharmaceutical toxicology laboratories. However, the Wistar and Fischer 344 strains are favored by some companies, while the Long Evans and CFE (Carworth) strains are rarely used [Pharmaceutical Manufacturers Association (PMA), 1988].

With respect to mice, the CD-1 is by far the most commonly used strain in the pharmaceutical industry. Other strains used less frequently are the B6C3F1,

CF-1, NMRI, C57B1, Balb/c, and Swiss (PMA, 1988; Rao et al., 1988). Swiss is the generic term since most currently used inbred and outbred strains were originally derived from the "Swiss" mouse.

If either the mouse or the rat is considered to be an inappropriate species for a carcinogenicity study, the hamster is usually chosen as the second species.

The use of two species in carcinogenicity studies is based on the traditional wisdom that no single species can be considered an adequate predictor of carcinogenic effects in humans. Absence of carcinogenic activity in two different species is thought to provide a greater level of confidence that a compound is "safe" for humans than data derived from a single species.

One may question this reasoning on the basis that data from two "poor predictors" may not be significantly better than data from a single species. It is also reasonable to expect that the ability of one rodent species to predict a carcinogenic effect in a second rodent species should be at least equal to, if not better than, its ability to predict carcinogenicity in humans. The concordance between mouse and rat carcinogenicity data has been investigated and a summary of the results is presented in the next paragraph.

A review of data from 250 chemicals found 82% concordance between results of carcinogenicity testing in the mouse and the rat (Purchase, 1980). Haseman et al. (1984a) reported a concordance of 73% for 60 compounds studied in both species. However, 30–40% of 186 National Cancer Institute (NCI) chemicals were found to be positive in one species and negative in the other (Gold et al., 1984). It is reasonable to conclude that neither rodent species will always predict the results in the other rodent species or in humans and that the use of two species will continue until we have a much better understanding of the mechanisms of carcinogenesis.

The choice of species and strain to be used in a carcinogenicity study is based on various criteria, including susceptibility to tumor induction, incidence of spontaneous tumors, survival, existence of an adequate historical database, and availability.

Susceptibility to tumor induction is an important criterion. There would be little justification for doing carcinogenicity studies in an animal model that did not respond when treated with a "true" carcinogen. Ideally, the perfect species/strain would have the same susceptibility to tumor induction as the human. Unfortunately, this information is usually unavailable, and the tendency has been to choose animal models that are highly sensitive to tumor induction to minimize the probability of false negatives.

The incidence of spontaneous tumors is also an important issue. Rodent species and strains differ greatly in the incidence of various types of spontaneous tumors. The Sprague Dawley stock, although preferred by most pharmaceutical companies, has a very high incidence of mammary tumors in aging females, which results in substantial morbidity during the second year of a carcinogenicity study. If one chooses the Fischer 344 (F344) strain, the female mammary tumor incidence will be lower, but the incidence of testicular tumors will be higher (close to 100%) than that in Sprague Dawley rats.

A high spontaneous tumor incidence can compromise the results of a carcinogenicity study in two ways. If a compound induces tumors at a site that already has a high spontaneous tumor incidence, it may be impossible to detect an increase above the high background "noise." Conversely, if a significant increase above control levels is demonstrated, one may question the relevance of this finding to humans on the basis that the species is "highly susceptible" to tumors of this type.

The ability of a species/strain to survive for an adequate period is essential for a valid assessment of carcinogenicity. Poor survival has caused regulatory problems for pharmaceutical companies and is, therefore, an important issue (PMA, 1988). The underlying concept is that animals should be exposed to the drug for the greater part of their normal life span to make a valid assessment of carcinogenicity. If animals in a study die from causes other than drug-induced tumors, they may not have been at risk long enough for tumors to have developed. The sensitivity of the bioassay would be reduced and the probability of a false-negative result would be increased.

The availability of an adequate historical database is often cited as an important criterion for species/strain selection. Historical control data can sometimes be useful in evaluating the results of a study. Although such data are not considered equal in value to concurrent control data, they can be helpful if there is reason to believe that the concurrent control data are "atypical" for the species/strain.

Although outbred stocks (e.g., Sprague Dawley rats and CD-1 mice) are generally favored in the pharmaceutical industry, inbred strains are also used (e.g., F344 rats and B6C3F1 mice). Inbred strains may offer greater uniformity of response, more predictable tumor incidence, and better reproducibility than outbred strains. However, their genetic homogeneity may also result in a narrower range of sensitivity to potential carcinogens than exists in random-bred animals. In addition, extrapolation of animal data to humans is the ultimate goal of carcinogenicity studies, and the human population is anything but genetically homogenous.

The ideal species for carcinogenicity bioassays should absorb, metabolize, and excrete the compound under study exactly as humans do. Unfortunately, because of the small number of species that meet the other criteria for selection, there is limited practical utility to this important scientific concept as applied to carcinogenicity studies.

Before concluding this discussion of species/strain selection, it may be worthwhile to take a closer look at the animals preferred by pharmaceutical companies to determine to what extent they meet the conditions described above. Advantages of the CD-1 mouse are (1) a good historical database including various routes of exposure, (2) demonstrated susceptibility to induction of tumors, and (3) relatively low spontaneous incidence of certain tumors to which other strains are highly susceptible, especially mammary and hepatic tumors. Disadvantages are (1) lack of homogeneity, (2) relatively low survival, (3) moderate to high incidence of spontaneous pulmonary tumors and

leukemias, and (4) high incidence of amyloidosis in important organs, including the liver, kidney, spleen, thyroid, and adrenals (Sher et al., 1982).

There has recently been a reduction in survival of Sprague Dawley rats and rats of other strains (FDA, 1993). This reduction may be the result of ad libitum feeding, as preliminary results suggest that caloric restriction may improve survival. Leukemia appears to be the major cause of decreasing survival in the F344 rat. The problem of reduced survival may necessitate a reevaluation of the survival requirements for carcinogenicity studies by regulatory agencies.

12.4 ANIMAL HUSBANDRY

Because of the long duration and expense of carcinogenicity studies, the care of animals used in these studies is of paramount importance. Various physical and biological factors can affect the outcome of these studies. Some important physical factors include light, temperature, relative humidity, ventilation, atmospheric conditions, noise, diet, housing, and bedding (Rao and Huff, 1990). Biological factors include bacteria and viruses that may cause infections and diseases.

The duration, intensity, and quality of light can influence many physiological responses, including tumor incidence (Greenman et al., 1984; Wiskemann et al., 1986). High light intensity may cause eye lesions, including retinal atrophy and opacities (Bellhorn, 1980; Greenman et al., 1982). Rats housed in the top row and the side columns of a rack may be the most severely affected.

The influence of light on the health of animals may be managed in several ways. The animals may be randomly assigned to their cages on a rack such that each column contains animals of a single dose group. The location of the columns on the rack may also be randomized so that the effect of light is approximately equal for all dose groups. In addition, the cages of each column of the rack may be rotated from top to bottom when the racks are changed.

Room temperature has been shown to influence the incidence of skin tumors in mice (Weisbrode and Weiss, 1981). Changes in relative humidity may alter food and water intake (Fox, 1977). Low humidity may cause "ringtail," especially if animals are housed in wire mesh cages (Flynn, 1960).

Diets for rodents in carcinogenesis studies should ideally be nutritionally adequate while avoiding excesses of nutrients that may have adverse effects.

Types of caging and bedding have been shown to affect the incidence and latency of skin tumors in mice. In a study by DePass et al. (1986), benzo[*a*] pyrene-treated mice were housed either in stainless steel cages or polycarbonate shoebox cages with hardwood bedding. The mice housed in shoebox cages developed tumors earlier and with higher frequency than those housed in steel cages.

Housing of rats in stainless steel cages with wire mesh floors may result in decubitous ulcers on the plantar surfaces. This condition may be a significant

clinical problem associated with high morbidity and may affect survival of the animals if euthanasia is performed for humane reasons. Ulcers are particularly frequent and severe in older male Sprague Dawley rats, perhaps because of their large size and weight compared with females and rats of other strains.

Common viral infections may affect the outcome of carcinogenicity studies by altering survival or tumor incidence. Nevertheless, viral infections did not cause consistent adverse effects on survival or tumor prevalence in control F344 rats from 28 NCI/National Toxicalogy Program (NTP) studies, though body weights were reduced by Sendai and pneumonia viruses of mice (Rao et al., 1989). The probability of such infections can be minimized by using viral-antibody-free animals, which are readily available.

12.5 DOSE SELECTION

12.5.1 Number of Dose Levels

In the pharmaceutical industry, most carcinogenicity studies have employed at least three dose levels in addition to the controls, but four levels have occasionally been used (PMA, 1988). The use of three or four dose levels satisfies regulatory requirements (Speid et al., 1990) as well as scientific and practical considerations. If a carcinogenic response is observed, information on the nature of the dose–response relationship will be available. If excessive mortality occurs at the highest dose level, a valid assessment of carcinogenicity is still possible when there is adequate survival at the lower dose levels.

12.5.2 Number of Control Groups

Pharmaceutical companies have most frequently favored the use of two control groups of equal size (PMA, 1988). A single control group of the same size as the treated groups is also used and, less frequently, one double-sized control group may be used. The diversity of study designs reflects the breadth of opinion among toxicologists and statisticians on this issue.

Use of two control groups has the advantage of providing an estimate of the variation in tumor incidence between two groups of animals in the absence of a drug effect. If there are no significant differences between the control groups, the data can be pooled, and the analysis is identical to that using a single, double-sized group. When significant differences occur between the control groups, one must compare the data from the drug-treated groups separately with each control group.

There will be situations in which the incidence of a tumor in one or more drug-treated groups is significantly higher than that of one control group but similar to that of the other control group. In such a situation, it is often helpful to compare the tumor incidences in the control groups to appropriate historical control data. One may often conclude that, for this tumor, one of the

control groups is more "typical" than the other and should therefore be given more weight in interpreting the differences in tumor incidence.

In spite of its current popularity in the pharmaceutical industry, the use of two control groups is opposed by some statisticians on the grounds that a significant difference between the two groups may indicate that the study was compromised by excessive, uncontrolled variation. Haseman et al. (1986), however, analyzed tumor incidence data from 18 color additives tested in rats and mice and found that the frequency of significant pairwise differences between the two concurrent control groups did not exceed that which would be expected by chance alone.

The use of one double-sized group is sometimes preferred because it may provide a better estimate of the true control tumor incidence than that provided by a smaller group. Nevertheless, more statistical power would be obtained by assigning the additional animals equally to all dose groups rather than to the control group only if power is a primary consideration.

12.5.3 Criteria for Dose Selection

Dose selection is one of the most important activities in the design of a toxicology study. It is especially critical in carcinogenicity studies because of their long duration. Whereas faulty dose selection in an acute or subchronic toxicity study can easily be corrected by repeating the study, this situation is much less desirable in a carcinogenicity study, especially since such problems may not become evident until the last stages of the study.

The information used for dose selection usually comes from subchronic toxicity studies, but other information about the pharmacological effects of a drug and its metabolism and pharmacokinetics may also be considered. The maximum recommended human dose (MRHD) of the drug may be an additional criterion if this is known when the carcinogenicity studies are being designed.

For most pharmaceutical companies, the doses selected are as follows. The highest dose is selected to be the estimated maximum tolerated dose (MTD). The lowest dose is usually a small multiple (1–5 times) of the MRHD, and the middose approximates the geometric mean of the other two doses (PMA, 1988; McGregor, 2000).

The MTD is commonly estimated to be the maximum dose that can be administered for the duration of the study that will not compromise the survival of the animals by causes other than carcinogenicity. It should be defined separately for males and females. ICH (1997b) states that the MTD is "that dose which is predicted to produce a minimum toxic effect over the course of the carcinogenicity study, usually predicted from the results of a 90-day study." Factors used to define minimum toxicity include no more than a 10% decrease in body weight gain relative to controls, target organ toxicity, and/or significant alterations in clinical pathology parameters. If the MTD has been chosen appropriately, there should be no adverse effect on survival, only a modest

decrement in body weight gain and minimal overt signs of toxicity. The procedures for dose selection described above are generally consistent with major regulatory guidelines for carcinogenicity studies (Speid et al., 1990; FDA, 1993).[1] There are, however, exceptions to the general approach described above. For example, for nontoxic drugs, the difference between the high and the low doses may be many orders of magnitude if the high dose is set at the estimated MTD and the low dose is a small multiple of the clinical dose. Some guidelines request that the low dose be no less than 10% of the high dose (Speid et al., 1990). In this situation, it may be acceptable to set the high dose at 100 times the MRHD even if the MTD is not achieved (Speid et al., 1990). Similarly, when a drug is administered in the diet, the highest concentration should not exceed 5% of the total diet whether or not the MTD is achieved (Japanese Ministry of Health and Welfare, 1989).

Metabolism and/or pharmacokinetic data, when available, should also be considered in the dose selection process. It is desirable that a drug not be administered at such a high dose that it is excreted in a different manner than at lower doses, such as the MRHD. Similarly, the high dose should not lead to the formation of metabolites other than those formed at lower (clinical) doses. If data show that a given dosage produces maximum plasma levels, administration of higher doses should be unnecessary. These considerations may be very useful when interpreting the results of the study or attempting to extrapolate the results to humans.

12.6 GROUP SIZE

The minimum number of animals assigned to each dose group in pharmaceutical carcinogenicity studies is 50 of each sex (PMA, 1988). Most companies, however, use more than the minimum number, and some use up to 80 animals per sex per group. The most important factor in determining group size is the need to have an adequate number of animals for a valid assessment of carcinogenic activity at the end of the study. For this reason, larger group sizes are used when the drug is administered by daily gavage because this procedure may result in accidental deaths by perforation of the esophagus or aspiration into the lungs. Larger group sizes are also used when the carcinogenicity study is combined with a chronic toxicity study in the rat. In this case, serial sacrifices are performed at 6 and 12 months to evaluate potential toxic effects of the drug.

In the final analysis, the sensitivity of the bioassay for detecting carcinogens is directly related to the sample size. Use of the MTD has often been justified based on the small number of animals at risk compared to the potential human

[1] Note the *FDA Redbook* applies, strictly speaking, only to food additives. It is cited here because it is a well-known toxicology guideline routinely applied to animal pharmaceuticals to which humans may be exposed. The *Redbook* has recently been updated by the FDA (1993).

population, in spite of the difficulties inherent in extrapolating effects at high doses to those expected at much lower clinical doses. A reasonable compromise may be the use of doses lower than the MTD combined with a larger group size than the 50 per sex minimum accepted by regulatory agencies.

12.7 ROUTE OF ADMINISTRATION

In the pharmaceutical industry, the two most common routes of administration are via diet and gavage (PMA, 1988). Some compounds are given by drinking water, topical (dermal) application, or injection, depending on the expected clinical exposure route, which is the primary criterion for determining the route of administration in carcinogenicity studies. When more than one clinical route is anticipated for a drug, the dietary route is often chosen for practical reasons.

Dietary administration is often preferred over gavage because it is far less labor intensive. Another advantage is that the MTD has rarely been overestimated in dietary studies, whereas it has often been overestimated in gavage studies, according to data from the NTP (Haseman, 1985). The dietary route is unsuitable for drugs that are unstable in rodent chow or unpalatable. The dietary route is also disadvantaged by the fact that dosage can only be estimated based on body weight and food intake data, in contrast with gavage, by which an exact dose can be given. Disadvantages of gavage testing are the likelihood of gavage-related trauma, such as puncture of the trachea or esophagus, and possible vehicle (e.g., corn oil) effects.

When doing studies by the dietary route, the drug may be administered as a constant concentration at each dose level or the concentration may be increased as body weight increases to maintain a constant dose on a milligram-per-kilogram basis. The latter method allows greater control of the administered dose and avoids age- and sex-related variations in the dose received, which occur with the former method. Both methods are acceptable to regulatory agencies.

12.8 STUDY DURATION

The duration of carcinogenicity studies for both rats and mice is 2 years in most pharmaceutical laboratories (PMA, 1988). Occasionally, rat studies are extended to 30 months, while come companies terminate mouse studies at 18 months. The difference in duration between mouse and rat studies is based on the belief that rats have a longer natural life span than mice. Recent data indicate, however, that this is not the case. The most commonly used strains, the Sprague Dawley rat and the CD-1 mouse, have approximately equal survival at 2 years based on industry data (PMA, 1988). The same is true for the most popular inbred strains, the F344 rat and the B6C3F1 mouse (PMA, 1988).

Data from NCI studies confirm that the 2-year survival of the B6C3F1 mouse is at least equal to, if not greater than, that of the F344 rat (Cameron et al., 1985)

12.9 SURVIVAL

As stated earlier, adequate survival is of primary importance in carcinogenicity studies because animals must be exposed to a drug for the greater part of their life span to increase the probability that late-occurring tumors can be detected. Early mortality, resulting from causes other than tumors, can jeopardize the validity of a study because dead animals cannot get tumors.

In general, the sensitivity of a carcinogenicity bioassay is increased when animals survive to the end of their natural life span because weak carcinogens may induce late-occurring tumors. The potency of a carcinogen is often inversely related to the time to tumor development. By analogy, as the dose of a carcinogen is reduced, the time to tumor occurrence is increased (Littlefield et al., 1979; DePass et al., 1986).

Why do we not allow all animals on a carcinogenicity study to live until they die a natural death if by so doing we could identify more drugs as carcinogens? In fact, the sensitivity of a bioassay may not be improved by allowing the animals to live out their natural life span because the incidence of spontaneous tumors tends to increase with age. Thus, depending on the tumor type, the ability of the bioassay to detect a drug-related increase in tumor incidence may actually decrease, rather than increase, with time. Therefore, the optimum duration of a carcinogenicity study is that which allows late-occurring tumors to be detected but does not allow the incidence of spontaneous tumors to become excessive.

Reduced survival in a carcinogenicity study may or may not be drug related. Sometimes, the MTD is exceeded and increased mortality occurs at the highest dose level and, occasionally, at the middose level as well. This situation may not necessarily invalidate a study; in fact, the protocol may be amended to minimize the impact of the drug-induced mortality. For example, cessation of drug treatment may enhance the survival of the animals in the affected groups and allow previously initiated tumors to develop. As shown by Littlefield et al. (1979) in the National Center for Toxicological Research (NCTR) ED01 study, liver tumors induced by 2-acetylaminofluorene, which appeared very late in the study, were shown to have been induced much earlier and not to require the continuous presence of the carcinogen to develop. By contrast, bladder tumors that occurred in the same study were dependent on the continued presence of the carcinogen.

Whether drug treatment is terminated or not, drug-related toxicity may also be managed by performing complete histopathology on animals in the lower dose groups rather than on high-dose and control animals only. If there is no increase in tumor incidence at a lower dose level that is not compromised by

reduced survival, the study may still be considered valid as an assessment of carcinogenicity.

When reduced survival is related to factors other than excessive toxicity, the number of animals at risk for tumor development may be inadequate, and the validity of the study may be compromised even in the absence of a drug effect on survival. Obviously, the adjustments described above for excessive, drug-related toxicity are not relevant to this situation.

There is no unanimity of opinion among regulatory agencies as to the minimum survival required to produce a valid carcinogenicity study or as to the best approach for dealing with survival problems. Even within a single agency such as the FDA, different opinions exist on these issues. For example, the recently issued *FDA Redbook II Draft Guideline* (FDA, 2000) requires that rats, mice, or hamsters be treated for 24 months. Early termination due to decreased survival is not recommended. The European Economic Commission (EEC) guidelines differ in that they suggest termination of the study when survival in the control group reaches 20%, while the Japanese guideline suggests termination at 25% survival in the control or low-dose groups (Speid et al., 1990). These provisions make good sense in that they do not request termination of the study when drug-related mortality may be present only at the highest dose.

12.10 ENDPOINTS MEASURED

A carcinogenicity study is more focused than a chronic toxicity study—fewer endpoints are evaluated, and as such it is a simpler study. The key endpoints are actually few:

- Pathology (limited to neoplastic and preneoplastic tissue transformations
- Body weight (to ensure that toxicity is not so great as to invalidate the assays and also that it is just sufficient to validate the assay)
- Survival (key to determining when to terminate the study)
- Clinical pathology (limited to evaluating the morphology of white blood cells, and usually this is actually deferred until there are indications that such data are needed)
- Food consumption (actually measured to ensure that dietary administration doses are accurate)

Only pathology will be considered in detail.

The primary information for carcinogenicity evaluation is generated by pathologists. Table 12.1 lists the tissues normally collected, processed, and evaluated. These professionals, like any other group of professionals, vary in their training and experience, and these are characteristics which may influence the evaluation in a number of ways. Some of these are listed below:

TABLE 12.1 Standard Tissue List

Kidney	Urinary bladder	Aorta
Heart	Trachea	Lungs
Liver	Gall bladder	Pancreas
Fat	Salivary gland	Spleen
Cervical lymph node	Mesenteric lymph node	Thymus
Tongue	Esophagus	Stomach
Duodenum	Jejunum	Ileum
Cecum	Colon	Mammary gland
Skin	Skeletal muscle	Sciatic nerve
Parathyroid	Thyroid	Adrenal
Pituitary	Prostate	Seminal vesicles
Testes	Epididymides	Ovaries
Oviducts	Uterine horns	Uterine body
Cervix	Vagina	Brain
Spinal cord	Sternum	Rib/bone
Eyes	Harderian glands	BM smear
Nares	Clitoral/preputial gland	Zymbal's gland
Gross lesions		

1. Differences in terminology may be important when considering controversial lesions.
2. Lack of consistency throughout a study is likely when a pathologist has only recently become involved with rodent carcinogenicity. Training is often in a clinical situation (especially in Europe), where each animal or person is unique and there is in a rodent carcinogenicity study consisting of 500 animals.
3. Unfamiliarity with the observed lesion in a particular species may cause problems in interpretation.

Possible bias introduced by knowledge of treatment can be corrected in several ways, but the use of a two-stage process would seem to be most efficient:

1. An initial evaluation is performed with full knowledge of the animal's history, including treatment.
2. A second evaluation of specific lesions is then carried out. This should be done blind, either by the same pathologist or, preferably, by the same and a second pathologist.

Differences in evaluation between pathologists should always be discussed by them to resolve the differences; they may be due to subtle differences in diagnosis and do not indicate incompetence in one of the pathologists. It is unacceptable for a study sponsor to shop around until he or she finds a pathologist who gives, for whatever reason, the expected result without giving

an opportunity for interaction with all of the other evaluators. Sometimes these diagnoses are given years apart, during which time understanding of the pathogenesis of lesions may change, and even the first pathologist may not arrive at the same conclusion as he or she did some years ago.

Evaluation of the data is not purely a statistical exercise. A number of important factors should be considered: (1) dose–effect relationship; (2) a shift toward more anaplastic tumors in organs where tumors are common; (3) earlier appearance of tumors; and (4) presence of preneoplastic lesions.

The language used to describe the carcinogenic response has masked its complexity and presents a stumbling block to its understanding among nonhistopathologists. Benign or malignant neoplasms do not arise without some precursor change within normal tissue. An important concept in carcinogenicity evaluation is that of neoplastic progression, which was derived from studies on skin tumors (Berenblum and Shubik, 1947) and expanded to a number of other tissues (Foulds, 1969, 1975). There is, on many occasions, a far from clear distinction between hyperplastic and "benign" neoplasia and between benign and malignant neoplasia.

Hyperplasia and benign and malignant neoplasia are convenient medical terms with prognostic significance. Hyperplasia can occur either as a regenerative response to injury, with no neoplastic connotations, or as a sustained response to a carcinogenic agent. It is an increase in the number of normal cells retaining normal intercellular relationships within a tissue. This normally may break down, resulting in altered growth patterns and altered cellular differentiation—a condition which may be described as atypical hyperplasia or presumptively as preneoplastic lesions. Possible sequelae to hyperplasia are (1) persistence without qualitative change in either structure or behavior; (2) permanent regression; (3) regression with later reappearance; and (4) progression to develop new characteristics indicating increased probability of malignancy. The last of these in the least likely to occur in experimental multistage models, such as in mouse skin or rat liver, where large numbers of hyperplastic lesions may occur but notably fewer carcinomas develop from them.

Benign neoplasms in most rodent tissues apparently arise in hyperplastic foci, for example, squamous cell papillomas of the skin and forestomach. Furthermore, these papillomas seldom demonstrate autonomous growth and even fewer progress to squamous cell carcinomas (Burns et al., 1976; Colburn, 1980). This decisive progression to carcinoma, when it occurs, provides powerful evidence for the multistage theory of carcinogenesis: the new, malignant cells arising as a focus within the papilloma or even in an area of hyperplasia since the papilloma is not a necessary intermediate stage. In other organs, benign neoplasia is usually characterized by well-differentiated cell morphology, a fairly uniform growth pattern, clear demarcation from surrounding tissues, and no evidence of invasion. The progression toward malignancy involves anaplasia (loss of differentiation) and pleomorphism (variety of phenotypic characteristics within the neoplasm). These changes may be focal in an otherwise

TABLE 12.2 Tumor-Bearing Animals in Control Groups from Rodent Studies

Control Animals for 2-Year NTP Bioassay	No. of Animals	Percent with Tumors		
		Malignant	Benign	Total
B6C3F1 mice				
Male	1692	42	35	64
Female	1689	45	33	64
F344 rats				
Male	1596	55	95	98
Female	1643	38	76	88
Osborne–Mendel rats				
Male	50	26	68	78
Female	50	12	80	88
Sprague Dawley rats				
Male	56	9	36	39
Female	56	30	68	79

Source: Haseman, unpublished summary of U.S. NTP data.

benign neoplasm and may vary in degree and extent. Evidence of invasion of the surrounding tissues or of metastasis is not an essential characteristic of malignancy, although their presence strengthens the diagnosis.

The grouping together of certain tumor types can aid statistical analysis, but it must be done carefully, with full appreciation of the biology and whatever is known of the pathogenesis of the lesions. Grouping for analysis of all animals showing neoplasia, irrespective of the tumor type, is inappropriate because the incidence in most treatment control groups can be very high and, in U.S. NTP studies, approaches 100% in rats and 50–70% in mice (Table 12.2).

There may be similar incidences of tumors in aging people, but the real prevalence of tumors in human populations is uncertain. In the United States, where autopsies are uncommon, over one-third reveal previously undiagnosed cancers when they are conducted (Silverberg, 1984). A single type of neoplasm, renal adenoma, is present in 15–20% of all adult kidneys (Holm-Nielson and Olsen, 1988), although it is unclear whether these 2–6-mm foci of proliferating tubular and papillary epithelium represent small carcinomas or benign precursors of renal cell carcinomas. Irrespective of the significance of these lesions in human pathology, the presence of similar foci in a rodent carcinogenicity experiment would trigger the recording of renal tumor–bearing animals and, hence, their consideration in the statistical and pathological evaluation processes. Evaluation is further complicated by the increased background incidences of tumors as animals get older.

The independent analysis of every different diagnosis in rodent studies would also mask significant effects in many cases while enhancing them in others. Benign and malignant neoplasms of a particular histogenesis are often grouped because the one is seen as a progression from the other. However, this grouping may result in a nonsignificant difference from the controls

because there has been an acceleration of progression toward malignancy, the incidence of benign neoplasms decreasing while the malignant neoplasms increase. Guidelines are available for "lumping" or "splitting" tumor types, but in using them, the basis for the classification of neoplastic lesions should be clarified, especially when data generated over several or many years are coupled, since diagnostic criteria and ideas regarding tumor histogenesis may have changed. Reliance on tabulated results alone can lead to serious misinterpretation by those not closely connected with a particular study. For this very important reason, the pathology and toxicology narrative should be full and clear. If it is not, then there will always be doubts about future interpretations, even if these doubts are not, in reality, justified.

12.11 STATISTICAL ANALYSIS

Irrespective of the specific protocols used, all carcinogenicity studies end with a statistical comparison of tumor proportions between treated and control groups. This analysis is necessary because the control incidence of most tumor types is rarely zero. In the unlikely case that a type of tumor is found in treated animals but not in concurrent or appropriate historical controls, it is reasonable to conclude that the tumor is drug related without statistical analysis.

Most pharmaceutical companies analyze tumor data using mortality-adjusted methods (PMA, 1988). Peto/International Agency for Research on Cancer (IRC) methodology is most commonly used, perhaps because this method is currently favored by the FDA (Peto et al., 1980). The use of life table methods is most appropriate for "lethal" tumors, that is, those that cause the death of the animals. Various statistical methods are available for analyzing the incidence of the lethal and nonlethal tumors (e.g., Gart et al., 1979, 1986; Chu et al., 1981; Dinse and Lagakos, 1983; McKnight, 1988; Portier and Bailer, 1989; Gaylor and Kodell, 2001). These methods are especially useful when there are drug-related differences in mortality rates. When there is no drug effect on survival, unadjusted methods will generally give the same results.

As a general approach, most pharmaceutical statisticians begin by testing for the presence of a dose-related trend in tumor proportions. If the trend test is significant, that is the p value is less than or equal to 0.05, pairwise comparisons are performed between the treated and control groups. Trend and pairwise analyses may be adjusted for mortality as stated earlier or performed without mortality adjustment using such simple methods as chi-square or Fisher's exact tests.

Although in most cases the use of trend tests is appropriate since most biological responses are dose related, there are exceptions to this rule. Certain drugs, especially those with hormonal activity, may not produce classical dose responses and may even induce inverse dose–response phenomena. In these cases, a pairwise comparison may be appropriate in the absence of a significant positive trend.

Most (70%) pharmaceutical companies use one-tailed comparisons, and a substantial number use two-tailed methods (PMA, 1988). Since regulatory agencies are primarily interested in identifying carcinogenic drugs, as opposed to those that inhibit carcinogenesis, the use of one-tailed tests is generally considered more appropriate. Some companies prefer two tailed comparisons because, in the absence of a true carcinogenic effect, there is an equal probability of seeing significant decreases as well as significant increases by chance alone.

One of the most important statistical issues in the analysis of carcinogenicity data is the frequency of "false positives," or type I errors. Because of the multiplicity of tumor sites examined and the number of tests employed, there is concern that noncarcinogenic drugs may be erroneously declared carcinogens. If an $p < 0.05$ increase in tumor incidence is automatically regarded as a biologically meaningful result, then the false-positive rate may be as high as 47–50% (Haseman et al., 1986).

Several statistical procedures designed to correct for the multiplicity of significance tests have been published (and reviewed by Haseman, 1990). One approach to the problem of multiple tumor site/type testing is a procedure attributed to Tukey by Mantel (1980). This method is used to adjust a calculated p value based on the number of tumor types/sites for which there are minimum number of tumors in the particular study. The reasoning here is that, for tumor sites, the number of tumors found is so small that it is impossible to obtain a significant result for that tumor site no matter how the tumors might have been distributed among the dose groups. Only those sites for which a minimum number of tumors is present can contribute to the false-positive rate for a particular study.

A method proposed by Schweder and Spjotvoll (1982) is based on a plot of the cumulative distribution of observed p values. Farrar and Crump (1988) have published a statistical procedure designed not only to control the probability of false-positive findings but also to combine the probabilities of a carcinogenic effect across tumor sites, sexes, and species.

Another approach to controlling the false-positive rate in carcinogenicity studies was proposed by Haseman (1983). Under this "rule," a compound would be declared a carcinogen if it produced an increase significant at the 1% level in a common tumor or an increase significant at the 5% level in a rare tumor. A rare neoplasm was defined as a neoplasm that occurred with a frequency of less than 1% in control animals. The overall false-positive rate associated with this decision rule was found to be not more that 7–8% based on control tumor incidences from NTP studies in rats and mice. This false-positive rate compares favorable with the expected rate of 5%, which is the probability at which one would erroneously conclude that a compound was a carcinogen. The method is notable for its simplicity and deserves serious consideration by pharmaceutical statisticians and toxicologists. Without resorting to sophisticated mathematics, this method recognizes the fact that tumors differ in their spontaneous frequencies and, therefore, in their contribution to

the overall false-positive rates in the carcinogenicity studies. False-positive results are much less likely to occur at tissue sites with low spontaneous tumor incidences than at those with high frequencies.

As a final point that has special relevance to pharmaceutical carcinogenicity studies, one may question whether the corrections for multiple comparisons and their effect on the overall false-positive rate are appropriate for all tumor types. For example, if a compound is known to bind to receptors and produce pharmacological effects in a certain organ, is it justified to arbitrarily correct the calculated p value for the incidence of tumors in that organ using the methods described above? It is difficult to justify such a correction considering that the basis for correcting the calculated p value is that the true probability of observing an increased incidence of tumors at any site by chance alone may be much higher than the nominal α level (usually 0.05). It is reasonable to expect that, when a drug has known pharmacological effects on a given organ, the probability of observing an increased tumor incidence in that organ by chance alone is unlikely to be higher than the nominal 5% α level.

Although most pharmaceutical statisticians and toxicologists agree on the need to control the probability of false-positive results, there is no consensus as to which method is most appropriate or most acceptable to regulatory agencies. The FDA and other such agencies will accept a variety of statistical procedures but will often reanalyze the data and draw their own conclusions based on their analyses.

12.12 TRANSGENIC MOUSE MODELS

Sine the early 1970s, the standard for adequate evaluation of the carcinogenic potential of a candidate pharmaceutical has been the conduct of lifetime, high-dose assays in two species—almost always the rat and the mouse.

The relevance (and return on investment) for the bioassays performed in mice has been questioned for some time. In 1997, ICH opened the possibility for the substitution of some form of short- or medium-term mouse test as an alternative to the traditional lifetime mouse bioassay. The FDA has subsequently stated that it would accept "validated" forms of a set of medium-term mouse studies based on transgenic models, and significant effort has since gone into such validation.

The huge advances made in molecular biology since the late 1980s have provided the possibility of approaches to evaluating chemicals and potential drugs for carcinogenic potential in approaches which are different, less expensive, and take a shorter period of time than traditional long-term bioassays. This work has also been stimulated by dissatisfaction with the performance of traditional test systems.

The traditional long-term bioassays use highly inbred animals developed with the goal of reducing the variability in background tumor incidences as a means of increasing the statistical sensitivity of the bioassays. This inbreeding

has led to narrowing of the allelic pool in the strains of animals that are currently used for testing, as opposed to the wild-type populations (of humans) that the tests are intended to protect (Festing, 1979). Transgenic models should serve to improve the identification of carcinogens by providing the gene-specific mechanistic data, minimizing the influence of spontaneous tumors and strain-specific effects, and reducing time required. Cost and animal usage should also be reduced (Eastin et al., 1998).

As it has become possible to transfer new or engineered genes to the germ lines of mammals, the results have been transgenic mice that can be used in shorter term in vivo assays for carcinogenicity and which are also useful for research into the characterization of genotoxic events and mechanisms in carcinogenesis. By coupling reporter phenotypes (such as papilleomas in the Tg·AC mouse, the task of "reading" results in test animals is made much less complex.

There are four transgenic mouse models that have been broadly evaluated—the TSPp53$^{+/-}$, the TgAC, the Hras2, and the XPA$^{-/-}$. Each of these has its own characteristics. Each of these merits some consideration. They are each made by either zygote injection or specific gene targeting in embryonic cells (McAnulty, 2000; French et al., 1999).

12.12.1 Tg·AC Mouse Model

This was the earliest of the models to be developed, and its use in mouse skin carcinogenicity studies was first reported in 1990. The mice have four copies of the v-H-*ras* oncogene in tandem on chromosome 11, and the transgene is fused with a fetal ξ-globin gene which acts as a promoter. The transgene codes for a switch protein which is permanently "on," and this results in the mice having genetically initiated skin. The application of tumor promoters to the surface of the skin causes the rapid induction of pedunculate papillomas that arise from the follicular epithelium. This is despite the fact that the transgene is not expressed in the skin, although it is present in the papillomas that form and also in the focal follicular hyperplastic areas that are the precursors to the papillomas. In about 40% of the mice, the papillomas become malignant skin tumors—mainly squamous cell carcinomas and sarcomas.

The first assessments of this model as an alternative to traditional carcinogenicity studies were performed by the U.S. National Institute of Environmental Health Sciences (NIEHS) and NTP, and the results with over 40 chemicals have been published. The majority of studies were performed by skin painting, regardless of whether the product was a dermal or systemic carcinogen. However, a good correlation was found with the known carcinogenicity of the test compounds, and both mutagens and nonmutagens were identified. It was found that great care had to be taken with the skin because damage could also induce papillomas, which means that these animals cannot be identified using transponder chips. This sensitivity may also explain some of the false positive results that have occurred with resorcinol and rotenone. Of more concern is

that there have been false-negatives with known carcinogens, namely ethyl acrylate and *N*-methyl-*O*-acrylamide. The model was designed for use in the context of the two-stage model of carcinogenesis with the underlying mechanistic pathway involving specific transcription factors, hypomethylation, and cell-specific expression of the results. Along with the p53, this model has seen the widest use and evaluation (in terms of number of agents evaluated) so far. The carrier mouse strain employed, the FVB/N, is not commonly employed in toxicology and is prone to sound-induced seizures. It may be that the dermal route is not suitable for all systemic carcinogens, and this is the reason that in the International Life Sciences Institute (ILSI) program both the dermal and systemic routes are being investigated in this model.

Another problem with this model was the occurrence of a nonresponder genotype to positive control agents. This was found to be attributable to a rearrangement of the ζ-globin promoter region, but it is claimed that this problem has been resolved. However, this has considerably delayed the ILSI studies with this model, but all data should be available in time for the November meeting. It is already clear that the model gives a robust response to the positive control agent, 12-*O*-tetradecanoylphorbol 13-acetate (TPA).

12.12.2 Tg.rasH2 Mouse Model

This model was developed at the Central Institute for Experimental Animals (CIEA) in Japan, and the first information about the mouse was published in 1990. The mice have five or six copies of the human H-*ras* protooncogene inserted in tandem into their genome surrounded by their own promoter and enhancer regions. This transgene has been very stabile, with no loss of responsiveness sine the model was developed. The transgene codes for a molecular switch protein in the same way as the previous model, but the transgene is expressed in all organs and tissues. Thus the response endpoint is not primarily dermal.

The initial studies with this model revealed a rapid appearance of forestomach papillomas with *N*-methyl-*N*-nitrosourea (MNU), and this compound has already been used as the positive control agent in subsequent studies with this strain. A study duration of six months is sufficient to obtain a positive response, and longer periods should be avoided because the mice start to develop various spontaneous tumors, such as splenic hemangiosarcomas, forestomach and skin papillomas, lung and Harderian gland adenocarcinomas, and lymphomas. It has a high level of constitutive expression and some spontaneous tumors even when the animals are younger. It is, however, very responsive to carcinogens—one gets a rapid onset after exposure and a higher response incidence than with the other models. The underlying mechanism is still not certain.

A large number of studies have been run in this strain in Japan in advance of the ILSI program. The model is sensitive to both mutagenic and nonmutagenic carcinogens, although cyclophosphamide and furfural have

given equivocal results in each category, respectively. The majority of noncarcinogens have also been identified correctly, although, again, there are a small number of compounds that have given equivocal results. In the ILSI program, 24 of the 25 studies will have been completed in time for the November meeting, and the final studies will be completed during 2001.

12.12.3 $p53^{+/-}$ Mouse Model

The thrombospondin (TSP) $p53^{+/-}$, hereafter referred to as the *p53* (the designation of the tumor suppressor gene involved), is a heterozygous knockout with (up to seven or so months of age) a low spontaneous tumor incidence. It is responsive to the genotoxic carcinogens by a mechanism based on the fact that many (but not all) tumors show a loss of the wild-type allele. The p53 has been extensively worked on by Tennant's group at NIEHS (Tennant et al., 1995, 1999). This model was developed in the United States and carries a hemizygous knockout of the *p53* gene which was developed by integrating a mutated copy of the gene into the genome of mice. The *p53* gene is known as a tumor suppressor gene, and it is the most commonly mutated gene in human malignancies. It searches for a protein transcription factor which activates multiple genes when damage to DNA strands occurs, and this in turn leads to either the arrest of the cell cycle while DNA repair occurs or to apoptosis (programmed cell death), which removes the damaged cell. The heterozygote is used because homozygotes show a very high incidence of spontaneous tumors within a few months of birth. The heterozygotes have a low background incidence of tumors up to 12 months, but during this time there is a high chance of a second mutagenic event occurring—following exposure to a carcinogen, for example—and this would result in a loss of suppressor function or an increase in transforming activity.

The initial studies with this model as an alternative in traditional carcinogenicity testing were performed at the NIEHS, and these suggested that it was sensitive to mutagenic carcinogens such as benzene and *p*-cresidine within six months. Nonmutagenic carcinogens were negative in the assay, as were mutagenic noncarcinogens. However, subsequent studies and some parts of the ILSI program have shown clear indications that a six-month duration may be insufficient. In particular, benzene has given negative or equivocal results within six months, although positive results have been obtained by extending the study to nine months. It will be very important to assess the results of the ILSI program when deciding the best study duration for this model. This is the most popular model in the United States.

12.12.4 $XPA^{-/-}$ Mouse Model

This was the last of the models to be developed and was created using a knockout technique after the *XPA* gene had been cloned. The first data were published by the National Institute for Public Health and the Environment

(RIVM) in the Netherlands in 1995. Both alleles of the *XPA* gene have been inactivated by a homologous recombination in ES cells, resulting in a homozygous deletion of the gene spanning exons 3 and 4. The protein coded by this gene is essential for the detection and repair of DNA damage using the nucleotide excision repair (NER) pathway. This model only has between 2 and 5% of residual NER activity.

The initial studies at RIVM demonstrated that exposure of these mice to UV-B radiation or 7,12-dimethylbenz[*a*]anthracene resulted in the rapid induction of skin tumors. It was also shown that various internal tumors could be induced following oral administration of mutagenic carcinogens such as benzo[*a*]pyrene (B[a]P) and 2-acetylaminofluorine (2-AAF). The early studies suggested that this response could occur within six months, but further experience has indicated that a nine-month treatment period is essential in order to obtain a response with positive control agents such as B[a]P, 2-AAF, and *p*-cresidine.

All of the 13 studies that have been undertaken with this model were available for review at the November 2000 meeting. The model is sensitive to both UV and genotoxic carcinogens and also to some nonmutagenic carcinogens, such as diethylstilbestrol (DES), Wy-14,643, and cyclosporin A. There have been no false positives with noncarcinogens. Some laboratories have also investigated a double transgenic $XPA^{-/-}$ $p53^{+/-}$ model, and this seems to increase the sensitivity of the assay. For example, in a DES study, seven animals with metastasizing osteosarcomas were found in the double transgenic group, compared with one in the *XPA* group and none among the wild-type animals. There remains concern (as with any new model) that these models may be overly sensitive or (put another way) that the relevance of positive findings to risk in humans may not be clear. The results of the ILSI Health and Environmental Sciences Institute (HESI) workshop seem to minimize these concerns.

It is generally proposed that while such models can improve the identification of carcinogens in three ways (providing gene-specific mechanistic data, minimizing the influence of spontaneous tumors and strain-specific effects, and reducing the time, cost, and animal usage involved), they have two potential uses in pharmaceutical development. These are in lieu of either the mouse two-year cancer bioassay or subchronic toxicity assessments prior to making a decision to commit to a pair of two-year carcinogenicity bioassays.

As performance data have become available on these strains, ICH (1997a) has incorporated their use into pharmaceutical testing guidelines in lieu of the second rodent species tests (that is, to replace the long-term mouse bioassay when the traditional rat study has been performed). The FDA has stated that it would accept such studies when "performed in a validated code." In fact, the Center for Biologics Evaluation and Research (CBER) has accepted such studies as a sole carcinogenicity bioassay in some cases where there was negative traditional genotoxicity data and strong evidence of a lack of a mechanistic basis for concern.

A joint ILSI and HESI validation program has been completed looking at the results of the four prime candidate models in identifying carcinogens as compared to the results of traditional long-term rodent bioassays. This validation program involved 51 different laboratories and imposed protocol standards to allow comparison of results. Three dose levels were studied per chemical, with 15 males and 15 females being used for each dose group. A vehicle and high-dose control in wild-type animals was also included, with information from NTP bioassays and 4-week range-finding assays being used to help set doses. Animals were dosed for 26 weeks. The issues coming in and out of these validation programs bear consideration (Tennant et al., 1999):

- Is there proper comparator data for evaluating the performance of human or rodent bioassays? It should be kept in mind that there are sets of rodent bioassay data (particularly those involving liver tumors in mice) that are widely accepted as irrelevant in the prediction of human risk.
- How will the data from these assays be incorporated into any weight-of-evidence approach to assessing human health risk?
- What additional mechanistic research needs to be undertaken to improve our understanding of the proper incorporation and best use of the data from these assays?
- How can the results of current validation efforts be best utilized in the timely evaluation of the next generation of assays?
- Given that, at least under some conditions, assays using these models tend to "blow up" (have high spontaneous tumor rates) once the animals are more than eight or nine months of age, how critical are age and other not currently apprehended factors to optimizing both sensitivity and specificity?
- How wide and unconditional will FDA (and other regulatory bodies) acceptance be of these models in lieu of the traditional two-year mouse bioassay?

12.13 INTERPRETATION OF RESULTS: CRITERIA FOR POSITIVE RESULT

There are three generally accepted criteria for a positive result in a carcinogenicity study. The first two are derived directly from the results of the statistical analysis: (1) a statistically significant increase in the incidence of a common tumor and (2) a statistically significant reduction in the time to tumor development. The third criterion is the occurrence of very rare tumors, that is, those not normally seen in control animals, even if the incidence is not statistically significant. Table 12.3 presents an evaluation matrix for these factors.

TABLE 12.3 Interpretation of Analysis of Tumor Incidence and Survival Analysis (Life Table)

Outcome Type	Tumor Association with Treatment[a]	Mortality Association with Treatment	Interpretation
A	–	+	Unadjusted test[b] may underestimate tumorigenicity of treatment.
B	+	+	Unadjusted test gives valid picture of tumorigenicity of treatment.
C	+	–	Tumors found in treated groups may reflect longer survival of treated groups. Time-adjusted analysis is indicated.
D	–	+	Apparent negative findings on tumors may be due to the shorter survival in treated groups. Time-adjusted analysis and/or a retest at lower doses is indicated.
E	–	0	Unadjusted test gives a valid picture of the possible tumor-preventive capacity of the treatment.
F	–	–	Unadjusted test may underestimate the possible tumor-preventive capacity of the treatment.
G	0	+	High mortality in treated groups may lead to unadjusted test missing a possible tumorigen. Adjusted analysis and/or retest at lower doses is indicated
H	0	0	Unadjusted test gives a valid picture of lack of association with treatment.
I	0	–	Longer survival in treated groups may mask tumor-preventive capacity of treatment.

[a]+ = Yes, – = No, and 0 = no bearing on discussion.
[b]The unadjusted test referred to here is a contingency table type of analysis of incidence, such as Fisher's exact test.

12.14 STATISTICAL ANALYSIS

The actual statistical techniques used to evaluate the results of carcinogenicity bioassays basically utilize four sets of techniques, three of which have been presented earlier in this book:

- Exact tests
- Trend tests
- Life tables (such as log rank techniques)
- Peto analysis

These are then integrated into the decision-making schemes discussed earlier in this chapter. The methods themselves and alternatives are discussed elsewhere in detail (Chow and Lin, 1998; Gad, 2006).

12.14.1 Exact Tests

The basic forms of these (the Fisher exact test and chi square) have previously been presented, and the reader should review these. Carcinogenicity assays are, of course, conducted at doses that are at least near those that will compromise mortality. As a consequence, one generally encounters competing toxicity producing differential mortality during such a study. Also, often, particularly with certain agricultural chemicals, latency of spontaneous tumors in rodents may shorten as a confounded effect of treatment with toxicity. Because of such happenings, simple tests on proportions, such as χ^2 and Fisher–Irwin exact tests on contingency tables, may not produce optimal evaluation of the incidence data. In many cases, however, statisticians still use some of these tests as methods of preliminary evaluation. These are unadjusted methods without regard for the mortality patterns in a study. Failure to take into account mortality patterns in a study sometimes causes serious flaws in interpretation of the results. The numbers at risk are generally the numbers of animals histopathologically examined for specific tissues.

Some gross adjustments on the numbers at risk can be made by eliminating early deaths or sacrifices by justifying that those animals were not at risk to have developed the particular tumor in question. Unless there is dramatic change in tumor prevalence distribution over time, the gross adjusted method provides fairly reliable evidence of treatment effect, at least for nonpalpable tissue masses.

12.14.2 Trend Tests

Basic forms of the trend tests [such as that of Tarone (1975)] have previously been presented in this text.

Group comparison tests for proportions notoriously lack power. Trend tests, because of their use of prior information (dose levels), are much more powerful. Also, it is generally believed that the nature of true carcinogenicity (or toxicity for that matter) manifests itself as dose–response. Because of the above facts, evaluation of trend takes precedence over group comparisons. In order to achieve optimal test statistics, many people use ordinal dose levels (0,1,2, …) instead of the true arithmetic dose levels to test for trend. However, such a decision should be made a priori. Example 12.1 demonstrates the weakness of homogeneity tests.

As is evident from this example, often group comparison tests will fail to identify significant treatment but trend tests will. The same arguments apply to survival-adjusted tests on proportions as well. In an experiment with more than one dose group ($K > 1$), the most convincing evidence for carcinogenicity

Example 12.1 Trend Versus Heterogeneity

Number at Risk	Number with Tumor	Dose Level
50	2	0
50	4	1
50	6	2
50	7	3

Cochran–Armitage Test for Trend

	Calculated χ^2 Subgroup	Degrees of Freedom	α	Two-tailed *p*
Trend	3.3446	1	0.0500	0.0674
Departure	0.0694	2	0.0500	0.9659
Homogeneity	3.4141	3	0.0500	0.3321

One-Tail Tests for Trend

Type	Probability[a]
Uncorrected	0.0337
Continuity corrected	0.0426
Exact	0.0423

Multiple Pairwise Group Comparisons by Fisher–Irwin Exact Test

Groups Compared	α	One-Tailed Probability
1 vs. 2	0.0500	0.33887
2 vs. 3	0.0500	0.13433
1 vs. 4	0.0500	0.07975

[a]Direction = +.

is given by tumor incidence rates that increase with increasing dose. A test designed specifically to detect such dose-related trends is Tarone's (1975) trend test.

Letting $\mathbf{d}=(O, d_1, d_2 \ldots d_k)^{\mathrm{T}}$ be the vetor of dose levels in *all* $K+1$ groups and letting

$$(\mathbf{O}-\mathbf{E})=(O_o-E_0,,,O_k-E_k)^{\mathrm{T}} \quad \text{and} \quad \mathbf{V}=\begin{pmatrix} V_{00} \ldots V_{0K} \\ . \quad . \\ . \qquad . \\ V_{K0} \ldots V_{KK} \end{pmatrix}$$

contain elements as described in the previous section but for *all* $K+1$ *groups*, the trend statistic is given by

$$X_T^2=\frac{\left[\mathbf{d}^{\mathrm{T}}(\mathbf{O}-\mathbf{E})\right]^2}{\mathbf{d}^{\mathrm{T}}\mathbf{V}\mathbf{d}}$$

The statistic X^2_T will be large when there is evidence of a dose-related increase or decrease in the tumor incidence rates and small when there is little difference in the tumor incidence between groups or when group differences are not dose related. Under the null hypothesis of no differences between groups, X^2_T has approximately a chi-squared distribution with one degree of freedom.

Tarone's trend test is most powerful at detecting dose-related trends when tumor onset hazard functions are proportional to each other. For more power against other dose-related group differences,weighted versions of the statistic are also available; see Breslow (1984) or Crowley and Breslow (1984) for details.

These tests are based on the generalized logistic function (Cox, 1972). Specifically one can use the Cochran–Armitage test (or its parallel, Mantel–Haenszel version) for monotonic trend as the heterogeneity test.

12.14.3 Life Table and Survival Analysis

These methods are essential when there is any significant degree of mortality in a bioassay. They seek to adjust for the differences in periods of risk individual animals undergo. Life table techniques can be used for those data where there are observable or palpable tumors. Specifically, one should use Kaplan–Meier product limit estimates from censored data graphically, Cox–Tarone binary regression (log rank test), and the Gehan–Breslow modification of Kruskal–Wallis test (Thomas et al., 1977) on censored data.

The Kaplan–Meier estimates produce a step function for each group and are plotted over the lifetime of the animals. Planned, accidentally killed, and lost animals are censored. Moribund deaths are considered to be treatment related. A graphical representation of Kaplan–Meier estimates provide excellent interpretation of survival-adjusted data except in the cases where the curves cross between two or more groups. When the curves cross and change direction, no meaningful interpretation of the data can be made by any statistical method because the proportional-odds characteristic is totally lost over time. This would be a rare case where treatment initially produces more tumor or death and then, due to repair or other mechanisms, becomes beneficial.

In Cox–Tarone binary regression (Tarone, 1975; Thomas et al., 1977) censored survival and tumor incidence data are expressed in a logistic model in dose over time. The log rank test (Peto, 1974), tests based on the Weibull distribution, and the Mantel–Haenszel (1952) test are very similar to this test when there are no covariates or stratifying variables in the design. The logistic regression–based Cox–Tarone test is preferable because one can easily incorporate covariates and stratifying variables which one cannot in the IARC methods.

The Gehan–Breslow modification of the Kruskal–Wallis test is a nonparametric test on censored observations. It assigns more weight to early incidences compared to the Cox–Tarone test.

For survival-adjusted tests on proportions, as mentioned earlier, in the case of survival-adjusted analyses, instead of having a single $2k$ table, one has a

series of such 2*k* tables across the entire lifetime of the study. The numbers at risk for such analyses will depend on the type of tumor one is dealing with. These are shown below:

1. Palpable or Lethal Tumors Number at risk at time t = number of animals surviving at the end of time t–1
2. Incidental Tumors Number at risk at time t = number of animals that either died or were sacrificed whose particular tissue was examined histopathologically

The methods of analyzing the incidences once the appropriate numbers at risk are assigned for these tumors are rather similar, either binary regression based or by pooling evidence from individual tables (Gart et al., 1986).

12.14.4 Peto Analysis

The Peto method of analysis of bioassay tumor data is based on careful classification of tumors into five different categories as defined by IARC:

1. Definitely incidental
2. Probably incidental

Comment: Combine 1 and 2:

3. Probably lethal
4. Definitely lethal

Comment: These categories may be combined into one (otherwise a careful cause-of-death determination is required):

5. Mortality independent (such as mammary, skin, and other observable or superficial tumors)

Interval Selection for Occult (Internal Organ) Tumors

1. *FDA* 0–50, 51–80, 81–104 weeks, interim sacrifice, terminal sacrifice
2. *NTP* 0–52, 53–78, 79–92, 93–104 weeks, interim sacrifice, terminal sacrifice
3. *IARC* ad hoc selection method (Peto et al., 1980)

Comment: Any of the above may be used. Problems with IARC selection method include two sexes and two or more strains that will have different intervals for the same compound. Different interval selection methods will produce different statistical significance levels. This may produce bias and requires an isotonic tumor prevalence for ready analysis.

Logistic Regression Method for Occult (Internal Organ) Tumors (Dinse, 1985) Tumor prevalence is modeled as a logistic function of dose and polynomial in age.

Comment: The logistic tumor prevalence method is unbiased. It requires maximum-likelihood estimation and allows for covariates and stratifying variables. It may be time consuming and have convergence problems with sparse tables (low tumor incidences) and clustering of tumors.

12.14.5 Methods To Be Avoided

The following methods and practices should be avoided in evaluation of carcinogenicity:

1. Use of only the animals surviving after one year in the study
2. Use of a two-strata approach; separate analyses for animals killed during the first year of the study and the ones thereafter
3. Exclusion of all animals in the study that died on test and analysis of only the animals that are sacrificed at the end of the study
4. Exclusion of interim sacrifice animals from statistical analyses
5. Evaluation of number of tumors of all sites as opposed to number of animals with tumors for specific sites of specific organs

Another issue is subjectivity in slide reading by most pathologists who do not want to read them in a coded fashion whereby they will not know the dose group an animal is coming from. This is not under the statisticians' control but they should be aware of it in any case.

Often a chemical being tested is both toxic as well as potentially carcinogenic. When competing toxicity causes extreme differences in mortality or there is a clustering effect in tumor prevalence in a very short interval of time, none of the adjusted methods works. One then must use biological intuition to evaluate the tumor data.

Use of historical control incidence data for statistical evaluation is controversial. There are too many sources of variation in these data. For example, different pathologists use different criteria for categorizing tumors (in fact, the same pathologist may change his or her opinion over time), there is laboratory-to-laboratory variation, there may be genetic drift over time; the location of suppliers may make a difference, and, finally, these data are not part of randomized concurrent control. Regulatory agencies and pathologists generally use these data for qualitative evaluation. My personal view is that is where they belong.

12.14.6 Use of Historical Controls

When the study is over, the data analyzed, and the p values corrected, as appropriate, one may find that one or more tumor types increased in drug-

treated groups relative to concurrent controls. Although the FDA and other regulatory agencies play down the importance of historical control data, it is common practice in the pharmaceutical industry to use historical data in the interpretation of tumor findings. The first and most appropriate comparison of a treated group is with concurrent control group(s), but it is of interest to see how tumor incidences in the treated groups compare with the historical incidence, and such a comparison is an accepted practice in toxicology and biostatistics (Gart et al., 1979; Hajian, 1983; Haseman et al., 1984b). A treated group may have a tumor incidence significantly higher than that of the concurrent control groups(s) but comparable to or lower than the historical incidence. Occasionally, a small number of tumors may be found in a treated group and the incidence may be significant because of the absence of this tumor in the concurrent controls. Review of appropriate historical control data may reveal that the low tumor incidence in the treated group is within the "expected" range for this tumor.

The role of historical control data in interpreting carcinogenicity findings depends on the "quality" of the historical data. Ideally, the data should be derived from animals of the same age, sex, strain, and supplier and housed in the same facility, and the pathology examinations should have been performed by the same pathologist or using the same pathological criteria for diagnosis. Since genetic drift occurs even in animals of a given strain and supplier, recent data are more useful than older data. The value of historical control data is directly proportional to the extent to which these conditions are fulfilled.

Although methods are available for including historical control data in the formal statistical analysis (Tarone, 1982; Dempster et al., 1983), this is usually not done and for good reason. The heterogeneity of historical data requires that they be used qualitatively and selectively to aid in the final interpretation of the data after completion of the formal statistical analysis. Table 12.4 presents a summary of background tumor incidences for the most commonly employed rodent strains.

12.14.7 Relevance to Humans

After statistical analyses have been performed and historical data consulted, the final interpretation may be that a drug appears to cause tumors at one or more tissue sites in the mouse or the rat. But what does this mean for the species to which the drug will be administered, namely, the human? Extrapolation of rodent carcinogenicity data to humans remains one of the greatest challenges of modern toxicology. There is no simple formula, and each case must be evaluated on its own merits. Very generally speaking, the FDA and other major regulatory agencies consider compounds that are tumorigenic in one or more animal species to be "suspect" tumorigens in humans. The actual impact of this conclusion on the approval of a drug depends on the target population and the indication. For example, even a suspicion of carcinogenic

TABLE 12.4 Comparative Percent Incidence of Pertinent Neoplasia in Different Strains of Rats and Mice (104 weeks old)

	F344 Rats		SD Rats		Wistar Rats		$B6C3F_1$ Mice		CD-1 Mice	
Types of Neoplasia	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
Hepatocellular adenoma	4	<1	5	<1	1	2	29	30	26	5
Hepatocellular carcinoma	2	0	2	0	<1	<1	26	16	10	1
Pancreas islet adenoma	12	2	8	9	4	2	2	0	<1	<1
Pancreas islet carcinoma	3	0	<1	5	<1	<1	0	0	0	0
Pancreas acinar adenoma	6	0	1	0	13	<1	2	0	<1	0
Pheochromocytoma	21	4	23	5	10	2	0	2	<1	<1
Adrenocortical adenoma	0	2	3	0	8	9	<1	0	1	<1
Pituitary adenoma	49	42	62	85	34	55	2	8	0	5
Thyroid C-cell adenoma	17	8	7	6	6	8	0	0	0	0
Thyroid follicular adenoma	0	0	4	2	2	1	2	6	1	<1
Mammary gland fibroadenoma	4	57	2	54	3	36	0	0	<1	1
Mammary gland carcinoma	0	4	<1	26	1	13	0	0	0	6
Skin fibroma	10	2	2	<1	5	1	1	2	<1	<1
Skin papilloma	6	0	2	0	2	<1	0	0	<1	0
Pulmonary adenoma	4	4	<1	<1	<1	0	22	6	15	15
Preputial gland neoplasia	10	NA	>1	NA	<1	NA	<1	NA	<1	NA
Leydig cell neoplasia	89	NA	7	NA	11	NA	0	NA	1	NA
Clitoral gland neoplasia	NA	14	NA	<1	NA	<1	NA	<1	NA	0
Uterine polyps	NA	14	NA	6	NA	16	NA	1	NA	<1
Ovarian neoplasia	NA	6	NA	1	NA	8	NA	6	NA	1
Mononuclear cell leukemia	62	42	0	0	<1	<1	0	0	2	2
Lymphoma	0	0	2	1	3	5	14	24	8	22
Forestomach papilloma	0	2	<1	<1	0	<1	4	2	<1	<1
Scrotal mesothelioma	5	NA	1	NA	2	NA	0	NA	0	NA

Note: F344, Fischer 244; SD, Sprague Dawley; $B6C3F_1$, $(C57BL/6N+C3H/HeN)F_1$; CD-1, 1CRCr: CD-1 mice; NA, not applicable; the average number used by species/strain/gender was in excess of 750 animals.

activity may be fatal for a potential contraceptive drug intended for use in a very large population of healthy people. By contrast, clear evidence of carcinogenic activity may be overlooked in a drug being considered for use in a restricted population with a life-threatening disease.

Regardless of the target population and indication, the FDA and other agencies have, in recent years, attempted to consider the mechanism of tumor induction in rodents and its relevance for humans. If a drug is known to cause tumors in a rodent via a mechanism that does not exist in humans, the importance of the tumor findings may be markedly reduced. For example, drugs that cause tumors by a secondary hormonal mechanism shown to be inapplicable to humans may be given special consideration. It is the sponsor's responsibility to provide pertinent data on the mechanism of tumor induction and its relevance, or irrelevance, for humans. If the sponsor can show that an apparently drug-related tumor is species specific, the importance of the tumor in the overall evaluation of the drug will be greatly minimized. Table 12.5 presents a list of neoplastic /tumorigenic responses seen in rodents which have limited relevance to human safety. Part of the consideration must also be recognition of the main characteristics of nongenotoxic carcinogens. These are recognized to be dose-dependent responses with operative thresholds. The major characteristics are (Spindler et al., 2000):

- Specificity (of species, sex, and organ).
- A threshold is operative and must be exceeded for cell proliferation and tumor development to occur.
- There is a stepwise dose–response curve/relationship between exposure, cell proliferation, and tumor development.
- The response is reversible with a cessation of dosing unless a point of no return has been passed.

12.15 CONCLUSIONS

The design, conduct, and interpretation of carcinogenicity studies is one of the major challenges for the pharmaceutical toxicologist, pathologist, biostatistician, and regulator. This is a rapidly changing field generating more questions than answers. The largest question continues to be the extrapolation of rodent data to humans, especially when data on mechanisms of tumor induction are unavailable or controversial. Much has been written on the difficulties inherent in extrapolating results from rodents treated with MTDs of a compound to humans who will be exposed to much lower doses and often for shorter periods. A discussion of these and other aspects of carcinogenic risk assessment is beyond the scope of this chapter.

Regulatory agencies are very aware of these challenges and deserve credit for attempting to respond to changes in the state of knowledge while still

TABLE 12.5 Examples of Neoplastic Effects in Rodents with Limited Significance for Human Safety

Neoplastic Effect	Pathogenesis (Agents)
Renal tubular neoplasia in male rats	$\alpha_{2\mu}$-Globulin nephropathy/hydrocarbons (*d*-limonene, *p*-dichlorobenzene)
Hepatocellular neoplasia in rats and mice	Peroxisome proliferation (clofibrate, phthalate esters, phenoxy agents) Phenobarbital-like promotion
Urinary bladder neoplasia in rats	Crystalluria, carbonic anhydrase inhibition, urine pH extremes, melamine, saccharine, carbonic anhydrase inhibitors, dietary phosphates
Hepatocellular neoplasia in mice	Enzymatic-metabolic activation (in part unknown)/phenobarbital-like promotion
Thyroid follicular cell neoplasia in rats	Hepatic enzyme induction, thyroid enzyme inhibition/axazepam, amobarbital, sulfonamides, thioureas
Gastric neuroendocrine cell neoplasia mainly in rats	Gastric secretory suppression, gastric atrophy induction (climetidine, omeprazole, butachlor)
Adenohypophysis neoplasia in rats	Feedback interference/neuroleptics (dopamine inhibitors)
Mammary gland neoplasia in female rats	Feedback interference/neuroleptics, antiemetics, antihypertensives (calcium channel blockers), serotonin agonists, anticholinergics, exogenous estrogens
Pancreatic islet cell neoplasia in rats	Feedback interference/neuroleptics
Harderian gland neoplasia in mice	Feedback interference/misoprostol (PGE_1), nalidixic acid, aniline dyes
Adrenal medullary neoplasia in rats	Feedback interference (lactose, sugar alcohols)
Forestomach neoplasia in rats and mice	Stimulation of proliferation/butylated hydroxyanisole, phthalate esters, proprionic acid
Lymphomas in mice	Immunosuppression/cyclosporin
Mononuclear cell leukemia in rats (mainly F344)	Immunosuppression (in part unknown)/furan, iodinated glycerol
Splenic sarcomas in rats	Methemoglobinemia (in part unknown)/dapsone
Osteomas in mice	Feedback interference/lactose, sugar alcohols, H_2 antagonists, carbamazepine, vidarabine, isradipine, dopaminergics, finasteride
Leydig cell testicular neoplasia in mice	Feedback interference (proestrogens, finasteride, methoxychlor, cadmium)
Endometrial neoplasia in rats	Feedback interference (proestrogens, dopamine agonists)
Uterine leiomyoma in mice	Feedback interference (β_1 antagonists)
Mesovarial leiomyoma in rats (occasionally in mice)	Feedback interference (β_2 agonists)
Ovarian tubulostromal neoplasia in mice	Feedback interference (cytotoxic agents, nitrofurantoin)

discharging their responsibility to protect the public health. For example, the latest version of the Japanese guidelines (Speid et al., 1990) acknowledges that the highest does in a carcinogenicity study may be set at 100 times the clinical dose, instead of requiring that the MTD be achieved. It is also noteworthy that the FDA Center for Drug Evaluation and Research has announced the formation of a carcinogenicity assessment committee representing all drug review divisions. This group will advise all the divisions on issues related to carcinogenicity. Creation of such a group reflects the importance that the agency places on carcinogenicity data in evaluating the safety of new drug candidates.

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13

Histopathology in Nonclinical Pharmaceutical Safety Assessment

13.1 INTRODUCTION

Toxicological pathology is the study of the molecular, cellular, tissue, and/or organ responses of a living organism when exposed to injurious chemical or physical agents. These responses represent a spectrum of cellular changes ranging from cell death to malignant transformations, tissue and organ responses (including regeneration inflammation), and organization and overall response as identified by clinical changes and alterations in body fluids (Arnold et al., 1990; MHLW, 1990; EEC, 1992; EMEA, 2000; Rousseaux et al., 2002; Prince and Wilson, 2003; ICH, 2005, 2006). It starts from recognition of the fact that the cell constitutes the basic unit of life. Accordingly, morphological changes in organs and tissues arise as a result of injury beginning with the responses of underlying cells to the toxic insult. A proper evaluation and understanding of related pathology must start at the cellular level. Some cellular components whose alterations have been reported to be critically associated with cell injury include the plasma membrane, site of osmotic, electrolyte,

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and water regulation as well as signal transduction; the mitochondrion (site of energy storage and aerobic respiration); the endoplasmic reticulum (site of much protein synthesis); and the nucleus, which contains the genetic information and where transcription of the genetic code takes place (Hamm, 1974; Wallig, 2002).

Biochemical changes such as enzyme induction and gene expression occur at the earlier stages of the exposure–disease continuum. The degrees of cellular injury in different target tissue depend on the metabolic rate. Cells with high metabolic rates such as neurons, myocardial cells, and renal proximal convoluted tubule epithelial cells frequently suffer from injury more quickly than low-metabolizing ones. These high-metabolism cells depend on a continuous flow of oxygen to conduct the aerobic metabolism necessary to provide required energy in the form of adenosine triphosphate (ATP) for the maintenance of membrane polarity and membrane integrity (neurons), for the continual muscular contraction/relaxation and calcium transport (myocardium), and for the transport of fluids, electrolytes, and metabolites [renal proximal tubule cells (PTCs)]. Hence, any depletion in oxygen supply is likely to have a significant impact on their survival.

In contrast, cells with low metabolic activity such as fibroblasts and adenocytes are less affected by the low supply of oxygen, and they have a prominent role in regeneration and scarring. Homeostasis is one of the most remarkable and most typical properties of highly complex biological systems. It defines the ultimate environment under which cells maintain the physiochemical conditions (intracellular pH, cytosolic osmolarity, ion gradients) necessary to perform their biological functions. In biological systems, homeostasis is maintained by means of a multiplicity of dynamic equilibriums rigorously controlled by interdependent regulation mechanisms. Hence, the homeostatic system reacts to changes or disturbances in response to various insults by exerting a series of modifications or adjustments to maintain the internal balances and conditions within tolerable limits.

Pathology [including all the aspects of anatomic (histopathology) clinical chemistry and clinical pathology (hematology)] is generally considered the single most significant portion of data to come out of systemic toxicity studies (particularly the repeat dose, with versions going from 14 days to 2 years in duration) (Roz and Andrews, 2004; Perez and Barthold, 2007; Frame and Mann, 2008; Tehounwan and Centeno, 2008). Anatomic pathology evaluations actually consist of three related sets of data (gross pathology observations, organ weights, and microscopic pathology) that are collected during termination of the study animals. At the end of the study, a number of tissues are collected during termination of all surviving animals (test and control). Organ weights and terminal body weights are recorded at study termination, so that absolute and relative (to body weight) values can be statistically evaluated. Bindhu et al. (2007) have provided a review of practices of how such organ weight information is evaluated and utilized in the overall evaluation of pathology and adverse effects. In general, with the exception of brain weights,

TABLE 13.1 Tissues for Histopathology

Adrenals[a]	Mainstream bronchi
Body and cervix	Major salivary gland
Brain, all three levels[a]	Mesenteric lymph nodes
Cervical lymph nodes	Ovaries and tubes
Cervical spinal cord	Pancreas
Duodenum	Pituitary
Esophagogastric junction	Prostate
Esophagus	Skeletal muscle from proximal hind limb
Eyes with optic nerves	Spleen[a]
Femur with marrow	Strenebrae with marrow
Heart[a]	Stomach
Ileum	Testes with epididymides[a]
Kidneys[a]	Thymus and mediastinal contents[a]
Large bowel	Thyroid with parathyroid[a]
Larynx with thyroid and parathyroid	Trachea
Liver[a]	Urinary bladder
Lungs[a]	Uterus including horns

[a]Organs to be weighed.

relative (to body weight) changes are considered more relevant to identifying target organ toxicities.

These "standard list" tissues, along with the organs for which weights are determined, are listed in Table 13.1. All tissues collected are typically processed for microscopic observation, but only those from the high-dose and control groups are necessarily evaluated microscopically. If a target organ is discovered in the high-dose group, then the effect is "followed" in successively lower dose groups until a NOEL (no observed effect level) is determined.

In theory, all microscopic evaluations should be performed in a blind manner (without the pathologist knowing from which dose group a particular animal came), but this is difficult to do in practice and such an approach frequently actually limits (degrades) the quality of the evaluation. Like all the other portions of data in the study, proper evaluation benefits from having access to all data that address the relevance, severity, timing, and potential mechanisms of a specific toxicity. Blind examination is best applied in peer review or consultations on specific findings after a primary evaluation.

In addition to the "standard" set of tissues specified in Table 13.1, observations during the course of the study or in other or previous studies may dictate that special examination or tissue preparation procedures such as polarized light or electron microscopy, immunocytochemistry, or quantitative morphometry be undertaken to evaluate the relevance of such findings and help or understand the mechanisms underlying certain observations.

The evaluation of the pathological alterations induced in laboratory animals by new drugs represents the cornerstone of their safety assessment before they can be first tried in patients. This preliminary assessment, which is based largely on conventional histopathological techniques, represents a major con-

TABLE 13.2 Principles of Drug Testing before Trials in Humans

1. Exact composition of drug should be known; if not, method of preparation
2. Acute toxicity studies in animals of different species
3. Chronic toxicity experiments at varying doses in different species for cumulative effects
4. Careful and frequent observations of animals to develop a composite picture of clinical effects
5. Careful pathological examination of tissues with appropriate stains
6. Effects of drugs on excretory or detoxifying organs, especially kidney and liver
7. Rate of absorption and elimination, path and manner of excretion, concentration in blood and tissues at varying times
8. Possible influence of other drugs and foodstuffs
9. Careful examination for any syncrasies or untoward reactions

Note: As Defined by Geiling and Cannon (1938).

tribution to the development of new treatments for both human and animal diseases.

Although there have been many changes over the past few decades in the details of study design and conduct, the principles of drug testing prior to trial in humans are the same as those expounded by Geiling and Cannon (1938) after they studied the pathological effects and causes of death of patients treated with toxic elixir of sulphanilamide over 60 years ago (Table 13.2). The basic paradigm of dosing laboratory animals with various doses of new drug for increasing periods of time accompanied by careful clinical observations, biochemical and hematological monitoring, followed by histopathological examination of the tissue remains essentially unaltered. The pathologist is required to not only evaluate alterations to organs and tissues and any relationship that they might have to drug treatment but also to assess the likely relevance any treatment-related findings might have for patients.

The use of animals to study the pathological effects of chemicals and therapeutic agents has a long history. In the eighteenth century Morgagni reported his attempts to compare pathological changes produced by accidental ingestion by people of chemicals such as arsenic. A thorough and systematic review of pathology induced by toxins in humans and animals was published by Orfila as long ago as 1815 (Orfila, 1815). Although in the modern era drug safety evaluation has been widely practiced in rodent and nonrodent species since before World War II, there have been very few critical comparisons of the effects of drugs in humans and those seen in laboratory animals. Much potentially useful information still awaits data mining in the archives of pharmaceutical companies and government agencies. Nevertheless, the available data suggest that the traditional approach using experimental pharmacology alongside conventional toxicology studies with pathology is usually sufficient to predict important adverse effects and to support the safe conduct of the first clinical studies in humans (Turton and Hoasen, 1998; Greaves et al., 2004). Such a degree of concordance varies between different organs and tissues. Therefore each observed drug-induced pathological finding needs to be assessed on a case-by-case basis for its likely clinical relevance. For some

systems, histopathology remains critical for some organ systems but of lesser importance for others. Traditional animal studies are poor predictors of subjective neurological symptoms, but histopathological examination of the nervous system in laboratory animals treated with cancer drugs does well at identifying potential serious clinical neurotoxic effects. Results from nonclinical studies frequently fail to predict renal and hepatic toxicity (largely because of a "formula" approach to evaluation), but there is generally a good correlation for gastrointestinal effects, and histopathology still seems to represent one of the most sensitive techniques to detect effects on the reproductive system. Though the relevance of such findings can be confounded by general systemic toxicity, the pathologist also needs to be aware that some minor inflammatory alterations in certain organs, such as the liver, may have greater significance for the use of a drug in humans than other types of severe damage such as subendocardial necrosis in the myocardium mediated by exaggerated hemodynamic effects.

Treatment-induced findings in conventional toxicity studies found in different laboratory animal species also seem to possess different degrees of relevance for humans. Although the data are fragmentary, findings in beagle dog studies are not often better predictors of human adverse effects than data from rodents or, surprisingly, from primates (Greaves et al., 2004). Dog gastrointestinal and cardiovascular physiology appear to model particularly well for humans, though the pig is generally better yet.

Another long-standing issue most recently recognized again due to findings with cyclooxygenase 2 (COX-2) inhibitors is the importance of evaluating the adverse effects of some therapies with specific human diseases. COX-2 inhibitors were used for inflammatory disorders because of their perceived lower adverse effect profile on the gastrointestinal tract compared with conventional drugs, but this benefit is outweighed by an increased incidence of cardiovascular disease in some patients. Such effects are difficult, if not impossible, to predict from nonclinical safety studies in "normal" healthy animals. Unfortunately the detection of an increased incidence of a common event such as heart attack or stroke is difficult in patients for it requires careful collection and analysis of data, even though it may have a big impact on public health (Dragen, 2005). Such interactions usually require randomized controlled trials specifically designed to look for such risks. It has to be remembered that aspirin was in use for over 100 years before it became generally acknowledged about 30 years ago to be associated with Reye's syndrome, a devastating toxicity in children (Monto, 1999). While the actual mechanism involved in Reye's syndrome is still unknown, it is often preceded by a viral infection and displays a strong correlation with the subsequent ingestion of aspirin.

Histopathology testing is a terminal procedure, and, therefore, sampling of any single animal is a one-time event (except in the case of a tissue collected by biopsy). Because it is a regulatory requirement that the tissues from a specific minimum number of animals be examined at the stated end of the study, any evaluation of effects in tissues at another time course (most commonly, to investigate recovery from effects at study termination) requires that

additional numbers of animals be incorporated into that study at startup. Such animals are randomly assigned at the beginning of the study and are otherwise treated exactly the same as the rest of their group/cohort animals.

Anatomic pathology evaluation occurs only after the in-life portion of a study is complete and is typically the rate-limiting step in producing a report on the study. At the end of the study animals are euthanized, final blood and urine samples are taken, and tissues are collected with a prespecified set weighed while still wet and evaluated grossly (see Table 13.1) as to whether they are other than normative in appearance. They are then preserved in appropriate manners (Olsen et al., 2000; Haschek et al., 2002; Greaves, 2000, 2007) and processed so as to optimize evaluation (Gray, 1964). Experimental design may call for an interim necropsy (to allow for evaluation of progression of lesions or observation of indications of adaptive change by animals) and/or a recovery group (usually additional numbers of high-dose and control animals in which treatment is discontinued at the time of the main necropsy). Such recovery animals are maintained without further manipulation or treatment for a period of time after the termination of the main study animals (usually a month), allowing for an assessment of treatment-free regression or progression of conditions seen at the end of the main study. Once gathered, tissues must be processed, mounted, stained, and examined with great care. The steps involved in anatomic pathology represent a significant portion of the time required to complete a study and add from 30 to 50% to the cost of the shorter (14-, 28-, and 90-day) studies.

Nevertheless, the available data suggest that the traditional approach using experimental pharmacology alongside conventional toxicology studies with pathology are usually sufficient to predict important adverse effects and to support the safe conduct of the first clinical studies in humans. (Greaves et al., 2004). Indeed, the dosing rodent and nonrodent species with a new drug up to one month identifies over 90% of adverse effects that will ever be detected in the usual nonclinical safety assessment studies. However, more generally these studies do not detect all adverse drug effects that can occur in clinical practice and there remains significant over- and underprediction of human toxicity. Overall, the true positive concordance rate (sensitivity) is on the order of 70% with perhaps 30% of human adverse effects not predicted by safety pharmacology or conventional toxicity studies (Olsen et al., 2000). Moreover, this concordance varies between different organs and tissues. Therefore each drug-induced pathological finding needs to be assessed on a case-by-case basis for its likely clinical relevance.

13.2 PATHOLOGICAL TECHNIQUES

Over the past few years a number of excellent reviews of standardized techniques for use in the histopathology evaluation of toxicology studies have been produced covering tissue selection, blocking and sectioning procedures, immunocytochemical stains for laboratory animals, and other basic techniques

(Bregman et al., 2003; Mikaelian et al., 2004). In addition, the scientific literature is full of interesting techniques and novel reagents that can be applied to tissue sections. Some of these can be very useful in the analysis of pathological alterations in toxicity studies and some fail to work in routinely fixed material. However, it is important that these techniques are used in a judicious manner with clear aims following careful analysis of conventional hematoxylin and eosin–stained sections. This is particularly true for the application of microarray and bioinformatics technology. While undoubtedly useful in toxicology, these techniques should not be applied in isolation but in combination with other information, particularly pathology.

13.3 ORGAN WEIGHTS

Regulatory guidelines indicate that certain organs should be weighed during the course of the necropsy in repeat-dose toxicity studies (Alder and Zbinden, 1988). The extent to which organs are weighed varies between laboratories, but organ weighing is a useful adjunct to macroscopic assessment. Therefore, the selection of organs for weighing is the primary responsibility of the study pathologist. Weighing helps to focus the histopathological examination on key target organs, such as the liver and kidney, the weights of which are frequently altered upon administration of xenobiotics (Peters and Boyd, 1996).

Heart weight is a guide to potential cardiac alterations and is especially important in the assessment of cardiovascular drugs. Likewise, the lungs are weighed in inhalation studies as this can provide a useful indication of the extent of edoma or accumulation of exudate. Brain weight is employed as a stable reference point in adult animals as it is fairly independent of body weight changes. The weights of endocrine organs are useful guides to alterations in the endocrine status of laboratory animals (Pfeiffer, 1998). However, weighing a small and firmly attached organ such as the thyroid can severely disrupt its quality and orientation in the sections and thus offset any apparent advantage (Sellers et al., 2007).

Testicular weights correlate with testicular toxicity and weights can be compared with in-life measurement of testicular size (Heywood and James, 1978; Creasy, 2003). Weighing the testes is a useful precaution at the early phase of development of a novel drug prior to any assessment of male fertility. By contrast, ovarian weight is highly variable as a consequence of cyclical ovarian development and is therefore a less sensitive indicator of treatment-induced changes in the female reproductive system (Long et al., 1998).

13.4 CLINICAL PATHOLOGY

Clinical pathology is the evaluation of changes (or lack of changes) in the formed blood elements and their characteristics—the most common of these

TABLE 13.3 Examples of Basic Tests Applicable to Most Rat, Dog, and Monkey Studies

Hematology and Coagulation	Clinical Chemistry	Urinalysis
Red blood cell (RBC) count	Glucose	Color and clarity
Hemoglobin	Urea nitrogen (or urea)	Overnight volume (e.g., 16 h)
Hematocrit	Creatinine	Urine specific gravity
Mean corpuscular volume	Total protein	Reagent strip test, pH, protein, glucose, ketones, bilirubin, urobilinogen, blood
Mean corpuscular hemoglobin	Albumin	
Mean corpuscular hemoglobin concentration	Globulin (calculated)	
RBC morphology	Albumin/Globulin ratio (calculated)	Microscopic examination of sediment: cells, casts, crystal, bacteria, sperm
White blood cell (WBC) count	Cholesterol	
WBC differential count	Total bilirubin	
Platelet count	Alanine aminotransferase	
Blood and bone marrow smears	Aspartate aminotransferase	
Prothrombin time (PT)	Alkaline phosphatase	
Activated partial thromboplastin time (APTT)	γ-Glutamyltransferase	
	Creatine kinase	
	Calcium	
	Inorganic phosphorus	
	Sodium	
	Potassium	
	Chloride	

parameters are listed in Table 13.3. These parameters reflect the homeostasis and function of both the hematopoietic system and associated metabolic systems. Beutler et al. (1995) provides an extensive and detailed overview of these systems, though primarily from the perspective of the human system. These measures have the advantage that samples can be taken (and therefore evaluations made) at multiple points over the course of drug administration to drugs and at points subsequent to the discontinuation of such administration (that is, during "recovery").

At the same time, there is the disadvantage that these are frequently indirect measures of what is happening at specific target sites (primarily the bone marrow). The actual target organ effects can generally only be evaluated after termination of the test animals.

Actual evaluation of meaning and relevance and mechanism of cause of observed changes requires, of course, consideration of not individual parameters in isolation but rather of the entire set of measures and the relationships/correlations of these changes. This evaluation is discussed in the earlier chapter on repeat-dose toxicity studies.

13.5 CLINICAL CHEMISTRY

One of the portions of the information employed in a pathological evaluation which is not terminally collected comes from samples of blood and urine.

These can be analyzed for the presence and activity of enzyme and endogenous physiological components (such as electrolytes), including those listed in Table 13.3. Which are collected and how they are analyzed largely simply follow what is done in human beings during clinical evaluation. Some adaptations have been made, and indeed the measurement methods (and their validation) and interpretation are essentially modified for the specific species in question.

The interpretation of these parameters is primarily addressed in Chapter 8 on repeat-dose studies, though there are some excellent references on the field (Loeb and Quimby, 1999; Lewandrowski, 2003; Burtis et al., 2005). The last of these best addresses modern biomasses for organ damage. The issue of sampling in nonclinical safety studies is a multifaceted one. First, each species presents limitations on how much (and how often) sample can be drawn. Mice are the most limiting and large nonrodent species (dogs, primates, and pigs) the least. The proximity of sample collection, the time of drug to dosing, or organ damage means that there is a strong emphasis on a need for frequent sample collection. One must not lose sight of the fact that we only see where we look, that is, only where we sacrifice.

13.6 TARGET ORGAN TOXICITY BIOMARKERS

As this is written, the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMEA) are qualifying organ toxicities. These serve to significantly improve the performance of nonclinical safety studies in identifying potential drug-related toxicities.

The prototype set is for nephrotoxicity, where the two agencies have qualified a set of seven biomarkers—Kim-1, albumin, total protein, cystatin C, B_2-microglobulin, urinary clusterin, and urinary trefoil factor. Two of these (albumin and total protein) have been part of the clinical chemistry parameter set for decades. As with traditional clinical chemistry and clinical pathology measures, these biomarkers should be considered as sets, not in isolation.

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14

Irritation and Local Tissue Tolerance in Pharmaceutical Safety Assessment

Both irritation and local tolerance studies assess the short-term hazard of pharmaceutical agents in the immediate region of their application or installation. In particular, these studies are done (expected) to assess topically or parenterally administered drug formulations (Gad and Chengelis, 1998). Note that these are hazard tests properly performed using the clinical formulation.

Topical local tolerance effects are almost entirely limited to irritation. Though this usually means dermal irritation, it can also be intracutaneous, mucosal, penile, perivascular, vaginal, bladder, rectal, nasal, or ocular, depending on the route of drug administration. All but ocular irritation use some version of a common subjective rating scale (see Table 14.1) to evaluate responses. The outcome of all of these tests primarily evaluates the response of the first region of tissue (which is exposed to the highest concentration) to an administered drug substance. In general, any factor which enhances absorption through the contacted tissue is likely to decrease tissue tolerance. Zhai et al. (2008) should be referred to for a more detailed coverage of the subject of topical tissue toxicology.

For the skin, this scale is used in the primary dermal irritation test, which is performed for those agents that are to be administered to patients by application

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TABLE 14.1 Evaluation of Local Tissue Reactions in Tissue Irritation Studies

Skin Reaction	Value
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
Necrosis (death of tissue)	+N
Eschar (sloughing or scab formation)	+E
Edema formation	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond area of exposure)	4
Total possible score for primary irritation	8

to the skin. As with all local tolerance tests, it is essential that the material be evaluated in "condition of use"—that is, in the final formulated form, applied to test animals in the same manner that the agent is to be used clinically.

14.1 PRIMARY DERMAL IRRITATION TEST

A. *Rabbit Screening Procedure*

1. A group of at least four to six New Zealand white rabbits are screened for the study.
2. All rabbits selected for the study must be in good health; any rabbit exhibiting sniffles, hair loss, loose stools, or apparent weight loss is rejected and replaced.
3. One day (at least 18 h) prior to application of the test substance, each rabbit is prepared by clipping the hair from the back and sides using a small animal clipper. A size no. 10 blade is used to remove long hair and then a size no. 40 blade is used to remove the remaining hair.
4. Six animals with skin sites that are free from hyperemia or abrasion (due to shaving) are selected. Skin sites that are in the telogen phase (resting stage of hair growth) are used; those skin sites that are in the anagen phase (stage of active growth, indicated by the presence of a thick undercoat of hair) are not used.

B. *Study Procedure*

1. As many as four areas of skin, two on each side of the rabbit's back, can be utilized for sites for administration.
2. Separate animals are not required for an untreated control group. Each animal serves as its own control.

3. Besides the test substance, a positive control substance (a known skin irritant—1% sodium lauryl sulfate in distilled water) and a negative control (untreated patch) are applied to the skin. When a vehicle is used for diluting, suspending, or moistening the test substance, a vehicle control patch is required—especially if the vehicle is known to cause any toxic dermal reactions or if there is insufficient information about the dermal effects of the vehicle.
4. The intact (free-of-abrasion) sites of administration are assigned a code number. Up to four sites can be used as follows:
   1: Test substance
   2: Negative control
   3: Positive control
   4: Vehicle control (if required)
5. Application sites should be rotated from one animal to the next to ensure that the test substance and controls are applied to each position at least once.
6. Each test or control substance is held in place with a 1 × 1-in. 12-ply surgical gauze patch. The gauze patch is applied to the appropriate skin site and secured with 1-in.-wide strips of surgical tape at the four edges, leaving the center of the gauze patch nonoccluded.
7. If the test substance is a solid or a semisolid, a 0.5-g portion is weighed and placed on the gauze patch. The test substance patch is placed on the appropriate skin site and secured. The patch is subsequently moistened with 0.5 mL of physiological saline.
8. When the test substance is in flake, granule, powder, or other particulate form, the weight of the test substance that has a volume of 0.5 mL (after compacting as much as possible without crushing or altering the individual particles, such as by tapping the measuring container) is used whenever this volume is less than 0.5 g. When applying powders, granules, and the like, the gauze patch designated for the test sample is secured to the appropriate skin site with one of the four strips of the tape at the most ventral position of the animal. With one hand, the appropriate amount of sample measuring 0.5 mL is carefully poured from a glycine weighing paper onto the gauze patch that is held in a horizontal (level) position with the other hand. The patch containing the test sample is then carefully placed into position onto the skin and the remaining three edges secured with tape. The patch is subsequently moistened with 0.5 mL of physiological saline.
9. If the test substance is a liquid, a patch is applied and secured to the appropriate skin site. A 1-mL tuberculin syringe is used to measure and apply 0.5 mL of test substance to the patch.
10. The negative control site is covered with an untreated 12-ply surgical gauze patch (1 × 1 in.).
11. The positive control substance and vehicle control substance are applied to a gauze patch in the same manner as a liquid test substance.

12. The entire trunk of the animal is covered with an impervious material (such as saran wrap) for a 24-h period of exposure. The saran wrap is secured by wrapping several long strips of athletic adhesive tape around the trunk of the animal. The impervious material aids in maintaining the position of the patches and retards evaporation of volatile test substances.
13. An Elizabethan collar is fitted and fastened around the neck of each test animal. The collar remains in place for the 24-h exposure period. The collars are utilized to prevent removal of wrappings and patches by the animals while allowing the animals food and water ad libitum.
14. The wrapping is removed at the end of the 24-h exposure period. The test substance skin site is wiped to remove any test substance still remaining. When colored test substances (such as dyes) are used, it may be necessary to wash the test substance from the test site with an appropriate solvent or vehicle (one that is suitable for the substance being tested). This is done to facilitate accurate evaluation for skin irritation.
15. Immediately after removal of the patches, each 1×1-in. test or control site is outlined with indelible marker by dotting each of the four corners. This procedure delineates the site for identification.

C. *Observations*

1. Observations are made of the test and control skin sites 1 h after removal of the patches (25 h postinitiation of application). Erythema and edema are evaluated and scored on the basis of designated values presented in Table 14.1.
2. Observations are again performed 48 and 72 h after application and scores are recorded.
3. If necrosis is present or the dermal reaction is unusual, the reaction should be described. Severe erythema should receive the maximum score (4), and +N should be used to designate the presence of necrosis and +E the presence of eschar.
4. When a test substance produces dermal irritation that persists 72 h postapplication, daily observations of test and control sites are continued on all animals until all irritation caused by the test substance resolves or until day 14 postapplication.

D. *Evaluation of Results*

1. A *subtotal irritation value* for erythema or eschar formation is determined for each rabbit by adding the values observed at 25, 48, and 72 h postapplication.
2. A subtotal irritation value for edema formation is determined for each rabbit by adding the values observed at 25, 48, and 72 h postapplication.
3. A total irritation value is calculated for each rabbit by adding the subtotal irritation value for erythema or eschar formation to the subtotal irritation value for edema formation.

4. The *primary dermal irritation index* (PDII) is calculated for the test substance or control substance by dividing the sum of the total irritation scores by the number of observations (three days × three animals = nine observations).
5. The categories of the PDII are as follows [this categorization of dermal irritation is a modification of the original classification described by Draize et al. (1944)]:

PDII	Rating
0.0	Nonirritant
>0.0–0.5	Negligible irritant
>0.5–2.0	Mild irritant
>2.0–5.0	Moderate irritant
>5.0–8.0	Severe irritant

Other abnormalities, such as atonia or desquamation, should be noted and recorded.

14.2 OTHER NONPARENTERAL ROUTE IRRITATION TESTS

The design of vaginal, rectal, and nasal irritation studies is less formalized but follows the same basic pattern as the primary dermal irritation test. The rabbit is the preferred species for vaginal and rectal irritation studies, but the monkey and dog have also been used for these (Eckstein et al., 1969). Both the rabbit and rat have commonly seen use for nasal irritation evaluations. Defined quantities (typically 1.0 mL) of test solutions or suspensions are instilled into the orifice in question. For the vagina or rectum inert bungs are usually installed immediately thereafter to continue exposure for a defined period of time (usually the same period of hours as future human exposure). The orifice is then flushed clean, and 24 h after exposure it is examined and evaluated (graded) for irritation using the scale in Table 14.1.

14.3 FACTORS AFFECTING IRRITATION RESPONSES AND TEST OUTCOME

The results of local tissue irritation tests are subject to considerable variability due to relatively small differences in test design or technique. Weil and Scala (1971) arranged and reported on the best known of several intralaboratory studies to clearly establish this fact. Though the methods presented above have proven to give reproducible results in the hands of the same technicians over a period of years (Gad et al., 1986) and contain some internal controls (the positive and vehicle controls in the PDII) against large variabilities in

results or the occurrence of either false positives or negatives, it is still essential to be aware of those factors that may systematically alter test results. These factors are summarized below:

A. In general, any factor that increases absorption through the stratum corneum or mucous membrane will also increase the severity of an intrinsic response. Unless this factor mirrors potential exposure conditions, it may, in turn, adversely affect the relevance of test results.

B. The physical nature of solids must be carefully considered both before testing and in interpreting results. Shape (sharp edges), size (small particles may abrade the skin due to being rubbed back and forth under the occlusive wrap), and rigidity (stiff fibers or very hard particles will be physically irritating) of solids may all enhance an irritation response.

C. Solids frequently give different results when they are tested dry than if wetted for the test. As a general rule, solids are more irritating if moistened (going back to item A, wetting is a factor that tends to enhance absorption). Care should also be taken as to moistening agent—some (few) batches of U.S. Pharmacopeia (USP) physiological saline (used to simulate sweat) have proven to be mildly irritating to the skin and mucous membrane on their own. Liquids other than water or saline should not be used.

D. If the treated region on potential human patients will be a compromised skin surface barrier (e.g., if it is cut or burned), some test animals should likewise have their application sites compromised. This procedure is based on the assumption that abraded skin is uniformly more sensitive to irritation. Experiments, however, have shown that this is not necessarily true; some materials produce more irritation on abraded skin while others produce less (Guillot et al., 1982; Gad et al., 1986).

E. The degree of occlusion (in fact, the tightness of the wrap over the test site) also alters percutaneous absorption and therefore irritation. One important quality control issue in the laboratory is achieving a reproducible degree of occlusion in dermal wrappings.

F. Both the age of the test animal and the application site (saddle of the back vs. flank) can markedly alter test outcome. Both of these factors are also operative in humans, of course (Mathias, 1983), but in dermal irritation tests, the objective is to remove all such sources of variability. In general, as an animal ages, sensitivity to irritation decreases. For the dermal test, the skin middle of the back (other than directly over the spine) tends to be thicker (and therefore less sensitive to irritations) than that on the flanks.

G. The sex of the test animals can also alter study results because both regional skin thickness and surface blood flow vary between males and females.

H. Finally, the single most important (yet also most frequently overlooked) factor that influences the results and outcome of these (and in fact most) acute studies is the training of the staff. In determining how test materials are prepared and applied and in how results are "read" against a subjective scale, both

accuracy and precision are extremely dependent on the technicians involved. To achieve the desired results, initial training must be careful and all-inclusive. As important, some form of regular refresher training must be exercised, particularly in the area of scoring results. Use of a set of color photographic standards as a training and reference tool is strongly recommended; such standards should clearly demonstrate each of the grades in the Draize dermal scale.

I. It should be recognized that the dermal irritancy test is designed with a bias to preclude false negatives and, therefore, tends to exaggerate results in relation to what would happen in humans. Findings of negligible irritancy (or even in the very low mild irritant range) should therefore be of no concern unless the product under test is to have large-scale and prolonged dermal contact.

14.4 PROBLEMS IN TESTING (AND THEIR RESOLUTIONS)

Some materials, by either their physicochemical or toxicological natures, generate difficulties in the performance and evaluation of dermal irritation tests. The most commonly encountered of these problems are presented below:

A. *Compound Volatility* One is sometimes required or requested to evaluate the potential irritancy of a liquid that has a boiling point between room temperature and the body temperature of the test animal. As a result, the liquid portion of the material will evaporate off before the end of the testing period. There is no real way around the problem; one can only make clear in the report on the test that the traditional test requirements were not met, though an evaluation of potential irritant hazard was probably achieved (for the liquid phase would also have evaporated from a human that it was spilled on).

B. *Pigmented Material* Some materials are strongly colored or discolor the skin at the application site. This makes the traditional scoring process difficult or impossible. One can try to remove the pigmentation with a solvent; if successful, the erythema can then be evaluated. If use of a solvent fails or is unacceptable, one can (wearing thin latex gloves) feel the skin to determine if there is warmth, swelling, and/or rigidity—all secondary indicators of the irritation response.

C. *Systemic Toxicity* On rare occasions, the dermal irritation study is begun only to have the animals die very rapidly after test material is applied.

14.5 OCULAR IRRITATION TESTING

Ocular irritation is significantly different from the other local tissue irritation tests on a number of grounds. For the pharmaceutical industry, eye irritation testing is performed when the material is intended to be put into the eye as a

means or route of application for ocular therapy. There are a number of special tests applicable to pharmaceuticals or medical devices that are beyond the scope of this volume since they are not intended to assess potential acute effects or irritation. In general, however, it is desired that an eye irritation test that is utilized by this group be both sensitive and accurate in predicting the potential to cause irritation in humans. Failing to identify human ocular irritants (lack of sensitivity) is to be avoided, but of equal concern is the occurrence of false positives.

The primary eye irritation test was originally intended to predict the potential for a single splash of chemical into the eye of a human being to cause reversible and/or permanent damage. Since the introduction of the original Draize test more than 60 years ago (Draize et al., 1944), ocular irritation testing in rabbits has both developed and diverged. Indeed, clearly there is no longer a single test design that is used, and different objectives are pursued by different groups using the same test. This lack of standardization has been recognized for some time, and attempts have been made to address standardization of at least the methodological aspects of the test, if not the design aspects.

One widely used study design, which begins with a screening procedure as an attempt to avoid testing severe irritants or corrosives in animals, goes as follows:

A. *Test Article Screening Procedure*
   1. Each test substance will be screened in order to eliminate potentially corrosive or severely irritating materials from being studied for eye irritation in the rabbit.
   2. If possible, the pH of the test substance will be measured.
   3. A primary dermal irritation test will be performed prior to the study.
   4. The test substance will not be studied for eye irritation if it is a strong acid (pH of 2.0 or less) or strong alkali (pH of 12.0 or greater) and/or if the test substance is a severe dermal irritant (with a PDII of 5–8) or causes corrosion of the skin.
   5. If it is predicted that the test substance does not have the potential to be severely irritating or corrosive to the eye, continue to Section B, Rabbit Screening Procedure.

B. *Rabbit Screening Procedure*
   1. A group of at least six New Zealand white rabbits of either sex are screened for the study. The animals are removed from their cages and placed in rabbit restraints. Care should be taken to prevent mechanical damage to the eye during this procedure.
   2. All rabbits selected for the study must be in good health; any rabbit exhibiting sniffles, hair loss, loose stools, or apparent weight loss is rejected and replaced.
   3. One hour prior to installation of the test substance, both eyes of each rabbit are examined for signs of irritation and corneal defects with a

hand-held slit lamp. All eyes are stained with 2.0% sodium fluorescein and examined to confirm the absence of corneal lesions.

Fluorescein staining: Cup the lower lid of the eye to be tested and instill one drop of a 2% (in water) sodium fluorescein solution onto the surface of the cornea. After 15 s, thoroughly rinse the eye with physiological saline. Examine the eye, employing a hand-held long-wave ultraviolet illuminator in a darkened room. Corneal lesions, if present, appear as bright yellowish-green fluorescent areas.

4. Only three of the six animals are selected for the study. The three rabbits must not show any signs of eye irritation and must show either a negative or minimum fluorescein reaction (due to normal epithelial desquamation).

C. *Study Procedure*

1. At least 1 h after fluorescein staining, the test substance is placed in one eye of each animal by gently pulling the lower lid away from the eyeball to form a cup (conjunctival cul-de-sac) into which the test material is dropped. The upper and lower lids are then gently held together for 1 s to prevent immediate loss of material.
2. The other eye remains untreated and serves as a control.
3. For testing liquids, 0.01 mL of the test substance is used.
4. For solids or pastes, 100 mg of the test substance is used.
5. When the test substance is in flake, granular, powder, or other particulate form, the amount that has a volume of 0.01 mL (after gently compacting the particles by tapping the measuring container in a way that will not alter their individual form) is used whenever this volume weighs less than 10 mg.
6. For aerosol products, the eye should be held open and the substance administered in a single 1-s burst at a distance of about 4 in. directly in front of the eye. The velocity of the ejected material should not traumatize the eye. The dose should be approximated by weighing the aerosol can before and after each treatment. For other liquids propelled under pressure, such as substances delivered by pump sprays, an aliquot of 0.01 mL should be collected and instilled in the eye as for liquids.
7. The treated eyes of the three rabbits are not washed following the instillation of the test substance.
8. The treated eyes of the remaining three rabbits are irrigated for 1 min with room temperature tap water starting 20 s after instillation.
9. To prevent self-inflicted trauma by the animals immediately after instillation of the test substance, the animals are not immediately returned to their cages. After the test and control eyes are examined and graded at 1 h postexposure, the animals are returned carefully to their respective cages.

D. *Observations*

1. The eyes are observed for any immediate signs of discomfort after instilling the test substance. Blepharospasm and/or excessive tearing are

indicative of irritating sensations caused by the test substance, and their duration should be noted. Blepharospasm does not necessarily indicate that the eye will show signs of ocular irritation.

2. Grading and scoring or ocular irritation is performed in accordance with Table 14.2. The eyes are examined and grades of ocular reactions are recorded.

TABLE 14.2 Scale of Weighted Scores for Grading Severity of Ocular Lesions[a]

Reaction Criteria	Score
I. Cornea	
A. Opacity degree of density (area that is most dense is taken for reading)	
1. Scattered or diffuse area, details of iris clearly visible	1
2. Easily discernible translucent area, details of iris slightly obscured	2
3. Opalescent areas, no details of iris visible, size of pupil barely discernible	3
B. Area of cornea involved	
1. One-quarter (or less) but not zero	1
2. Greater than one-quarter, less than one-half	2
3. Greater than one-half, less that whole area	3
4. Greater than three-quarters up to whole area	4
Scoring equals $A \times B \times 5$; total maximum = 80[b]	
II. Iris	
A. Values	
1. Folds above normal, congestion, swelling, circumcorneal ingestion (any one or all of these or combination of any thereof), iris still reacting to light (sluggish reaction possible)	1
2. No reaction to light, hemorrhage, gross destruction (any one or all of these)	2
Scoring equals $A \times B$ (where B is the area of the iris involved, graded as "under cornea"); total maximum = 10	
III. Conjunctivae	
A. Redness (refers to palpebral conjunctivae only)	
1. Vessels definitely injected above normal	1
2. More diffuse, deeper crimson red, individual vessels not easily discernible	2
3. Diffuse beefy red	3
B. Chemosis	
1. Any swelling above normal (includes nictitating membrane)	1
2. Obvious swelling with partial eversion of lids	2
3. Swelling with lids about half closed	3
4. Swelling with lids about half closed to completely closed	4
C. Discharge	
1. Any amount different from normal (does not include small amount observed in inner canthus of normal animals)	1
2. Discharge with moistening of lids and hair just adjacent to lids	2
3. Discharge with moistening of lids and considerable area around eye	3
Scoring $(A + B + C) \times 2$; total maximum = 20	

[a]The maximum total score is the sum of all scores obtained for cornea, iris, and conjunctivae.
[b]All $A \times B = \sum(1–3) \times \sum(1–4)$ for three animals.

3. If signs or irritation persists at day 7, readings are continued on days 10 and 14 after exposure or until all signs of reversible toxicity are resolved.
4. In addition to the required observation of the cornea, iris, and conjunctiva, serious effects (such as pannus, rupture of the globe, or blistering of the conjunctivae) indicative of a corrosive action are reported.
5. Whether or not toxic effects are reversible depends on the nature, extent, and intensity of damage. Most lesions, if reversible, will heal or clear within 21 days. Therefore, if ocular irritation is present at the 14-day reading, a 21-day reading is required to determine whether the ocular damage is reversible or nonreversible.

14.6 VAGINAL IRRITATION

Few, if any, products are administered via the vagina that are intended for systemic absorption. Thus, this route has not been as widely studied and characterized as others. On the other hand, large numbers of different products (douches, spermicides, antiyeast agents, etc.) have been developed that require introduction into the vagina in order to assert their localized effects. Increased research into different birth control and antiviral prophylaxis will result in more vaginal products in the future. All these must be assessed for vaginal irritation potential, and this serves as an example of the other tissue tolerance issues.

Considerable research (Eckstein et al., 1969; Auletta, 1994) has indicated that the rabbit is the best species for assessing vaginal irritation. There are those investigators, however, who consider the rabbit too sensitive and recommend the use of ovariectomized rats. Ovariectomy results in a uniformly thin, uncornified epithelium which is more responsive to localized effects. This model is used when the results from a study with rabbits are questionable (Auletta, 1994). The routine progression of studies consists of first doing an acute primary vaginal irritation study, then a 10-day repeated dose study in rats. These protocols are summarized below. Longer term vaginal studies have been conducted in order to assess systemic toxicity of the active agents when administered by these routes (while the intended effects may be local, one cannot assume that there will be no systemic exposure).

14.6.1 Acute Primary Vaginal Irritation Study in Female Rabbit

1. *Overview of Study Design* One group of three adult rabbits received a single vaginal exposure and were observed for three days with periodic examination (1, 24, 48, and 72 h postdosing) of the genitalia. Animals are then euthanized and the vagina is examined macroscopically.
2. *Administration*
   Route The material is generally introduced directly into the vagina using a lubricated 18 French rubber catheter attached to a syringe for

quantification and delivery of the test material. Gentle placement of the catheter is important because one needs to ensure complete delivery of the dose without mechanical trauma. For rabbits, the depth of insertion is about 7.5 cm and the catheter should be marked to about that depth. After deliver is completed, the tube is slowly withdrawn. No attempt is made to close or seal the vaginal orifice. Alternative methods may be used to administer more viscous materials. The most common is to backload a lubricated 1-mL tuberculine syringe, then warm the material to close to body temperature. The syringe is then inserted into the vagina and the dose administered by depressing the syringe plunger.

Dosage The test material should be one (concentration, vehicle, etc.) that is intended for human application.

Frequency Once.

Duration One day.

Volume One milliliter per rabbit.

3. *Test System* Species, age, and weight range: Sexually mature New Zealand white rabbits are generally used weighing between 2 and 5 kg. The weight is not as important as the fact that the animals need to be sexually mature.

Selection: Animals should be multiparous and nonpregnant. Animals should be healthy and free of external genital lesions.

Randomization: Because there is only one group of animals, randomization is not a critical issue.

4. *In-Life Observations*

Daily Observations At least once daily for clinical signs.

Detailed Physical Examination Once during the week prior to dosing.

Body Weight Day of dosing.

Vaginal Irritation Scored at 1, 24, 48, and 72 h postdosing. Scoring criteria are shown in Table 14.3.

5. *Postmortem Procedures* Rabbits are euthanized by lethal dose of a barbiturate soon after the last vaginal irritation scores are collected. The vagina is opened by longitudinal section and examined for evidence of mucosal damage such as erosion, localized hemorrhage, and so on. No other tissues are examined. No tissues are collected. After the macroscopic description of the vagina is recorded, the animal is discarded.

14.6.2 Repeated-Dose Vaginal Irritation in Female Rabbit

1. *Overview of Study Design* Four groups of three adult rabbits each receive a single vaginal exposure daily for 10 days. The genitalia are examined daily. Animals are then euthanized and the vagina is examined macroscopically and microscopically.

TABLE 14.3 Scoring Criteria for Vaginal Irritation

Value	
	Erythema
0	No erythema
1	Very slight erythema (barely perceptible)
2	Slight erythema (pale red in color)
3	Moderate to severe erythema (definite red in color)
4	Severe erythema (beet or crimson red)
	Edema
0	No edema
1	Very slight edema (barely perceptible)
2	Slight edema (edges of area well defined by definite raising
3	Moderate edema (raised approximately 1 mm)
4	Severe edema (raised more than 1 mm and extending beyond area of exposure)
	Discharge
0	No discharge
1	Very slight discharge
2	Slight discharge
3	Moderate discharge
4	Severe discharge (moistening of considerable area around vagina)

2. *Administration*

   Route The test materials are introduced directly into the vagina with a lubricated 18 French rubber catheter using the techniques described previously for acute studies.

   Dosage The test material should be one (concentration, vehicle, etc.) that is intended for human application. There will also be a sham-negative control (catheter in place but nothing administered), a vehicle control, and a positive control (generally 2% nonoxynol-9).

   Frequency Once daily.

   Duration Ten days.

   Volume One milliliter per rabbit for each material.

3. *Test System*

   Species, Age, and Weight Range Sexually mature New Zealand white rabbits are generally used weighing between 2 and 5 kg. The weight is not as important as the fact that the animals need to be sexually mature.

   Selection Animals should be nulliparous and nonpregnant. Animals should be healthy and free of external genital lesions.

   Randomization At least 14 animals should be on pretest. Randomization to treatment groups is best done using a computerized blocking by body weight method or a random-number generation method.

4. *In-Life Observations*

   Daily Observations At least once daily for clinical signs.

Detailed Physical Examination Once during the week prior to dosing and immediately prior to necropsy.

Body Weight First, fifth, and last day of dosing.

Vaginal Irritation Scored once daily. Scoring criteria are shown in Table 14.3.

5. *Postmortem Procedures* Rabbits are euthanized by lethal dose of a barbiturate soon after the last vaginal irritation scores are collected. The vagina is isolated using standard prosection techniques and then opened by longitudinal section and examined for evidence of mucosal damage such as erosion, localized hemorrhage, and so on. No other tissues are examined. The vagina and cervix are collected and fixed in 10% neutral buffered formalin. Standard hematoxylin/eosin-stained, paraffin-embedded histological glass slides are prepared by routine methods. Three levels of the vagina (low, mid, and upper) are examined and graded using the scoring system shown in Table 14.4. Each level is cored separately and an average is calculated. Irritation is rated as follows:

TABLE 14.4 Microscopic Scoring Procedure for Vaginal Sections

Section	Value
Epithelium	
Intact—normal	0
Cell degeneration or flattening of epithelium	1
Metaplasia	2
Focal erosion	3
Erosion or ulceration, generalized	4
Leukocytes	
Minimal: <25 per high-power field	1
Mild: 25–50 per high-power field	2
Moderate: 50–100 per high-power field	3
Marked: >100 per high-power field	4
Injection	
Absent	0
Minimal	1
Mild	2
Moderate	3
Marked with disruption of vessels	4
Edema	
Absent	0
Minimal	1
Mild	2
Moderate	3
Marked	4

Source: Eckstein et al., 1969.

Score	Rating
0	Nonirritating
1–4	Minimal irritation
5–8	Mild irritation
9–11	Moderate irritation
12–16	Marked irritation

The score for each rabbit is then averaged and acceptability ratings are given as follows:

Average Score	Acceptability Rating
0–8	Acceptable
9–10	Marginal
11 or greater	Unacceptable

14.6.3 Repeated-Dose Vaginal Irritation in Ovariectomized Rats

This study is very similar in design to that described previously for rabbits, with the following (sometimes obvious) exceptions. Mature ovariectomized female rats can be obtained from a commercial breeder. A 15% surplus should be obtained. Ten animals per group should be used (40 total for the study). The vaginal catheter is placed to a depth of approximately 2.5 cm and the treatment volume should be 0.2 mL.

14.7 PARENTERAL IRRITATION/TOLERANCE

There are a number of special concerns about the safety of materials that are routinely injected (parenterally administered) into the body. By definition, these concerns are all associated with materials that are the products of the pharmaceutical and (in some minor cases) medical device industries. Such parenteral routes include three major ones—IV (intravenous), IM (intramuscular), and SC (subcutaneous)—and a number of minor routes (such as intra-arterial) that are not considered here.

These unusual concerns include irritation (vascular, muscular, or subcutaneous), pyrogenicity, blood compatibility, and sterility (Avis, 1985). The background of each of these, along with the underlying mechanisms and factors that influence the level of occurrence of such an effect, are briefly discussed below.

Irritation Tissue irritation upon injection and the accompanying damage and pain are concerns that must be addressed for the final formulation, which is to be either tested in humans or marketed, rather than for the active ingredient. This is because most irritation factors are either due to or influenced by

aspects of formulation design [see Avis (1985) for more information or parenteral preparations]. These factors are not independent of the route (IV, IM, or SC) that will be used and, in fact (as discussed later), are part of the basis for selecting between the various routes (USP 1995a).

The lack of irritation and tissue damage at the injection site is sometimes called *tolerance*. Some of the factors that affect tolerance are not fully under the control of an investigation and are also unrelated to the material being injected. These include body movement, temperature, and animal age. Factors that can be controlled but that are not inherent to the active ingredient include solubility, tonicity, and pH. Finally, the active ingredient and vehicle can have inherent irritative effects and factors such as solubility (in the physiological milieu into which they are being injected), concentration, volume molecular size, and particle size. Gray (1978) and Ballard (1968) discuss these factors and the morphological consequences that may occur if they are not addressed.

Pyrogenicity Pyrogenicity is the induction of a febrile (fever) response induced by the parenteral (usually IV or IM) administration of exogenous material (USP 1995b). Pyrogenicity is usually associated with microbiological contamination of a final formulation, but it is now of increasing concern because of the growing interest in biosynthetically produced materials. Generally, ensuring sterility of product and process will guard against pyrogenicity for traditional pharmaceuticals. For biologically produced products, the U.S. Food and Drug Administration (FDA) has promulgated the general guideline that no more than 5.0 units of endotoxin may be present per milligram of drug substance.

Blood Compatibility It is important that cellular components of the blood are not disrupted and that serum- or plasma-based responses are not triggered by parenteral administration. Therefore, two mechanisms must be assessed regarding the blood compatibility of component materials. These include the material's effect on cellular components that cause membrane destruction and hemolysis and the activation of the clotting mechanism resulting in the formation of the thromboemboli.

Many of the nonactive, ingredient-related physicochemical factors that influence irritation (tonicity, pH, and particle size, for example) also act to determine blood compatibility. But the chemical features of a drug entity itself—its molecular size and reactivity—can also be of primary importance.

Sterility Sterility is largely a concern to be answered in the process of preparing a final clinical formulation, and it is not addressed in detail in this chapter. However, it should be clear that it is essential that no viable microorganisms are present in any material to be parenterally administered (except for vaccines).

14.7.1 Parenteral Routes

There are at least 13 different routes by which to inject material into the body:

1. Intravenous
2. Subcutaneous
3. Intramuscular
4. Intra-arterial
5. Intradermal
6. Intralesional
7. Epidural
8. Intrathecal
9. Intracisternal
10. Intracardiac
11. Intraventricular
12. Intraocular
13. Intraperitoneal

Only the first three are discussed in any detail here. Most of these routes of administration place a drug directly or indirectly into systemic circulation. There are a number of these routes, however, by which the drug exerts a local effect, in which case most of the drug does not enter systemic circulation (e.g., intrathecal, intraventricular, intraocular, intracisternal). Certain routes of administration may exert both local and systemic effects depending on the characteristics of the drug and excipients (e.g., subcutaneous).

The choice of a particular parenteral route will depend on the required time of onset of action, the required site of action, and the characteristics of the fluid to be injected, among other factors.

Bolus versus Infusion Technically, for all the parenteral routes (but in practice only for the IV route), there are two options for injecting a material into the body. The bolus and infusion methods are differentiated on the single basis of rate of injection, but they actually differ on a wide range of characteristics.

The most commonly exercised option is the bolus, or "push," injection, in which the injection device (syringe or catheter) is appropriately entered into the vein and a defined volume of material is introduced through the device. The device is then removed. In this operation, it is relatively easy to restrain an experimental animal and the stress on the animal is limited. Though the person doing the injection must be skilled, it takes only a short amount of time to become so. And the one variable to be controlled in determining dosage is the total volume of material injected (assuming dosing solutions have been properly prepared).

There are limitations and disadvantages to the bolus approach, however. Only a limited volume may be injected, which may prohibit the use of bolus when volumes to be introduced are high (due to, e.g., low active compound solubility or a host of other reasons). Only two devices (syringe and catheter) are available for use in the bolus approach. If a multiple-day course of treatment is desired (say, every day for 15 days), separate injections must be made at discrete entry sites.

The infusion approach involves establishing a fixed entry point into the vein, then slowly passing the desired test material through that point over a

period of time (30 min is about minimum, while continuous around-the-clock treatment is at least therapeutically possible). There are a number of devices available for establishing their entry point: catheter, vascular port (Garramone, 1986), or osmotic pump (Theeuwes and Yum, 1976). Each of these must, in turn, be coupled with a device to deliver the dosing solution at a desired rate. The osmotic pump, which is implanted, is also its own delivery device. Other options are gravity driven "drips," hand-held syringes (not practical or accurate over any substantial period of time), or syringe pumps. Very large volumes can be introduced by fusion over a protracted period of time, and only a single site need be fitted with an entry device.

However, infusions also have their limitations. Skilled labor is required, and the setup must be monitored over the entire period of infusion. Larger animals must be restrained, while there are special devices that make this requirement unnecessary for smaller animals. Restraint and protracted manipulation are very stressful on animals. Over a period of time, one must regularly demonstrate patency of a device—that is, that entry into the vascular system continues to exist. Finally, one is faced with having to control two variables in controlling the dose—both total volume and rate.

When are the two approaches (bolus and infusion) interchangeable? And why select one over the other? The selection of infusion is usually limited to two reasons: (1) when a larger volume must be introduced than is practical in a bolus injection or (2) when tolerance is insufficient if the dose is given all at once (i.e., an infusions will "clear" a higher daily dose than will a bolus injection). For safety studies, when a bolus can be used to clear a human, infusion dosing is a matter of judgment. If the planned clinical infusion will take less than half an hour, practicality dictates that the animal studies be accomplished by bolus. In other situations, pharmacokinetics (in particular, the half-life of the drug entity) should be considered in making the decision.

14.7.2 Test Systems for Parenteral Irritation

There are no regulatory guidelines or suggested test methods for evaluating agents for muscular or vascular irritation. Since such guidelines are lacking but the evaluation is necessary, those responsible for these evaluations have tried to develop and employ the most scientifically valid procedures.

Hagan (1959) first suggested a method for assessing IM irritation. His approach, however, did not include a grading system for evaluation of the irritation, and the method used the sacrospinalis muscles, which are somewhat difficult to dissect or repeatedly inject.

Shintani et al. (1967) developed and proposed the methodology that currently seems to be more utilized. It uses the lateral vastus muscle and includes a methodology for evaluation, scoring, and grading of irritation. Additionally, Shintani et al. investigated the effects of several factors, such as pH of the solution, drug concentration, volume of injection, effect of repeated injections, and time to maximum tissue response.

Acute Intramuscular Irritation in Male Rabbit (USP 1985)

1. *Overview of Study Design* Each rabbit is injected as follows:

Site	Treatment
Muscle vastus lateralis	1.0 mL/site
Left	Test article
Right	Vehicle

Day 1: Injection of all treatment groups—nine rabbits
Day 2: Sacrifice and evaluation: 24 h posttreatment group—three rabbits
Day 3: Sacrifice and evaluation: 48 h posttreatment group—three rabbits
Day 4: Sacrifice and evaluation: 72 h posttreatment group—three rabbits

2. *Administration*
   - 2.1. Route: The test article is injected into the vastus lateralis of each rabbit.
   - 2.2. Dose: The dose selected is chosen to evaluate the severity of irritation and represents a concentration that might be used clinically. This volume has been widely used in irritation testing.
   - 2.3. Frequency: Once only.
   - 2.4. Duration: One day
   - 2.5. Volume: One milliliter per site.
3. *Test System*
   - 3.1. Species, age, and weight range: Male New Zealand white rabbits weighing 2–5 kg are used. The New Zealand white rabbit has been widely used in muscle irritation research for many years and is a reasonable sized, even-tempered animal that is well adapted to the laboratory environment.
   - 3.2. Selection: Animals to be used in the study are selected on the basis of acceptable findings from physical examinations and body weights.
   - 3.3. Randomization: Animals are ranked by body weight and assigned a number between 1 and 3. The order of number assigned (e.g., 1–3–2) is chosen from a table of random numbers. Animals assigned number 1 are in the 24-h-posttreatment group; those assigned number 2 are in the 48-h-posttreatment group; and those assigned number 3 are in the 72-h-posttreatment group.
4. *In-Life Observations*
   - 4.1. Daily observations: Once daily following dosing.
   - 4.2. Physical examinations: Once within the two weeks before the first dosing day.
   - 4.3. Body weight: Should be determined once before the start of the study.
   - 4.4. Additional examinations may be done by the study director to elucidate any observed clinical signs.

5. *Postmortem Procedures*

5.1. Irritation is evaluated as follows: Three rabbits are sacrificed by a lethal dose of barbiturate at approximately 24, 48, or 72 h after dosing. The left and right lateral vastus of each rabbit are excised. The lesions resulting from injection are scored for muscle irritation on a numerical scale of 0–5 as follows (Shintani et al., 1967):

Reaction Criteria	Score
No discernable gross reaction	0
Slight hyperemia	1
Moderate hyperemia and discoloration	2
Distinct discoloration in comparison with color of surrounding area	3
Brown degeneration with small necrosis	4
Widespread necrosis with appearance of "cooked meat" and occasionally an abscess involving major portions of muscle	5

The average score for the nine rabbits is then calculated, and a category of irritancy is then assigned based on the following table:

Average Score	Grade
0.0–0.4	None
0.5–1.4	Slight
1.5–2.4	Mild
2.5–3.4	Moderate
3.5–4.4	Marked
4.5 or greater	Severe

Acute Intravenous Irritation in Male Rabbit The design here is similar to the intramuscular assay, except that injections are made into the veins in specific muscle masses.

1. *Overview of Study Design* Rabbits will be injected as follows:

Group	No. of Animals	Treatment Site	Evaluation
1	2	Muscles vastus lateralis (left) and cervicodorsal subcutis (left)	24 h
		Muscles vastus lateralis (right) and cervicodorsal subcutis (right)	24 h
2	2	Muscles vastus lateralis (left) and cervicodorsal subcutis (left)	72 h
		Muscles vastus lateralis (right) and cervicodorsal subcutis (right)	72 h
3	2	Auricular vein, left and right (both evaluated)	24 and 72 h

Day 1: Injection of all groups (six rabbits).
Day 2: Evaluation of group 3 (two rabbits). Sacrifice and evaluation of group 1 (two rabbits).
Day 4: Evaluation of group 3 (two rabbits). Sacrifice and evaluation of group 2 (two rabbits).

2. *Administration*
   2.1. Intramuscular: Vastus lateralis.
   2.2. Subcutaneous: Cervicodorsal subcutis.
   2.3. Intravenous: Auricular vein.
   2.4. Dose: The doses and concentration selected are chosen to evaluate the severity of irritation. The dose volumes have been widely used in irritation testing.
   2.5. Frequency: Once only.
   2.6. Duration: One day.
   2.7. Volume: Muscles vastus lateralis and cervicodorsal subcutis: 1.0 mL per site; auricular vein: 0.5 mL per site.
3. *Test System*
   3.1. Species, age, and weight range: Male New Zealand white rabbits weighing 2–5 kg are used.
   3.2. Selection: Animals to be used in the study are selected on the basis of acceptable findings from physical examinations.
   3.3. Randomization: Animals are ranked by body weight and assigned a number between 1 and 3. The order of numbers assigned (e.g., 1–3–2) is chosen from a table of random numbers. Animals assigned number 1 are in group 1, those assigned number 2 are in group 2, and those assigned number 3 are in group 3.
4. *In-Life Observations*
   4.1. Daily observations: Once daily following dosing.
   4.2. Physical examinations: Once within the two weeks before the first dosing day.
   4.3. Body weight: Determined once before the start of the study.
   4.4. Additional examinations may be done by the study director to elucidate any observed clinical signs.
5. *Postmortem Procedures*
   5.1. Intramuscular irritation is evaluated as follows: Rabbits are sacrificed by lethal dose of barbiturate approximately 24 and 72 h after dosing. The left and right lateral vastus muscles of each rabbit are excised. The reaction resulting from injection is scored for muscle irritation using the scale shown in section 1 above.
   5.2. Subcutaneous irritation is evaluated as follows: Rabbits are scarified by a lethal dose of barbiturate approximately 24 and 72 h after dosing. The subcutaneous injection sites are exposed by dissection, and the reaction is scored for irritation on a scale of 0–5 as follows (Shintani et al., 1967):

Reaction Criteria	Score
No discernible gross reaction	0
Slight hyperemia and discoloration	1
Moderate hyperemia and discoloration	2
Distinct discoloration in comparison with color of surrounding area	3
Small areas of necrosis	4
Widespread necrosis, possibly involving underlying muscle	5

Average Score per Site	Irritancy Grade
0.0–0.4	None
0.5–1.4	Slight
1.5–2.4	Mild
2.5–3.4	Moderate
3.5–4.4	Marked
4.5 or greater	Severe

5.3. Intravenous irritation is evaluated as follows: Rabbits are sacrificed by a lethal dose of barbiturate following the 72-h irritation evaluation. The injection site and surrounding tissue are grossly evaluated at approximately 24 and 72 h after dosing on a scale of 0–3 as follows:

Reaction Criteria	Score
No discernible gross reaction	0
Slight erythema at injection site	1
Moderate erythema and swelling with some discoloration of vein and surrounding tissue	2
Severe discoloration and swelling of vein and surrounding tissue with partial or total occlusion of vein	3

Average Score per Site	Irritancy Grade
0.0–0.4	None
0.5–1.4	Slight
1.5–2.4	Moderate
2.5 or greater	Severe

5.4. Additional examinations may be done by the study director to elucidate the nature of any observed tissue change.

14.7.3 Alternatives

Intramuscular and IV injection of parenteral formulations of pharmaceuticals can produce a range of discomfort including pain, irritation, and/or damage to muscular or vascular tissue. These are normally evaluated for prospective formulations before use in humans by histopathological evaluation of damage in intact animal models, usually the rabbit. Attempts have been made to make this in vivo methodology both more objective and quantitative based on measuring the creatinine phosphokinase released in the tissue surrounding the

injection site (Sidell et al., 1974). Currently, a protocol utilizing a cultured skeletal muscle cell line (L6) from the rat as a model has been evaluated in an interlaboratory validation program among 11 pharmaceutical laboratories. This methodology (Young et al., 1986) measures creatine kinase levels in media after exposure of the cells to the formulation of interest and predicts in vivo IM damage based on this endpoint. It is reported to give excellent rank-correlated results across a range of antibiotics (Williams et al., 1987). The current multilaboratory evaluation covers a broader structural range of compounds and has shown a good quantitative correlation with in vivo results for antibiotics and a fair correlation for a broader range of parenteral drug products. Likewise, Kato et al. (1992) have proposed a model that uses cultured primary skeletal muscle fibers from the rat. Damage is evaluated by the release of creatinine phosphokinase. An evaluation using six parenterally administered antibiotics [ranking their median effective concentration (EC_{50}) values] showed good relative correlation with in vivo results.

Another proposed in vitro assay for muscle irritancy for injectable formulations is the red blood cell hemolysis assay (Brown et al., 1989). Water-soluble formulations in a 1:2 ratio with freshly collected human blood are gently mixed for 5 min. The percentage of red blood cell survival is then determined by measuring differential absorbance at 540 nm; this value is then compared to values for known irritants and nonirritants. Against a very small group of compounds (four), this assay reportedly accurately predicts muscle irritation.

14.8 PHOTOTOXICITY

The potential for sunlight (or selected other light frequencies) to transform a drug or product is both a useful tool for activating some drugs and a cause of significant adverse effects for others [such as the quinolone antibiotics (Horio et al., 1995; Lambert et al., 1996)].

14.8.1 Theory and Mechanisms

The portion of the solar spectrum containing the biologically most active region is from 290 to 700 mm.

The ultraviolet (UV) part of the spectrum includes wavelengths from 200 to 400 nm. Portions of the UV spectrum have distinctive features from both the physical and biological points of view. The accepted designations for the biologically important parts of the UV spectrum are UVA, 400–315 nm; UVB, 315–280 nm; and UVC, 280–220 nm. Wavelengths less than 290 nm (UVC) do not occur at the earth's surface because they are absorbed, predominantly by ozone in the stratosphere. The most thoroughly studied photobiological reactions that occur in skin are induced by UVB. The quinolones, for example, absorb light strongly in the 300–400-nm wavelength range. Although UVB wavelengths represent only approximately 1.5% of the solar energy received

at the earth's surface, they elicit most of the known chemical phototoxic and photoallergic reactions. The visible portions of the spectrum, representing about 50% of the sun's energy received at sea level, include wavelengths from 400 to 700 nm. Visible light is necessary for such biological events as photosynthesis, the regulation of circadian cycles, vision, and pigment darkening. Furthermore, visible light in conjunction with certain chromophores (e.g., dyes, drugs, and endogenous compounds which absorb light and therefore "give" color) and molecular oxygen induces photodynamic effects.

Understanding the toxic effects of light impinging on the skin requires knowledge of both the nature of sunlight and the skin's optical properties. Skin may be viewed as an optically heterogeneous medium composed of three layers that have distinct refractive indices, chromophore distributions, and light-scattering properties. Light entering the outermost layer, the stratum corneum, is in part reflected—4–7% for wavelengths between 250 and 3000 nm (Anderson and Parrish, 1981)—due to the difference in refractive index between air and the stratum corneum. Absorption by urocanic acid (a deamination product of histidine), melanin, and proteins containing the aromatic amino acids tryptophan and tyrosine in the stratum corneum produces further attenuation of light, particularly at shorter UV wavelengths. Approximately 40% of UVB is transmitted through the stratum corneum to the viable epidermis. The light entering the epidermis is attenuated by scattering and, predominantly, absorption. Epidermal chromophores consist of proteins, urocanic acid, nucleic acids, and melanin. Passage through the epidermis results in appreciable attenuation of UVA and UVB radiation. The transmission properties of the dermis are largely due to scattering, with significant absorption of visible light by melanin, β-carotene, and the blood-borne pigments bilirubin, hemoglobin, and oxyhemoglobin. Lightly traversing these layers of the skin is extensively attenuated, most drastically for wavelengths less than 400 nm. Longer wavelengths are more penetrating. It has been noted that there is an "optical window"—that is, greater transmission—for light at wavelengths of 600–1300 nm, which may have important biological consequences.

Normal variations in the skin's optical properties frequently occur. The degree of pigmentation may produce variations in the attenuation of light, particularly between 300 and 400 nm, by as much as 1.5 times more in blacks than in Caucasians (Pathak, 1967). Alterations in the amount or distribution of other natural chromophores account for further variations in skin optical properties. Urocanic acid deposited on the skin's surface during perspiration (Anderson and Parrish, 1981) and UV-absorbing lipids excreted in sebum may significantly reduce UV transmission through the skin. Epidermal thickness, which varies over regions of the body and increases after exposure to UVB radiation, may significantly modify UV transmission.

Certain disease states also produce alterations in the skin's optical properties. Alterations of the skin's surface, such as by psoriatic plaques, decrease

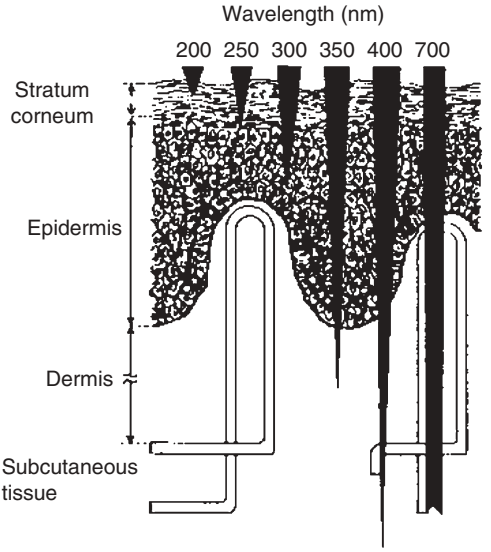

Figure 14.1 *Schematic penetration of light of varying wavelengths into skin.*

transmitted light. The effect may be lessened by application of oils whose refractive index is similar to that of skin (Anderson and Parrish, 1981). Disorders such as hyperbilirubinemia, porphyrias, and blue skin nevi result in increased absorption of visible light due to accumulation or altered distribution of endogenous chromophoric compounds.

The penetration of light into and through dermal tissues has important consequences. This penetration is demonstrated in Figure 14.1. Skin, as the primary organ responsible for thermal regulation, is overperfused relative to its metabolic requirements (Anderson and Parrish, 1981).

It is estimated that the average cutaneous blood flow is 20–30 times that necessary to support the skin's metabolic needs. The papillary boundaries between epidermis and dermis allow capillary vessels to lie close to the skin's surface, permitting the blood and important components of the immune system to be exposed to light. The equivalent of the entire blood volume of an adult may pass through he skin and potentially be irradiated in 20 min. This corresponds to the time required to receive one or two MEDs (the MED is defined as the minimal dose of UV irradiation that produces definite, but minimally perceptible, redness 24 h after exposure). The accessibility of incidence radiation to blood has been exploited in such regimens and phototherapy of hyperbilirubinemia in neonates, where light is used as a therapeutic agent. However, in general, there is a potential for light-induced toxicity due to irradiation of blood-borne drugs and metabolites.

14.8.2 Factors Influencing Phototoxicity/Photosensitization

There are a number of factors which can influence an agent acting as either a phototoxin or a photoallergen. In addition to the factors reviewed in Chapter 5, there are the following:

1. The quantity and location of photoactive material present in or on the skin
2. The capacity of the photoactive material to penetrate into normal skin by percutaneous absorption as well as into skin altered by trauma, such as maceration, irritation, and sunburn
3. The pH, enzyme presence, and solubility conditions at the site of exposure
4. The quantity of activating radiation to which the skin is exposed
5. The capacity of the spectral range to activate the materials on or within the skin
6. The ambient temperature and humidity
7. The thickness of the horny layer
8. The degree of melanin pigmentation of the skin
9. The inherent "photoactivity" of the chemical, whether it weakly or strongly absorbs light

Basically, any material that has both the potential to absorb ultraviolet light (in the UVA or UVB region) and the possibility of dermal exposure or distribution into the dermal region should be subject to some degree of suspicion as to potential phototoxicity. As shown in Table 14.5, a large number of agents have been identified as phototoxic or photoallergenic agents. Of these, tetrachlorosalicylanilide (TCSA) is the most commonly used as a positive control in animal studies.

14.8.3 Predictive Tests for Phototoxicity

Before we start on our description of the different methods, we will first cover some basics on light dosimetry. The intensity of the irradiation used in phototoxicity testing is determined with a light meter, which provides output as watts per square meter. The shelves on which the animals rest during the exposure periods are normally adjustable in order to control the dose of light to the exposure area. The irradiation from fluorescent lights will vary somewhat from day to day, depending on, for example, temperature and variations in line current. The dose the animals receive is generally represented as joules per square centimeter. A joule is equal to one watt per second. Therefore, the dose of light is dependent on the time of exposure. For example, in their review, Lambert et al. (1996) discuss dosages of UVA light of 9 or 10 $J\,cm^{-2}$ in the UVA spectral region. If the irradiation from the light is found to be

TABLE 14.5 Known Phototoxic Agents

In Humans		In Animals	
Compounds	Route	Compound	Route
Aminobenzoic acid derivatives	Topical	Acradine	Topical
		Amiodarone	Oral
Amyldimethylamino benzoate, mixed ortho and para isomers	Topical	Anthracine	Topical
		Bergapten (5-methoxypsolaren)	Topical
Anthracene acridine	Topical	Bithionol	Topical
Bergapten (5-methoxypsoralen)	Topical	Chlordiazepoxide	IP
Cadmium sulfide	Tattoo	Chlorprothiazide	IP
Chlorothiazides	Oral	Chlorpromazine	Topical
Coal tar (multicomponent)	Topical	Demeclocycline	IP
Dacarbazine	Infusion	Griseofulvin	IP
Disperse blue 35 (anthaquinone-base dye)	Topical	Kynuremic acid	Oral
Nalidixic acid	Oral	Nalidixic acid	Oral
Padimate A or Escolol 506 (amyl-*p*-dimethylamino benzoate)	Topical	Prochlorperazine	IP
		Quinokine methanol	IP
		Quinolone (antibacterial)	Oral
Psoralens	Oral, topical	Tetracyclines	IP, topical
Quinolone (antibacterial)	Oral	Xanthotoxin (8-methoxypsoralen)	Oral, IP, IM
Tetracyclines	Oral		
Xanthotoxin (8-methoxypsoralen)	Topical, oral		

$20\,\mathrm{W\,m^{-2}}$ at the exposure site, then the time of exposure required to obtain the target dose of light (in joules) is calculated as

$$\text{Time of exposure} = \frac{\mathrm{Ws}}{\mathrm{J}}\frac{9\,\mathrm{J}}{\mathrm{cm^2}}\frac{\mathrm{m^2}}{20\,\mathrm{W}}\frac{10^4\,\mathrm{cm^2}}{\mathrm{m^2}}\frac{\mathrm{min}}{60\,\mathrm{s}} = 75\,\mathrm{min}$$

If, with the same set of lights, two weeks later the irradiation is determined to be $19\,\mathrm{W\,m^{-2}}$, then the exposure period would have to be 79 min.

There are four recommended protocols for assessing topical phototoxicity potential—3T3 cells, rabbit, guinea pig, and mouse.

The first is an in vitro alternative success story—the 3T3 NRU (neutral red uptake) phototoxicity test: uses the BALB/c 3T3 (murine) cell line with cytotoxicity determination based on neutral red uptake to measure cell viability. While not a direct replacement alternative (as there is no in vivo equivalent test), it is an accepted screen for phototoxicity potential by the FDA.

The next test uses the rabbit. The traditional methodology for a predictive test for phototoxicity has been an intact rabbit test (Marzulli and Maibach, 1970). This test is conducted as follows (and illustrated diagrammatically in Figure 14.2):

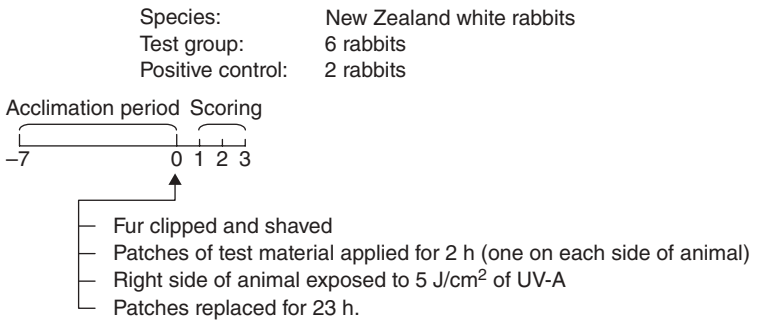

Figure 14.2 *Line chart for design and conduct of phototoxicity assay using rabbits.*

A. *Animals and Animal Husbandry*
   1. Strain/species: Female New Zealand white rabbits
   2. Number: Six rabbits per test; two rabbits for positive control
   3. Age: Young adult
   4. Acclimation period: At least seven days prior to study
   5. Food and water: Freely available commercial laboratory feed and water

B. *Test Article*
   1. A dose of 0.5 mL of liquid or 500 mg of a solid or semisolid will be applied to each test site.
   2. Liquid substances will be used undiluted.
   3. For solids, the test article will be moistened with water (500 mg test article/0.5 mL water or another suitable vehicle) to ensure good contact with the skin.
   4. The positive-control material will be a lotion containing 1% 8-methoxypsoralen.

C. *Experimental Procedures*
   1. Animals will be weighed on the first day of dosing.
   2. On the day prior to dosing, the fur of the test animals will be clipped from the dorsal area of the trunk using a small animal clipper, then shaved clean with a finer bladed clipper.
   3. On the day of dosing, the animals will be placed in restraints.
   4. One pair of patches (approximately 2.5 × 2.5 cm) per test article will be applied to the skin of the back, with one patch on each side of the backbone.
   5. A maximum of two pairs of patches may be applied to each animal and the patches must be at least 2 in. apart.
   6. The patches will be held in contact with the skin by means of an occlusive dressing for the 2-h exposure period.

7. After the 2-h exposure period, the occlusive dressing as well as the patches on the right side of the animal will be removed (aluminum foil).
8. The left side of the animal will be covered with opaque material.
9. The animal will then be exposed to approximately 5 J cm^{-2} of UVA (320–400 nm).
10. After exposure to the UVA light, the patches on the right side of the animal as well as the occlusive dressing will be replaced.
11. The dressing will again be removed approximately 23 h after the initial application of the test article. Residual test article will be carefully removed, where applicable, using water (or another suitable vehicle).
12. Animals will be examined for signs of erythema and edema and the responses scored at 24, 48, and 72 h after the initial test article application according to the Draize reaction grading system previously presented in this volume.
13. Any unusual observation and mortality will be recorded.

D. *Analysis of Data* The data from the irradiated and nonirradiated sites are evaluated separately. The scores from erythema and eschar formation and edema at 24, 48, and 72 h, are added for each animal (six values). The six values are then divided by 3, yielding six individual scores. The mean of the six individual animal irritation scores represents the mean primary irritation score (maximum score = 8, as in the primary dermal irritation study). This method was developed after a human model had been developed.

14.8.4 Guinea Pig

Recently, a standardized protocol for using the guinea pig for phototoxicity testing has been proposed (Nilsson et al., 1993) and has been the subject of an international validation exercise. This is detailed in Figure 14.3.

A. Animals and animal husbandry
   1. Strain/species: Male Hartley guinea pigs
   2. Number: At least 10 (two groups)
      Irradiation control: 4 animals
      Test material treated: 6 animals
   3. Age: Young adult, 300–500 g
   4. Acclimation period: At least five days
   5. Feed/water: Ad libitum

B. Test material
   1. Vehicle: Test assumes that material will be in solution. Use the most volatile, nonirritating organic solvent possible, for example, ethanol, acetone, dimethylacetamide, or some combination.
   2. Treatment: There can be up to four sites per animal, each measuring 1.5 × 1.5 cm (2.25 cm^2). In general, one side should be for a vehicle

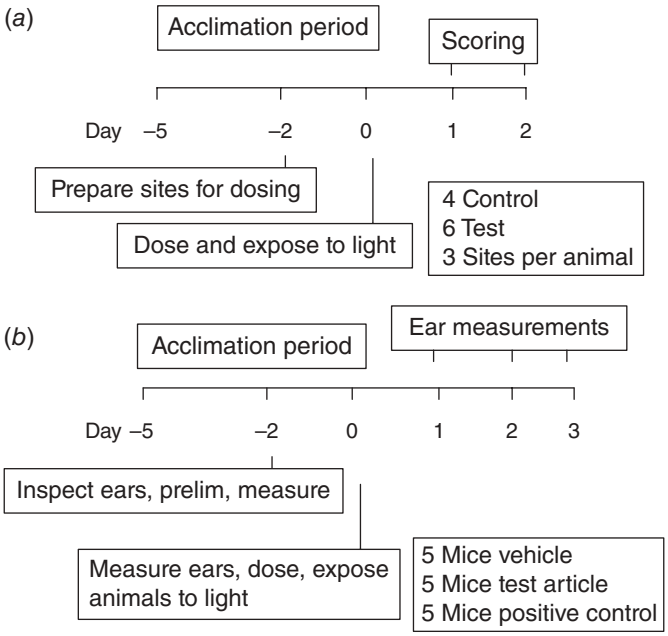

Figure 14.3 *(a) Guinea pig and (b) mouse for phototoxicity testing.*

control and another for a positive control [8-methoxypsoralen (8-MOP), 0.005% in ethanol].

3. Dosage: A dose of 0.025–0.050 mL is applied using a micropipette to each site.

C. Experimental procedure

1. Animals will be weighed on the first day of dosing.
2. Preparation: Approximately 48 h prior to treatment, the hair is removed from a 6 × 8-cm area on the back with a fine clipper. On the day of dosing, animals are dosed as described previously. Tests are situated to prevent mixing of test solutions after application. No patches or wraps are used.
3. Immediately after the dose application, the animals are placed in a restraint while keeping the test sites uncovered. Prior to irradiation the heads are covered to prevent ocular damage from light exposure.
4. Thirty minutes after dosing, animals are exposed to a nonerythmogenic dose of light in the UVA band (should have peak intensity between 335 and 365 nm). The dose of light should be 9 or 10 J cm^{-2} for UVA and 0.1–0.3 J/cm^{-2} for UVB.
5. Immediately after light exposure, the animals are wiped clean if necessary and returned to their home cages.

6. Animals are inspected and scored at 24 and 48 h postexposure according to the following:
   0: No reaction
   1: Slight erythema
   2: Moderate erythema
   3: Severe erythema, with or without edema

   The reader should note that this scoring scheme is the same one used for dermal sensitization scoring, whereas the scoring method for the rabbit model discussed previously is that used for dermal irritation studies.
7. Any unusual clinical signs noted during exposure should be noted. The following descriptive parameters can be calculated from the data.

$$\text{Phototoxic irritation index (PTII)} = \frac{\text{number of positive sites} \times 100}{\text{number of exposure sites}}$$

$$\text{Phototoxicity severity index (PSI)} = \frac{\text{total of scores}}{\text{total of observations}}$$

Lovell and Sanders (1993) had previously proposed a similar model of assessing topical phototoxicity potential in the guinea pig. Their model differed from the one proposed by Nilsson et al. (1993) with regard to the following:

- Only one test site per animal was used.
- Test sites were smaller (about 1.6 cm^2).
- Amounts applied were less (about 10 µL).
- Light intensity was set at 15 J cm^{-2}.
- Their paper made no reference to the use of a restrainer.
- Assessments were conducted at 4, 24, 48, and 72 h.

The scoring system was as follows:

0: Normal
2: Faint/trace erythema
4: Slight erythema
6: Definite erythema
8: Well-developed erythema

(Intermediate scores were indicated by odd numbers.) They recommended the use of acidine (weak phototoxin) or anthracene (strong phototoxin) for positive controls.

14.8.5 Pyrogenicity

The USP describes a pyrogen test using rabbits as a model (1995b). This test, which is the standard for limiting risks of a febrile reaction to an acceptable level, involves measuring the rise in body temperature in a group of three rabbits for 3 h after injection of 10 mL of test solution.

1. *Apparatus and Diluents* Render the syringes, needles, and glassware free of pyrogens by heating at 250 °F for not less than 30 min or by any other suitable method. Treat all diluents and solutions for washing and rinsing of devices or parenteral injection assemblies in a manner that will ensure that they are sterile and pyrogen free. Periodically perform control pyrogen tests on representative portions of the diluents and solutions that are used for washing or rinsing of the apparatus.

2. *Temperature Recording* Use an accurate temperature-sensing device, such as a clinical thermometer or thermistor or similar probe, that has been calibrated to ensure an accuracy of ±0.1 ° and has been tested to determine that a maximum reading is reached in less than 5 min. Insert the temperature-sensing probe into the rectum of the test rabbit to a depth of not less than 7.5 cm and, after a period of time not less than that previously determined as sufficient, record the rabbit's temperature.

3. *Test Animals* Use healthy, mature rabbits. House the rabbits individually in an area of uniform temperature (between 20 and 23 °C) free from disturbances likely to excite them. The temperature should vary no more than ±3 °C from the selected temperature. Before using a rabbit for the first time in a pyrogen test, condition it for not more than seven days before use by a sham test that includes all of the steps as directed in item 4 below, except injection. Do not use a rabbit for pyrogen testing more frequently than once every 48 h or prior to two weeks following a maximum rise in its temperature of 0.6 °C or more while being subjected to the pyrogen test or following its having been given a test specimen that was adjusted to be pyrogenic.

4. *Procedure* Perform the test in a separate area designated solely for pyrogen testing and under environmental conditions similar to those under which the animals are housed. Withhold all food from the test rabbits during the period of the test. Access to water is allowed at all times but may be restricted during the test. If probes measuring rectal temperature remain inserted throughout the testing period, restrain the rabbits with loose-fitting Elizabethan collars that allow the rabbits to assume a natural resting posture. Not more than 30 min prior to the injection of the test dose, determine the "control temperature" of each rabbit; this is the base for the determination of any temperature increase resulting from the injection of a test solution. In any one group of test rabbits, use only those rabbits whose control temperatures do not vary by more than 1 °C from each other and do not use any rabbit having a temperature exceeding 39.8 °C.

Unless otherwise specified in the individual protocol, inject 10 mL of the test solution per kilogram of body weight into an ear vein of each of three rabbits, completing each injection within 10 min after the start of administration. The test solution is either the product, constituted if necessary as directed in the labeling, or the material under test. For pyrogen testing of devices or injection assemblies, use washings or rinsings of the surfaces that come in contact with the parenterally administered material or with the injection site or internal tissues of the patient. Ensure that all test solutions are protected from contamination. Perform the injection after warming the test solution to a temperature of 37 ± 2 °C. Record the temperature at 1, 2, and 3 h subsequent to the injection.

5. *Test Interpretation and Continuation* Consider any temperature decreases as zero rise. If no rabbit shows an individual rise in temperature of 0.6 °C or more above its respective control temperature and if the sum of the three individual maximum temperature rises does not exceed 1.4 °C, the product meets the requirements for the absence of pyrogens. If any rabbit shows an individual temperature rise of 0.6 °C or more or if the sum of the three individual maximum temperature rises exceeds 1.4 °C, continue the test using five other rabbits. If not more than three of the eight rabbits show individual rises in temperature of 0.6 °C or more and if the sum of the eight individual maximum temperature rises does not exceed 3.7 °C, the material under examination meets the requirements for the absence of pyrogens.

In Vitro Pyrogenicity In vitro pyrogenicity testing (or bacterial endotoxin testing) is one of the great success stories for in vitro testing. Some 15 years ago, the limulus amebocyte lysate (LAL) test was developed, validated, and accepted as an in vitro alternative (Cooper, 1975; Wearly and Baker, 1977) to the rabbit test. An in vitro test for estimating the concentration of bacterial endotoxins that may be present in or on a sample of the article(s) to which the test is applied uses LAL that has been obtained from aqueous extracts of the circulating amebocytes of the horseshoe crab, *Limulus polyphemus*, and that has been prepared and characterized for use as an LAL reagent for gel-clot formation. The test's limitation is that it detects only the pyrogens of gram-negative bacteria. This is generally not significant, since most environmental contaminants that gain entrance to sterile products are gram negative (Develeeshouwer et al., 1985).

Where the test is conducted as a limit test, the specimen is determined to be positive or negative to the test judged against the endotoxin concentration specified in the individual monograph. Where the test is conducted as an assay of the concentration of endotoxin, with calculation of confidence limits of the result obtained, the specimen is judged to comply with the requirements if the result does not exceed (1) the concentration limit specified in the individual monograph and (2) the specified confidence limits for the assay. In either case the determination of the reaction endpoint is made with parallel dilutions of redefined endotoxin units.

Since LAL reagents have also been formulated to be used for turbidimetric (including kinetic) assays or colorimetric readings, such tests may be used if shown to comply with the requirements for alternative methods. These tests require the establishment of a standard regression curve and the endotoxin content of the test material is determined by interpolation from the curve. The procedure includes incubation for a preselected time of reacting endotoxin and control solutions with LAL reagent and reading the spectrophotometric light absorbance at suitable wavelengths. In the case of the turbidimetric procedure the reading is made immediately at the end of the incubation period. In the kinetic assays, the absorbance is measured throughout the reaction period and rate values are determined from those readings. In the colorimetric procedure the reaction is arrested at the end of the preselected time by the addition of an appropriate amount of acetic acid solution prior to the readings. A possible advantage in the mathematical treatment of results, if the test is otherwise validated and the assay suitably designed, could be the confidence interval and limits of potency from the internal evidence of each assay itself.

14.8.6 Blood Compatibility

The standard test (and its major modifications) currently used for this purpose is technically an in vitro one, but it requires a sample of fresh blood from a dog or other large donor animal. The test was originally developed by the National Cancer Institute for use in evaluating cancer chemotherapeutic agents (Prieur et al., 1973) and is rather crude, though definitive.

The variation described here is one commonly utilized. It uses human blood from volunteers, eliminating the need to keep a donor colony of dogs. The test procedure is described below.

1. *Test System* Human blood. Collect 30 mL heparinized blood for whole blood and plasma (three tubes) and 30 mL clotted blood for serum (two tubes) from each of six donors.
2. *Precipitation Potential*
   - 2.1. For each donor, set up and label eight tubes 1–8.
   - 2.2. Add 1 mL serum to tubes 1–4.
   - 2.3. Add 1 mL plasma to tubes 5–8.
   - 2.4. Add 1 mL formulation to tubes 1–5.
   - 2.5. Add 1 mL vehicle to tubes 2 and 6.
   - 2.6. Add 1 mL physiological saline to tubes 3 and 7 (negative control).
   - 2.7. Add 1 mL 2% nitric acid to tubes 4 and 8 (positive control).
   - 2.8. Observe tubes 1–8 for qualitative reactions (e.g., precipitation or clotting) before and after mixing.
   - 2.9. If a reaction is observed in the formulation tubes (1 and/or 5), dilute the formulation in an equal amount of physiological saline (½ dilution) and test 1 mL of the dilution with an equal amount of plasma

and/or serum. If a reaction still occurs, make serial dilutions of the formulation in saline (i.e., ¼, ⅛, etc.).

2.10. If a reaction occurs in the vehicle tubes (2 and/or 6), repeat in a manner similar to that in step 2.9.

3. *Hemolytic Potential*
   3.1. For each donor, set up and label eight tubes 1–8.
   3.2. Add 1 mL whole blood to each tube.
   3.3. Add 1 mL formulation to tube 1.
   3.4. Add 1 mL vehicle to tube 2.
   3.5. Add 1 mL of ½ dilution of formulation in saline to tube 3.
   3.6. Add 1 mL of ½ dilution of vehicle in saline to tube 4.
   3.7. Add 1 mL of ¼ dilution of formulation in saline to tube 6.
   3.8. Add 1 mL of ¼ dilution of vehicle in saline to tube 6.
   3.9. Add 1 mL of physiological saline to tube 7 (negative control).
   3.10. Add 1 mL of distilled water to tube 8 (positive control).
   3.11. Mix by gently inverting each tube three times.
   3.12. Incubate tubes for 45 min at 37 °C.
   3.13. Centrifuge 5 min at 1000 *g*.
   3.14. Separate the supernatant from the sediment.
   3.15. Determine hemoglobin concentrations to the nearest 0.1 g dL^{-1} on the supernatant (plasma).
   3.16. If hemoglobin concentrations of the above dilutions are 0.2 g dL^{-1} (or more) greater than the saline control, repeat the procedure, adding 1 mL of further serial dilutions (⅛, 1/16, etc.) of formulation or vehicle to 1 mL of blood until the hemoglobin level is within 0.2 g dL^{-1} of the saline control.

There are two proposed, true in vitro alternatives to this procedure (Mason et al., 1974; Kambic et al., 1976), but neither has been either widely evaluated or accepted.

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15

Pharmacokinetics and Toxicokinetics in Drug Safety Evaluation

15.1 INTRODUCTION

Among the cardinal principles of both toxicology and pharmacology is that the means by which an agent comes in contact with or enters the body (i.e., the route of exposure or administration) does much to determine the nature and magnitude of its effects (Goldstein et al., 1974; Pratt and Taylor, 1990). Accordingly, an understanding of route(s) of administration and their implications for absorption is essential. The therapeutic index (calculated based on plasma and therefore absorbed levels) is the ratio between what levels cause adverse effects and the levels which have the desired therapeutic effect.

Safety assessment studies usually involve a control group of animals (untreated and/or dosed with formulation only) and at least three treated groups receiving "low," "intermediate," and "high" dose levels of the drug entity of interest via a route approximately that used in humans (as closely as possible). Frequently there will also be recovery groups to determine if any observed effects are reversible (and if so, to what extent). In most instances the high dose level is expected to elicit some toxic effects in the animals, often expressed as decreased food consumption and/or below-normal body weight gain, and has been selected after consideration of earlier data, perhaps from dose range-finding studies, or at least to a dose as high as possible by the intended route. The other two dose levels are anticipated not to cause toxic effects. Generally, but not always (e.g., nonsteroidal anti-inflammatory drugs

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in rodents), the low dose level is a several-fold multiple of the expected human therapeutic or exposure level (generally more so in rodents than in nonrodents). However, without knowing the true relationship of these dose levels to each other with respect to the absorption, distribution, and elimination of the new chemical entity as reflected by its pharmacokinetics, it is difficult to see how meaningful extrapolations concerning safety margins can be made from the toxicity data obtained. Also, without pharmacokinetic data from the positive-control group, its inclusion is of limited value and the results obtained could lead to erroneous conclusions (Yacobi et al., 1989).

Pharmacokinetic studies can provide information on several aspects, knowledge of which greatly facilitates assessment of safety of the chemical entity. Six such aspects can be mentioned:

(i) Relationship between the dose levels used and the relative extent of absorption of the test compound
(ii) Relationship between the protein binding of the test compound and the dose levels used
(iii) Relationship between pharmacological or toxicological effects and the kinetics of the test compound
(iv) Effect of repeated doses on the kinetics of the test compound
(v) Relationship between the age of the animal and the kinetics of the test compound
(vi) Relationship between the dose regimens of the test compound used in the toxicity studies and those employed clinically in humans

International Conference on Harmonisation (ICH) guidelines (ICH, 2000a,b) dictate a clearly defined set of objectives for toxicokinetic studies:

- *Primary* To describe the systemic exposure achieved in animals and its relationship to dose level and the time course of toxicity studies.
- *Secondary*
  1. To relate the exposure achieved in toxicity studies to toxicological findings and contribute to the assessment of the relevance of these findings to clinical safety.
  2. To support the choice of species and treatment regimen in nonclinical toxicity studies.
  3. To provide information which, in conjunction with the toxicity findings, contributes to the design of subsequent nonclinical toxicity studies.

These data may be obtained from all animals on a toxicity study, from representative subgroups, from satellite groups, or from separate studies.

If toxicology can be described as being the study of the effects of a chemical on an organism, metabolism can be described as the opposite—the effects of the organism on the chemical. Metabolism refers to a process by which a drug

(xenobiotic) is chemically modified by an organism. It is part of the overall process of disposition of xenobiotic (ADME)—the process by which a chemical gains access to the inner working of an organism (absorption), how it moves around inside an organism (distribution), how it is changed by the organism (metabolism), and how it is eventually eliminated from the organism (elimination). The U.S. Environmental Protection Agency (EPA) definition of biotransformation or metabolism is "the sum of processes by which a xenobiotic (foreign chemical) is handled by a living organism." The mathematical formulae used to describe and quantify these processes are collectively known as pharmacokinetics. The EPA definition of pharmacokinetics is the "quantitation and determination of the time course and dose dependency of the absorption, distribution, biotransformation and excretion of chemicals." The acronym ADME (for absorption, distribution, metabolism, and excretion) has been used to describe the multifaceted biological process. The term metabolism has also come into common jargon to describe the entire process. This science has long played a central role in pharmaceutical development but has played less of a role in the development of other types of products. The purpose of this chapter is to introduce the basic concepts of ADME and practices of studies conducted to study it as described in regulations of the EPA and Organisation for Economic Co-operation and Development (OECD), which require such data for nonpharmaceutical products and to give some real-world examples.

15.2 REGULATIONS

The FDA believes that data from studies on the ADME of a chemical provide insight into mechanisms of toxicity and are essential in the design and evaluation of results from other toxicity studies (FDA, 2005). Such data should be provided for all drugs and significant impurities. Recommendations for obtaining data on the metabolism and pharmacokinetics of these substances are presented in ICH guidelines and the FDA *Redbook II* (2000). In general, it is required that this information be obtained as part of initial and subsequent repeat-dose studies with a drug. The European Medicines Agency (EMEA) has promulgated separate guidelines (EMEA, 2006) for evaluating the pharmacokinetics of protein therapeutics.

15.3 PRINCIPLES

An understanding of the design and analysis of pharmacokinetic studies requires a broad understanding of the underlying concepts and principles inherent in the ADME process and in our current technology for studying such. Each of these four principal areas is overviewed from a practical basis as it relates to toxicology. First, however, one should consider the fundamental terminology used in pharmacokinetic studies (Table 15.1).

TABLE 15.1 Fundamental Terms Used in Pharmacokinetic Studies

Absolute bioavailability	Bioavailability of dosage form relative to intravenous administration
Absorption	Process by which xenobiotic and its metabolites are transferred from site of absorption to blood circulation
Accumulation	Progressive increase of chemical and/or metabolites in body. Accumulation is influenced by the dosing interval and half-life of the chemical. The process can be characterized by an "accumulation factor," which is the ratio of the plasma concentration at steady state to that following the first dose in a multiple dosing regimen.
Analyte	Drug entity assayed in biological samples
Area under the curve (AUC)	Concentration of chemical and/or metabolites in blood (or plasma/serum) integrated over time. This is typically considered the best indicator of exposure.
Bioavailability	Rate and extent to which xenobiotic entity enters systemic circulation intact following oral or dermal administration. It is sometimes expanded to include therapeutically active metabolites. Also known as comparative bioavailability.
Biotransformation	Process by which xenobiotic is structurally and/or chemically changed in body by either enzymatic or nonenzymatic reactions. The product of the reaction is a different composition of matter or different configuration than the original compound.
Clearance	Volume of biological fluid which is totally cleared of xenobiotic in unit time
C_{max}	Maximum mean concentration of chemical in plasma. Also known as peak plasma concentration.
Concomitant toxicokinetics	Toxicokinetic measurements performed in toxicity study either in all animals or in representative subgroups or in satellite groups
Disposition	All processes and factors involved from time chemical enters body to time when it is eliminated from body either intact or in metabolite form
Distribution	Process by which absorbed xenobiotic and/or its metabolites partition between blood and various tissues/organs in body
Dosage form	Formulation (diet, lotion, capsule, solution, etc.) administered to animals or humans
Dose proportionality	Relationship between doses of chemical and measured parameters, usually including tests for linearity
Enterohepatic circulation	Process by which xenobiotics are emptied via bile into small intestine and then reabsorbed into hepatic circulation
Enzyme induction	Increase in enzyme content (activity and/or amount) due to xenobiotic challenge which may result in more rapid metabolism of chemical
Enzyme inhibition	Decrease in enzymatic activity due to effect of xenobiotic challenge
Excretion	Process by which administered compound and/or its biotransformation product(s) are eliminated from body

TABLE 15.1 ***Continued***

Absolute bioavailability	Bioavailability of dosage form relative to intravenous administration
Exposure	Exposure is represented by pharmacokinetic parameters demonstrating the local and systemic burden on the test species with the test compound and/or its metabolites. The area under the matrix level concentration–time curve (AUC) and/or the measurements of matrix concentrations at the expected peak-concentration time C_{max} or at some other selected time $C_{(time)}$ are the most commonly used parameters. Other parameters might be more appropriate in particular cases.
First-order kinetics	Kinetic processes the rate of which is proportional to concentration
First-pass effect	Phenomenon whereby xenobiotics may be extracted or metabolized following enteral absorption before reaching systemic circulation
Flux	Term (that takes area into consideration) used to describe movement of chemical across a barrier. Most typically used to describe the absorption of a chemical across the skin as micrograms per square centimeter per hour.
Half-life	Time elapsed for given chemical entity concentration or amount to be reduced by factor of 2
Hepatic clearance	Rate of total body clearance accounted for by liver
K_{el}	Elimination constant for chemical in plasma. Typically calculated using the formula $K_{el} = -1n[10] \times b$, where b is the slope of the linear regression line of the log of the mean plasma concentrations vs. time from t_{max} to 24 h.
Lag time	Interval between compound administration and when compound concentration is measurable in blood
Metabolite characterization	Determination of physiochemical characteristics of biotransformation product(s)
Metabolite identification	Structural elucidation of biotransformation product(s)
Metabolite profile	Chromatographic pattern and/or aqueous/nonaqueous partitioning of biotransformation products of administered compound
Monitor	To take a small number of matrix samples (e.g., 1–3) during dosing interval to estimate $C_{(time)}$ and/or C_{max}.
Nonlinear kinetics (saturation kinetics)	Kinetic processes the rate of which is not directly proportional to concentration
Presystemic elimination	Loss of that portion of dose that is not bioavailable. This would include, among others, loss through intestinal and gut-wall metabolism, lack of absorption, and first-pass hepatic metabolism.
Profile	To take (e.g., 4–8) matrix samples during dosing interval to estimate C_{max} and/or $C_{(time)}$ and area under matrix concentration–time curve (AUC)
Protein binding	Complexation of xenobiotic and/or its metabolite(s) with plasma or tissue proteins
Relative bioavailability	Bioavailability relative to reference or standard formulation or agent

TABLE 15.1 ***Continued***

Absolute bioavailability	Bioavailability of dosage form relative to intravenous administration
Renal clearance	Rate of total body clearance accounted for by kidney. Its magnitude is determined by the net effects of glomerular filtration, tubular secretion and reabsorption, renal blood flow, and protein binding.
Satellite	Groups of animals included in design and conduct of toxicity study treated and housed under conditions identical to those of main study animals but used primarily for toxicokinetics
Steady state	Equilibrium state where rate of chemical input is equal to rate of elimination during given dose interval
Support	In context of toxicity study to ratify or confirm design of toxicity study with respect to pharmacokinetic and metabolic principles. This process may include two steps: a. Confirmation using toxicokinetic principles that the animals on a study were exposed to appropriate systemic levels of the administered compound and/or its metabolite(s). b. Confirmation that the metabolic profile in the species used was acceptable; data to support this will normally be derived from metabolism studies in animals and humans.
T_{max}	Sampling time point at which C_{max} occurs.
Total clearance	Volume of biological fluid totally cleared of xenobiotic per unit time and usually includes hepatic clearance and renal clearance
Toxicokinetics	Study of kinetics of absorption, distribution, metabolism, and excretion of toxic or potentially toxic chemicals
Validate	In context of analytical method to establish accuracy, precision, reproducibility, response function, and specificity of analytical method with reference to biological matrix to be examined and analyte to be quantified
Volume of distribution (V_d)	Hypothetical volume of body fluid into which chemical distributes. It is not a "real" volume but is a proportionality constant relating the amount of chemical in the body to the measured concentration in blood or plasma.

15.3.1 Absorption

Absorption describes the process by which a chemical crosses a biological membrane to gain access to the inner workings of an organism. For mammals, this process results in the entry of the chemical into the bloodstream, or systemic circulation. In this case the process is also called systemic absorption. Pharmaceutical products, procedures, and devices, such as hypodermic needles or catheters, can be used to by-pass biological barriers. Other products gain access to the systemic circulation via the oral, dermal, buccal, or inhalatory route of administration.

For a material to be toxic (local tissue effects are largely not true toxicities by this definition), the first requirement is that it be absorbed into the organism [for which purpose being in the cavity of the gastrointestinal (GI) tract does not qualify]. Most pharmaceuticals are intended to gain such access.

There are characteristics which influence absorption by the different routes, and these need to be understood by any person trying to evaluate and/or predict the toxicities of different moieties. Some key characteristics and considerations are summarized below by route:

A. Oral and rectal routes (gastrointestinal tract)
   1. Lipid-soluble compounds (nonionized) are more readily absorbed than water-soluble compounds (ionized).
      a. Weak organic bases are in the nonionized, lipid-soluble form in the intestine and tend to be absorbed there.
      b. Weak organic acids are in the nonionized, lipid-soluble form in the stomach and one would suspect they would be absorbed there, but the intestine is more important because of time and area of exposure.
   2. Specialized transport systems exist for some moieties: sugars, amino acids, pyrimidines, calcium, and sodium.
   3. Almost everything is absorbed—at least to a small extent (if it has a molecular weight below 10,000).
   4. Digestive fluids may modify the structure of a drug.
   5. Dilution increases toxicity because of more rapid absorption from the intestine, unless stomach contents bind the moiety.
   6. Physical properties are important—for example, dissolution of metallic mercury is essential to allow absorption.
   7. Age—neonates have a poor intestinal barrier.
   8. The effect of fasting on absorption depends on the properties of the chemical of interest.

B. Inhalation (lungs)
   1. Aerosol deposition:
      a. Nasopharyngeal—5 μm or larger in humans, less in common laboratory animals
      b. Tracheobronchiolar—1–5 μm
      c. Alveolar—1 μm
   2. If a solid, mucociliary transport may serve to clear from the lungs to the GI tract.
   3. Lungs are anatomically good for absorption.
      a. Large surface area (50–100 m^2)
      b. High blood flow
      c. Close to blood (10 μm between gas media and blood)
   4. Absorption of gases is dependent on solubility of the gas in blood.
      a. Chloroform, for example, has high solubility and is all absorbed; respiration rate is the limiting factor.
      b. Ethylene has low solubility and only a small percentage is absorbed—blood flow limited absorption.
      c. Parenteral routes.
      d. Dermal routes.

As a generalization, there is a pattern of relative absorption rates which extends between the different routes that are commonly employed. This order of absorption (by rate from fastest to slowest and, in a less rigorous manner, in degree to absorption from most to least) is intravenous (IV) > inhalation > intramuscular (IM) > intraperitoneal (IP) > subcutaneous (SC) > oral > intradermal (ID) > other dermal.

Absorption (total amount and rate), distribution, metabolism, and species similarity in response are the reasons for selecting particular routes in toxicology. In acute studies, however, these things are rarely known to us. So the cardinal rule for selecting routes of use in acute testing is to use those routes which mirror the intended route for human exposure. If this route of human exposure is uncertain or if there is the potential for either a number of routes or the human absorption rate and pattern being greater, then the common practice becomes that of the most conservative approach. This approach stresses maximizing potential absorption in the animal species (within the limits of practicality) and selecting from among those routes commonly used in the laboratory that which gets the most material into the animal's system as quickly and completely as possible to evaluate the potential toxicity.

In general, drugs cross biological barriers by one of three mechanisms: active transport, facilitative transport, and passive transport. In active transport, the chemical is specifically recognized by the organism, which then expends energy to take the chemical up, even against a concentration gradient. In facilitative transport, the organism produces a carrier molecule which reacts with the target molecule to form a complex which more easily traverses the membrane, but no energy is expended to take up the complex. Such complexes do not flow against a concentration barrier. The simplest mechanism is passive transfer or diffusion. Here, a drug flows down a concentration gradient (from high concentration to a lower concentration) and must passively (no energy expended by organism) cross a biological membrane. Passive transfer or diffusion is the most common (if not the only) mechanism involved in the absorption of the vast majority of approved drugs. It should be remembered that for purposes here "concentration gradient" must be considered in relationship to partition coefficient. That is, a gradient will reflect the relative solubilities of drug in polar (water) and nonpolar (lipid) matrices or tissues. The other mechanisms involved in absorption will not be further discussed here.

Drugs in solution have a natural tendency (more rigorously defined by the laws of thermodynamics) to move down a concentration gradient. That is to say, the individual molecules of solute tend to move from a region of high concentration toward regions of lower concentration. Also, the movement of a chemical across a permeable barrier, such as a biological membrane, is a process called diffusion, as illustrated by Figure 15.1. For most products, these biological barriers are the wall of the GI tract, the lining of the pulmonary system, and/or the skin.

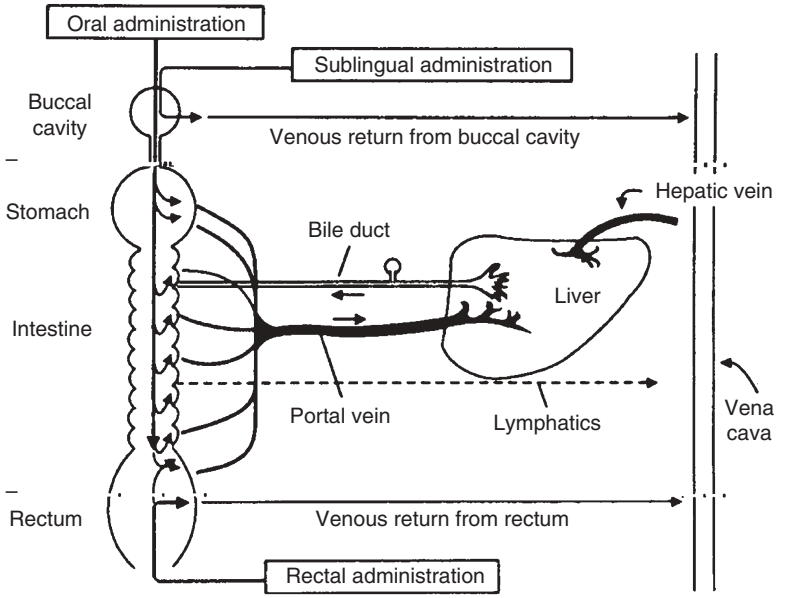

Figure 15.1 *Passage of chemical moieties from GI tract into bloodstream.*

Absorption from the GI tract is controlled by a variety of factors. These include the acid–base characteristics of the chemical (described as the pK_a), the solubility, the nature of the delivery (e.g., diet vs. gavage), the nature of any vehicle (suspensions vs. solution or aqueous vs. nonaqueous), and the GI tract of the species under study. Gad (2007b) provides much greater detail on this subject.

Ionized or charged organic moieties do not readily pass through the lipophilic cell membranes of the epithelial cells that line the GI tract. Thus more acidic molecules tend to be more readily absorbed from the stomach while more alkaline materials tend to be absorbed from the small intestine. This is because at the acidic pH of the stomach acidic chemicals tend to be nonionized. More alkaline chemicals tend to be more ionized in the stomach and less ionized in the gut. The equilibrium reaction for acidic dissociation can be represented by the equation

$$\begin{array}{l} \text{X-C-OH} + \text{H}_2\text{O} == \text{X-C-O} + \text{H}_3\text{O}^+ \\ \quad // \qquad\qquad\quad // \\ \quad \text{O} \qquad\qquad\quad \text{O} \end{array}$$

Like all chemical equations, this one has an equilibrium constant. The discussion of basic chemistry is outside the purview of this book. Readers who may need a refresher are referred to Tse and Jaffe (1991). For every chemical,

TABLE 15.2 Receptors Slowing Gastric Emptying

Receptor (Site)	Stimulus	Example	Sensitivity
Osmoreceptor (Duodenum)	Osmotic Effect (except tryptophan)	Glucose Amino Acids Electrolytes	Least
Acid Receptor (Proximal Duodenum & Jejunum)	Acids with pKa < 5	Citric Acid HCl	Intermediate
Fat Receptor (Jejunum)	Fatty Acids	Sodium Myristate	Most
Tryptophan Receptor (Duodenum & Jejunum)	Tryptophan	Tryptophan	—

Adapted from Hunt & Knox, 1968 and Minami & MacCallum, 1984.

a pK_a can be calculated based on the equilibrium constant, which represents the proportion of ionized and un-ionized material in solution. The lower the pK_a of a chemical the more likely it is to be nonionized in the stomach.

One can manipulate movement of a drug through the GI tract (and particularly the stomach) by formulation and/or by feeding concurrent with drug administration. Some of the factors which slow gastric emptying (and therefore increase drug absorption) are presented in Table 15.2.

Absorption from Pulmonary System Of the three routes discussed here, absorption from the pulmonary system is perhaps the most rapid. Systemic absorption of inhaled materials is highly dependent on the physical properties of the inhaled materials which dictate how easily the materials reach the alveoli of the deep lung. Proteins may be readily absorbed when instilled in the deep lungs—as an example, note the inhalable insulins. Gases and vapors easily penetrate into the deep lung. For mists and dusts, absorption will be highly dependent on particle size. In general, the larger the particles, the less they will penetrate the pulmonary system. The term impaction describes the deposition of particles in the respiratory tract. Particles of less than 0.2 μm are preferentially deposited in the pulmonary portion of the respiratory system and particles over 2 μm do not reach the alveolar epithelium in great number. Particles from 1 to 4 μm tend to be distributed over the length of the system and particles over 4 μm tend to be deposited in the nasal region. Aerosolized particles of greater than 20 μm do not commonly occur in nature. Tidal volume will also influence impaction. In general, the larger the tidal volume, and thus the more forceful the inhalatory process, the more deeply particles of all sizes tend to be driven into the lung.

Once deposited, materials must be in solution before they can be absorbed. Hence, materials in an aerosolized solution will be more readily absorbed than materials that are delivered as solid (e.g., dusts) particles. Solid materials must be able to go into solution in situ in order to be absorbed. Particle size influences dissolution rate. Large particles dissolve more slowly (for any given material) than small particles due to the differences in surface area. Once in

solution, the same laws of passive diffusion apply to materials in the lung as apply to material in the GI tract. The large surface area and the rich blood flow at the alveoli make for ideal conditions for rapid absorption into the systemic circulation. Absorption across the mucosa lining the upper airways is less rapid. Materials that do not dissolve are ingested by pulmonary macrophages and are either broken down there or moved out of the lungs by the upward movement of the bronchociliary tree.

For gases and vapors, the amount absorbed is highly dependent on the partial pressure of the gas and the solubility of the gas in blood. Take the simple case of a gas that is not metabolized and is excreted by exhalation (e.g., an anesthetic gas or a Halon-type fire-extinguishing agent). At any given concentration (or partial pressure) in the atmosphere, the concentration in the blood will reach a steady state in the blood. Accordingly, prolonged exposure does not lead to continual buildup.

At equilibrium, the concentration in the blood is depicted by the formula (also known as the Ostwald coefficient) $X_b/X_a = S$, where X_b is the concentration in the blood and X_a is the concentration in the inspired air. Thus, if one knows S for a given chemical and the target concentration for a given exposure, one can predict what the resulting concentration may be at equilibrium. Additionally, the lower the S value (i.e., the lower the solubility in blood), the more rapidly the chemical will achieve equilibrium.

Absorption across Skin An aqueous carrier may be used for a variety of dermal products. In fact, carriers can be designed to limit the transportation of the penetration of the active ingredient (such as an insect repellent) if the desired effect is to keep the active ingredient on the surface of the skin. Once again, however, only those materials that are dissolved will be available for penetration across the skin to gain access to the systemic circulation. For almost all drugs in or about to enter clinical trials, dermal penetration is a passive process. The relative thickness of the skin makes absorption (into the systemic circulation) slower than the absorption across the GI or pulmonary barriers. This is compounded by the fact that the stratum corneum function is to be impervious to the environment. One of the skin's major functions is protection from infection. Once a drug penetrates into the dermis, it may partition into the subcutaneous fat. Essentially, absorption across the skin is a two-step process with the first being penetration and deposition into the skin and the second being release from the skin into the systemic circulation. The pattern of blood levels obtained via dermal penetration is generally one with a delayed-absorption, slow buildup to more of a plateau than a peak. Blood levels of chemicals absorbed via the dermal route are generally low (Garner and Matthews, 1998).

Given the overwhelming influence of the physical properties of skin in determining bioavailabilities via the dermal route, assessment of dermal penetration is one area in metabolism and toxicology where the use of in vitro methods can be effectively used to predict in vivo results and to screen chemi-

cals. Apparatus and equipment exist that one can use to maintain sections of skin (obtained from euthanized animals or from human cadavers or surgical discard) for such experiments (Holland et al., 1984; Bronaugh, 1998). These apparatus are set up to maintain the metabolic integrity of the skin sample between two reservoirs: the one on the stratum corneum side, called the application reservoir and the one on the subcutaneous side, called the receptor reservoir. One simply places radiolabeled test material in the application reservoir and collects samples at various time points from the receptor fluid.

The rate of penetration can be presented by the traditional kinetic formulas to obtain a penetration rate constant. Given that exposed surface area also plays a role in the amount of material absorbed, the concept of flux is also important.

Determining the quantity of material that is absorbed into the skin and eventually released into the systemic circulation is primarily dependent upon three factors: the surface area exposed, the volume of material applied, and the concentration of the material applied:

Surface Area All things being equal, it is clear that the greater the surface exposed, the higher the achieved internal dose.

Volume The volume of material will obviously play a role in total dose, but it is not as straightforward as the relationship to surface area. Theoretically, the maximum absorption is obtained when the material is spread as thin and uniform as possible; piling material on so that it is literally rolling off the animal serves no practical purpose. In fact, it is not sound practice when dealing with an in vivo animal experiment as it makes it more likely for the material to be available for oral ingestion.

Concentration The higher the concentration in a formulation, the higher the flux achieved of drug molecules across the skin.

Of course, the nature of the vehicle that the drug is being carried in may also have a profound influence on absorption.

Parameters Controlling Absorption The absorption of a chemical into the skin is a function of the nature of the molecule, the behavior of the vehicle, and the status of the skin. Three major variables account for differences in the rate of absorption or flux of different topical chemicals or of the same molecule in different vehicles: the concentration of the molecule in the vehicle, the partition coefficient of chemical between the stratum corneum and the vehicle, and the diffusion coefficient of the molecule in the stratum corneum.

The rate of diffusion is proportional to the concentration of molecule in the vehicle. The relationship is linear only at low molecule concentrations and only applies to the soluble molecule in the vehicle. The latter factor may explain the variable therapeutic effects of different formulations of the same drug molecule. The partition coefficient is a measure of the molecule's ability to escape from the vehicle and is defined as the equilibrium solubility of the

molecule in the surface of the stratum corneum relative to its solubility in the vehicle. Increased lipid solubility favors penetration of the molecule through the skin by increasing the solubility in the relatively lipophilic stratum corneum. The diffusion coefficient indicates the extent to which the matrix of the barrier restricts the mobility of the molecule. Increases in molecular size of the molecule will increase frictional resistance and decrease the diffusion coefficient (Bronaugh, 1998); molecules over 1000 Da usually will not be absorbed easily into normal adult skin.

Finally, intact stratum corneum is an excellent barrier, but in disease states that compromise the skin barrier, the resistance to absorption is rapidly lost and absorption can be facilitated. Such compromised skin can be humanly simulated by using either a dermatome or tape striping the skin site in question.

15.3.2 Distribution

Once the chemical gains access to the body, it is carried by the bloodstream and distributed to the different organs. The preferential organ of deposition is determined by a variety of factors: The two most important are blood flow to the organ and the affinity of the chemical for that organ. Affinity is governed by two general characteristics. First, the product may be designed to have a specific affinity for a specific molecular entity in a target cell. For example, an anticholinesterase insecticide will tend to accumulate in the cells that have the highest concentration of cholinesterase. Second, the product may have a nonspecific or general chemical attraction for a specific cell type. The more highly lipophilic a chemical, the more likely it is to distribute and remain in adipose tissue. Blood flow will also have a major impact on distribution, as chemicals will be distributed more readily to those organs that are more highly perfused. A highly lipophilic chemical may first be deposited in the brain due to the fact that it is richly perfused and then be distributed to body fat with time.

Once a material is absorbed, distribution of a compound in most early toxicology studies is usually of limited interest. This is unfortunate, as it is the preferential distribution of the drug to the therapeutic target that is desired. Some factors which can serve to alter distribution are listed in Table 15.3.

For most drugs, the rate of disposition or loss from the biological system is independent of rate and input once the agent is absorbed. Disposition is defined as what happens to the active molecule after it reaches a site in the blood circulation where concentration measurements can be made (the systemic circulations, generally). Although disposition processes may be independent of input, the inverse is not necessarily true because disposition can markedly affect the extent of availability. Agents absorbed from the stomach and the intestine must first pass through the liver before reaching the general circulation (Figure 15.1). Thus, if a compound is metabolized in the liver or excreted in bile, some of the active molecule absorbed from the GI tract will be inactivated by hepatic processes before it can reach the systemic circulation

TABLE 15.3 Selected Factors That May Affect Chemical Distribution to Various Tissues

Factors relating to chemical and its administration
- Degree of binding of chemical to plasma proteins (i.e., agent affinity for proteins) and tissues
- Chelation to calcium, which is deposited in growing bones and teeth (e.g., tetracyclines in young children)
- Whether chemical distributes evenly throughout body (one-compartment model) or differentially between different compartments (two-or-more-compartment model)
- Ability of chemical to cross blood–brain barrier
- Diffusion of chemical into tissues or organs and degree of binding to receptors that are and are not responsible for drug's beneficial effects
- Quantity of chemical given
- Route of administration/exposure
- Partition coefficients (nonpolar chemicals are distributed more readily to fat tissues than are polar chemicals)
- Interactions with other chemicals that may occupy receptors and prevent drug from attaching to receptor, inhibit active transport, or otherwise interfere with drug's activity
- Molecular weight of chemical

Factors relating to test subject
- Body size
- Fat content (e.g., obesity affects distribution of drugs that are highly soluble in fats)
- Permeability of membranes
- Active transport for chemicals carried across cell membranes by active processes
- Amount of proteins in blood, especially albumin
- Pathology or altered homeostasis that affects any of the other factors (e.g., cardiac failure and renal failure)
- Presence of competitive binding substances (e.g., specific receptor sites in tissues bind drugs)
- pH of blood and body tissues
- pH of urine[a]
- Blood flow to various tissues or organs (e.g., well-perfused organs usually tend to accumulate more chemical than less well perfused organs)

[a]The pH of urine is usually more important than the pH of blood.

and be distributed to its sites of action. If the metabolizing or biliary excreting capacity of the liver is great, the effect on the extent of availability will be substantial. Thus, if the hepatic blood clearance for the chemical is large, relative to hepatic blood flow, the extent of availability for this chemical will be low when it is given by a route that yields first-pass metabolic effects.

Likewise, metabolism is generally of only limited concern in most acute studies. There are some special cases, however, in which metabolic considerations must be factored in seeking to understand differences between routes and the effects which may be seen.

The first special case is parenteral routes, where the systemic circulation presents a peak level of the moiety of interest to the body at one time tempered only by the results of a single pass through the liver.

The second special case arises from inhalation exposures. Because of the arrangements of the circulatory system, inhaled compounds enter the full range of systemic circulation without any "first-pass" metabolism by the liver. Keberle et al. (1971) and O'Reilly (1972) have published reviews of absorption, distribution, and metabolism that are relevant.

Protein Binding The degree to which a drug binds to plasma proteins will highly influence its distribution. Albumin, the most prominent of the many proteins found in mammalian plasma, carries both positive and negative charges with which a polar compound can associate by electrostatic attraction. As with all such reactions it can be described by the following equations. The more avidly bound the material, the less will be distributed to surrounding fluids as part of a solution and only that portion that is free in solution will be available for diffusion into the tissues.

Water Solubility The solubility of a chemical has a direct bearing on its distribution. Recall that only molecules that are in solution will be available for absorption.

As mentioned above, only that portion that is free in solution will be available for diffusion into the tissues. Hence, the more material that is in solution, the more will be available for diffusion.

Volume of Distribution If one takes the dose administered (in milligrams) and divides it by the plasma concentration of the test material (milligrams per milliliter), the result is a volume number:

$$\frac{\text{Dose}}{\text{Concentration}} = \text{volume}$$

One can take this process a step further and extrapolate back from a plasma time curve to the y axis. This is theoretically the plasma concentration (C_0) that would occur if, upon being administered, the material is instantly distributed throughout the body. The volume number obtained with the above equation becomes

$$\frac{\text{Dose}}{C_0} = V_D$$

where V_D represents the apparent volume of distribution, a proportionality constant that reflects the relation of the concentration of a xenobiotic in plasma to the total amount of the entity in the body. Materials that are avidly bound to plasma proteins will have a high volume of distribution, while materials that are avidly taken by the tissues (deposit fat, for example) will have a low one. The V_D is a parameter that is simple to calculate yet gives an important piece of information about the distribution of the chemical under investigation.

TABLE 15.4 Volume and Half-Life of Body Water in Selected Species

Species	Sex	Exchangeable Body Water (% of Body Weight)	Half-Life (Days)
Mouse	F	58.5	1.13
Rat	M	59.6	2.53
Rabbit	F	58.4	3.87
Dog	M	66.0	5.14
Cynomolgus monkey	M	61.6	7.80
Rhesus monkey	M	61.6	7.80
Humans	M, F	55.3	9.46

TABLE 15.5 Typical Organ Weights in Adult Laboratory Animals

	Percent of Body Weight				
Organ	Rat	Mouse	Dog	Rabbit	Monkey
Liver	3.5	6	3.5	3	2.5
Kidney	0.8	1.6	0.5	0.8	0.5
Heart	0.4	0.4	0.8	0.3	0.4
Spleen	0.3	0.5	0.3	0.04	0.1
Brain	0.5	0.6	0.8	0.4	3
Adrenals	0.02	0.01	0.01	0.02	0.03
Lung	0.6	0.6	1	0.6	0.7

The available volumes and masses for distribution vary from species to species, as summarized in Tables 15.4 and 15.5.

15.3.3 Metabolism/Biotransformation

Metabolism describes the process by which chemicals are changed by the body. In fact, very few foreign chemicals that come to enter the body are excreted unchanged. Most are chemically modified. In general, metabolism results in chemicals that are more polar and water soluble and more easily excreted (La Du et al., 1972). Examples of more common metabolic conversions are shown in Table 15.6. In general, the vast majority of lipophilic chemicals are first oxidized via the cytochrome P-450 (CYP)–dependent mixed-function oxidase system of the liver. This is the process classically called phase I metabolism. Cytochrome P-450 exists as a family of isozymes (the CYP gene superfamily) with varying but overlapping substrate affinity and responses to different inducing agents. For a review of the molecular biology of the CYP gene superfamily the reader is referred to Meyer (1994). Induction is the process whereby exposure to a chemical leads to increased activity of the mitochondrial mixed-function oxidase (MMFO) due to an increase in CYP. The isoenzymes induced by a variety of different chemicals are given in Table 15.7, and example compounds which inhibit specific CYPs are presented in

TABLE 15.6 Summary of Prominent Phase I Biotransformation Reactions

Reaction	Enzyme	Location	Example/Comments
Hydrolysis	Carboxylesterase	Ubiquitous	Vinyl acetate to acetate and acetaldehyde
	Peptidase	Blood, lysomes	Amino-, carboxy-, and endopeptidases which cleave peptides at specific amino acid linkages
	Epoxide hydroplase	Microsomes, cytosol	Conversion of styrene 7,8-epoxide to styrene 7,8-glycol
Reductions	Azo and nitro reduction	Gut microflora	Sequential conversion of nitrobenzene to aniline
	Carbonyl reductase	Cytosol	Conversion of haloperidol to reduced haloperidol (secondary alcohol)
	Disulfide reduction	Cytosol	Glutathione-dependent reduction of disulfiram to diethyldithiocarbamate
	Sulfoxide reduction	Cytosol	Thioredoxin-dependent sulindac to sulindac sulfide
	Quinone reduction	Cytosol, microsomes	DT diaphorase reduction of menadione to hydroquinone
	Reductive dehalogenation	Microsomes	Conversion of pentabromoethane to tetrabromoethane (releasing free bromide ion)
Oxidation	Alcohol dehydrogenase	Cytosol	Conversion of ethanol to acetaldehyde [Drosophila alcohol dehydrogenase (DAD)/DADH-dependent reversible reaction]
	Aldehyde dehydrogenase	Mitochondria/ cytosol	Conversion of acetaldehyde to acetate
	Aldehyde oxidase	Liver cytosol	Flavin adenine dinucleotide (FAD)–dependent metalloenzyme, oxidation of benzaldehyde to benzoic acid
	Xanthene oxidase	Cytosol	Oxidation of purine derivative, conversion of allopurinol to alloxanthene
	Monoamine oxidase	Mitochondria	FAD-dependent oxidative deamination of monoamines, e.g., primaquine
	Diamine oxidase	Cytosol	Pyridoxal-dependent, copper-containing enzyme; conversion of allylamine to acrolein
	Prostaglandin oxidase	Microsomes	Cooxidation reaction, can "activate" chemical in tissues low in cytochrome P-450, e.g., nephrotoxicity of acetaminophen, oxidation of phenylbutazone
	Flavin-monooxygenase	Microsomes	FAD-dependent oxidation of nucleophilic nitrogen, sulfur, and phosphorus heteroatoms, e.g., conversion of nicotine to nicotine 1′-*N*-oxide, cimetidine to cimetidine *S*-oxide
	Cytochrome P-450	Microsomes	

TABLE 15.7 Examples of Xenobiotics Metabolized by Human P450

CYP1A1: Benzo[*a*]pyrene and other polycyclic aromatic hydrocarbons
CYP1A2
- Acetaminophen
- 2-Acetylaminofluorene
- 4-Aminobiphenyl
- 2-Aminofluorene
- 2-Naphthylamine
- Amino acid pyrolysis products (DiMeQx, MeIQ, MeIQx, Glu P-1, Glu P-2, IQ, PhIP, Trp P-1, Trp P-2)

CYP2A6
- 6-Aminochursene
- Cyclophosphamide
- Isosphamine
- *N*-Nitrosodiethylamine

CYP2B6
- 6-Aminochrysene
- Cyclophosphamide
- Ifosphamine

CYP2C8, 9, 18 (Note: 2C9 is absent in 15–30% of Asians)
- Talbutamide
- Taxol

CYC2C19
- Diazepam
- Diphenylhydantoin
- Hexaburbitol
- Propanolol

CYP2D6 (Note: absent in 7% of Caucasians): Bufuralol

CYP2E1
- Acetaminophen
- Acrylonitrile
- Benzene
- Carbon tetrachloride
- Chloroform
- Chlorzoxzone
- Dichloromethane
- 1,2-Dichloropropane
- Ethylene dibromide
- Ethylene dichloride
- Ethyl carbamate
- *N*-Nitrosodimethylamine
- Styrene
- Trichloroethylene
- Vinyl chloride

CYP3A4 (50% of all marketed drugs are marketed here)
- Acetaminophen
- Aflatoxin B_1 and G_1
- 6-Aminochrysene
- Benzo[*a*]pyrene 7,8-dihydrodiol
- Cyclophosphamide
- Ifosphamide
- Nidazolam
- Nifedipine
- Testosterone
- 1-Nitropyrene
- Sterigmatocystin
- Senecionine
- Tris(2,3-dibromopropyl) phosphate

CYP4A9/11: None known

Table 15.7. In a practical sense, a drug can induce its own metabolism. Hence, repeated dosing with a chemical may lead to lower blood levels at the end, for example, of a 13-week study than at the beginning. There could also be alterations in the spectrum of metabolites produced such that an agent could become more or less toxic with repeated dosing depending on the nature of the metabolites. It is not unusual during a subchronic or chronic toxicity test for tolerance to occur. There may be signs of toxicity early in the study, but even with continued daily dosing, the signs abate. This phenomenon, particularly in rodents, is frequently due to microsomal induction, whereby the chemical has induced its own metabolism and more rapid clearance of the parent chemical occurs. It should be noted the CYP system is not the only drug-metabolizing system. As Table 15.8 summarizes, there are at least five major metabolic systems in mammals.

TABLE 15.8 A Comparison of the key *in vitro* drug metabolizing experimental systems (liver microsomes), liver postmitochondrial supernatant (S9), liver cytosol (cytosol) and hepatocytes in their contents of the major drug metabolizing enzymes (cytochrome P450 isoforms (P450); monoamine oxidase (MAO); UDP-glucuronsyl transferase (UGT); sulfotransferase (ST); and glutathione-S-transferase (GST)

In Vitro System	P450	MAO	UGT	ST	GST
Microsomes	+	–	+*	–	+**
S9	+	–	+*	+*	+
Cytosol	–	–	–*	+*	+***
Hepatocytes	+	+	+	+	+

*activity of this drug metabolizing enzyme requires the addition of specific cofactors, for instance UDP-glucuronic acid (UDPGA) for UGT activity, and 3′-phosphoadenosine 5′-phosphosulfate (PAPS) for ST activity.
**membrane-bound GST but not the soluble GST are found in the microsomes.
***soluble GST but not membrane-bound GST are found in the cytosol.

After the drug has been metabolically oxidized, it can in fact be further metabolized. In fact, it is possible for the metabolites to also be substrates of the MMFO and to be metabolized themselves.

The route of metabolic activation of the classic carcinogen benzo[*a*]pyrene is due to such a mechanism. The biology of these reactive intermediates has been extensively studied. Glutathione is among the most common organic intracellular chemicals in all mammalian species, being present at a concentration of up to 10 mm and glutathione *S*-transferase is very active. Glutathione is a tripeptide (glutamine–cysteine–glycyne). The sulfhydryl group of cysteine is the business end of the molecule where the reaction with the nucleophilic reactive intermediate takes place. After that, the glutathione conjugate is further metabolized to a cytinyl-acetyl moiety. These moieties are called mercapturic acids and are generally found in the urine. The relative predominance of mercapturic acid over other metabolites may be considered a rough indication of how "reactive" the intermediates may have been. Teleologically, it is tempting to speculate that it is a very well designed protective mechanism. So long as intracellular glutathione concentrations remain above a critical level, the destructive actions of active metabolites can be held in check. Thus, a small dose of a chemical (bromo-benzene is a good example) may cause no liver damage while a large dose may. This is also a good example of one of the aspects of toxicokinetics versus pharmacokinetics where a high dose of a chemical will become toxic due to saturation of a detoxification pathway.

The glutathione *S*-transferase pathway is sometimes in biochemical competition with the epoxide hydratase pathway, in that both deactivate intermediates of the MMFO. Epoxide hydratase is a microsomal enzyme that acts specifically to deactivate epoxide intermediates by the addition of water across the C–O bond to form a diol. As a very broad generality, the glutathione *S*-transferase pathway tends to be more prominent in rodents, while the epoxide hydratase pathway tends to be more dominant in nonrodents.

The hydroxyl- or diol-containing metabolites of the MMFO can be further metabolized by so-called phase II (synthetic) metabolism whereby they are conjugated to/from glucuronides and/or sulfates (so-called etherial sulfates). Amines can also be substrates. The net effect of phase II reactions is to create a more polar molecule that is more readily excretable. While there are species differences, glucuronides are actively transported and excreted in the bile into the GI tract. Sulfates are excreted more predominantly in the urine. Both glucuronides and sulfates, however, can be found in both the urine and the feces. Like the MMFO pathway, glutathione 3-transferase, uridine 5′-diphospho (UDP)-glucuronyl transferase, and eopoxide hydratase are inducible, that is, treatment with exogenous chemicals will increase the amount of enzyme protein present.

Outside of the MMFO-mediated (phase I) reactions there are a few other major reactions that are worthy of note. The two major ones involve ester hydrolysis and alcohol and aldehyde dehydrogenases. All mammalian species have an extensive ability to hydrolyze the ester bond. The products of the reactions then can go on to be further metabolized. In the pharmaceutical industry, this property has been utilized to synthesize prodrugs, that is, chemicals that have desirable pharmaceutical properties (generally increased water solubility) that are not converted to their active moiety until hydrolyzed in the body.

The activity of alcohol dehydrogenase is one with which we should all be familiar. It oxidizes alcohols to aldehydes. The aldehydes produced by this reaction can go on to be further metabolized to a carbocylic acid if they are not sterically hindered. Side-chain constituents of aromatic compounds can also be a substrate for this reaction sequence, producing side-chain carboxylates. The oxidation of alcohols to aldehydes can also be a form of metabolic activation as aldehydes can have potent physiological actions. Fortunately, aldehyde dehydrogenase has a very high activity when compared to alcohol dehydrogenase, so that the aldehydes do not accumulate. Inhibition of aldehyde dehydrogenase by disulfiram (Antabuse) leads to the accumulation of acetaldehyde, causing nausea, dizziness, and flushing. Like disulfiram, some pesticides contain dithiocarbamates and have the potential of causing this type of reaction.

Hopefully, this brief description of the major metabolic pathways has given one some appreciation of the richness of the processes. The different sites of oxidation, the possibility of additional oxidative metabolism of metabolites, and differences in phase II reactions all lead to a multiplicity of possible metabolites. Over 100 different metabolites of the human pharmaceutical chlorpromazine have been isolated and identified. When analyzed by high-performance liquid chromatography (HPLC), for example, the parent chemical and the different (detectable) metabolites will form a pattern of different peaks. This is referred to as the metabolic fingerprint or profile of a chemical. Different species will have different profiles. Ideally, in doing a risk assessment, one would like to know the similarity in this pattern between the animals used

in the toxicology studies and that produced by human beings. This is only infrequently available for most nonpharmaceutical products, as pesticides (for example) are rarely given intentionally to human subjects for the purposes of study. The technology now exists, however, to address this potential problem. Cell lines with human CYP have been developed that can provide some indication of the similarities of human metabolism of a chemical to that of experimental animals. At least they may be able to assist in identifying the major oxidative metabolite. For nonpharmaceutical products, it may be an unusual circumstance that would require one to identify potential human metabolites as part of a marketing application; however, it may be useful for one to know that the technology exists to do so.

The processes of metabolic conversion are frequently involved in the mechanisms of toxicity and carcinogenicity.

Metabolic Activation As mentioned, most nonnutritive chemicals pass through the GI tract by passive absorption and then enter the mesenteric circulation. The venous circulation from the mesentery flows through the portal vein into the liver. The metabolic action of the liver literally sits between the GI tract and the general systemic circulation. Thus, even chemicals that may be highly absorbed from the GI tract could appear only sparingly in systemic circulation if they are highly metabolized by the liver. The combination of absorption from the GI tract and metabolism from the liver leads to what is called the first-pass effect. An extension of this is the fact that the gut flora contain glucuronidases that can cleave glucuronides of chemicals and/or metabolites that are then available to be reabsorbed. This process is called enterohepatic circulation.

Induction of CYP Metabolism and Isoenzymes When organisms are exposed to certain xenobiotics, their ability to metabolize a variety of chemicals is increased. This phenomenon can produce either a transitory reduction in the toxicity of a drug or an increase (if the metabolite is the more toxic species). However, this may not be the case with compounds that require metabolic activation. The exact toxicological outcome of such increased metabolism is dependent on the specific xenobiotic and its specific metabolic pathway. Since the outcome of a xenobiotic exposure can depend on the balance between those reactions that represent detoxification and those that represent activation, increases in metabolic capacity may at times produce unpredictable results.

The ability of different drugs to differentially inhibit and/or induce individual CYP isoenzymes has become critical in assessing the potential safety of drug molecules. Table 15.9 presents a summary overview of some of what we have come to know about differential metabolism by CYP isoenzymes. Draper et al. (1998) have published work on the use of human liver microsomes for determining the levels of activity or inhibition a drug has the formation of 6-testosterone as a model for CYP3A activity (1) and chlorzoxazone

TABLE 15.9 Examples of Xenobiotics Activated by Human Cytochrome P-450 Isoenzymes

CYP1A1	Codeine
Benzo[*a*]pyrene and other polycyclic aromatic hydrocarbons	Timolol
CYP1A2	Metoprolol
Acetaminophen	CYP2E1
2-Acetylaminofluorine	Acetaminophen
4-Aminobiphenyl	Acrylonitrile
2-Aminofluorene	Benzene
2-Naphthylamine	Carbon tetrachloride
CYP2A6	Chloroform
N-Nitrosodiethylamine	Dichloromethane
Butadiene	1,2-Dichloropropane
Coumarin	Ethylene dibromide
CYP2B6	Ethylene dichloride
6-Aminochrysene	Ethyl carbamate
Cyclophosphamide	*N*-Nitrosodimethylamine
Ifosphamine	Styrene
CYP2C8: Taxol	Trichloroethylene
CYP2C9	Vinyl chloride
Diclofenac	CYP3A4
Phenytoin	Acetaminophen
Piroxicam	Aflatoxin B_1 and G_1
Tolbutamide	6-Aminochrysene
CYP2C19	Benzo[*a*]pyrene 7,8-dihydrodiol
Diazepam	Cyclophosphamide
Diphenylhydantoin	Ifosphamide
Hexabarbitol	1-Nitropyrene
Propanolol	Sterigmatocystin
CYP2D6	Senecionine
Buforolol	Tris(2,3-dibromopropyl) phosphate
	CYP4A9/11: None known

Source: Adapted in part from Parkinson, 1996.

for CYP2E1 activity (2). If, for example, a chemical under study competitively inhibits the metabolism of these model substrates in these systems, then it is a substrate for that human isozyme. Using these more recently available in vitro systems, it is much easier to perform cross-species comparisons with regard to biotransformation. It is now easier to determine how similar the routes of metabolism are in the experimental animals with comparison to that in humans without having to administer the chemical to human subjects. Human and animal model microsome preparations may be used as models to identify patterns of metabolites in vitro, allowing for better selection of model species for safety studies, and competition for or inhibition activation of specific isoenzymes can be evaluated to identify potential problems of drug–drug interaction in patients (Levy et al., 2000).

Species Differences Species differences in metabolism are among the principal reasons that there are species differences in toxicity. A difference in CYP

is one of the most common reasons for a difference in metabolism. For example, Monostory et al. (1997) recently published a paper comparing the metabolism of panomifene (a tamoxifen analogue) in four different species. These data serve to address that the rates of metabolism in the nonhuman species was most rapid in the dog and slowest in the mouse. Thus, one should not a priori make any assumptions about which species will have the more rapid metabolism. Of the seven metabolites, only one was produced in all four species. Both the rat and the dog produced the two metabolites (M5 and M6) produced by human microsomes. So how does one decide which species best represents the humans? One needs to consider the chemical structure of the metabolites and the rates at which they are produced. In this particular case, M5 and M6 were relatively minor metabolites in the dog which produced three other metabolites in larger proportion. The rat produced the same metabolites at a higher proportion, with fewer other metabolites than the dog. Thus, in this instance the rat, rather than the dog, was a better model. Likewise, Table 15.10 offers a comparison of excretion patterns between three species for a simple inorganic compound. Table 15.11 presents a summary of interspecies differences between species in drug metabolism and pharmacokinetics.

TABLE 15.10 Differences in Disposition of 2,4-Dichlorophenoxyacetic acid

Species	Sex	Urine	Feces
Rat	M	31.2	2.7
	F	16.5	1.1
Mouse	M	12.7	2.8
	F	26.8	6.7
Hamster	M	4.9	2.5
	F	33.9	14.5

Note: All animals dosed orally with radiolabeled 2.4-D, 200 mg/kg. Results are expressed as percent of 14C dose recovered. Urine was collected for 8 h and feces for 24 h.

TABLE 15.11 "General Rules" on interspecies differences in DMPK

Species	DMPK Characteristics
Human	Polymorphisms (e.g., CYP 2C9, CYP 2C19, CYP 2D6, NAT1, NAT2)
Dog	Low acetylation, high capacity for deacetylation
	Different absorption due to higher pH in gastro-intestinal tract than in humans (consider use of synthetic gastric fluid to mimic human situation)
Rat	Often gender differences which are not observed in other species
	Abundant tetrahydrofolate (protects, e.g., against methanol ocular damage)
Rabbit	Low sulfation
(Mini)Pig	Low sulfation
	Gastro-intestinal conditions similar to humans
Cat	Low glucuronidation
	High sulfation

More thorough reviews on species differences in pharmacokinetics have been presented by Smith (1991), Gad and Chengelis (1997), and Gad (2007a).

Sex-Related Differences in Rodents Not only are there differences in absorption, distribution, biotransformation, and metabolism between species, there may also be differences between sexes within a species (Mugfor and Kidderis, 1998). Griffin et al. (1997), for example, has demonstrated sex-related differences in the metabolism of 2,4-dichlorophenoxyacetic acid (2,4-D). They noted that while there were differences between sexes they tended to be quantitative (rates), not qualitative (metabolites). Differences between species were greater than sex-related differences. With regard to sex-related differences, it is noteworthy that males do not always have the higher rates, as Griffen et al. have shown; in hamsters, the female metabolizes 2,4-D more rapidly than males. In general, male rats tend to have higher activity than female rats, especially with regard to CYP-dependent activity.

In the case of 2,4-D, the only urinary metabolite is 2,4-D glucuronide, but the half-life of 2,4-D was 138 min in males and 382 min in females.

15.3.4 Excretion

Excretion encompasses the process by which chemicals or their metabolites are transported out of the body. There are three possible major routes of excretion and a handful of minor ones. The major routes of excretion for chemicals, in particular their metabolites, are as follows:

Urine The kidneys filter the entire cardiac output multiple times each day and thus provide a large opportunity for the removal of chemicals from the bloodstream. How much of a xenobiotic is actually excreted is dependent on three factors or processes:

1. The glomerular membrane has pores of 70–80 Å; under the positive hydrostatic conditions in the glomerulus, all molecules smaller than about 20,000 Da are filtered. Proteins and protein-bound compounds thus remain in the plasma, and about 20% of the nonbound entity is carried with 20% of the plasma water into the glomerular filtrate.
2. Because the glomerular filtrate contains many important body constituents (e.g., glucose), there are specific active uptake processes for them. Also, lipid-soluble chemicals diffuse back from the tubule into the blood, especially as the urine becomes more concentrated because of water reabsorption. The pH of the urine is generally lower than that of the plasma, and therefore pH partitioning tends to increase the reabsorption of weak acids. The pH of the urine can be altered appreciably by treatment with ammonium chloride (decreases pH) or sodium carbonate (increases pH); the buffered plasma shows little change.

3. Xenobiotics may be secreted actively into the renal tubule against a concentration gradient by anion and cation carrier processes. These processes are saturable and of relatively low specificity; many basic or acidic compounds and their metabolites (especially conjugation products) are removed by them. Because the dissociation rate for the chemical–albumin complex is rapid, it is possible for highly protein-bound compounds to be almost completely cleared at a single passage through the kidney.

Feces The most important mechanism allowing circulating foreign compounds to enter the gut is in the bile. The biological aspects of this mechanism have been reviewed, and certain pertinent points have emerged. The bile may be regarded as a complementary pathway to the urine, with small molecules being eliminated by the kidney and large molecules in the bile. Thus the bile becomes the principal excretory route for many drug conjugates. Species differences exist in the molecular weight requirement for significant biliary excretion, which has been estimated as 325 ± 50 in the rat, 440 ± 50 in the guinea pig, and 475 ± 50 in the rabbit. In the rat, small molecules (less than 350 Da) are not eliminated in the bile or large molecules (more than 450 Da) in the urine, even if the principal excretory mechanism is blocked by ligation of the renal pedicles or bile duct, respectively. Compounds of intermediate molecular weight (350–450 Da) are excreted by both routes, and ligation of one pathway results in increased use of the other.

Foreign compounds may also enter the gut by direct diffusion or secretion across the gut wall, elimination in the saliva, pH partitioning of bases into the low pH of the stomach, and elimination in the pancreatic juice.

Expired Air Volatile compounds or metabolites can be extensively excreted by passage across pulmonary membranes into the airspace of the lungs, then expulsion from the lungs in expired air.

Minor routes for excretion can include tears, saliva, sweat, exfoliated keratinocytes, hair, and nasal discharge. These are of concern or significance only in rare cases. Accordingly, quantitation of excretion typically requires collection of urine and feces (and occasionally expired air) over a period of time.

15.3.5 Pharmacokinetics

The interplay of the processes of ADME result in changes in concentration of the test chemical in different organs with time (Shargel and Yu, 1999; Renwick, 2000). With regard to the practical concerns of monitoring human exposure, the organ of interest is the blood. Blood is generally considered the central compartment. Determining the concentration of the chemical in plasma gives one an assessment of exposure. Mathematical formulas are used to quantitatively describe this exposure (Bauer, 2001).

Physiologically Based Pharmacokinetic Modeling Pharmacokinetic parameters are descriptive in nature. They quantitatively describe the manner in which a test material is absorbed and excreted such that a specific blood or tissue level is achieved or maintained. In the past, experiments had to be done by every route of administration to gather the data appropriate for describing the pharmacokinetic behavior of a chemical administered by different routes. The development of more sophisticated and readily accessible computers has led to the development of a different approach, that of pharmacokinetic modeling (Connally and Anderson, 1991). In this computerized model, different compartments are represented as shown in boxes and the movement of the material in and out of the compartments is defined by the rate constants. These can be determined either in vivo or in vitro. Other physiological parameters are brought into play as well, such as octanol–water partition coefficient, blood flow through an organ, respiration rate (for the inhalation route of exposure), rate of microsomal metabolism, and so on.

15.4 LABORATORY METHODS

The actual means by which pharmacokinetic information is collected is through the conduct of one or more specific studies employing a wide range of available analytical techniques. Administered therapeutic molecules can be identified and quantified in relevant samples collected in accordance with carefully designed and executed protocols.

15.4.1 Analytical Methods

There are three broad categories of analytical techniques now available—instrumental (cold chemical), radiolabeled, and immunological. Each of these have advantages and disadvantages. Only an overview of these techniques will be given here—detailed explanations are beyond the scope of this text. These methodologies are all directed at being able to identify and/or quantify a chemical (and/or its metabolites) in various biological matrices.

Instrumental Methods These bioanalytical methods are also sometimes called cold-chemistry methods. These generally start from a place of isolating the compound or compounds of interest, for which the work horse methodology is HPLC. A wide variety of specialized columns are used to achieve desired separation. At the end of the column, where separation of molecular entities has been achieved, the outflow of the column can be directed to any of a wide variety of detection instruments, including various forms of detectors intrinsic to the HPLC. In general, all of the cold-chemistry methodologies have less sensitivity (higher detection limits) than do radiochemical or immunological methods (Caldwell et al., 1994).

Mass spectrometry (MS); nuclear magnetic resonance (NMR); electron skin resonance (ESR); ultraviolet, infrared, and visible spectrophotometry; and mass spectroscopy are all well-established detection methodologies.

Radiochemical Methods The massive expansion of our understanding of toxicokinetics since the late 1970s is to a large degree a reflection of the wide use of radioactive isotopes as tracers of chemical and biological processes. Appropriately radiolabeled test compounds are commonly used in toxicokinetic studies, providing a simple means of following the administered dose in the body. This is particularly important when specific analytical methods are unavailable or too insensitive. The use of total radioactivity measurements allows an estimation of the total exposure to drug-related material and facilitates the achievement of material balance.

The most commonly used radionuclides in drug metabolism and disposition studies are carbon-14 (^{14}C) and tritium (^{3}H), both of which are referred to as beta emitters. Since these beta-emitting isotopes have relatively long half-lives, their radioactive decay during an experiment is insignificant. Additionally, they provide sufficient emission energy for measurement and are relatively safe to use, as indicated by the data in Table 15.12. Although individual beta particles can have any energy up to the maximum, E_{max}, the basic quantity in determining the energy imparted to tissues by beta emitters is the average energy E_{β}. The range is the maximum thickness the beta particles can penetrate. Beta particles present virtually no hazard when they originate outside the body (Shapiro, 1981).

During the synthesis of radiolabeled compounds, the label is usually introduced as part of the molecular skeleton in a metabolically stable and, with tritium, nonexchangeable position. The in vivo stability of ^{14}C labels is often reflected by the extent of [^{14}C] carbon dioxide formation. The biological stability of ^{3}H labels can be estimated by the extent of tritiated water formation. The tritiated water concentration (dpm mL^{-1}) in urine samples collected during a designated time interval after dosing, assumedly after equilibrium is reached between urine and the body water pool, is determined. This value is extrapolated from the midpoint of the collection interval to zero time based on the known half-life of tritiated water in the given species. The percentage of the radioactive dose that is transformed to tritiated water (% $^{3}H_2O$) can be calculated using the equation

TABLE 15.12 Properties of Primary Radioisotopes Employed in Pharmacokinetics

Property	^{3}H	^{51}Cr	^{14}C	^{125}I
Half-life	12.3 years	27.8 days	5730 years	13 days
Maximum beta energy (MeV)	0.0186	0.752	0.156	2.150
Average beta energy (MeV)	0.006	0.049		
Range in air (mm)	6	300		
Range in unit density material (mm)	0.0052	0.29		

$$\text{Percent } {}^3H_2O = \frac{{}^3H_2O \text{ concentration at zero time} \times \text{exchangeable body water volume}}{\text{radioactivity dose}} \times 100\%$$

Values for the exchangeable body water content as well as the half-life of tritiated water in some mammalian species that can be applied to the above equation were shown earlier in Table 15.3. If the molecule is likely to or is known to fragment into two major portions, it may be desirable to monitor both fragments by differential labeling (^3H and ^{14}C).

The chemical and radiochemical purity of the labeled compound must be ascertained prior to use. In practice a value of 95% or greater is usually acceptable. The desired specific activity of the administered radioactive compound depends on the dose to be used as well as the species studied. Doses of ^{14}C on the order of $5\,\mu Ci\,kg^{-1}$ for the dog and $20\,\mu Ci\,kg^{-1}$ for the rat have been found adequate in most studies, while doses of ^3H are usually two to three times higher owing to lower counting efficiency of this isotope.

Liquid scintillation counting is the most popular technique for the detection and measurement of radioactivity. In order to count a liquid specimen such as plasma, urine, or digested blood or tissues directly in a liquid scintillation spectrometer, an aliquot of the specimen is first mixed with a liquid scintillant. Aliquots of blood, feces, or tissue homogenates are air-dried and ash-free filter papers and combusted in a sample oxidizer provided with an appropriate absorption medium and a liquid scintillant prior to counting. The liquid scintillant plays the role of an energy transducer, converting energy from nuclear decay into light. The light generates electrical signal pulses which are analyzed according to their timing and amplitude and are subsequently recorded as a count rate, for example, counts per minute (cpm). Based on the counting efficiency of the radionuclide used, the count rate is then converted to the rate of disintegration, for example, disintegrations per minute (dpm), which is a representation of the amount of radioactivity present in the sample.

Immunoassay Methods Radioimmunoassay (RIA) allows measurement of biologically active materials which are not detectable by traditional cold-chemistry techniques. RIAs can be used to measure molecules that cannot be radiolabeled to detectable levels in vivo. They also are used for molecules unable to fix complement when bound to antibodies or they can be used to identify cross-reacting antigens that compete and bind with the antibody.

Competitive inhibition of radiolabeled hormone antibody binding by unlabeled hormone (either as a standard or an unknown mixture) is the principle of most RIAs. A standard curve for measuring antigen (hormone) binding to antibody is constructed by placing known amounts of radiolabeled antigen and the antibody into a set of test tubes. Varying amounts of unlabeled antigen are added to the test tubes. Antigen–antibody complexes are separated from the antigen and the amount of radioactivity from each sample is measured to detect how much unlabeled antigen is bound to the antibody. Smaller amounts

of radiolabeled antigen–antibody complexes are present in the fractions containing higher amounts of unlabeled antigen. A standard curve must be constructed to correlate the percentage of radiolabeled antigen bound with the concentration of unlabeled antigen present.

Two methods are commonly employed in RIAs to separate antigen–antibody complexes. The first, the double-antibody technique, precipitates antigen–antibody complexes out of solution by utilizing a second antibody, which binds to the first antibody. The second most commonly used method is the dextran-coated activated charcoal technique. Addition of dextran-coated activated charcoal to the sample followed immediately by centrifugation absorbs free antigen and leaves antigen–antibody complexes in the supernatant fraction. This technique works best when the molecular weight of the antigen is 30 kDa or less. Also, sufficient carrier protein must be present to prevent adsorption of unbound antibody.

Once a standard curve has been constructed, the RIA can determine the concentration of hormone in a sample (usually plasma or urine). The values of hormone levels are usually accurate using the RIA, but certain factors (e.g., pH or ionic strength) can affect antigen binding to the antibody. Thus similar conditions must be used for the standard and the sample.

Problems of RIAs include lack of specificity. This problem is usually due to nonspecific cross-reactivity of the antibody. RIA represents an analytical approach of great sensitivity. Unlike assays that often require large amounts of tissue (or blood), the greater sensitivity of the RIAs or monoclonal antibody techniques can be achieved using small samples of biological fluids. Some of these RIA methodologies are more useful than others and to some extent depend on the degree of hormonal cross-reactions or, in the case of monoclonal antibody methods, their degree of sensitivity.

Enzyme-linked immunosorbent assay (ELISA) is comparable to the immunoradiometric assay except that an enzyme tag is attached to the antibody instead of a radioactive label. ELISAs have the advantage of no radioactive materials and produce an end product that can be assessed with a spectrophotometer. The molecule of interest is bound to the enzyme-labeled antibody, and the excess antibody is removed for immunoradiometric assays. After excess antibody has been removed or the second antibody containing the enzyme has been added (two-site assay), the substrate and cofactors necessary are added in order to visualize and record enzyme activity. The level of molecule of interest present is directly related to the level of enzymatic activity. The sensitivity of the ELISAs can be enhanced by increasing the incubation time for producing substrate.

Immunoradiometric assays (IRMAs) are like RIAs in that a radiolabeled substance is used in an antibody–antigen reaction, except that the radioactive label is attached to the antibody instead of the hormone. Furthermore, excess of antibody, rather than limited quantity, is present in the assay. All the unknown antigen becomes bound in an IRMA rather than just a portion, as in a RIA; IRMAs are more sensitive. In the one-site assay, the excess antibody

that is not bound to the sample is removed by addition of a precipitating binder. In a two-site assay, a molecule with at least two antibody-binding sites is adsorbed onto a solid phase, to which one of the antibodies is attached. After binding to this antibody is completed, a second antibody labeled with ^{125}I is added to the assay. This antibody reacts with the second antibody-binding site to form a "sandwich" composed of antibody-hormone-labeled antibody. The amount of hormone present is proportional to the amount of radioactivity measured in the assay.

With enzyme-multiplied immunoassay technique (EMIT) assays, enzyme tags are used instead of radiolabels. The antibody binding alters the enzyme characteristics, allowing for measurement of target molecules without separating the bound and free components (i.e., homogeneous assay). The enzyme is attached to the molecule being tested. This enzyme-labeled antigen is incubated with the sample and with antibody to the molecule. Binding of the antibody to the enzyme-linked molecule either physically blocks the active site of the enzyme or changes the protein conformation so that the enzyme is no longer active. After antibody binding occurs, the enzyme substrate and cofactor are added, and enzyme activity is measured. If the sample contains subject molecules, it will compete with enzyme-linked molecules for antibody binding, enzyme will not be blocked by antibody, and more enzyme activity will be measurable.

Most protein drug entities can now be assessed using monoclonal antibody (MAb) techniques. It is possible to produce antisera containing a variety of polyclonal antibodies that recognize and bind many parts of the molecule. Polyclonal antisera can create some nonspecificity problems such as cross-reactivity and variation in binding affinity. Therefore it is oftentimes desirable to produce a group of antibodies that selectively bind to a specific region of the molecule (i.e., antigenic determinant). In the past, investigators produced antisera to antigenic determinants of the molecule by cleaving the molecule and immunizing an animal with the fragment of the hormone containing the antigenic determinant of interest. This approach solved some problems with cross-reactivity of antisera with other similar antigenic determinants, but problems were still associated with the heterogeneous collection of antibodies found in polyclonal antisera.

The production of MAbs offers investigators a homogenous collection of antibodies that could bind selectively to a specific antigenic determinant with the same affinity. In addition to protein isolation and diagnostic techniques, MAbs have contributed greatly to RIAs.

While MAbs offer a highly sensitive, specific method for detecting antigen, sometimes increasing MAb specificity compromises affinity of the antibody for the antigen. In addition, there is usually decreased complement fixation, and costs are usually high for preparing and maintaining hybridomas that produce MAbs (Table 15.13).

The monoclonal antibody techniques provide a means of producing a specific antibody for binding antigen. This technique is useful for studying protein

TABLE 15.13 Advantages and Disadvantages of Monoclonal Antibodies Compared to Polyclonal Antisera

Advantages	Disadvantages
Sensitivity	Overly specific
Quantities available	Decreased affinity
Immunologically defined	Diminished complement fixation
Detection of neoantigens on cell membrane	Labor intensive; high cost

structure relations (or alterations) and has been used for devising specific RIAs.

Metabolism and Elimination Biologics are usually not excreted unchanged in urine. They are degraded to small peptides and individual amino acids with pathways equally and generally understood for endogenous compounds. Their metabolites (amino acids) are reutilized in the endogenous amino acid pool for the de novo biosynthesis of structural or functional body proteins.

The metabolism of biologics is highly dependent on structure (including sugars), charge (density and distribution), size, and hydrophilicity/lipophilicity. Sites of metabolism of biologics are the liver, the kidneys, and the blood and the extravascular sites of administration. In the liver, hepatocytes are mainly responsible for the catabolism of biologics using carrier-mediated membrane transport as well as endocytosis/pinocytosis for transport process. Kidneys play a major role in the catabolism of many small polypeptides. After being filtered by the glomeruli, some proteins are reabsorbed by the proximal tubule by endocytosis, while small amino acid chains are hydrolyzed at the brush border. Controversy exists surrounding glomerular filtration selectivity regarding size, molecular conformation, and charge of the protein (Tang et al., 2004). The often-observed incomplete bioavailability of biologics after extravascular injection can be attributed to local metabolism. Catabolism at extravascular sites has been observed, for example, for insulin, calcitonin, and interferon-β (for review see, Mohler et al., 1992).

Several biologics, especially antibodies, show high interindividual variation of pharmacokinetics parameters, which is mainly clearance driven. It is now clear that MAbs which target cellular antigens have far more complex, non-linear pharmacokinetics such that the half-life of these drugs can be both dose and time dependent (Tobo et al., 2004). When antigen concentration is high, half-life is short because the MAb is rapidly cleared from the blood through antigen–MAb interaction. As the antigen is depleted, clearance decreases and half-life is consequently prolonged. As the MAb accumulates, a new steady state is reached. Eventually, the target is totally depleted, at which time the clearance of MAb will be at its slowest. At this point, half-life will be at its longest, approaching the half-life of endogenous immunoglobulin G (IgG)

(~21 days). More probable than total target depletion is saturation of the target–MAb binding with similar consequences.

Due to catabolism of proteins to (mostly) endogenous amino acids, classical biotransformation studies for small molecules are not needed. Additionally, limitations of current analytical methods to detect and distinguish metabolites and the putative lack of pharmacological or toxicological activity of the metabolites remain obstacles. Similarly, mass balance studies usually used to determine the excretion pathways of small molecules (and their metabolites) are not used for biologics.

The majority of therapeutic biologics, especially after chronic administration, elicit an immune response in test animal species and often also in humans. This is an inherent property when administering nonhuman sequence proteins of sufficient size. Initial success in reducing immunogenicity has been achieved by replacing biologics obtained from nonhuman sources with human sequences. Antibody formation can also occur in immunocompetent recipients after treatment with products derived from human sera and tissues and also with recombinant human proteins that are identical or nearly identical in sequence to native human proteins. The mechanism for generation of antibodies of recombinant human proteins is not well understood. In most cases, the underlying mechanism is the breaking of immune tolerance that typically exists in self-antigens. Other reasons for immunogenicity relate to manufacturing, formulation, and storage (e.g., aggregates). These are especially addressed when modifications of these processes are performed which might influence the physicochemical properties of the product. An immune response to a product does not mean it cannot be developed. However, the development and use of a product may be complicated and, in rare cases, also impossible. In certain cases, some patients develop antibodies which neutralize the biological activity of the therapeutic product and become unresponsive to treatment. Alterations in the pharmacokinetic profile due to immune-mediated clearance mechanisms may affect the pharmacokineticprofiles and the interpretation of the preclinical toxicity data. Last but not least, safety issues like immunomediated toxicity may be raised. Detection and characterization of the immune response in patients are expected by the authorities [U.S. Food and Drug Administration (FDA) (2002)].

The development and usefulness of appropriate animal models for testing immunogenicity are still unclear. Conventional animal models have poorly predicted immunogenicity problems in humans. One limitation of traditional animal models is that tolerance, a key aspect of the immune response, is highly species specific. However, characterization of the immune response in research and preclinical development is necessary to get a valid interpretation of the preclinical efficacy and safety data.

Not only the compound structure itself but also the administration route may affect immunogenicity. Extravascular injection is known to stimulate antibody formation more than IV application. This is most likely due to

increased immunogenicity of protein aggregates and precipitates formed at the injection site.

To lower the systemic clearance and increase elimination half-lives, several strategies have been developed, including polyethylene glycol (PEG) attachment (PEGylation), glycosylation, or fusion to proteins with decreasing clearance and prolonged serum half-lives. PEGylation improves the pharmacokinetic behaviors by increasing the effective size of the protein, with most significant effects for proteins smaller than 70 kDa. However, conjugation of the protein may also alter receptor affinity and biodistribution, changing the concentration–response profile for the protein independent of effects on pharmacokinetics. PEGylation can also reduce immunogenicity and aggregation.

15.4.2 Sampling Methods and Intervals

1. *Blood* Since blood (plasma and serum) is the most easily accessible body compartment, the blood concentration profile is most commonly used to describe the time course of drug disposition in the animal. With the development of sensitive analytical methods that require small volumes (100–200 μL) of blood, ADME data from individual rats can be obtained by serial sample collection. Numerous cannulation techniques have been utilized to facilitate repeated blood collection, but the animal preparation procedures are elaborate and tedious and are incompatible with prolonged sampling periods in studies involving a large number of animals. In contrast, noncannulation methods such as collection from the tail vein, orbital sinus, or jugular vein are most practical. Significant volumes of blood can be obtained from the intact rat by cardiac puncture, although this method can cause shock to the animal system and subsequent death.

Blood collection from the tail vein is a simple and rapid, nonsurgical method which does not require anesthesia. A relatively large number of serial samples can be obtained within a short period of time. However, this method is limited to relatively small sample volumes (~250 μL per sample). Although larger volumes can be obtained by placing the rat in a warming chamber, this procedure could significantly influence the disposition of the test compound and therefore is not recommended for routine studies. Blood collected from the cut tail has been shown to provide valid concentration data for numerous compounds.

The rat is placed in a suitable restrainer with the tail hanging freely. The tail is immersed in a beaker of warm water (37–40 °C) for 1–2 min to increase the blood flow. Using surgical scissors or a scalpel, the tail is completely transected approximately 5 mm above the tip. The tail is then gently "milked" by sliding the fingers down the tail from its base. It should be noted that excessive "milking" could cause damage to the blood capillaries or increase the white cell count in the blood. A heparinized micropipette of desired capacity (25–

250 μL) is held at a 30°–45° downward angle in contact with the cut end of the tail. This allows blood to fill the micropipette by capillary action. Application of gentle pressure with a gauze pad for approximately 15 s is sufficient to stop bleeding. A sufficient number of serial blood samples may be obtained to adequately describe the blood level profile of a compound.

If plasma is required, the blood may be centrifuged after sealing one end of the filled micropipette and placing it in a padded centrifuge tube. The volume of plasma is determined by measuring the length of plasma as a fraction of the length of the micropipette multiplied by the total capacity of the pipette. The tube is then broken at the plasma–red blood cell interface and the sample is expelled using a small bulb. If serum is needed, the blood should be collected without using anticoagulants in the sampling tube.

Serial blood samples can also be collected from the orbital sinus, permitting rapid collection of larger (1–3-mL) samples.

2. *Excreta* Excretion samples commonly collected from the rat include urine, feces, bile, and expired air. By using properly designed cages and techniques, the samples can be completely collected so that the mass balance is readily determined. These samples also serve to elucidate the biotransformation characteristics of the compound.

These samples can be easily collected through the use of suitable metabolism cages. Since rodents are coprophagic, the cage must be designed to prevent the animal from ingesting the feces as it is passed. Other main features of the cage should include the ability to effectively separate urine from feces with minimal cross-contamination, a feed-and-water system that prevents spillage and subsequent contamination of collected samples, and collection containers that can be easily removed without disturbing the animal. Also, the cage should be designed so that it can be easily disassembled for cleaning or autoclaving.

Following dose administration, rats are placed in individual cages. The urine and feces that collect in containers are removed at predetermined intervals. The volume of urine and the weight of feces are measured. After the final collection, the cage is rinsed, normally with ethanol or water, to assure complete recovery of excreta. If the rats are also used for serial blood sampling, it is important that bleeding be performed inside the cage to avoid possible loss of urine or feces.

3. *Bile* The bile is the pathway through which an absorbed compound is excreted in the feces. In order to collect this sample, surgical manipulation of the animal is necessary (Wang and Reuning, 1994).

4. *Expired Air* For ^{14}C-labeled chemicals, the tracer carbon may be incorporated in vivo into carbon dioxide, a possible metabolic product. Therefore, when the position of the radiolabel indicates the potential for biological instability, a pilot study to collect expired air and monitor its radioactivity content should be conducted prior to initiating a full-scale study. Expired air studies should also be performed in situations where the radiolabel has been

postulated to be stable but analyses of urine and feces from the toxicokinetic study fail to yield complete recovery (mass balance) of the dose.

Following drug administration, the rat is placed in a special metabolism cage. Using a vacuum pump, a constant flow of room air (~500 mL min^{-1}) is drawn through a drying column containing anhydrous calcium sulfate impregnated with a moisture indicator (cobalt chloride) and passed into a second column containing Ascarite II, where it is rendered carbon dioxide free. The air is then drawn in through the top of the metabolism cage. Exhaled breath exiting the metabolism cage is passed through a carbon dioxide adsorption tower, where the expired $^{14}CO_2$ is trapped in a solution such as a mixture of 2-ethoxyethanol and 2-aminoethanol (2:1). The trapping solution is collected, replaced with fresh solution, and assayed at designated times postdose so that the total amount of radioactivity expired as labeled carbon dioxide can be determined.

5. *Milk* The study of passage of a xenobiotic into milk serves to assess the potential risk to breast-fed infants in the absence of human data. The passage into milk can be estimated as the milk–plasma ratio of drug concentrations at each sampling time or that of the area under the curve (AUC). Approximately 30 rats in their first lactation are used. The litter size is adjusted to about 10 within 1–2 days following parturition. The test compound is administered to the mothers 8–10 days after parturition. The rats are then divided into groups for milk and blood collection at designated times postdose. All sucklings are removed from the mother rats several hours before milking. Oxytocin, 1 IU per rat, is given intramuscularly 10–15 min before each collection of milk to stimulate milk ejection. The usual yield of milk is about 1 mL from each rat. Blood is obtained immediately after milking. In order to minimize the number of animals used, the sucklings can be returned to the mother rat, which can then be milked again 8–12 h later.

In all the fluid-sampling techniques above, the limitations of availability should be kept in mind. Table 15.14 presents a summary of such availability for the principal model species.

For topical exposures, determining absorption (into the skin and into the systemic circulation) requires a different set of techniques. For determining how much material is left, skin washing is required. There are two components

TABLE 15.14 Approximate Volumes of Pertinent Biological Fluids in Adult Laboratory Animals

Fluid	Rat	Mouse	Dog	Rabbit	Monkey
Blood (mL kg^{-1})	75	75	70	60	75
Plasma (mL kg^{-1})	40	45	40	30	45
Urine (mL kg^{-1} day^{-1})	60	50	30	60	75
Bile (mL kg^{-1} day^{-1})	90	100	12	120	25

to skin washing in the recovery of chemicals. The first component is the physical rubbing and removal from the skin surface. The second component is the surfactant action of soap and water. However, the addition of soap affects the partitioning. Some compounds may require multiple successive washing with soap and water applications for removal from skin.

Skin tape stripping can be used to determine the concentration of drug in the stratum corneum at the end of a short application period (30 min) and by linear extrapolation predicts the percutaneous absorption of that chemical for longer application periods. The chemical is applied to the skin of animals or humans, and after a 30-min skin contact application time, the stratum corneum is blotted and then removed by successive tape applications. The tape strippings are assayed for chemical content. There is a linear relationship between this stratum corneum reservoir content and percutaneous absorption. The major advantages of this method are (1) the elimination of urinary and fecal excretion to determine absorption and (2) the applicability to nonradiolabeled determination of percutaneous absorption because the skin strippings contain adequate chemical concentrations for nonlabeled assay methodology.

Finally, a complete determination of the distribution and potential departing of a chemical and its metabolites requires some form of measurement or sampling of tissues/organs. Autoradiography provides a nonquantitative means of doing such, but quantitation requires actual collection and sampling of tissues. Table 15.7 provided guidance as to the relative percentage of total body mass that the organs constitute in the common model species.

Sampling Interval To be able to perform valid toxicokinetic analysis, it is necessary to properly collect not only samples of appropriate biological fluids but also a sufficient number of samples at the current intervals. Both of these variables are determined by the nature of the answers sought. Useful parameters in toxicokinetic studies are C_{max}, which is the peak plasma test compound concentration; T_{max}, which is the time at which the peak plasma test compound concentration occurs; C_{min}, which is the plasma test compound concentration immediately before the next dose is administered; AUC, which is the area under the plasma test compound concentration–time curve during a dosage interval; and $t½$, which is the half-life for the decline of test compound concentrations in plasma. The samples required to obtain these parameters are shown in Table 15.15. The C_{min} requires one blood sample immediately before a dose is given and provides information on accumulation. If there is no accumulation in plasma, the test compound may not be detected in this sample.

Several C_{min} samples are required at intervals during the toxicity study to check whether accumulation is occurring. Assume C_T is a blood sample taken at a chosen time after dosing and provides proof of absorption as required by the good labor practice (GLP) regulations, but little else. In addition, C_{max} requires several blood samples to be taken for its accurate definition, as does

TABLE 15.15 Blood Samples Required so That Certain Toxicokinetic Parameters Can be Obtained and Calculated

Parameter	Blood Sample Required	Information Obtained
C_{min} (C_{24})	24 h	Accumulation
CT	T hours	Proof of absorption
C_{max} (C peak)	Several[a]	Rate of absorption
T_{max} (T peak)	Several[a]	Rate of absorption
AUC	Several[a]	Extent of absorption
$t_{½}$	Several[a]	Various
Accumulation ratio	Several after first and repeated doses	Extent of accumulation

[a]Several samples to define concentration–time profile.

T_{max}: these two parameters provide information on rate of absorption. The AUC also requires several blood samples to be taken so that it can be calculated: It provides information on extent of absorption. The half-life t½ requires several samples to be taken during the terminal decline phase of the test compound concentration–time curve: This parameter provides information on various aspects, such as change in the kinetics of the test compound during repeated doses or at different dose levels. Depending on the other parameters obtained, the accumulation ratio can be calculated from C_{min}, C_{max}, and/or AUC when these are available after the first dose and after several doses to steady state.

Operational and metabolic considerations generally make urine sampling and assay of limited value for toxicokinetic purposes.

Study Type Metabolic and pharmacokinetic data from a rodent species and a nonrodent species (usually the dog) used for repeat-dose safety assessments (14, 28, or 90 days or 6 months) are recommended. If a dose dependency is observed in metabolic and pharmacokinetic or toxicity studies with one species, the same range of doses should be used in metabolic and pharmacokinetic studies with other species. If human metabolism and pharmacokinetic data also are available, this information should be used to help select test species for the full range of toxicity tests and may help to justify using data from a particular species as a human surrogate in safety assessment and risk assessment.

Metabolism and pharmacokinetic studies have greater relevance when conducted in both sexes of young adult animals of the same species and strain used for other toxicity tests with the test substance. The number of animals used in metabolism and pharmacokinetic studies would be sufficient to reliably estimate population variability. This usually means a separate (but parallel) set of groups of animals in rodent studies. A single set of IV and oral dosing results from adult animals, when combined with some in vitro kinetic results, may provide an adequate data set for the design and interpretation of short-term, subchronic and chronic toxicity studies.

Studies in multiple species may clarify what appear to be contradictory findings in toxicity studies (i.e., equal milligram-per-kilogram body weight doses having less effect in one species that in another). If disposition and metabolite profiles are found to be similar, then differences in responses among species could more reliably be attributed to factors other than differences in metabolism. Studies of the pharmacokinetics and metabolism of a substance in neonatal and adolescent animals provide information about any changes in metabolism associated with tissue differentiation and development. Animals with fetuses of known gestational age should be used for determining the disposition of the test substance in the fetus. Dosage is by (to the maximum extent possible) the intended clinical route.

An acute IV study can provide accurate rates of metabolism—without interference from intestinal flora—plus rates of renal and biliary elimination if urine and bile are collected. This route also avoids the variability in delivered dose associated with oral absorption and ensures that the maximum amount of radiolabel is excreted in the urine or bile for purposes of detection. Once IV data and parameters are available, they can be used with plasma concentrations from limited oral studies to compute intestinal absorption via the ratio of areas under the (plasma and/or urine) curves or via simulations of absorption with GI absorption models.

In single-dose pharmacokinetic studies of oral absorption, the primary concerns are with the extent of absorption and peak plasma or target tissue concentrations of the test substance. If the test vehicle affects gastric emptying, it may be necessary to use both fasted and nonfasted animals for pharmacokinetic studies.

Blood [red blood cells (RBCs), plasma, and serum], urine, and feces are the most commonly collected samples. In addition, a few representative organ and tissue samples should be taken, such as liver, kidney, fat, and suspected target organs. Sampling times should depend on the substance being tested and the route of administration. In general, an equal number of blood samples should be taken in each phase of the concentration-versus-time curve. Intravenous studies usually require much shorter and more frequent sampling than is required for oral dosing. Time spacing of samples will depend on the rates of uptake and elimination. In a typical IV study, blood and tissue samples are taken in a "powers of 2" series, that is, samples at 2, 4, 8, 16, and 30 min and 1, 2, 4, 8, and 16 h. Similar coverage could be obtained with only seven time points by using a "powers of 3" series: 3, 9, and 30 min and 1, 3, 9, and 24 h. Oral dosing studies usually extend to at least 72 h. Such a sampling scheme would provide data coverage for evaluation of absorption, elimination, enterohepatic recirculation, and excretion processes.

The number of animals used in metabolism and pharmacokinetic studies should be large enough to reliably estimate population variability. In the case for rats and mice, tissue and/or blood sample size is usually the limiting factor: Analysis of the substance may require 1 mL or more blood, but it is difficult to obtain multiple blood samples of this size from one animal. As a consequence,

a larger number of animals is required (three to four per time point, seven to nine time points) when small rodents are used. Such an approach has the advantage of allowing limited sampling of critical tissues (e.g., liver, fat) at each time point, an option which is usually unavailable with large animals. The use of humans and large animals generally permits collection of multiple (serial) blood samples. For outcrossing populations like humans and large animals, individual differences in the rates of biotransformation are likely to be greater than those of inbred rodent populations; under these circumstances, more samples per sex per group may be needed to reliably estimate variability.

Individual metabolism cages are recommended for collecting urine and feces in oral dosing studies. Excreta should be collected for at least five elimination half-lives of the test substance. When urine concentrations will be used to determine elimination rates, sampling times should be less than one elimination half-life (taken directly from the bladder in IV studies); otherwise, samples should be taken at equal time intervals.

The results of the preliminary biotransformation/kinetic study together with the current regulatory metabolism studies and the 28- and 90-day studies should allow the selection of a relatively small number of appropriate tissues and/or fluids for monitoring purposes. Satellite groups of animals will provide the material for analysis. Methods must be developed to analyze nonradioactive test chemical. Obviously it is important to monitor blood. It is accessible and convenient, and in certain circumstances sequential sampling from the same animal may be important. The most useful aspect of blood is that the results can be compared with those obtained in humans (see below). It is important, however, not to be constrained by this aspect. The most relevant tissues and body fluids should also be analyzed. These are target organs (if known) and indicator organs, tissues, or fluids, that is, those in which the concentration of pesticide or metabolite is a measure of that in the whole animal. In cases where distribution varies with dose (if shown in the preliminary study), a larger number of organs/tissues would be chosen for monitoring.

Whether the parent drug or metabolite (or both) is chosen for analysis depends on the preliminary study. In principle, analysis for the parent compound should always be carried out; however, there are situations (e.g., rapid metabolism) when this is quite futile and a major retained metabolite should be used. Covalently bound metabolites are addressed below.

Four occasions may be adequate for monitoring:

(i) One month (equilibrium between intake of chemical and elimination of metabolites should be established; the time relates to the 28-day preliminary study)
(ii) Three months (confirmation of results at one month; relates to the 90-day study)
(iii) One year (coincides with the interim kill)
(iv) Two years (effects of age; coincides with termination of study)

Consideration should be given to the analysis of moribund animals.

In Vitro Studies In vitro measurements employing enzymes, subcellular organelles, isolated cells, and perfused organs may be used to augment the dose–response information available from less extensive metabolic and pharmacokinetic studies. Because in vitro systems generally are less complex than whole animals, elucidation of a test compound's metabolic pathways and the pathways' kinetic characteristics may be facilitated. Such systems can be used to measure binding, adduct, and conjugate formation, transport across cell membranes, enzyme activity, enzyme substrate specificity, and other singular objectives. Biochemical measurements that can be made using in vitro systems include intrinsic clearances of enzymes in an organ or tissue, kinetic constants for an enzyme, binding constants, and the affinity of the test compound and its metabolites for the target macromoecules. The activity of a hepatic drug-metabolizing enzyme in vivo may be approximated by kinetic constants that are calculated from in vitro studies; when a first-order approximation is used, the ratio of V_{max} to K_m is equal to the intrinsic clearance of the drug. In vitro measurements made using readily accessible tissues and body fluids from animals and humans may also be useful in elucidating mechanisms of toxicity.

Analysis of Data Data from all metabolism and pharmacokinetic studies should be analyzed with the same pharmacokinetic model and results should be expressed in the same units. Concentration units are acceptable if the organ or sample size is reported, but percent of dose/organ is usually a more meaningful unit. In general, all samples should be analyzed for metabolites that cumulatively represent more than 1% of the dose.

A variety of rate constants and other parameters can be obtained from IV and oral dosing data sets provided that good coverage of the distribution, elimination, and absorption (oral dose) phase is available. Typical parameters calculated to characterize the disposition of a test substance are half-lives of elimination and absorption; area under the concentration-versus-time curve for blood; total body, renal, and metabolic clearance(CL); volume of distribution (V_d); bioavailability (F); and mean residence and absorption times (MAT, MRT). Some of these parameters, such as half-lives and elimination rates, are easily computed from one another; the half-life is more easily visualized than the rate constant.

Computation of oral absorption (k_a) and elimination (E) rates is often complicated by the "flip-flop" of the absorption and elimination phases when they differ by less than a factor of 3. Because of these analysis problems, computation of absorption and elimination rates should not be attempted on the basis of oral dosing results alone.

Blood/tissue uptake rates (k_{jl}) can often be approximated from data at early ($t < 10$ min) time points in IV studies provided that the blood has been washed from the organ (e.g., liver) or the contribution from blood to the tissue residue is subtracted (fat). High accuracy is not usually required since these parameters can be optimized to fit the data when they are used in more complex models. Tissue/blood recycling rates (k_{lj}) and residence times can

be computed from partition coefficients if estimates of uptake rates are available.

Tissue/blood partition coefficients (R_{jl}) should be determined when a steady state has been achieved. Estimates based on samples obtained during the elimination phase following a single dose of the test substance may lead to underestimates of this ratio in both eliminating and noneliminating tissues unless its half-life is very long. Correction of these values for elimination has been described by several authors.

It may be important to determine the degree of plasma protein and RBC binding of the test substance; calculation of blood clearance rates using plasma or serum concentrations of the substance that have not been adjusted for the degree of binding may under- or overestimate the true rate of clearance of the test substance from the blood. This is usually done through experiments in vitro.

Two classical methods used in the analysis of pharmacokinetic data are the fitting of sums of exponential functions (two- and three-compartment mammillary models) to plasma and/or tissue data and, less frequently, the fitting of arbitrary polynomial functions to the data (noncompartmental analysis).

Noncompartmental analysis is limited in that it is not descriptive or predictive; concentrations must be interpolated from data. The appeal of noncompartmental analysis is that the shape of the blood concentration-versus-time curve is not assumed to be represented by an exponential function and, therefore, estimates of metabolic and pharmacokinetic parameters are not biased by this assumption. In order to minimize errors in parameter estimates that are introduced by interpolation, a large number of data points that adequately define the concentration-versus-time curve are needed (Gabrielsson and Weiner, 1997).

Analysis of data using simple mammillary, compartmental models allows the estimation of all of the basic parameters mentioned above if data for individual tissues are analyzed with one- or two-compartment models and combined with results from two- to three-compartment analyses of blood data. "Curve-stripping" analysis can be applied to such simple models through the use of common spreadsheet programs (i.e., LOTUS 1-2-3 or Excel) as long as a linear regression function is provided in the program. Optimization of the coefficients and exponents estimated may require the use of more sophisticated software: A number of scientific data analysis packages such as RS/1 and SigmaPlot have the necessary capabilities. Specialized programs such as NONLIN, CONSAM, or SIMUSOLV will be needed when more complex models must be analyzed. Coefficients and exponents from mammillary models can be used to calculate other parameters; however, they should not be taken too literally, since mammillary models assume that all inputs are to a central pool (blood) which communicates without limitation into other compartments. This approach does not include details such as blood flow limitations, anatomical volumes, or other physiological limits in the animal.

Physiologically based pharmacokinetic (PBPK) models were developed to overcome the limitations of simple mammillary models. Physiologically based models describe the disposition of test substances via compartmental models which incorporate anatomical, biochemical, and physiological features of specific tissues in the whole animal. The types of information added include organ-specific blood flows, volumes, growth models, and metabolism rates. Metabolic parameters often are obtained from in vitro studies (e.g., enzyme reaction rates in cultured hepatocytes, and plasma protein binding), while other parameters are becoming available as standard parameters in the literature. Parameters from mammillary models can be used to compute the value of parameters used in physiological pharmacokinetic models using tissue-specific blood flows, anatomical volumes, and other information (literature values). Estimation of parameters for a simple mammillary model is often the first data reduction step in creating a physiological model.

Because PBPK models are based on physiological and anatomical measurements and all mammals are inherently similar, they provide a rational basis for relating data obtained from animals to humans. Estimates of predicted disposition patterns for test substances in humans may be obtained by adjusting biochemical parameters in models validated for animals; adjustments are based on experimental results of animal and human in vitro tests and by substituting appropriate human tissue sizes and blood flows. Development of these models requires special software capable of simultaneously solving multiple (often very complex) differential equations, some of which were mentioned above. Several detailed descriptions of data analysis have been reported.

Use of Data from Metabolism and Pharmacokinetic Studies Information from metabolism and pharmacokinetic studies can be used in the design and analysis of data from other toxicity studies. Some examples are described below.

Design of Toxicity Studies The concentration-versus-time curve and peak and steady-state concentrations of the test substance in blood or plasma provide information on the distribution and persistence of the substance in the animal which may suggest essential elements in the design of the toxicity studies. For example, when metabolic and pharmacokinetic studies indicate that the test compound accumulates in the bone marrow, long-term toxicity tests should include evaluation of the test compound's effect on hematopoietic function and morphology. If a test compound is found to accumulate in milk, an investigator may need to plan to perform reproductive toxicity studies with in utero exposure and a nursing phase (cross-fostering study). In addition, information from metabolic and pharmacokinetic studies can be used to predict the amount of test compound that enters biological compartments (tissues, organs, etc.) that may not suffer a toxic insult but may serve as depots for indirect or secondary exposure.

15.4.3 Whole-Body Autoradiography

Autoradiography is the production of an image in a photographic emulsion by the emission from a radioactive element. The term autoradiography is preferred to that of radioautography. Prefixes are added to words to further classify the concept. Therefore, the process is "auto-" radiography for a "self-" radiograph and not a "radio-" autograph for one's transmitted signature (Waddell, 1972).

Whole-body autoradiography (WBA) has been used with increasing frequency as a means of identifying tissues which concentrate test substances. This technique allows a small number of animals (5–10) to be used for screening purposes with a minimal investment in manual labor. The FDA encourages the use of WBA with IV dosing as a means of screening and selecting tissues of greatest relevance for later oral dosing studies. Animals used for WBA should generally be sacrificed during primary consideration in selecting specific tissues.

The most comprehensive technique currently available for the initial survey of the distribution of a drug is that of WBA. The species of animals used include mice, rats, hamsters, monkeys, pigs, dogs, and ferrets. The most widely used animal has been the mouse, which has the advantages of requiring less isotope and being easier to section.

The animals are anesthetized and then frozen by immersion at various times after administration of the labeled compound in hexane or acetone cooled with dry ice. Since the freezing in the interior of the animals occurs slowly, large ice crystals form within these tissues, and hence subcellular localization of compounds is not possible.

The selection of times for freezing an animal after injection of a drug must be based on the information available on the rate of elimination of the compound from the animal by metabolism and excretion. In general, a geometric increase in time intervals is most useful. In order to have time intervals for comparison, we routinely have employed freezing times which are approximately multiples of three, namely 2, 6.5, and 20 min and 1, 3, 9, and 24 h. In certain cases, rapid elimination of the drug by the kidneys must be circumvented by ligation of the renal pedicles to avoid apparent localization from failure of the agent to reach equilibrium. An example would be the clearance of urea-^{14}C in pregnant mice.

The frozen animal is frozen into a block of carboxymethylcellulose ice on the microtome stage. Although the Jung, type K, microtome has been used, the Leitz, model 1300, sledge microtome is more suitable, for its smaller size allows it to be mounted in an ordinary commercial freezer instead of a walk-in freezer. The microtome stage must be designed for mounting in the vice of the front end of the stage.

Sections from 5 μm to approximately 80 μm thick are taken onto No. 800 Scotch tape (Minnesota Mining & Mfg. Co.). Before removal from the freezer, the sections must be allowed to dry thoroughly so that no ice remains which melts and allows movement of the isotope. After drying, if covered to prevent

condensation of moisture on the sections, the sections may be transferred from the freezer to room temperature.

X-ray films which produce the most satisfactory autoradiograms are Kodak industrial type AA and Gevaert Structurix D-7. Both are fine-grain films which have been demonstrated not to produce chemical artifacts. Approximately six times faster, Kodak No Screen and Kodirex may be used for rapid screening and timing of autoradiograms. However, they occasionally produce artifacts and should not be relied on for interpretation. Some investigators have used photographic emulsions such as Ilford G-5, 10 μm thick, preapplied to glass plates. The increased cost and likelihood of breakage, however, hardly justify the small improvement in resolution for whole-body sections.

Exposure of the photographic emulsion by the radioactivity of the tissue section should be at freezer temperatures to prevent autolysis of the tissue. After exposure of the X-ray film, sections with isotopes which have a long half-life may be placed against fresh X-ray film for additional sets of autoradiograms with either a longer or shorter exposure time. This procedure is useful for revealing relative concentrations of radioactivity for areas that have either very high or very low concentrations after the first exposure. When no further autoradiograms are needed, the section can be stained with histological dyes to verify localizations of radioactivity.

Compounds that fluoresce under ultraviolet light can be visualized in the tissue sections and their locations recorded with color film. Whole-body tissue sections can be used for histochemical localizations for comparison with the autoradiograms. Furthermore, the areas can be removed and extracted and the extract chromotographed to identify the chemical nature of the radioactivity revealed by the autoradiogram.

Although the whole-body technique will allow localization of an increased concentration of an isotope in a tissue or occasionally a cell type, other techniques must be used for single cells and subcellular localization. A nuclear tract plate is prepared by dipping the plate in a 12% solution of glycerine in absolute ethyl alcohol and allowing it to drain for 10 min in a vertical position before approximating the section on tape. After the emulsion is exposed, soaking in xylene removes the tape but leaves the section attached to the nuclear tract plate. The Ilford G-5 nuclear tract plates with 10-μm emulsions are most satisfactory. The increased resolution gained by the finer grained Ilford K and L emulsions is warranted only for tissues that are well preserved and relatively free of ice crystal artifacts. Kodak NTB emulsions seem to produce more pressure artifacts than the Ilford plates.

Comparison of various techniques of autoradiography for diffusible compounds clearly demonstrates that no solutions can be used in processing the tissue. These investigators have dried thin sections of liver and uterus at temperatures below –60 °C. These freeze-dried sections were dry mounted on microscope slides which had been precoated with either Kodak NTB-3 or NTB-10 emulsion. Other techniques which thawed the frozen section, embedded the tissue in paraffin, or dipped the section in liquid emulsion were

demonstrated to translocate diffusible compounds. Many other similar attempts have been and are currently being made to localize diffusible compounds by autoradiography at the electron microscope level.

15.5 PHYSIOLOGICALLY BASED PHARMACOKINETIC (PBPK) MODELING

Pharmacokinetic modeling is the process of developing mathematical explanations of absorption, distribution, metabolism, and excretion of chemicals in organisms. Two commonly used types of compartmental pharmacokinetic models are (a) data based and (b) physiologically based. The data-based pharmacokinetic models correspond to mathematical descriptions of the temporal change in the blood/tissue level of a xenobiotic in the animal species of interest. This procedure considers the organism as a single homogeneous compartment or as a multicompartmental system with elimination occurring in specific compartments of the model. The number, behavior, and volume of these hypothetical compartments are estimated by the type of equation chosen to describe the data and not necessarily by the physiological characteristics of the model species in which the blood/tissue concentration data were acquired.

Whereas these data-based pharmacokinetic models can be used for interpolation, they should not be used for extrapolation outside the range of doses, dose routes, and species used in the study on which they were based. In order to use the data-based models to describe the pharmacokinetic behavior of a chemical administered at various doses by different routes, extensive animal experimentation would be required to generate similar blood–time course data under respective conditions. Even within the same species of animal, the time-dependent nature of critical biological determinants of the disposition (e.g., tissue glutathione depletion and resynthesis) cannot easily be included or evaluated with the data-based pharmacokinetic modeling approach. Further, due to the lack of actual anatomical, physiological, and biochemical realism, these data-based compartmental models cannot easily be used in interspecies extrapolation, particularly to predict pharmacokinetic behavior of chemicals in humans. These various extrapolations, which are essential for the conduct of dose–response assessment of chemicals, can be performed more confidently with a physiologically based pharmacokinetic modeling approach. This chapter presents the principles and methods of physiologically based pharmacokinetic modeling as applied to the study of toxicologically important chemicals.

PBPK modeling is the development of mathematical descriptions of the uptake and disposition of chemicals based on quantitative interrelationships among the critical biological determinants of these processes. These determinants include partition coefficients, rates of biochemical reactions, and physiological characteristics of the animal species. The biological and mechanistic basis of the PBPK models enable them to be used, with limited animal experimentation, for extrapolation of the kinetic behavior of chemicals from high

dose to low dose, from one exposure route to another, and from test animal species to people.

The development of PBPK models is performed in four interconnected steps: model representation, model parameterization, model stimulation, and model validation. Model representation involves the development of conceptual, functional, and computational descriptions of the relevant compartments of the animal as well as the exposure and metabolic pathways of the chemical. Model parameterization involves obtaining independent measures of the mechanistic determinants, such as physiological, physicochemical, and biochemical parameters, which are included in one or more of the PBPK model equations. Model simulation involves the prediction of the uptake and disposition of a chemical for defined exposure scenarios using a numerical integration algorithm, simulation software, and a computer. Finally, the model validation step involves the comparison of the a priori predictions of the PBPK model with experimental data to refute, validate, or refine the model description and the characterization of the sensitivity of tissue dose to changes in model parameter values. After appropriate testing and validation PBPK models can be used to conduct extrapolations of the pharmacokinetic behavior of chemicals from one exposure route/scenario to another, from high dose to low dose, and from one species to another.

The PBPK model development for a chemical is preceded by the definition of the problem, which in toxicology may often be related to the apparent complex nature of toxicity. Examples of such apparent complex toxic responses include nonlinearity in dose–response, sex/species differences in tissue response, differential response of tissues to chemical exposure, qualitative and/or quantitative difference responses for the same cumulative dose administered by different routes/scenarios, and so on. In these instances, PBPK modeling studies can be utilized to evaluate the pharmacokinetic basis of the apparent complex nature of toxicity induced by the chemical. One of the values of PBPK modeling, in fact, is that accurate description of target tissue dose often resolves behavior that appears complex at the administered dose level.

The principal application of PBPK models is in the prediction of the target tissue dose of the toxic parent chemical or its reactive metabolite. Use of the target tissue dose of the toxic moiety of a chemical in risk assessment calculations provides a better basis of relating to the observed toxic effects than the external or exposure concentration of the parent chemical. Because PBPK models facilitate the prediction of target tissue dose for various exposure scenarios, routes, doses, and species, they can help reduce the uncertainty associated with the conventional extrapolation approaches. Direct application of modeling includes:

- High-dose/low-dose extrapolation
- Route–route extrapolation
- Exposure scenario extrapolation
- Interspecies extrapolation

15.6 POINTS TO CONSIDER

Stereoisomerism will influence metabolism and toxicity. For example, Lu et al. (1998) reported a comparison of (*S*)-(–)ifosfamide and (*R*)-(+)-ifosfamide. They demonstrated that there were significant differences between the two stereoisomers with regard to pharmacokinetic behavior and major metabolite formation, as shown in Table 15.16.

In addition, treatment of animals with phenobarbital not only increased overall rates of metabolism and clearance but also shifted the metabolite patterns. One of the more common methods used for determining an exposure to (or the amount of a metabolite produced) is to determine an AUC for the metabolite. Further, one of the more common methods for representing a racemically preferred metabolite is to calculate the ratio of *R* to *S*. For example, the 3-decholoro metabolite of ifosfamide was produced in higher amounts from the *R* enantiomer while the 2-decholorometabolite was the major metabolite produced from the *R* enantiomer in naive animals. Treatment with phenobarbital shifted the metabolism so that the 3-dechloro metabolite was no longer the major metabolite for the *S* enantiomer.

15.7 BIOLOGICALLY DERIVED MATERIALS

The progress and products of biotechnology have brought some new challenges to the assessment of pharmacokinetics and toxicokinetics. While the reasons for needing these data (demonstrating exposure, displaying dose dependency, correlating any findings of toxicity to exposure, and determining steady state for systemic agent levels) are certainly as compelling as with traditional drugs, there are a whole set of special problems involved (Baumann, 2006; Dennis et al., 2002).

These special concerns for biologically derived products are:

TABLE 15.16 Example of Stereoselective Differences in Metabolism (*R*) versus (*S*) Ifosfamide

Parameter	Phenobarb	*R*	*S*	*R*/*S*
Term half-life	–	34.3	41.8	.820
(min)	+	19.8	19.41	1.02
AUC	–	4853	6259	.820
(μM·min)	+	1479	1356	1.03
2-Dehloro metabolite	–	799	2794	.287
AUC	+	229	1205	.186
3-Dehloro metabolite	–	1380	996	1.41
AUC	+	192	1175	.159

Note: Animals were pretreated with phenobarbital (80 mg·kg) for four days.

Source: Adapted from Lu et al., 1998.

Assay Sensitivity/Specificity

- Needs to be at $1\,ng\,mL^{-1}$ or lower.
- Cross-reactivity to native protein may confound results.
- If test article is the same as native protein, how do you tell the difference?
- Western blot can be used to demonstrate specificity.
- Antibody interference may occur with assay.

Low Systemic Levels

- Rapid metabolism: Metabolites may be endogenous proteins or amino acids.
- Extensive metabolism: Metabolites may be incorporated into cell structures rapidly.
- Rapid distribution
- Rapid hepatic clearance
- Route of administration may bypass first-pass metabolism:
  Subcutaneuos
  Intracerebroventricular, intrathecal
  Buccal

Endogenous Protein

- May cross react and lead to false-positive blood levels.
- Can radiolabel to tell difference between administered molecule and endogenous molecule:
  However, the label may lead to different distibution.
  What is the specific activity if diluted with unlabeled endogenous material?

Sample Volume

- May need to be large to increase sensitivity.
- May also need to be small because of competing assays:
  Immune factors (antibodies, globulins)
  Hormones
  Disease state modifiers
  In humans, concomitant medications

Distribution

- Rapidly cleared from blood
- Frequently distributed via lymphatics

- Target and off-target receptor found portion of drug molecule population in the body rapidly predominate
- Pharmacodynamic very different from pharmacokinetics
- Delivery rarely by oral route
- Available test material supply very limited in early development

The upshot of these points is that it may not be practical to follow established guidelines for ADME evaluation. Binding proteins, immunoreactive metabolites, and antibodies could interfere with the immunoassays used to measure the activity of biotechnologically derived pharmaceuticals. The link between immunoreactivity and pharmacological activity may be difficult to establish, making the data difficult to interpret. In radiolabeled distribution studies, if the label alters the physicochemical and biological properties of the test material, its pharmacokinetic behavior may change. These analytical difficulties may preclude accurate characterization of the distribution, metabolism, and excretion of a protein.

AUC and C_{max} are commonly measured to identify safety ratios for new chemical entities. Since the analytical methods used for biotechnologically derived pharmaceuticals may lack specificity, a clinical marker of biological activity or efficacy may sometimes be more appropriate than exposure data.

It is therefore essential that before pivotal (repeat-dose) preclinical studies are initiated, bioanalytical assay development must be completed. This has to cover potential test species, normal (and diseased) humans. The assays must be validated in the sampling matrix of the toxicity test species, and one should also develop suitable assays for antibodies to the test article.

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16

Safety Pharmacology

Safety pharmacology is the evaluation and study of the potentially life threatening pharmacological effects of a potential drug which is unrelated to the desired therapeutic effect and therefore may present a hazard—particularly in individuals who already have one or more compromised or limited organ system functions. Unlike other nonclinical evaluations of the safety of a drug, these evaluations are usually conducted at doses not too much in excess of the intended clinical dose. This topic is another which has had to undergo significant change since the last edition because of a changed regulatory environment.

General/safety pharmacology was an emerging discipline within the pharmaceutical industry in 2002, when the International Conference on Harmonisation (ICH) guidance was promulgated and it became a major area of both concern and activity. It seeks to identify unanticipated effects of new drug candidates on major organ function (i.e., secondary pharmacological effects) and ensure that they are critically assessed in a variety of animal models. A survey was conducted to obtain customer input on the role and strategies of this emerging discipline. Overlooked in importance by all but a few (Zbinden, 1966, 1984) for many years, the Japanese clearly became the leaders in developing and requiring such information, while the United States was in a position behind Japan and the European Union (EU) in both having formal requirements and implementing industrial programs. While major companies were aware and largely addressing the need by the mid-1990s (Kintner et al.,

Drug Safety Evaluation, Second Edition, by Shayne Cox Gad

1994; Murphy et al., 1995; Sullivan and Kinter, 1995) and the European Medicines Agency (EMEA) promulgated guidelines in 2000, it was only with the ICH S7 guidance that U.S. and global regulatory interest came to play.

While historically companies have conducted evaluations of cardiovascular and central nervous system (CNS) functions, less evaluate respiratory, gastrointestinal, and renal functions; a few conduct a ligand-binding/activity panel as part of their pharmacological profiling. Since 2001, studies to evaluate the cardiovascular, pulmonary, and CNS safety pharmacological aspects of all but a few new drugs have been required (these exceptions will be discussed shortly) before they are evaluated clinically (i.e., had human exposure).

It is important that the tests employed detect bidirectional drug effects and that the tests performed be validated in both directions with appropriate reference (control) substances and be sensitive in the acute therapeutic range (Folke, 2000; Redfern et al., 2002). This requirement is less appropriate for multiparameter procedures. Blind testing could be an advantage. Ethical considerations are important, but the ultimate ethical criterion is the assessment of risk for humans. Safety pharmacology studies should not be overinclusive but should be performed to the most exacting standards, including good laboratory practice (GLP) compliance. It is important that safety pharmacological data be available during the planning stage for phase I studies, but this is often not the case. In part this arises from the viewpoint that human tolerance (particularly in a well-designed and executed phase I study in normal volunteers) is, in itself, an adequate assessment of safety pharmacology. This is, of course, backward—such human tolerance is rather, properly, an extension (and expression) of the nonclinical safety pharmacology.

The other point of view in the past has been that properly executed repeat-dose preclinical safety studies meeting the current design requirements will (or could) fill these needs. In addition, undesired pharmacological activities of novel drugs or biologicals are seen as limiting development of a therapeutic agent prior to the characterization of any toxicological effects. In rodent species, general pharmacological assays have traditionally been used to screen new agents for pharmacological effects on the central and peripheral nervous systems, the autonomic nervous system and smooth muscles, the respiratory and cardiovascular systems, the digestive system, and the physiological mechanisms of water and electrolyte balance. In large-animal species, such as dogs and nonhuman primates, smaller numbers of animals per study limit their use for screening assays, but these species may play an important role in more detailed mechanistic studies. For drugs and biologicals that must be tested in nonhuman primates because of species-specific action of the test agent, functional pharmacological data are often collected during acute or subacute toxicity studies. This requires careful experimental design to minimize any impact that pharmacological effects or instrumentation may have on the assessment of toxicity. In addition, with many new therapies targeted at immunological diseases, the pharmacological effect of therapeutics on the immune system presents new challenges for pharmacological profiling. The applications of pharmacological

assays by organ systems in both rodent and large-animal species are discussed as well as practical issues in assessing pharmacological endpoints in the context of toxicity studies (Martin et al., 1997; Matsuzawa et al., 1997).

Pharmacoepidemiological studies in Europe and the United States show that adverse drug reactions now may account for up to 10% of the admissions of patients to hospitals at a cost of hundreds of millions of U.S. dollars annually (Sjoquist, 2000). This represents a considerable increase compared to 20 years ago. A partial explanation is the many shortcomings of clinical trials and their relevance for health care. Adverse drug reactions are often poorly studied and documented in these studies and very seldom included in health economical analyses of the value of new drugs. Pharmacovigilance is product—rather than utilization—oriented and quite invisible in clinical medicine. This is regrettable, since up to 50% of adverse drug reactions (ADRs) are dose dependent and thus preventable. Hopefully, the rapid progress in molecular and clinical pharmacogenetics will provide new tools for clinicians to choose and dose drugs according to the individual needs of patients. A good starting point for those not well versed in pharmacology and the range of potential mechanisms of action and of interaction can be found in *Goodman & Gilman* (Brunton et al., 2006).

16.1 REGULATORY REQUIREMENTS

While the ICH guidelines promulgated in November 2000 (implemented in Europe in the three regions in June 2001) are the announced international standards for regulation, the actual situation in some countries remains very mixed (Hite, 1997; Olejiniczak, 1999; Fujimori, 1999).

Japan continues to operate in conformance to the Ministry of Health and Welfare (MHW) 1999 revision of its "Guidelines for Safety Pharmacology Studies." The basic principle of the revision is to harmonize the guideline with the international concepts. The working group decided to change the title "General Pharmacology" to "Safety Pharmacology" because the objective of this guideline is to assess the safety of a test substance in humans by examining the pharmacodynamic properties of the substance. The proposed guideline includes studies on vital functions as essential studies that should be performed prior to human exposure. Studies are also required to be conducted when predictable or unexpected observed effects are concerned. The working group recommends a case-by-case approach to select the necessary test items in consideration of the variable information available.

In the EU, the Committee for Proprietary Medicinal Products (CPMP) issued the draft "Note for Guidance on Safety Pharmacology Studies in Medicinal Product Development" in 1998, but it has not yet been finalized or put in force, and as of the middle of 2001, the U.S. Food and Drug Administration (FDA) has remained mute on guidelines.

The actual requirements of the initial November 8, 2000, ICH guidelines provided only broad outlines of requirements. They called for the conduct of

studies in a core battery to assess effects on the cardiovascular (Table 16.1), respiratory (Table 16.2), central nervous system (Table 16.3), and secondary organ system (Table 16.4) effects. Follow-up studies for the care battery are also required on a case-by-case basis for the three main organ systems:

- *Central Nervous System* Behavioral pharmacology, learning and memory, specific ligand binding, neurochemistry, visual, auditory, and/or electrophysiological examinations, and so on.
- *Cardiovascular System* Behavioral pharmacology, learning and memory, specific ligand binding, neurochemistry, visual, auditory, and/or electrophysiological examinations, and so on.

TABLE 16.1 Cardiovascular System Safety Pharmacology Evaluations: Core

Hemodynamics (blood pressure, heart rate)
Autonomic function (cardiovascular challenge)
Electrophysiology (ECG in dog)
QT Prolongation: Noncore
An additional guideline, ICH S7B, is in preparation which will address the assessment of potential for QT prolongation. In the meantime, CPMP 986/96 indicates the following preclinical studies should be conducted prior to first administration to humans:
• Cardiac action potential in vitro
• ECG (QT measurements) in cardiovascular study which would be covered in core battery
• hERG channel interactions (hERG expressed in HEK293 cells)

TABLE 16.2 Respiratory System Safety Pharmacology Evaluation

Respiratory functions: Measurement of rate and relative tidal volume in conscious animals
Pulmonary function: Measurement of rate, tidal volume, and lung resistance and compliance in anesthetized animals

TABLE 16.3 Central Nervous System Safety Pharmacology Evaluation

Irwin test: General assessment of effects on gross behavior and physiological state[a]
Locomotor activity: Specific test for sedative, excitatory effects of compounds
Neuromuscular function: Assessment of grip strength
Rotarod: Test of motor coordination
Anesthetic interactions: Test for central interaction with barbiturates
Anti-/proconvulsant activity: Potentiation or inhibition of effects of pentylenetetrazole
Tail flick: Tests for modulation of nociception (also hot plate, Randall Selitto, tail pinch)
Body temperature: Measurement of effects on thermoregulation
Autonomic function: Interaction with autonomic neurotransmitters in vitro or in vivo
Drug dependency: Test for physical dependence, tolerance, and substitution potential
Learning and memory: Measurement of learning ability and cognitive function in rats

[a]Usually a functional observational battery (FOB) is integrated into rodent (rat) repeat-dose toxicity studies to meet this requirement.

TABLE 16.4 Secondary Organ System Safety Pharmacology Evaluation

Renal system
Renal function—Measurement of effects on urine excretion in saline-loaded rats
Renal dynamics—Measurement of renal blood flow, glomerular filtration rate (GFR), and clearance
GI system
GI function—Measurement of gastric emptying and intestinal transit
Acid secretion—Measurement of gastric acid secretion (Shay rat)
GI irritation—Assessment of potential irritancy to gastric mucosa
Emesis—Nausea, vomiting
Immune system
Passive cutaneous anaphylaxis (PCA)—Test for potential antigenicity of compounds
Other
Blood coagulation
In vitro platelet aggregation
In vitro hemolysis

- *Respiratory System* Tidal volume, bronchial resistance, compliance, pulmonary arterial pressure, blood gases.

No testing is deemed necessary for:

- Locally applied agents (e.g., dermal or ocular) where systemic exposure or distribution to the vital organs is low
- Cytotoxic agents for treatment of end-stage cancer patients
- Biotechnology-derived products that achieve *highly specific* receptor targeting (refer to toxicological studies)
- New salts having similar pharmacokinetics and pharmacodynamics to already well-characterized drugs

16.2 STUDY DESIGNS AND PRINCIPALS

As a starting place, unlike older pharmacology studies, safety pharmacology studies are conducted as GLP studies unless performed as screens. At the same time, unlike other safety assessment studies, these do not need to vastly exceed intended therapeutic doses so as to identify signs of toxicity. In this sense, they are closer to hazard tests.

General guidance for dose (or concentration) section for such studies is as follows (ICH, 1997, 2001; Gad, 2004):

In vivo studies are designed to define the dose–response curve of the adverse effects:

- Doses should include and exceed primary pharmacodynamic or therapeutic range.

- In the absence of safety pharmacology parameters, the highest doses equal or exceed some adverse effects (toxic range).

In vitro studies are generally designed to establish an effect–concentration relationship (range of concentrations).

Considerations in the selection and design of specific studies are straightforward:

The following factors should be considered (selection):

- Effects related to the therapeutic class
- Adverse effects associated with members of the chemical/therapeutic class
- Ligand-binding or enzyme data suggesting a potential for adverse effects
- Data from investigations that warrant further investigation

A hierarchy of organ systems can be developed:

- Importance with respect to life-supporting functions: cardiovascular, respiratory, central nervous system
- Functions which can be transiently disrupted without causing irreversible harm

The absence of observed activity may represent either a true- or a false-negative effect. If an assay is valid for the particular test article and fails to indicate activity, it is an appropriate indicator of future events (Green, 1997). However, if the assay is insensitive or incapable of response, the test represents a form of bias, albeit unconscious. Many biological products demonstrate a specificity of response that limits the utility of commonly employed safety studies. Specificity for many biologicals arises from both their physicochemical properties and their similarity to endogenous substances which are regulated in a carefully controlled manner. To overcome the issue of a lack of predictive value, various approaches may be used. For example, a multiple-testing strategy of mutually reinforcing studies may be employed or safety studies may be adaptively fit to the biological circumstance.

A separate issue is how and when to consider isomers, metabolites, and the actual finished product:

- Generally a parent compound and its major metabolite(s) that achieve systemic exposure should be evaluated.
- It may be important to test active metabolites from humans.
- Testing of individual isomers should also be considered.
- Studies with the finished product are necessary only if kinetics/dynamics are substantially altered in comparison to the active substance previously tested.

There are also special considerations as to how to statistically evaluate specific aspects of these studies. Specifically, analysis of time to event becomes very important (Anderson et al., 2000).

16.3 ORGAN-SYSTEM SPECIFIC TESTS

16.3.1 General Considerations in Selection and Design of Safety Pharmacology Studies

The following factors should be considered (selection):

- Effects related to the therapeutic class (e.g., proarrhythmia is a common feature of antiarrhythmic drugs)
- Adverse effects associated with members of the chemical/therapeutic class (e.g., antipsychotics and QT prolongation)
- Receptor/enzyme/ion channel binding data suggesting a potential for adverse effects
- Any data from previous studies that warrant further investigation

A hierarchy of organ systems is considered:

- Importance with respect to life-supporting functions: cardiovascular, respiratory, central nervous
- Functions which can be transiently disrupted without causing irreversible harm (e.g., urinary system, GI tract)

16.3.2 Studies on Metabolites, Isomers, and Finished Products

Generally the parent compound and its major metabolite(s) that achieve systemic exposure need to be evaluated. This means that either the test species must be metabolically comparable to humans or that human metabolites must also be evaluated. The testing of individual isomers also needs to be considered.

16.4 CARDIOVASCULAR

While the initial greatest concern for cardiovascular risks was associated with QT prolongation (first raised by the CAST trial in 1989), it has become clear since then that there are a range of potentially life threatening cardiovascular pharmacological drug effects which must be evaluated (see Gad, 2008;

Braunwald, 2008) by drugs not intended to have cardiac effects (see Table 16.5). The cardiovascular system is one of the primary vital functions which has to be examined during safety pharmacology studies. Cardiovascular system functioning is maintained by cardiac electrical activity and by pump-muscle function, which contribute to hemodynamic efficacy.

The aim of cardiovascular safety pharmacology is to evaluate the effects of test substances on the most pertinent components of this system in order to detect potentially undesirable effects before engaging in clinical trials (Lacroix and Provost, 2000). In the basic program, a detailed hemodynamic evaluation is carried out in the anesthetized dog. It is completed by cardiac and/or cellular electrophysiology investigations in order to assess the arrhythmogenic risk. The basic program can be preceded by rapid and simple testing procedures during the early drug discovery stage. It should be completed, if necessary, by specific supplementary studies, depending on the data obtained during the early clinical trials. The current gold standard study is performed using unrestrained radiotelemetrized dogs (Gauvin et al., 2006) and can measure multiple endpoints continuously.

TABLE 16.5 Noncardiac Drugs Known to Induce or Worsen Heart Failure According to Suggested Mechanism(s) Implicated

Drug Class	Drug
Cardiomyopathy	
Cytotoxic drugs	Doxorubicin, epitubicin, and other anthracyclines; mitoxantrone, cyclophosphamide, 5-fluorouracil, capecitabine
Immunomodulating drugs/antibodies	Trastuxumab, interferon-α, interleukin-2, infliximab, etanercept
Antifungal drugs	Itraconazole, amphotericin B
Antipyschotic drugs	Clozapine
Pulmonary hypertension	
Antimigraine drugs	Methysergide, ergotamine
Appetite suppressants	Fenfluramine, fluramine, phentermine
Heart valve abnormalities	
Antimigraine drugs	Methysergide, ergotamine
Appetite suppressants	Fenfluramine, fluramine, phentermine
Antiparkinsonian drugs	Pergolide
Fluid overload	
Nonsteroidal anti-inflammatory drugs (NSAIDs), including cyclooxygenase-2 inhibitors	All
Antidiabetic drugs	Rosiglitazone, pioglitazone, troglitazone
Glucocorticoids	All
Herbal drugs	Herbal drugs containing liquorice or adulterated with NSAIDs

16.4.1 Hemodynamics, Electrocardiograms, and Respiration in Anesthetized Dogs or Primates

Anesthetized studies using Modular Instruments data capture systems are done to record six-lead electrocardiograms (ECGs) (I, II, III, aV_l, and aV_f), left ventricular pressure variables, arterial blood pressure and respiratory measurement of arterial blood flow in selected vascular beds, cardiac output, and arterial blood gas measurement. ECG intervals are measured from the lead II ECG and the QT interval can be corrected for heart rate using Bazett's, Friderecia's, or Van De Water's formulas. Different formulas are appropriate for different species (Solovien et al., 2006).

16.4.2 Cardiac Conduction Studies

In addition to the above hemodynamic measurements, intraventricular, intra-arterial, and atrioventricular conduction times and velocities can be measured using epicardial electrodes in the anesthetized and thoracotomized dog.

16.4.3 Conscious Dog, Primate, or Pig Telemetry Studies

Effects on blood pressure, heart rate, lead II ECG, core body temperature, and locomotor activity can be explored using DataSciences telemetry (or similar) implanted devices in guinea pigs, dogs, pigs, and primates. Effects on behavior can be captured on video using CCTV for dog and primate studies. Repeated administration and interaction studies may also be performed. This approach has the advantages of avoiding the effects of both anesthesia and restraint of the animal.

16.4.4 Six-Lead ECG Measurement in Conscious Dog

Conscious studies using integrated telemetry systems devices for measurement of blood pressure and six-chest-lead ECG measurements (V2, V4, V6, V10, rV2, rV4) (Hamlin, 2008). ECG interval analysis is performed on the V2 lead (RR, PR, QT, QTc intervals, QRS duration). QT dispersion can also be measured. Locomotor activity can be monitored and behavior captured on video using CCTV:

- In addition to validated systems for automatic measurement of ECG parameters, ECGs can be reviewed by veterinary cardiology services to detect any transparent abnormalities.
- Colonies of telemetered animals can be set up and maintained for repeat use.
- Respiration rate measurements can be taken from dogs in slings using a pneumograph system.

- An animal-specific correction of the QT interval can also be derived for each dog/primate based on individual variability of the QT interval with rate using the Framingham equation.

Recent concerns over the arrhythmogenic effects of a number of marketed compounds have resulted in the issue of the Points to Consider document CPMP 986/96 by the EMA (http://www.eudra/org/humandocs/PDFs/SWP/098696en.pdf).

Studies to assess the effects of a compound and any known metabolites on the ECG and cardiac action potentials are recommended. Changes in action potential duration and other parameters measured are a functional consequence of effects on the ion channels which contribute to the action potential. This in vitro test is considered to provide a reliable risk assessment of the potential for a compound to prolong the QT interval in humans.

16.4.5 Systems for Recording Cardiac Action Potentials

These include a range of currently available methodologies, some of which can be incorporated into existing study designs:

- Isolated ventricular Purkinje fibers from dog or sheep
- Isolated right ventricular papillary muscle from guinea pig
- Continuous intracellular recording of action potentials and online analysis of resting membrane potential, maximum rate of depolarization, upstroke amplitude, and action potential duration using the Notocord HEM data acquisition system
- Assessment of use-dependent and inverse-use-dependent actions by stimulation at normal, bradycardic, and tachycardic frequencies (e.g., see the discussion below of the inverse-use-dependent properties of sotalol in dog Purkinje fibers)

16.4.6 Special Case (and Concern)—QT Prolongation

Drugs that alter ventricular repolarization (generally recognized as drugs that prolong the QT interval) have been associated with malignant ventricular arrhythmias (especially the distinctive polymorphic ventricular tachycardia called torsade de pointes) and death. Many of the drugs now known to alter ventricular repolarization were developed as antiarrhythmics (e.g., dofetelide, sotalol), but others (e.g., cisapride, terfenadine) were developed without the expectation of any effect upon electrically excitable membranes. This has led to the ICH promulgating S7B (ICH, 2005) with specific guidance for evaluation.

The QT interval of the ECG is reflected in three main ways. First, electrophysiologically, it reflects the depolarization and repolarization phase of ven-

tricular myocytes. Second, mechanically, it represents the time of contraction of the ventricles. And last, physiologically, its duration is a function of numerous variables (heart rate, diseases, nutrition, diurnal cycle, etc.) Diagnostically, a prolongation of its duration indicates an enhanced risk for ventricular arrhythmias (torsade de pointes) and sudden cardiac death.

The association between abnormalities of repolarization and life-threatening arrhythmias is stronger than some other associations between laboratory abnormalities and clinical events. For example, there are drugs (tacrine) and inborn errors of metabolism (Gilbert's syndrome) that cause wild excursions in liver function tests but with no adverse consequences. In contrast, although the severity or proarrhythmia at a given QT duration varies from drug to drug and from patient to patient, no drug is known to alter ventricular repolarization without inducing arrhythmias,[1] and each of the several congenital long-QT syndromes is associated with an elevated incidence of malignant arrythmias.

With any given repolarization-altering drug, the risk of malignant arrhythmia seems to increase with increasing QT interval, but there is no well-established threshold duration below which a prolonged QT interval is known to be harmless. The extent of QT prolongation seen with a given drug and patient may be nonlinearly related to patient factors (sex, electrolyte levels, etc.) and to serum levels of the drug and/or its metabolites. The actual incidence of malignant arrhythmias, even in association with the drugs most known to induce them, is relatively low, so failure to observe malignant arrhythmias during clinical trials of ordinary size and duration does not provide substantial reassurance.

Abnormal repolarization and the associated arrhythmias are the end results of a causative chain that starts with alternations in the channels of ionic flux through cell membranes. Some cells (e.g., those of the Purkinje system or midmyocardium) seem especially susceptible to these changes. At a substrate level, the links on the chain are alterations in the time course of the action potential, alteration in the propagation of action potentials within a given cell, and alterations in the propagation of action potentials from cell to cell within syncitia and from tissue to tissue within the heart. At a higher level of aggregation, one sees "afterdepolarizations" in the terminal portion of the action potential, spontaneous beats triggered by afterdepolarizations, propagation of these beats to other cells, and reentrant excitation.

With these considerations in mind, the problem of altered repolarization should be integrated into drug development by (Malik and Camm, 2001):

[1] Some QT-prolonging drugs [e.g., amiodarone; see Hohnloser et al. (1994)] are not reported to have caused many arrhythmic deaths, but this observation must be interpreted carefully. In a population with a high incidence of life-threatening arrhythmias, a drug with both proarrhythmic and antiarrhythmic effects might cause a net reduction in arrhythmias, and the arrhythmias that it had induced might not be attributed to it. In a population whose native arrhythmias were not life threatening, the same drug might result in a net decrease in mortality.

- In vitro screening of the drug and its metabolites for effects on ion channels (especially I_{Kr})
- In vitro screening of the drug and its metabolites for effects on action potential duration
- Screening of the drug and its metabolites for altered repolarization in animal models
- Focused preclinical studies for proarrhythmia if altered repolarization is seen in preclinical screening or in patients

Some specific techniques which can be employed are as follows:

1. *Cloned Human Potassium Channels* Assessment of effects on cloned human ether-á-go-go (hERG) K^+ channels stably expressed in a cell line by measurement of whole-cell K current (I_{Kr}) using a voltage clamp. Other cloned human ion channels (e.g., KvLQT1/minK-IKs currents) are also possible.
2. *Cardiac Action Potential In Vitro—Purkinje Fibers* Intracellular recording of action potentials from cardiac Purkinje fibers isolated from dog or sheep ventricle. Measurement of maximum rate of depolarization and action potential duration to detect sodium and potassium channel interactions, respectively, according to recommendations in EMA Points to Consider document CPMP 986/96 (1997).
3. *Monophasic Action Potential in Anesthetized Guinea Pigs* Epicardia monophasic action potential recording using suction/contact pressure electrodes according to Carlsson et al. (1997). Simultaneous measurement of ECG.
4. *ECG by Telemetry in Conscious Guinea Pigs* Lead II ECG recording using DataSciences telemetry device. Repeated administration and interaction studies can be performed.
5. *Hemodynamics and ECG in Anesthetized or Conscious Dogs or Primates*
   - Conscious studies using DataSciences telemetry for blood pressure and lead II ECG or the ITS system for blood pressure and six-chest-lead ECG measurements (including QT dispersion)
   - Anaesthetized studies using MI^2 data capture system with additional measurement of blood flow in selected vascular beds, cardiac output, respiratory, and left ventricular function

16.4.7 Relevance of hERG to QT Prolongation

Compounds which are associated with ADRs of QT prolongation, arrhythmias such as torsade de pointes, and sudden death predominantly have a secondary pharmacological interaction with the rapidly activating delayed rectifier potassium channel I_{Kr}. The gene encoding this channel has been identified as *hERG*.

Testing of compounds for interactions with the hERG channel allows the identification of the potential risk of QT prolongation in humans and can be used as a screen in development candidate selection.

Expression and Recording Systems HEK-293 cells have been transfected with cDNA for hERG-1 to produce a stable expression system. The cell line has been obtained under license for the laboratory of Craig January at the University of Wisconsin (Mohammad et al., 1997).

16.5 CENTRAL NERVOUS SYSTEM

The primary screening tool for CNS safety pharmacology evaluation is the functional observational screen, which seeks to use objective but noninvasive methods for evaluating the pharmacological effects of a drug on peripheral and nervous system effects.

Initially, the starting basis for such screens were the Irwin screen (Irwin, 1968), used to screen for effects in mice and still one of the ICH-designated primary screens for fulfilling the regulatory requirements for S7 evaluation of new drugs.

More commonly used is the rat functional observational batter, initially developed by Gad (1982) and subsequently further modified (Haggerty, 1991; Mattson et al., 1996). Moscardo et al. (2007) present an excellent description of the rodent functional observation battery (FOB) as currently performed in the pharmaceutical industry. Other modifications/versions which are included under the ICH screening guidelines cover the use of the dog (Gad et al., 2003).

The neurobehavioral screens which meet the primary regulatory requirement for CNS safety pharmacology evaluation of necessity actually evaluate both central and peripheral nervous system function. Such evaluations used, to the maximum extent possible, semiquantitative evaluations of a wide range of endpoints which serve to determine if there are effects on the primary functional domains of the CNS. The methods are noninvasive and use basic instruments to get quantitative data where possible—an electronic thermometer (to measure rectal/core body temperature), a strain gauge with T-bar animal grip (to measure forelimb grip strength), a sand table (to measure hindlimb splay), an activity stage (to measure locomotor activity), and a rotarod (to evaluate motor coordination).

The only other required equipment usually includes a dicher for auditory startle, a pen light (for pupil response), and a blunt probe (for various touch-based reflexes). The complete screen should be performed at at least two (and preferably three) time intervals after a single dose of the drug.

There are four broad classes of approaches to any subsequent and more detailed assessment of nervous system effects of drugs in animals.

16.5.1 Isolated Tissue Assays

The classic approach to screening for nervous system effect is a series of isolated tissue preparation bioassays conducted with appropriate standards to determine if the material acts pharmacologically directly on neural receptor sites or transmission properties. Though these bioassays are normally performed by a classical pharmacologist, a good technician can be trained to conduct them. The required equipment consists of a Mangus (or similar style) tissue bath (Turner, 1965; Offermeier and Ariens, 1966; Nodine and Seigler, 1964), physiograph or kymograph, force transducer, glassware, stimulator, and bench spectrophotometer. The assays utilized in the screening battery are listed in Table 16.6 along with the original reference describing each preparation and assay. The assays are performed per the original author's descriptions with only minor modifications, except that control standards (as listed in Table 16.6) are always used. Only those assays that are appropriate for the neurological/muscular alterations observed in the screen are utilized. Note that all these are intact organ preparations, not minced tissue preparations as others (Bondy, 1979) have recommended for biochemical assays.

The first modification in each assay is that, where available, both positive and negative standard controls (pharmacological agonists and antagonists, respectively) are employed. Before the preparation is utilized to assay the test material, the tissue preparation is exposed to the agonist to ensure that the preparation is functional and to provide a baseline dose–response curve against which the activity of the test material can be quantitatively compared. After the test material has been assayed (if a dose–response curve has been generated), one can determine whether the antagonist will selectively block the activity of the test material. If so, specific activity at that receptor can be considered as established. In this assay sequence, it must be kept in mind that a test material may act to either stimulate or depress activity, and therefore the roles of the standard agonists and antagonists may be reversed.

Commonly overlooked when performing these assays is the possibility of metabolism to an active form that can be assessed in this in vitro model. The test material should be tested in both original and "metabolized" forms. The metabolized form is prepared by incubating a 5% solution (in aerated Tyrodes) or other appropriate physiological salt solution with strips of suitably prepared test species liver for 30 min. A filtered supernatant is then collected from this incubation and tested for activity. Suitable metabolic blanks should also be tested.

16.5.2 Electrophysiology Methods

There are a number of electrophysiological techniques available which can be used to detect and/or assess neurotoxicity. These techniques can be divided into two broad general categories: those focused on CNS function and those focused on peripheral nervous system function (Seppalainen, 1975).

TABLE 16.6 Isolated Tissue Pharmacological Assays

Assay System	Endpoint	Standards (Agonist/ Antagonist)	References
Rat ileum	General activity	None (side-spectrum assay for intrinsic activity)	Domer, 1971
Guinea pig vas deferens	Muscarinic nicotinic or muscarinic	Methacholine/atropine Methacholine/ hexamethonium Methacholine/atropine	Leach, 1956
Rat serosal strip	Nicotinic	Methacholine/hexamethonium	Khayyal et al., 1974
Rat vas deferens	Alpha adrenergic	Norepinephrine/ phenoxybenzamine	Rossum, 1965
Rat uterus	Beta adrenergic	Epinephrine/propranol	Levy and Tozzi, 1963
Rat uterus	Kinin receptors	Bradykinin/none	Gecse et al., 1976
Guinea pig tracheal chain	Dopaminergic	Dopamine/none	Domer, 1971
Rat serosal strips	Tryptaminergic	5-Hydroxytryptamine (serotonin)/dibenzyline or lysergic acid dibromide	Lin and Yeoh, 1965
Guinea pig tracheal chain	Histaminergic	Histamine/benadryl	Castillo and De Beer, 1947a,b
Guinea pig ileum (electrically stimulated)	Endorphin receptors	Methenkephaline/none	Cox et al., 1975
Red blood cell hemolysis	Membrane stabilization	Chloropromazine (not a receptor-mediated activity)	Seeman and Weinstein, 1966
Frog rectus abdominis	Membrane depolarization	Decamethonium iodide (not a receptor-mediated activity)	Burns and Paton, 1951

First, however, the function of the individual components of the nervous system, how they are connected together, and how they operate as a complete system should be very briefly overviewed.

Data collection and communication in the nervous system occur by means of graded potentials, action potentials, and synaptic coupling of neurons. These electrical potentials may be recorded and analyzed at two different levels depending on the electrical coupling arrangements: individual cells (i.e., intracellular and extracellular) or multiple cells [e.g., electroencephalogram (EEG), evoked potentials (EPs), slow potentials]. These potentials may be recorded in specific central or peripheral nervous system areas (e.g., visual cortex, hippocampus, sensory and motor nerves, muscle spindles) during various behavioral states or in in vitro preparations (e.g., nerve muscle, retinal photoreceptor, brain slice).

16.5.3 CNS Function: Electroencephalography

The EEG is a dynamic measure reflecting the instantaneous integrated synaptic activity of the CNS, which most probably represents, in coded form, all ongoing processes under higher nervous control. Changes in frequency, amplitude, variability, and pattern of the EEG are thought to be directly related to underlying biochemical changes, which are believed to be directly related to defined aspects of behavior. Therefore, changes in the EEG should be reflected by alterations in behavior and vice versa.

The human EEG is easily recorded and readily quantified, is obtained noninvasively (scalp recording), samples several regions of the brain simultaneously, requires minimal cooperation from the subject, and is minimally influenced by prior testing. Therefore, it is a very useful and recommended clinical test in cases in which exposure to drugs produces symptoms of CNS involvement and in which long-term exposures to high concentrations are suspected of causing CNS damage.

Since the EEG recorded using scalp electrodes is an average of the multiple activity of many small areas of cortical surface beneath the electrodes, it is possible that in situations involving noncortical lesions the EEG may not accurately reflect the organic brain damage present. Noncortical lesions following acute or long-term low-level exposures to toxicants are well documented in neurotoxicology (Norton, 1980). The drawback mentioned earlier can be partially overcome by utilizing activation or evocative techniques, such as hyperventilation, photic stimulation, or sleep, which can increase the amount of information gleaned from a standard EEG.

As a research tool, the utility of the EEG lies in the fact that it reflects instantaneous changes in the state of the CNS. The pattern can thus be used to monitor the sleep–wake cycle activation or deactivation of the brainstem and the state of anesthesia during an acute electrophysiological procedure. Another advantage of the EEG, which is shared by all CNS electrophysiological techniques, is that it can assess the differential effects of toxicants (or drugs) on various brain areas or structures. Finally, specific CNS regions (e.g., the hippocampus) have particular patterns of afterdischarge following chemical or electrical stimulation which can be quantitatively examined and utilized as a tool in neurotoxicology.

The EEG does have some disadvantages or, more correctly, some limitations. It cannot provide information about the effects of toxicants on the integrity of sensory receptors or of sensory or motor pathways. As a corollary, it cannot provide an assessment of the effects of toxicants on sensory system capacities. Finally, the EEG does not provide specific information at the cellular level and therefore lacks the rigor to provide detailed mechanisms of action.

Rats represent an excellent model for this as they are cheap, resist infection during chronic electrode and cannulae implantation, and are relatively easy to train so that behavioral assessments can be made concurrently.

Depending on the time of drug exposure, the type of scientific information desired, and the necessity of behavioral correlations, a researcher can perform acute and/or chronic EEG experiments. Limitations of the former are that most drugs that produce general anesthesia modify the pattern of EEG activity and thus can complicate subtle effects of toxicants. However, this limitation can be partially avoided if the effect is robust enough. For sleep–wake studies, it is also essential to monitor and record the electromyogram (EMG).

Excellent reviews of these electrophysiological approaches can be found in Fox et al. (1982) and Takeuchi and Koike (1985).

16.5.4 Neurochemical and Biochemical Assays

Though some very elegant methods are now available to study the biochemistry of the brain and nervous system, none has yet discovered any generalized marker chemicals which will serve as reliable indicators or early warnings of neurotoxic actions or potential actions. There are, however, some useful methods. Before looking at these, however, one should understand the basic problems involved.

Normal biochemical events surrounding the maintenance and functions of the nervous system centers around energy metabolism, biosynthesis of macromolecules, and neurotransmitter synthesis, storage, release, uptake, and degradation. Measurement of these events is complicated by the sequenced nature of the components of the nervous system and the transient and labile nature of the moieties involved. Use of measurements of alternations in functionality as indicators of neurotoxicity is further complicated by our lack of a complete understanding of the normal operation of these systems and the multitude of day-to-day occurrences (such as diurnal cycle, diet, temperature, age, sex, and endocrine status) which are constantly modulating the baseline system. For detailed discussions of these difficulties, the reader is advised to see Johnson (1975) and Damstra and Bondy (1980, 1982).

16.6 RESPIRATORY/PULMONARY SYSTEM

The known effects of drugs from a variety of pharmacological/therapeutic classes on the respiratory system and worldwide regulatory requirements support the need for conducting respiratory evaluations in safety pharmacology. The objective of the studies is to evaluate the potential for drugs to cause secondary pharmacological or toxicological effects that influence respiratory function. Changes in respiratory function can result from either alterations in the pumping apparatus that controls the pattern of pulmonary ventilation or changes in the mechanical properties of the lung that determine the transpulmonary pressures (work) required for lung inflation and deflation. Defects in the pumping apparatus are classified as hypo- or hyperventilation syndromes and are evaluated by examining ventilatory parameters in a conscious animal

model. The ventilatory parameters include respiratory rate, tidal volume, minute volume, peak (or mean) inspiratory flow, peak (or mean) expiratory flow, and fractional inspiratory time. Defects in the mechanical properties of the lung are classified as obstructive or restrictive disorders and can be evaluated in animal models by performing flow–volume and pressure–volume maneuvers, respectively. The parameters used to detect airway obstruction include peak expiratory flow, forced expiratory flow at 25 and 75% of forced vital capacity, and a timed forced expiratory volume, while the parameters used to detect lung restriction include total lung capacity, inspiratory capacity, functional residual capacity, and compliance. Measurement of dynamic lung resistance and compliance, obtained continuously during tidal breathing, is an alternative method for evaluating obstructive and restrictive disorders, respectively, and is used when the response to drug treatment is expected to be immediate (within minutes postdose). The species used in the safety pharmacology studies conducted in our laboratory are the same as those used in toxicology studies since pharmacokinetic and toxicological/pathological data are available in these species. These data can be used to help select test measurement intervals and doses and to aid in the interpretation of functional change. The techniques and procedures for measuring respiratory function parameters are well established in guinea pigs, rats, and dogs (Murphy, 1994):

Respiration and Pulmonary Function in Rats

Study design includes three doses and controls with eight animals per group and must be GLP compliant and a single dose by oral (depending on clinical route of administration) gavage.

Evaluations include respiratory rate, peak inspiratory flow, peak expiratory flow, inspiration time, expiration time, tidal volume, airway resistance, and blood oxygenation.

16.7 SECONDARY ORGAN SYSTEM

The kidneys are an important target for toxic effects of drug candidates. It is mandatory to select accurate, clinically relevant parameters in order to be in a position to detect putative nephrotoxic effects during the safety pharmacology program. The glomurelar filtration rate appears to be of major interest since it is associated with the definition of acute renal failure. Measurement of, for example, renal blood flow, proteinuria, enzymuria, and fractional excretion of sodium is also highly useful to detect any possible renal impact of a new compound. Although the rat is, by far, the most widely used animal species, there are no specific (clinically relevant) reasons to choose it. Various parameters may vary according to the species, sex, strain, age, and so on. Since in most cases acute renal failure occurs following administration of drugs in patients with preexisting risk factors, it is suggested that sensitized animal

models be validated and used (salt depletion, dehydration, coadministration of pharmacological agents, etc.).

The potential effects of new drugs on the digestive system can be examined in a number of model systems of which intestinal motility in the mouse and/or gastric emptying in the rat are examples recommended for safety pharmacology evaluation. Intestinal motility, assessed by the transit of carmine dye in the mouse, and gastric motility, assessed by stomach weight in the rat, were examined using a range of clinical drugs or potent pharmacological agents known to affect gastrointestinal function. Assessment of both models in the guinea pig was also evaluated. Activity was demonstrated with codeine, diazepam, atropine, and CCK-8 (all of which inhibited gastric function). However, neither model gave consistent and reliable results with the remaining reference compounds, namely metoclopramide, bethanechol, cisapride, deoxycholate, carbachol, and domperidone. In conclusion, this investigation questions the usefulness of simple models of gastrointestinal transport in the rodent as a means of detecting potential effects of a new drug on the digestive system. This finding should be of concern to the pharmaceutical industry as these simple models are routinely used as part of a regulatory safety pharmacology "package" of studies.

A number of classic assays have been designed to examine the effects of a test article on gastrointestinal function. Gastrointestinal transit rate is most often measured with a test employing a forced meal of an aqueous suspension of activated charcoal (Janssen and Jageneau, 1957). The test article is given via the appropriate route at a preset time prior to the charcoal meal. For example, a compound intended for use via intravenous injection would be injected intravenously in mice 30 min prior to delivering a charcoal meal by gavage. The distance traveled from the stomach by the black-colored charcoal meal to a specific anatomic location within the intestine is measured at a fixed time after this meal, usually 20 or 30 min later. In validating this procedure at Mason Laboratories, we tested the ability of a parasympatholytic agent, intravenous atropine sulfate, to inhibit gastrointestinal transit. In a dose-dependent fashion, 30 and 50 mg kg^{-1} atropine sulfate significantly decreased the distance traveled by the charcoal meal.

Another important safety assay of the gastrointestinal system is the influence of test article on the formation of ulcers (Shay et al., 1945). After overnight fasting, young rats are given the test article and euthanized 4 or 6 h later. The mucosal surface of the stomach and duodenum is scored for the presence of hyperemia, hemorrhage, and ulcers. The dose-dependent ulcerative properties of nonsteroidal anti-inflammatory drugs (NSAIDs) are clearly demonstrated in this assay, making it important in the development of other NSAIDs that are not as caustic to the gastrointestinal mucosa (Bramm et al., 1981; Cashin et al., 1977; Diadone et al., 1994; Darias et al., 1994).

Additional digestive system safety pharmacology tests include effects of test articles on gastric emptying rate and gastric secretion. Gastric emptying rate is measured in rats using a solution of phenol red (or Evans blue) deliv-

ered via oral gavage a preset time after administration of the test article (Megens et al., 1991). The dilution of phenol red after 30 min in the rat's stomach is determined colorimetrically at 558 nm in a spectrophotometer. This is compared to a group of control rats that are euthanized immediately after phenol red administration. The influence of test articles on gastric juice secretion is accomplished by ligating the pyloric sphincter under anesthesia in rats following a fasting period (Graf et al., 1982; Shay et al., 1945; Takasuna et al., 1992). Immediately after recovery from anesthesia, each rat is given a preset dose of the test article. The fluid content of the rat's stomach is recovered after a set period of time, usually 4 h. The volume and contents of the stomach are measured to determine the effect of the test article on gastric secretions. Electrolyte concentrations, pH, and protein content of gastric secretions can be measured in this assay (Takasuna et al., 1992).

16.7.1 Gastric Emptying Rate and Gastric pH Changes: New Model

Sometimes new technologies for safety pharmacology can come from clinical settings. The Heidelberg pH capsule (HC) was developed over 30 years ago at Heidelberg University in West Germany. H. G. Noller invented and first tested this device on over 10,000 adult patients over a three-year period. The HC is a pill-sized device containing an antimony–silver chloride electrode for measuring pH and a high-frequency transmitter operating at an average frequency of 1.9 MHz. The transmitter in the HC is activated by immersion in physiological saline by a permeable membrane enclosing the battery compartment. Thus, when a patient swallows the HC, the fluid contents of the stomach activate the transmitter. Transmitted signals are picked up via a belt receiver and can be displayed and recorded. The profile of changes in pH over time correlate with the movement of the HC through the different regions of the gastrointestinal tract (Mojaverian et al., 1989). The pH of the fasted human stomach is very acidic, on average about pH 1. When the HC moves through the pyloric sphincter and into the duodenum, there is a rapid increase in pH of over 4 pH units. Thus, one can get a fairly precise measure of gastric emptying rate in humans with this noninvasive technique. Additional pH changes have been correlated with transition of the HC through the duodenum, jejunum, and colon.

Mojaverian and colleagues (1989, 1991) have used the HC extensively to examine the influence of gender, posture, age, and content and frequency of food ingestion on the gastric emptying rate (or gastric residence time) in healthy volunteers. While developed for clinical use in people, the HC may be a useful tool for measuring important digestive system parameters in laboratory animals. The size of the HC, approximately the size of a No. 1 gelatin capsule (7 mm diameter, 20 mm long), prohibits its use in small animals (Mojaverian et al., 1989). It may be useful in studies with dogs and possibly in nonhuman primates. In particular, the HC could be used to measure gastric emptying rate in a totally noninvasive manner in dogs (Itoh et al., 1986). Dogs

are readily trainable to accept pills and to wear a receiver belt and could be tested after administration of a test compound (Lui et al., 1986; Vashi and Meyer, 1988). This technique for measuring gastric emptying rate in dogs is also advantageous in that it is not a terminal procedure. The influence of test articles on the pH within different portions of the gastrointestinal system could also be measured with the HC (Youngberg et al., 1985). The major drawback for using the HC for safety pharmacology screening is the price of the capsules and the receiver system.

16.8 SUMMARY

The initiative to all mandated safety pharmacology studies to the drug development process is overdue in arriving. However, its actual implementation and the use of the resulting data in risk–benefit decision will take some time to be worked out.

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17

Special Concerns for Preclinical Evaluation of Biotechnology Products

Although many assume that biotechnology is a recent concept, the application of this science has been understood for many years and utilized in its simplest form in the fermentation of beer, wine, and bread by microbial agents. Modern biotechnology can be divided into three research and development areas: recombinant DNA technology (rDNA), monoclonal antibody technology, and bioprocess technology (Mackett, 1993; Malinowski, 1999). The commercialization of these three processes is based on the premise that biotechnology can cost-effectively produce large quantities of a highly purified product.

Biotechnology as a promising source of new and more efficient source of more targeted therapeutics has been with us since the mid-1980s (Maulik and Patel, 1997). While (as one should expect) some of the early promises have not quite been met, biotechnology has turned out to be a valuable source of new and valuable therapeutics and currently accounts for one-third of all new therapeutics entering the marketplace. Table 17.1 lists the proteins approved in 2007 that have more than a billion dollars a year in global sales.

Protein and other biotechnology-derived therapeutics have some fundamental differences from traditional small (synthetic organic) molecules and so require modified or different approaches to characterize their toxicity and evaluate their safety. Table 17.2 presents a comparative summary of these differences.

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TABLE 17.1 Blockbuster Biotechnology Approvals (2007)

Company	Product	Indication	2007 Approval	Status	Peak Sales Potential ($ billions)
Cephalon	Nuvigil (amordafinil)	Daytime sleepiness	Approved	Approved June 18	$1–2
Onyx/Bayer	Nexavar (sorafenib)	Hepatocellular carcinoma	Likely	PDUFA* Dec. 28	$1+
ImClone	Erbitux (cetuximab)	Refractory colorectal cancer (survival claim)	Approved	Approved Oct. 2	$1
Genzyme	Renvela (sevelamer carbonate)	Serum phosphorus control in dialysis patients with chronic kidney disease	Approved	Approved Oct. 21	$1
Alexion	Soliris (eculizumab)	Paroxysmal nocturnal hemoglobinuria	Approved	Approved Mar. 19	$0.5–1+
Speedel/ Novartus	Tekturna	Hypertension	Approved	Approved Mar. 6	$0.5–1

*Prescription Drug User Fee Act.

TABLE 17.2 Comparison of Protein Therapeutic Agents with Small-Molecule Drugs

Parameter	Proteins	Small Molecules
Drug substance	Heterogeneous mixture; broad specifications during development; specifications may change during development	Single entity; high chemical purity; exception: racemic mixtures; specifications well defined early in development
Drug product	Usually intravenously or subcutaneously	Generally oral; few formulations during development
Impurities	Difficult to standardize	Purity standards well established
Bridging requirements	Significant for drug substance	Bioequivalence procedures
Biological activity	May mimic naturally occurring molecules; primary mechanism of toxicity; predictive based on mechanism	Less predictive
Nonspecificity	Variable significance	Usually significant; drug–drug interactions
Chronic toxicity	Lack of models because of species-determined biological specificity and antigenicity	Models sometimes relevant
Impurities	Toxicity not a major issue; may impact immunogenicity	May be significant; purity standards well established

Among all the other aspects of increasing understanding of what is involved in the evaluation and development of biologically derived therapeutics has been a very real evolution of what is needed to evaluate the safety of these products. In the beginning, there was a stark duality of expectations. On one

side, early advocates of biotechnology held that there were unlikely to be any safety concerns other than those due to hyperpharmacology overactivity at the target receptors (Thomas and Myers, 1998; Weissinger, 1989; Vallbracht et al., 1982) and contamination (such as occurred with the Cutter product early on in the history of the polio vaccine; Offit, 2005). On the other hand, there were those that cautioned against the possibility of extreme and unforeseeable toxicities. The truth, as is usually the case, has turned out to be in between. We have come to understand that the toxicity of protein moieties primarily arises from either overexpression of their desired therapeutic effects (i.e., largely disordering of the immune system) such as seen with interferons and interleukins (Fent and Zbinden, 1997) or "off-target effects" that are at other than the intended receptor. Additionally, there are cases of immune responses to therapeutics and of antibody neutralization products (Weissinger, 1989; Vallbracht et al., 1982).

The principal purposes of preclinical safety evaluation in this context remain:

(a) To detect harmful (toxic) effects
(b) To exclude other potentially harmful effects
(c) To determine their relationship to dose and duration of treatment
(d) If possible to discover their mechanism or at least pathogenesis

The information from (a)–(d) should be used to predict possible adverse actions in the target species in order to:

- Warn clinicians about unacceptable risks
- Warn clinicians about risks that should be monitored
- Remind clinicians and others of the possibility of toxic effects not detected because they could not be displayed by the test systems used (e.g., headache in a nonhuman species or carcinogenicity in a one-month experiment) or were not sought.

In addition, the toxicologist as a general biological scientist should always be alert to physiological and pharmacological effects manifested in his or her experiments because they may illuminate mechanisms of health and disease of both academic and practical importance (Folb, 2006). Table 17.3 points out the common differences between use and large-molecule safety assessment programs.

The objectives of the preclinical safety studies on biologically derived therapeutics are to identify the pharmacological and toxicological effects that are likely to be encountered throughout the course of clinical development and beyond. The selection and design of such studies should first consider what may be known about other products which are structurally and/or

TABLE 17.3 Differences between Chronic Use Nonclinical Safety Assessment Plans for Large and Small Molecules

Biopharmaceuticals	Small-molecule Pharmaceuticals
• "Case-by-case" approach to nonclinical safety evaluation • Pharmacology used to select species • Single species common for repeat-dose studies beyond 28 days • Dosing sequences may not be daily • Immunogenicity important • Genetic toxicology not required • Metabolism studies not appropriate • Longest repeat dose typically 6 months nonrodent • Two-year carcinogenicity rare • Developmental toxicity in relevant species may be required	• Similar nonclinical safety studies for most products • Metabolism used to select species • Rodent and nonrodent typical • Dosing daily unless justified • Generally not immunogenic • Genetic toxicology required • Metabolism studies required • Nonrodent may be up to 12 months • Two-year carcinogenicity required • Developmental toxicity in two species required

pharmacologically similar. The program and study design should then proceed to consider:

1. Intended manner of use, including dose, route of administration, and particulars of dosing regimen
2. Age of intended patient population
3. Selection of relevant model species
4. Stability of formulated drug substance under conditions of use
5. Physiological (disease) state of intended patients

In the area of bioengineered products, many of which are complex proteins of potent but sparsely studied activities in living systems, the investigative responsibilities of the toxicologist are likely to be very important because he or she may be the first observer able to study the effects of repeated administration of a range of doses on a living system (Griffiths and Lumley, 1998). It is now possible to frame a classification of the types of biologically derived therapeutic products (Table 17.4).

Each type of product has some specific considerations. The range of materials is enormous. The deciding factors for the toxicologist should be the precision with which the material can be characterized by physicochemical means, as that should be inversely related to the burden of repeated biological testing necessary to assure safety, and the extent of prior knowledge of its biological properties. The greater our ignorance of the latter, the more searching should the toxicologist's studies be in order to discern the biological (pharmacological) properties of the substance. Exposure of the individual must also be considered, as different criteria may apply to deliberate administration of a living organism, which could spread in the community.

TABLE 17.4 Classification of Bioengineered Products on Practical Grounds

Type	Bioengineering Involvement	Pharmacological Properties	PhysicoChemical Characterization	Example
1. Low-molecular-weight substance	New production route	Well known	Rigorous	Amino acid 6-APA
2. High-molecular-weight substance	New production route	Fairly well known	Extensive	Human hormones, e.g., HGH, hPTH (humanized parathyroid hormone)
3a. Endogenous high-molecular-weight substance	First ever production	Some knowledge	Moderate	IFN
3b. Endogenous high-molecular-weight substance	First ever production, perhaps gene splicing to make hybrid molecule	Scanty to limited knowledge	Moderate	Other lymphokines, tumor necrosis factor, etc.
4. Engineered antigen	Partly or totally synthetic antigen + rDNA production	Probably predictable	Rigorous	Synthetic vaccine for poliomyelitis or hepatitis B
5. Monoclonal antibody (or component)	Hybridoma human cell line	Probably predictable antigenicity	Moderate	Antitumor antibody for imaging anti-idiotype antibody as vaccine
6. Living organism	Removal of pathogenicity by genetic manipulation	Uncertain	Limited	As vaccine immunogen, e.g., *Salmonella typhimurium* TY21a, modified herpes, or to carry antigen, e.g., vaccinia

As in any safety evaluation, the planned work should be related to the intended use and treatment of humans, for example, one dose in a few gravely ill patients or multiple doses of the entire healthy community as prophylaxis against a trivial condition. Contrast, say, what might be appropriate for tumor necrosis factor (TNF), as in an experimental trial in a few sufferers from late-stage cancer, with the requirements for a candidate vaccine against dental caries to be widely administered to healthy children.

Because of the rapid development of new biotechnology products, toxicology and safety assessment departments at most chemical or pharmaceutical companies are presently or soon will be confronted with the development of testing protocols for the safety evaluation of rDNA products. Routine

toxicology assessment as performed in the past using standard protocols may not apply and may in fact represent unnecessary or inappropriate studies. Because of the relatively nontoxic nature and species specificity of many of the new biotechnology products, less evaluation in rodent species may be required than for some of the chemicals of the past. What is needed in dealing with the products of this new technology is the rethinking of traditional toxicology-testing approaches.

17.1 REGULATION

The regulation of biologically derived therapeutics actually has a long history and has also continued to evolve (see Table 17.5). This history led to the Public Health Service (PHS) Act providing a somewhat mixed description of the products under its authority, which in turn serves to define biologicals for the U.S. Food and Drug Administration (FDA) Center for Biologics Evaluation and Research (CBER): "[A biologic is] any virus, therapeutic serum, toxin, antitoxin, vaccine, blood, blood component or derivative, allergenic product, or analogous product, or arsphenamine or its derivative (or any other trivalent organic arsenic compound), applicable to the prevention, treatment, or cure of disease, or injuries in man. …" The Code of Federal Regulations (CFR), Title 21, Section 600.3 then goes on to state: "(h) Biological product means any virus, therapeutic serum, toxin, antitoxin, or analogous product applicable to the prevention, treatment or cure of diseases or injuries in man." Confusion

TABLE 17.5 Historical Perspectives of Biological Therapeutics Regulation

1902	Federal Virus, Serum and Toxin Act / PHS (after tetanus-contaminated diphtheria antitoxin led to deaths of 10 children); intent was to ensure safety, purity, and potency
1906	Pure Food and Drugs Act (Upton Sinclair's *The Jungle*)
1937	Division of Biologics Control/NIH
1938	FD&C Act (sulfanilamide elixir), biologicals exempt!
1955	Division of Biological Standards Established (poliovirus)
1962	FD&C Amendments (thalidomide)
1972	Bureau of Biologics / FDA
1978	Good manufacturing practices regulations
1979	Good laboratory practices regulations
1982	Bureau of Biologics merged with Bureau of Drugs ⇒ National Center for Drugs and Biologics
1983	Orphan Drug Act
1984	Drug Price Competition and Patent Term Restoration Act
1992	User fees
1997	ICH harmonized guidelines
2002	Responsibility for regulation of biological substances divided between CDER and CBER

of authority and responsibility between the three human health product centers of the FDA [Center for Drug Evaluation and Research (CDER), CBER, and Center for Devices and Radiological Health (CDRH)] led in 1992 to the promulgation of three intercenter agreements. The agreement between CDER and CBER states that the following biological products require licensure and come under the CBER's authority (Mathieu, 1997):

- Vaccines, regardless of the method of manufacture (vaccines were defined as agents administered for the purpose of eliciting an antigen-specific cellular or humoral response) (CBER, 1997)
- In vivo diagnostic allergenic products and allergens intended for use as "hyposensitization agents"
- Human blood or human blood–derived products, including placental blood–derived products, animal-derived procoagulant products, and animal- or cell culture-derived hemoglobin-based products intended to act as red blood cell substitutes
- Immunoglobulin products
- Products composed of or intended to contain intact cells or intact microorganisms
- Proteins, peptides, or carbohydrate products produced by cell culture, excluding antibiotics, hormones, and products previously derived from human or animal tissue regulated as drugs
- Protein products made in animal body fluid by genetic alteration of transgenic animals, animal venoms, or constituents of venoms

Other classes of products identified as CBER-regulated products include:

- Synthetically produced allergenic products intended to specifically alter the immune response to a specific antigen or allergen
- Certain drugs used in conjunction with blood banking or transfusion

As will be seen at the end of this chapter in the discussion of gene therapy products [where the National Institutes of Health (NIH) also has some regulatory role], there is still some ambiguity of authority in areas where technology has outrun regulatory foresight.

Within the United States, the regulation of therapeutics is split on relatively arbitrary grounds. This is presented in Table 2.5. This chapter reflects both current FDA practices and the International Conference on Harmonisation (ICH) guidelines. European Medicines Agency (EMEA) guidances started with that of the Committee on Proprietary Medicinal Products (CPMP, 1989) but subsequently has come from the EMEA. Most recently, subsequent to the TGN1412 near disaster, the EMEA has issued new guidances meant to issue a more conservative approach to clinical trials (EMEA, 2007).

17.2 PRECLINICAL SAFETY ASSESSMENT

Because of the complexity and wide diversity of biological products, their safety is evaluated on a case-by-case basis (in accordance with CBER's promulgated points to consider as summarized in Table 17.6) until such time as enough data on either specific products or a class of products are available.

Generally, in vivo nonclinical studies should be designed to include a sufficient number of animals per group to permit a valid estimation of a drug's toxicological and pharmacological effects in terms of incidence, severity, and the dose–response relationships involved. The latter point requires, as pointed

TABLE 17.6 Points to Consider in Preclinical Safety Assessment of Biologicals

Rationale
- In vitro or in vivo studies
- Potency assays
- Receptor characteristics (across species)
- Physiological modeling
- Scientific literature
- Scientific speculation

Indication
- Replacement therapy (long term)
- Nonpharmacodynamic treatment (prophylactic or diagnostic)
- Pharmacodynamic treatment (short or long term)

Pharmacological activity (pharmacodynamics)
- Primary endpoints
- Secondary endpoints

In vivo model selection
- Species-specific effects
- Effects independent of species
- Animal model of disease

Pharmacokinetics and absorption, distribution, metabolism, and excretion (ADME): correlation with pharmacodynamics
- Low dose
- High dose

General toxicity
- Single dose (acute)
- Repeated dose (subacute or subchronic)

Specific toxicity (may include one or more of the following studies)
- Local irritation (local reactogenicity)
- Antigenicity
- Chronic toxicity
- Reproduction toxicity, including teratogenic potential
- Mutagenicity
- Tumorigenicity
- Carcinogenicity
- Other toxicity concerns (e.g., neurotoxicity, immunotoxicity)

out throughout this text, thoughtful selection of doses. Comparable formulation, routes and regimens of administration, duration of exposure, and suitable time to allow expression of expected response are also important proper design features. Tables 17.7–17.9 summarize the basic testing requirements for three subsets of biologically derived drugs.

TABLE 17.7 Biotechnology-Derived Drug Test Matrix

Test Requirement	Species
Initial clinical trial/investigational new drug (IND) requirement	
1. Acute toxicity in rodents (oral and IV[a])	R/M
2. Acute toxicity in nonrodents (oral)	D/S/P
3. Seven-day dose range finder (DRF) toxicity in rodents (oral)	R/M
4. Seven-day DRF toxicity in nonrodents (oral)	D/S/P
5. Genotoxicity only if appropriate (species cases)	
6. Safety pharmacology: cardiovascular in vivo	D/P/S
7. Safety pharmacology: respiratory—rodent	R
8. Pivotal/repeat dose in rodents (14–28 days oral)	R/M
9. Pivotal/repeat dose	D/P/S
10. Five species microsome metabolic panel[a]	In vitro
11. Develop bioanalytical for three species (human/rodent/nonrodent)	NA
12. Antibody-based assay to select appropriate species	
To support continued clinical development	
13. Immunotoxicity	TBD
14. Pivotal/repeat dose in rodents (3/6 months oral)[b]	R/M
15. Pivotal/repeat dose in nondents (3/9–12 months oral)	D/P/S
To support marketing approval	
16. Reproductive toxicity, segment I[b]	R
17. Reproductive toxicity, segment III[b]	R

Note: Species: R = rat, M = mouse, D = dog, S = pig, P = primate, B = rabbit; TBD = to be determined. All studies described must be performed with GLP.
[a]Recommended.
[b]May be required.

TABLE 17.8 Vaccine Test Matrix

Test Requirement	Species
Initial clinical trial/IND requirement	
1. Acute toxicity in rodents (oral and IV[a])	R/M
2. Acute toxicity in nonrodents (oral)	D/S/P
8. Pivotal/repeat dose in rodents (14–28 days oral)[b]	R/M
9. Pivotal/repeat dose in nonrodents (14–28 days oral)	D/P/S
To support continued clinical development and to support marketing approval	
13. Immunotoxicity	TBD

Note: Abbreviations as in Table 17.7. All studies described must be performed with GLP.
[a]Recommended.
[b]May be required.

TABLE 17.9 Biological Test Matrix

Test Requirment	Species
Initial clinical trial/IND requirement	
1. Acute toxicity in rodents (oral and IV[a])	R/M
2. Acute toxicity in nonrodents (oral)	D/S/P
3. Seven-day DRF toxicity in rodents (oral)	R/M
4. Seven-day DRF toxicity in nonrodents (oral)	D/S/P
5. Genotoxicity: bacterial mutagenicity (Ames)[b]	In vitro
6. Pivotal/repeat dose in rodents (28 days oral)	R/M
7. Pivotal/repeat dose in nonrodents (28 days oral)	D/P/S
8. Develop bioanalytical for three species (human/rodent/nonrodent)	NA
To support continued clinical development	
9. Developmental toxicity (segment II), rat and rabbit pilots and rat and rabbit studies[b]	R/B
10. Neoantigenicity	B

Note: Abbreviations as in Table 17.7. All studies described must be performed with GLP.
[a]Recommended.
[b]May be required.

The number of species necessary in preclinical testing programs varies. However, there is no specific requirement for the routine use of two species (e.g., one rodent and one nonrodent) in toxicology studies of biological products. Proper species selection for use in these trials is essential. Table 17.10 summarizes the key considerations for species selection.

In each stage of product development, it is important to determine exposure by measuring pharmacokinetic (including ADME) or pharmacodynamic endpoints. This includes the following: (1) measurements of the biological in plasma or target organs; (2) distribution and persistence of cells for cellular therapies; (3) measurements of viral shedding and recovery of certain values; (4) localization of targeted novel delivery systems; and (5) tissue tropism, including germ line tissue, of vectors used in gene therapies.

Such studies provide important information for a better interpretation of the toxicity observed in animals and aid in the selection of not only the proposed initial human dose but also the dose escalation scheme and the frequency of dosing in the clinical trial(s). Further, once such exposure data are available in humans, the data can be used to better correlate the human and animal findings. Toxicity studies should be performed in the same species used to assess exposure. Oftentimes, exposure and toxicity are measured in the same study, particularly when nonrodents are used.

Toxicity studies should be designed to identify not only a safe dose but also a toxic dose(s) to anticipate the product's safety and to better define the therapeutic index in humans. Specific product considerations that may complicate the process of defining a toxic dose may include limits based on

TABLE 17.10 Factors to Consider in Species Selection for Protein Therapeutic Development

Cross-Reactivity	Immunogenicity
• Cross-reactivity alone is not sufficient to identify a relevant species. Suitable affinity and potency to give valid results is also necessary. How close does the level of potency have to be to the situation in humans to be meaningful? • How can alternative approaches be "front loaded" into the development pathway without leading to a standard two-species approach? • What is the possibility of assigning greater value to nonconventional preclinical studies, such as genetically altered rodents and surrogate antibodies, that are scientifically relevant? • If, after a variety of cross-reactivity testing that includes binding studies, functional activity in cell-based systems, sequence homology, and tissue cross-reactivity studies, the only relevant species is chimpanzees, is it justified, scientifically and ethically, to use the chimpanzees to study the effects of the MAb?	• An emerging issue as more MAb is developed for chronic use is the impact of neutralizing antibodies on repeat-dose studies. This is a significant scientific problem that might be partially overcome by the use of the surrogate antibodies. What is the potential for the regulatory acceptability of surrogate antibodies for immunogenicity reasons. • To what extent is immunogenicity considered in species selection for safety and toxicology studies? Should this be given more priority in the selection of relevant species for long-term toxicity studies for MAb intended for chronic indications?

formulation, lack of significant systemic absorption, or the amount of the product available. The lack of significant toxicity in animals does not necessarily mean that the product is safe. The margin of safety for the initial starting dose, however, will likely be adequate.

Historically, the goal of acute toxicity studies was to define a lethal dose range following a single administration or the administration of a few closely spaced doses. More recently, these studies have been designed to evaluate a high dose that causes significant toxicity but not necessarily lethality. If deaths occur, rarely are such studies expected to provide sufficient information to determine the cause of death.

Studies are often one to two weeks in duration and routinely include body weight determinations, clinical observations, and gross necropsy findings. Additional antemortem studies may be performed as appropriate, especially in large animals (e.g., observation of local reactogenicity, pharmacokinetic evaluations, hematological, and/or clinical biochemistry measurements). Histological evaluations may also be performed.

The duration of repeat-dose studies should be at least as long as the proposed clinical study. These studies are designed to establish a dose–response relationship, define target organ(s) of toxicity, and determine whether observed toxicities are reversible. Evaluation parameters should include not only those routinely performed in the acute studies but also those performed in the additional studies as well. Special tests, such as ophthalmoscopic, electrocardiograph,

body temperature, and blood pressure monitoring, are often included. Depending on the study duration, sampling at multiple time points may be necessary to better characterize the kinetics of response. As mentioned, a group of animals will be examined at term, and some may be reserved for at treatment-free or recovery period to evaluate the reversibility of any findings.

Specific (local tissue tolerance) toxicity studies may be necessary due to special characteristics of the product or the clinical indication. Adjuvant vaccines are routinely evaluated for local (injection site) reactions, and cellular therapies are routinely screened for tumorigenic potential. Research is also needed to better predict the sensitizing potential of biological products and to determine the relevance of serum antibody levels following repeat dosing in animals and humans.

While carcinogenicity studies have not been performed routinely for biological products, they may be appropriate for products proposed for chronic use. Reproductive toxicology studies will probably become more common, especially as more women of child-bearing potential participate in early clinical trials. In the past, such studies have not been conducted for biologicals. Reproductive toxicology studies have been performed with many of the recently approved therapeutics (e.g., interferons, interleukins, cytokines, growth factors). Such studies also have been conducted in the development of AIDS vaccines intended for use in pregnant women. The standard protocol designs were modified to address specific vaccine-related concerns, including dosing in relationship to immunological effects.

The recent development of biologicals to treat various nervous system diseases has involved additional, specific neurotoxicological studies on these products. However, despite the fact that most products regulated as biologicals have an immune component or impact directly or indirectly on the immune system, standardized immunotoxicity tests that are potentially useful in screening large numbers of chemicals for their ability to adversely affect the immune system have not proven essential in assessing the safety of biological products.

Throughout the various phases of product development, additional preclinical safety studies may be necessary due to unexpected toxicity, significant changes in manufacturing process or the final formulation, or changes in clinical indications. In some cases, the ideal assessment of the safety of novel biological therapies may require alternative approaches, such as in vitro or in vivo organogenesis model systems, animal models of tolerance, animal models of disease, or transgenic animal models.

17.3 RECOMBINANT DNA TECHNOLOGY

The concept of recombinant DNA technology is based on the premise that a gene sentence may be taken from an animal or human gene responsible for the production of a particular protein and inserted into the DNA of

Escherichia coli, a single-cell bacterium. The bacterial cells then divide very rapidly, making billions of copies of themselves, including a replica of the gene that has been inserted.

There are unique ways to insert human genes into bacteria. In addition to chromosomal DNA, bacteria have numerous copies of extrachromosomal circular DNA called plasmids, which are not attached to the bacterial chromosome. These plasmids can be transferred from one bacterium to another by conjugation (e.g., mating) and can be isolated from bacteria and easily purified. Through use of restriction enzymes (i.e., a family of enzymes that can cut DNA at specific base sequences), the gene sentence to be inserted can be isolated and the plasmid DNA can be opened. While in the open state, the desired piece of animal or human DNA can be inserted. Through the use of ligase enzymes, complementary ends of the plasmid can be connected, thereby producing a recombinant plasmid recombined by joining two heterologous pieces of DNA. This recombinant plasmid can then be put back into the bacteria, and the bacteria will express the new gene function that has been inserted.

A unique characteristic of plasmids is that thousands are produced within each bacterium, to the point that up to 40% of the total DNA of the bacterium may in fact be plasmid DNA. Hence, a single piece of human DNA that heretofore could only be obtained in low concentrations can be recombined with a plasmid and the DNA sequence multiplied a million- or a billionfold (i.e., cloned). Use of cloning techniques may produce many grams of a particular human protein, instead of the few molecules that are produced in normal cells.

Examples of the early application of recombinant DNA technology in medicine are the development of recombinant human growth hormone; human insulin; human interferons (IFNs), thought to have anticancer activity in addition to antiviral activity; interleukins (regulatory proteins from lymphocytes that are believed to be important in the treatment of immunodeficiency diseases and cancer); tumor necrosis factor; epidermal and bone marrow progenitor cell growth factors; and the production of vaccines (Table 17.1).

Human growth hormone and insulin produced by rDNA technology are already registered with the FDA for therapeutic use. The applications of rDNA technology agriculture should improve the quality of domesticated animals through the production of new and improved vaccines, growth--promoting hormones, and less expensive food additives. Seed crops will be produced that offer improved yields and better resistance to environmental conditions. Further applications may include the insertion of genes into plants or bacteria for production of toxins that can act as biochemical pesticides or allelophatic agents (chemicals that act as natural herbicides to prevent the growth of other plant species in the same geographical area).

17.3.1 General Safety Issues

Recombinant DNA technology represents one of the most innovative achievements in biology in the last century. Although the new technology has

generated much enthusiasm for its potential applications, it has also raised concerns among both scientists and the public in general. Many of the early fears of the inadvertent development of an "Andromeda strain" during the genetic engineering of a specific microbe have long since vanished. However, other concerns remain. Can a gene cloned for toxic production from an rDNA microbe be transferred into normal bacterial flora? Could antibiotic-resistant genes be cloned and inadvertently inserted into clinically relevant pathogens not presently antibiotic resistant? To reduce these possibilities, the NIH has only certified nonconjugative plasmids (e.g., nonmating) for use in recombinant DNA microbes.

Studies by Levine et al. (1983) have addressed the issue of plasmid mobilizations, the movement of plasmids between different host cells. Human volunteers fed tetracycline along with *E. coli* HS-4 (typical of the normal intestinal flora of humans) bearing highly mobilized plasmids (e.g., pJBK5) that carried resistance to chloramphenicol and tetracycline became cocolonized with *E. coli* HS-4 bearing the antibiotic-resistant plasmid. However, the use of a poorly mobilizable plasmid (pBR325) did not result in plasmid transfer.

Taken as a whole, these studies establish the safety of recombinant DNA research when poorly mobilizable cloning vectors are used, while supporting the rationale for biological containment of highly mobilizable plasmids. They also point out the need to protect laboratory workers on antibiotic therapy from potential exposure to DNA organisms carrying any sort of antibiotic-resistant genes. A reassuring point is the relatively poor survival of rDNA strains of *E. coli* in the intestinal environment. For example, in most successful studies, 50 billion *E. coli* HS-4 organisms were required to ensure survival within the harsh environment of a human's stomach.

17.3.2 Specific Toxicological Concerns

While rDNA techniques offer exciting possibilities, there are many unanswered questions about the potential toxicity that each new product represents. For example, acute clinical toxicities of IFNs include flulike syndrome, fever, chills, malaise, anorexia, fatigue, and headache. Chronic dose-limiting toxicities include neutropenia, thrombocytopenia, impairment of myeloid maturation, reversible dose-related hepatotoxicity, some neurological toxicity (stupor, psychosis, peripheral neuropathy), and gastrointestinal toxicity. Some of these toxicities would be difficult to ascertain in rodents and, in fact, may be species specific.

A particular toxicity associated with the administration of IFN to humans and experimental animals has been depression of the cytochrome P-450 monooxygenase (MFO) metabolizing enzymes. As a consequence of MFO inhibition following treatment with IFN, the sleep time of mice treated with hexabarbital is increased, as is the toxicity of acetaminophen (Stebbing and

Weck, 1984). Possible effects on the metabolism of chemotherapeutic agents or other drugs processed by the P-450 MFOs should be anticipated.

The in vivo antitumor effects of IFNs are believed to be related to both augmentation of natural killer cell activity and antiproliferative effects. Antiproliferative activity probably also accounts for the bone marrow suppression observed in some individuals given IFN and could potentially produce effects in a routine preclinical reproduction or teratology evaluation. Dosing studies performed in newborn mice with homologous IFN have resulted in death at high doses and a marked wasting syndrome when given over an extended period (Gresser and Bourali, 1970). Both effects were attributed to the antiproliferative activity of IFNs. Inhibition of proliferation and metabolism represent potential dose-limiting toxicities of this family of rDNA molecules.

17.4 IMMUNOGENICITY

Human biopharmaceuticals are commonly immunogenic (elicit and antibody response) in nonhuman species. Immunogenicity should be evaluated in repeat-dose nonclinical safety studies to help determine whether antibody may have influenced pharmacology, toxicity, or exposure. If immunogenicity decreases exposure or neutralizes the activity of the biopharmaceutical, it may not be appropriate to continue the study or conduct studies of longer duration in that species. It is important to confirm biological activity of administered biological in "no-effect" studies where the top dose is the no-observable-effect level (NOEL). The immunogenicity of a biopharmaceutical in nonclinical species is not necessarily predictive of a potential for antibody formation in humans, but the potential consequences (summarized in Table 17.11) must be considered. Table 17.12 summarizes the strategies to conduct a bioanalytical evaluation of immunogenicity responses.

TABLE 17.11 Consequences of Immunogenicity

Loss of efficacy	Enhancement of efficacy
• Insulin	• Growth hormone
• Salmon Calcitonin	
• Factor VIII	Neutralization of native protein
• IFN-α_2	• MDGF
• IFN-β	• EPO
• IL-2	
• GnRH	General immune effects
• TNFR55/IgG1	• Allergy
• HCG	• Anaphylaxis
• GM-CSF/IL3	• Serum sickness, etc.

Note: Immunogenicity in humans may be a major safety concern.

TABLE 17.12 Immunogenicity Bioanalytical Strategy for Animal Studies

Bioanalytical Scheme for Lower Risk Products	Bioanalytical Scheme for Medium- and Higher Risk Products
Frequency of Sampling within Study	
• Planned assessment of baseline and an appropriate, drug-free, end-of-study immunogenicty sampling time point; contingent analysis of dosing phase samples if required to support pharmacokinetic profiles	• Planned assessment of baseline and an appropriate, drug-free, end-of-study immunogenicty sampling time point; contingent analysis of dosing phase samples if required to support pharmacokinetic profiles
Assessment of ADAs	
• Detection of ADAs through screen and confirmatory immunoassays • Consideration of characterization of titer/relative concentration of ADAs	• Detection of ADAs through screen and confirmatory immunoassays • Characterization of titer/relative concentration of ADAs • Detection of cross-reactivity to endogenous counterpart through screen and confirm (and maybe titer) immunoassays • If antibody reactivity to endogenous counterpart is detected, characterization of neutralizing ability using target binding inhibition-based neutralizing antibody immunoassay or cell-based neutralizing antibody bioassay

Note: ADAs, anti-drug antibodies.

17.5 MONOCLONAL ANTIBODY TECHNOLOGY

Offering an impressive potential for human therapy, monoclonal antibodies have become the first commercialized products of the new biotechnology. They are now becoming widely used in diagnostic medicine and are very successful as therapeutic agents in cancer (Oldham, 1983). In clinical diagnostic medicine they have provided us with the sensitivity not heretofore available for specific and rapid diagnosis of a particular drug level or infectious disease process.

Antibodies are important in the body as defense against infectious agents. They are extremely specific proteins that are produced in response to a foreign material, or antigen, by lymphoid cells of the immune system and share the property of being able to bind specifically to the inducing antigenic epitope (a single antigenic determinant; that portion of the antigen which combines with the antibody paratope). Unfortunately, under most conditions of antigenic stimulation, a family of antibodies is produced, each with a slightly different antigenic specificity.

In 1975, Kohler and Milstein observed that if an antibody-producing cell was fused with a myeloma tumor cell, a rapidly dividing hybrid was produced

that synthesized a monospecific antibody. Each hybridoma formed then became a "factory," producing antibodies monospecific to a particular sensitizing antigenic epitope. Cell cloning allows selection of hybrids producing antibody with the desired characteristics.

Monoclonal antibodies are thought to represent a major advance in cancer therapy because they have a very high therapeutic-to-toxic index when compared with anticancer drugs or radiation therapy and should provide a greater degree of specificity for the tumor cell than other forms of therapy. The conjugation of toxins with monoclonal antibodies is theoretically very exciting because a high specific toxin activity could be achieved at the tumor target cell.

Clinically, monoclonal antibodies are also proposed as drug delivery vehicles in certain tumors where specific tumor-associated antigens are expressed. In this context, investigators have found that by conjugating toxins such as the A-chain polypeptide of the plant protein ricin or the bacterial toxin from *Corynebacterium diphtheriae* to monoclonal antibodies specific for certain tumor type, as few as one or two molecules of antibody–toxin conjugate can destroy a tumor cell in vitro. Some success has also been obtained in clinical trials with monoclonal antibody–toxin conjugates.

Monoclonal antibodies have also been proposed for detoxification of individuals suffering from drug overdose or chemical intoxication as well as for radioimaging of tumor burden or metastatic foci. In veterinary medicine, monoclonal antibodies are already being used to develop new rapid methods for diagnosis of infections in poultry, cattle, and other animals.

17.5.1 Toxicological Concerns with Monoclonal Antibodies

It is already clear that there are certain problems implicit in the use of monoclonal antibodies in therapeutic trials. First, for example, there may be modulation of the antigenic determinant on the target cell, so that the monoclonal antibody cannot recognize its appropriate antigenic epitope. Second, the tumor cell may release free antigens so that the monoclonal antibody is effectively neutralized before it can reach the target cell. Third, antibodies to mouse epitopes on the monoclonal antibody could be induced (this may be overcome in the future by the use of human–human hybrids or the use of immunosuppressive agents to prevent the development of antibodies). Fourth, monoclonal antibodies have an extremely short half-life in systemic circulation, which would require that they be intermittently infused to provide the beneficial effect. Last, there may be an unwanted release of the toxin from its conjugate or specificity problems may develop whereby the antibody–toxin conjugates end up in an inappropriate organ.

The clinical toxicology findings associated with the use of monoclonal antibodies in therapeutic trials have included fever, chills, flushing, dyspnea, hypotension and tachycardia, anaphylactic and anaphylactoid reactions, urticaria, rash, nausea, elevated creatinine levels, headache, bronchial spasm, and serum

sickness (Oldham, 1983). Few of these reactions might be predicted from safety evaluation in rodents. A major problem with using the intact ricin or diphtheria toxin molecule, containing both the A and B polypeptide chains, has been the dissociation of the parent molecule from the monoclonal antibody, leading to toxicity of the reticuloendothelial system. A promising solution to this problem comes from separating the A chain (toxic moiety) from the B chain (cell association moiety) and preparing only A-chain conjugates. This results in much lower toxicity if the A chain should become dissociated from the antibody conjugate because cellular association does not occur.

Since a monoclonal antibody is a fusion product of a malignant mouse cell and an antibody-producing cell, there is some concern about the safety of the production process itself (Petricciani, 1983). Methods for the production of monoclonal antibodies raise two general safety issues: (1) the theoretical risk of transferring in the product factors associated with malignancy (e.g., oncogene factors) and (2) the use of animals for antibody production that are known to harbor a number of microbial agents some of which can produce diseases in humans.

Preclinical studies should address the potential toxicity due to inappropriate release of the conjugated toxin. Preclinical toxicology of monoclonal antibodies may not require extensive animal studies but should examine for cross-reactivity with antigenic epitopes present on normal cells in vitro and for the presence of human or rodent viruses. Early clinical trial should involve biodistribution studies with radiolabelled material.

The level of regulatory concern with the safety of the products of biotechnology underwent a sea change in early 2006 with the near catastrophe with TGN1412, a superagonist anti-CD28 monoclonal antibody that induces the production of anti-inflammatory cytokines by directly simulating T cells.

A German company, Tegenero, had the first-in-man clinical trial of the product initiated by a clinical research organization (CRO) at a clinic within a hospital in London. Eight healthy young males were enrolled, and six received a dose of the drug while two received placebo. Within 90 min after receiving a single intravenous dose of the drug, all six volunteers had a systemic inflammatory response characterized by a rapid induction of proinflammatory cytokines and accompanied by headache, myalgias, nausea, diarrhea, erythema, vasodilatation, and hypotension. Within 12–16 h after infusion, they became critically ill, with pulmonary infiltrates and lung injury, renal failure, and disseminated intravascular coagulation. Severe and unexpected depletion of lymphocytes and monocytes occurred within 24 h after infusion. All six patients were transferred to the care of the authors at an intensive care unit at a public hospital, where they received intensive cardiopulmonary support (including dialysis), high-dose methylprednisolone, and an anti-interleukin-2 (IL-2) receptor antagonist antibody. Prolonged cardiovascular shock and acute respiratory distress syndrome developed in two patients, who required intensive organ support for 8–16 days. Despite evidence of multiple cytokine release syndrome (CRS), all six patients survived (Goodyear, 2006; Suntharalingam

et al., 2008). This response has been characterized as a "cytokine storm," similar to that seen earlier with OKT3.

The preclinical evaluation which occurred before the trial was conducted was a set piece approach to the then guideline requirements. Studies were conducted in rats, mice, and primates, with the repeat-dose studies in the rats and mice being once a week for four weeks at doses up to 50 mg kg^{-1}, with these doses being characterized as well tolerated with transient increases in CD4 and CD8 (which were expected) and of IL-2, IL-5, and IL-6 but with no signs of first-dose CRS. There was cross-reactivity in stained lymphoid tissue and astrocytes in both human and primate tissues, but no histopathology signs of central nervous system (CNS) toxicity were seen in safety studies. The no-observable-adverse-effect level (NOAEL) was set at 50 mg kg^{-1} in the primate (cynomolgus monkey), and with FDA HED (human equivalent doses) style allometric scaling and safety factor of 10 and then additional safety factors were applied, proposed 0.1 mg kg^{-1} for first-in-man clinical trials, which was the dose used in the trial.

What was not done for this highly humanized monoclonal antibody (MAb) was to use the appropriate model (either a knockout mouse responsive to the humanized molecule or evaluation of a suitable homologue molecule).

The EMEA response has been to put in place the MABEL (minimum active biological effective level) approach to setting first doses in clinical trials (EMEA, 2007). In the case of TGN1412, based on biological activity in the rat, a safe dose would have been set at 0.005 mg kg^{-1}.

17.6 BIOPROCESS TECHNOLOGY

In the chemical and pharmaceutical industry, DNA technology will allow for the synthesis of chemical that can only be practically achieved through a bioprocess. For example, methylation of a particular carbon in a chemical structure might be done quite easily with a recombinant-engineered bacteria. This technology will allow the synthesis of a family of isomers and the development of a synthetic process that cannot be achieved by strict physical chemical processes.

This area of DNA technology also has application in the degradation of solid waste materials: in wastewater recovery, in leaching minerals from ore containing rock, in improved oil recovery, and in the decontamination of chemical waste dumps through the engineering of microorganisms that can destroy specific toxic contaminants.

17.7 GENE THERAPY PRODUCTS

Gene therapy products, while holding tremendous promise, have so far delivered but limited (two cases as of this writing) positive outcomes. The concept involved—inserting functioning genes in place (or places) where

nonfunctional or malfunctioning genes have produced a disease state—is stunning. But the public outcry over the death of Jesse Gelsinger, an 18-year-old in a clinical trial at the University of Pennsylvania's Institute of Human Gene Therapy, in 2000 has led to a significant slowdown in the rate of evaluation. This tragic event, probably due to an innate immune response to a protein in the vector's protein coat (Stephenson, 2001), has led to increased restrictions.

Five aspects specific to gene therapy need to be evaluated to assess the safety of a therapy—DNA/RNA biodistribution, gene transfer and biological activity, risk of vertical transmission of the gene, the safety of the vector (the means of delivering the gene to the intended site), and the safety of the product protein:

1. Evaluate DNA/RNA biodistribution:
   - Radiolabeling
   - Souther blot
   - Polymerase chain reaction (PCR)
   - Real-time PCR
   - In situ PCR
2. Evaluate gene transfer and biological activity:
   - Immunohistochemistry
   - Western blot
   - Enzyme-linked immunosorbent assay (ELISA)
   - Flow cytometry
3. Evaluate risk of vertical transmission:
   - To gonads
   - If yes to risk to gonads, then semen/germ cells (which is probably required anyway)
   - To circulating blood; if so, how long (persistence)
4. Assess safety of the vector:
   - Identify a suitable model species.
   - Assess the acute toxicity of the vector particle (in rabbits): at high dose, potential for anaphylactic response (not seen in mice). Also look for neutrophil proliferation.
5. Assess safety of the product protein:
   - Use data from preclinical pharmacology toxicology ("safety") studies to support the safety of clinical trials.
   - Those not appropriate for genotox. Instead, assess integration/insertion frequency in a mammalian cell.
   - Standard rodent carcinogenicity is *probably* not appropriate.

Regulatory authority for gene therapy products is unique in that overlapping responsibilities extend to both the CBER and NIH:

CBER	Division of Cell and Gene Therapies: manufacturing Division of Clinical Trial Design and Analysis • Preclinical pharmacology and toxicology • Clinical trial design, safety and efficacy • 20% of CBER clinical protocols now for gene therapy
NIH	Recombinant DNA Advisory Committee (RAC): no authority for approval for clinical trials, but all adverse events must be reported

This duplicating authority has led to both misunderstanding and problems in trials, as investigators must report adverse responses to both. But each has a different definition of what constitutes a reportable adverse response.

Currently, gene therapy is restricted to life-threatening and severely disabling diseases, but when a larger safety database has been accumulated, there should be expanded opportunities for therapy. It is not possible or desirable to identify a uniform "recipe" for safety studies that should be conducted with gene therapy products to support either the first dose in humans or extended clinical evaluation. Each product should be treated on a case-by-case basis, taking into consideration a number of important factors, such as the clinical indication, the duration of expression of the gene, and whether DNA transfer will be in vivo or ex vivo. For example, elimination of a tumor may require short-term treatment such as the transient expression of a suicidal gene. On the other hand, treatment may be long term, such as the replacement of a missing enzyme in the liver, where the goal may be lifetime expression. Gene therapy is currently an area of limited but rapidly advancing knowledge, and study design should be based on previous experience together with ongoing feedback from the clinic throughout development. Considerable early thought should also be given to appropriate assays and their sensitivities. The choice of assay will need to be justified and the basic "tooklit" of assays properly considered and evaluated in advance.

17.7.1 Vectors

In gene therapy, genes typically are delivered using a vector which may be nonviral or viral. The complete construct should be tested; separate safety evaluation studies of vectors per se are not generally recommended except to explore mechanisms of action if potentially harmful effects have been demonstrated in a previous investigation, for example, red cell agglutination on intravenous administration. If a novel nonviral vector is to be used, evidence of its lack of toxicity and information on its basic pharmacokinetics will be an essential component of the preclinical package. The interaction between the vector and the gene is quite important, perhaps more so with nonviral gene therapeutics, where the physicochemical properties of the particles themselves very much determine which tissues take up the gene. For viral vectors it is important to have sufficient knowledge of how they replicate, how to render

the viruses replication incompetent, any inherent pathogenicity and immunogenicity, and any risk of recombination with wild-type virus.

Conventional pharmaceutical quality assurance procedures should be applied to gene therapy products as well as appropriate infectivity tests for self-replicating/living vectors.

17.7.2 Studies to Support First Dose in Humans

The most scientific approach is to replicate, in an appropriate animal, the type of dosing that would be expected to be used in humans, employing the dose for dose–animal to human principle. A single, suitable animal species should suffice. If viral vectors are used, the animal species should be sensitive to infection by the wild-type virus. Studies should not automatically be done in primates but, initially, the commonly used laboratory species should be utilized. Only if those are demonstrated to be unsuitable should the next step be to consider the use of a primate.

Based on these general principles, the first dose in humans should be supported by a single-dose study in an appropriate animal species by the intended clinical route. Several dose levels should be explored, as some gene therapy expression products have a narrow therapeutic index.

There are many circumstances when a single-dose intravenous (IV) study can provide useful information; for example, if the intended treatment route is intraperitoneal (IP) or if the product will be administered to an open wound or injected into a muscle or a tumor, it might accidentally enter a blood vessel, and the knowledge gained from an IV study would be of value as well as one by the clinical route. Hence, if the intended clinical route is not IV, the absence of an additional study with IV dosing would require specific justification.

The physiological consequences of the gene product should be explored in these studies, particularly with totally novel gene products. In addition, all the standard toxicological evaluations should be carried out, including examination of functional endpoints in vivo, including cardiovascular and respiratory effects.

17.7.3 Distribution of Gene and Gene Product

The distribution of the gene must be evaluated carefully in time point assays. The choice of assay used, with regard to specificity and sensitivity, must be justified. The objectives are to identify the tissues in which the gene is present, demonstrate whether or not the gene product is expressed in particular tissues, and demonstrate the time course of gene expression, that is, how long it persists.

Some regulatory authorities, including those in the United Kingdom, are particularly concerned that the possibility of alteration to germ line cells should be excluded. Both male and female gonads should therefore be examined. If the gene is found there, then it is necessary to examine the gonads at

a more detailed level to ascertain whether the gene is present in the actual germ cells. Where gene persistence is short as, for example, in nonintegrating nonreplicating vectors, assay of the gonads at an appropriate time point will minimize false-positive findings.

17.7.4 Studies to Support Multiple Doses in Humans

The animal studies should parallel the intended treatment regimen in humans. At their present stage of development, a single dose of a gene therapeutic may not be totally curative, so multiple cycles of treatment may be used clinically rather than a single period of administration. It is appropriate to explore this in animal safety work; that is, the cycle regimen should be paralleled in the animal up to a maximum of three cycles. The duration of follow-up in the test animals after completion of the last test cycle should be based on the duration of gene expression up to a maximum of six months. There may be situations, particularly if long-term gene expression is the goal, when there could be an argument for a longer follow-up, but that should be considered on a case-by-case basis.

There should not be blind adoption of a checklist of assays and observations, but appropriate investigations should be selected based on earlier findings in the single-dose studies. Increasing the number of doses raises more concern about the immune response. There may be indications of this, such as lymphocyte infiltration at the site of administration, and there are many markers from single-dose studies that would indicate when it might be appropriate to examine the immune response to the gene product and selection markers, for example, immunity to adenoviral vectors, or to expressed proteins, resulting in accelerated loss of the transgene.

17.7.5 Unnecessary Studies

Genotoxicity and carcinogenicity studies are not generally recommended or required. Eliminating the possibility of the gene being inserted in the germ line ensures that some elements of concern about reproduction toxicology have already been addressed; hence, classical reproduction and developmental toxicity studies are not generally recommended. They should be considered on a case-by-case basis, for example, if the treatment were to be intended to manage a long-term metabolic disease and the patients would then survive to reach reproductive competence.

Drug interaction studies are not generally appropriate, with the exception of gene-directed enzyme prodrug therapy (involving a gene expressing an enzyme that activates a prodrug given subsequently). In the latter case, it is necessary to demonstrate the presence of the prodrug as well as the gene in the animal and to consider the potential toxicity of the active metabolite(s) both locally (which is the desired pharmacological effect) and systemically (the undesirable effects).

17.7.6 Ex Vivo Procedures

Ex vivo procedures involve removing cells and transfecting or transducing them. The cells should be checked to confirm that they are all healthy and are still expressing their normal surface markers, for example; observations of normal growth characteristics can also be reassuring. Animal studies are of limited value to test the safety of transfected or transduced human cells.

17.7.7 Change of Gene or Vector

Currently, only a limited number of vectors are available, although there is a large array of inserted genes. If the therapy involves developing a construct of a new gene in a well-characterized vector, it is important to use existing information on the vector. Rather than regenerating data on the vector itself, bridging studies of the construct should be carried out, that is, additional studies involving a limited toxicology evaluation to specifically characterize the nature of the new gene. Since the safety of the vector is already known, this should drive more exploration of the effects of the gene and the gene product rather than the vector itself.

If the vector is changed, a full safety evaluation may be required. However, if the changes are minor compared to the structure of a fully evaluated vector, it is appropriate for safety to be addressed by bridging studies. For example, if there is only a minor change on one of the condensation peptides of a nonviral, self-assembling vector, then some simple bridging work, rather than a full evaluation, may be appropriate.

The possibility of abbreviated testing is referred to in a guidance document on somatic cell and gene therapy published by the FDA (1991). According to this guideline, if changes are made to the vector backbone which do not alter the safety properties of the vector and the same route of administration and a similar dosing regimen is used to that employed previously, then truncated testing may be appropriate, depending upon the gene being expressed. When a promoter sequence or a targeting sequence in a viral or nonviral vector is changed or the expression of viral gene products is considerably altered, the vector should be considered as a new vector, even though it may have the same gene as the previous version of that vector.

17.7.8 Change of Route

It is quite possible that a treatment may be initiated using, for example, intratumoral injections to deliver a gene, which may subsequently be administered systemically. As a considerable amount of relevant information will have already been generated to support the intratumoral route, this would be another case for doing bridging studies. Comparative distribution studies will help to identify how much more safety evaluation may be required.

17.7.9 Insertional Mutagenesis

The long-term management of genetic disorders will require integration of the therapeutic DNA into the host genome or the maintenance of a stable episomal gene. The target of homologous recombination is still not achievable and, until that time, the problem of insertional mutagenesis—that is, inappropriate insertion of DNA into the host genome—must be addressed. How can the risk be quantified? Characterization of gene expression over time is more important than the copy number of the gene. An increased copy number can equate to an increased risk of insertional mutagenesis, but it also equates to an increase in the desired product. Insertional mutagenesis is a safety problem, and it is important to advise and warn patients who receive genes which will become integrated into the genome of this potential risk associated with their treatment.

17.8 VACCINES

Vaccination against viral and bacterial diseases has been one of the success stories of human and veterinary medicine. Probably the most outstanding example of the effectiveness of vaccination is the eradication of smallpox. In 1967 between 10 and 15 million cases of smallpox occurred annually in some 33 countries. By 1977 the last naturally occurring case was reported in Somalia. Polio too has been controlled in developed countries; for example, the number of cases in the United States was reduced from over 40,000 per year in the early 1950s, before a vaccine was available, to only a handful of cases in the 1980s. Diphtheria is now almost unheard of, whereas over 45,000 cases in 1940 led to 2480 deaths from diphtheria in the United Kingdom (similar numbers to those who died from AIDS in the United Kingdom in the entire 1980s). This has been reduced in the United Kingdom to only 13 cases and no deaths from the bacterium between 1986 and 1991. The scale of the problem is enormous—over 10 million deaths worldwide per year are due to infectious disease. United Nations figures suggest that cancers, circulatory problems, and injuries cause fewer deaths in developing countries than infectious diseases.

The process of developing vaccines is becoming increasingly complex due both to the nature of the infections being protected against and the nature of the cultures in which the affected individuals live. Kaufman (1996) provided an excellent review of this process and the inherent problems.

17.8.1 Approaches to Vaccination

There are two classical strategies for vaccination. One involves vaccination with either killed pathogenic organisms or subunits of the pathogenic organism. The other utilizes live attenuated viruses or bacteria that do not cause disease but have been derived from the pathogenic parent organism.

Inactivated vaccines are made from virulent pathogens by destroying their infectivity, usually with β-propiolactone or formalin to ensure the retention of full immunogenicity. Vaccines prepared in this way are relatively see and stimulate circulating antibody against the pathogen's surface proteins thereby conferring resistance to disease. Two or three vaccinations are usually required to give strong protection and booster doses are often required a number of years later to boost flagging immunity.

Subunit vaccines can be seen as a subcategory of inactivated vaccines because similar considerations apply to subunits and whole organisms. Doses, routes, duration of immunity, and efficacy of these vaccines are all very comparable. In this case a part of the pathogen, such as a surface protein, is used to elicit antibodies that will neutralize the infectivity of the pathogenic agent. The widespread use of hepatitis B virus surface antigen purified from the blood of carriers (or more recently from recombinant yeast) shows that this can be a very effective way to immunize. Hepatitis B virus surface antigen, the product of a single gene, assembles into a highly antigenic 22-nm particle which if used in three 40-μg doses at zero, one, and six months gives virtually complete protection against infection with hepatitis B virus.

Another example that can be included in the subunit vaccine class is the use of bacterial toxoids. Many bacteria produce toxins which play an important role in the development of the disease caused by a particular organism. Thus, vaccines against some agents, for example, tetanus and diphtheria, consist of the toxin inactivated with formaldehyde conjugated to an adjuvant. Immunization protects from disease by stimulating antitoxin antibody which neutralizes the effects of the toxin.

A further type of vaccine included in the subunit category is the capsular polysaccharide vaccine, for example, those against *Haemophilus influenzae* and meningococcal meningitis. In this case an extract of the polysaccharide outer capsule of the bacterium is used as a vaccine and is sometimes conjugated to protein to improve immunogenicity. Antibody persists for several years and is able to protect against the bacterium.

About half of all vaccines have traditionally been from live attenuated mutants of parent pathogenic organisms (Walker and Gingold, 1993). In effect live vaccines mimic natural infection yet produce subclinical symptoms and elicit long-lasting immunity, often giving rise to resistance at the portal of entry. Most of today's attenuated vaccine strains have been derived by a tortuous, often emperical route involving passage in culture until the pathogen is found to lose its virulence. This loss of virulence is tested in animal model systems before being tested in human volunteers. For example, the vaccine used to immunize against tuberculosis was derived after 13 years' passage in bile-containing medium by Calmette and Guerin (hence the name BCG—bacille Calmette–Guerin).

There has been much debate over the past 40 years as to the relative merits of live and killed vaccines often generating more heat than light! The evidence is that both routes will give adequate vaccines that can be used to protect

TABLE 17.13 Relative Merits of Live versus Killed Vaccines

	Step	Live	Killed/Subunit
Production	Purification[a]	Relatively simple	More complex
	Cost	Low[b]	Higher
	Route	Natural or injection	Injection
	Dose	Low, often single	High, multiple
Administration	Adjuvant	None	Required[c]
	Heat lability	Yes	No
	Need for refrigeration[d]	Yes	Yes
	Antibody response	IgG; IgA	IgG
Efficacy	Duration of immunity	Many years	Often less
	Cell-mediated response	Good	Poor
	Interference	Occasional OPV only[e]	No
Safety	Reversion to virulence	Rarely[f]	
	Side effects	Low level[g]	No

[a]Increasing safety standards mean that for new vaccines some of the older methodologies would not be acceptable.
[b]The price for new vaccines will approach that of killed subunit vaccines as safety standards are increased.
[c]Very few adjuvants for human use are acceptable.
[d]The need for refrigeration increases the costs significantly.
[e]Especially in the Third World.
[f]At very low levels (less than 1 case per 10^6 vaccinations).
[g]This varies from occasional mild symptoms with rubella and measles vaccines to possible brain damage with pertussis vaccine.

against disease under the appropriate conditions. Table 17.13 summarizes the major points of debate. Many factors, including cost, safety, number of immunizations, ease of access to vaccines, politics, and social acceptance, will determine whether there is a high uptake of a particular vaccine and whether it is ultimately successful in eradicating the target disease. Even if a perfectly viable, relatively safe vaccine is available, uptake may be limited. For example, it has been estimated that vaccination against measles within the World Health Organization Extended Program on Immunisation (WHO EPI) has prevented over 60 million cases and 1.37 million deaths. Despite these efforts there are still some 70 million cases of measles annually, resulting in nearly 1.5 million deaths. Consequently a recent WHO congress adopted the following goals:

(i) Increasing immunization coverage
(ii) Improving surveillance
(iii) Developing laboratory services and improving vaccine quality
(iv) Training
(v) Promoting social mobilization
(vi) Developing rehabilitation services
(vii) Research and development

This again also serves to illustrate the importance of factors other than the efficacy of the vaccine itself in disease prevention.

The single most important issue in developed countries is the safety of a vaccine; a single death in a million vaccinations for a new vaccine would be unacceptable (except possibly if it were an effective AIDS vaccine). While this is obviously important in a third world country, other issues such as cost and how to deliver the vaccine are of paramount importance.

17.8.2 Genetic Engineering and Vaccine Development

Not all protective antigens are as simple to identify, clone, and express as the surface antigen gene of hepatitis. The entire sequence of the hepatitis B virus (HBV) genome became available, and as it is less than 10 kb, it was relatively simple to establish which open reading frame to express. It has been known for many years that irradiated malarial sporozoites protect against malaria. As the sporozoite stage in the life cycle of the malarial parasite can only be grown in small quantities, it was left to DNA technology to identify, clone, and express components of the sporozoite that might be of use in vaccine production. The genome of the malarial parasite is many thousands of times larger than the genome of HBV and therefore provides a different scale of problem. Not only was there little sequence data available, but there was also no idea of which gene products may be protective.

The starting point of any DNA work is to generate a library of DNA in *E. coli* which is representative of the organism under study. Once there is a complementary DNA (cDNA) bank or a genomic library, there are three basic ways of identifying and isolating a gene of interest.

DNA/Oligonucleotide Hybridization If there is some preexisting knowledge of the nucleic acid sequence or where purified messenger RNA (mRNA) is available, it is possible to detect recombinant clones by hybridization of ^{32}P-labeled DNA or RNA to bacterial colonies or bacteriophage plaques. Often a protein has been purified and some amino acid sequence is available which allows a corresponding nucleic acid sequence to be synthesized. Due to the degeneracy of the genetic code, a complex mixture of oligonucleotides is required to ensure that all possible sequences are represented. Labeling this mixture of oligonucleotides yields a probe that can be used to screen a cDNA (or possibly genomic) library that might be expected to contain the gene of interest.

Hybrid Selection and Cell-Free Translation A second approach is to use hybrid selection of mRNA coupled with cell-free translation. DNA clones from a library, either individually or in pools of clones, can be immobilized by binding to a solid support and mRNA hybridized to them. Only the mRNA that corresponds to the clones will bind, and this can then be eluted and translated to protein in a cell-free system. The protein can then be immunoprecipitated

with antisera to the gene product of interest or assayed for activity. An example that encompasses both this approach and the sequence route is in the development of a vaccine for Epstein–Barr virus (EBV). It had been known since 1980 that antibody to the major membrane antigen of the virus (gp350/220) would neutralize the virus. Around 1983 a fragment of the virus genome was cloned and sequenced; using computer predictions the gp340/220 gene was identified. The experimental evidence that confirmed this prediction was published in 1985 and came from experimental work that managed to hybrid select EBV mRNA using genomic DNA clones. This was followed by cell-free translation of the eluted mRNA and immunoprecipitation of gm350/220 with a high-titer antibody. The DNA clone that hybridized with the gm340/220 mRNA was the one predicted to encode the gp340/220 gene by computer analysis. The hybrid selection approach is rather labor intensive and has for the most part been superseded by one of the forms of expression cloning.

Expression Cloning This approach is invaluable when the only means of identification are antisera against the protein or pathogen of interest.

Probably the most laborious form of this approach is its use in conjunction with a biological assay. Complementary DNA libraries are cloned into a plasmid that will allow expression in eukaryotic cells, for example, simian virus 40 (SV40) or EBV vectors. Clones or pools of clones are then transferred to appropriate cell types; for example, COS cells for SV40 vectors and cell extracts or cell supernatant is assayed for biological activity. If a pool of clones gives the biological activity, then the individual clones can be reassayed and the desired cDNA clone identified. This methodology, although tedious, has allowed many of the interleukin genes to be cloned probably because the assays for these proteins are very sensitive.

Other gene products or vaccine antigens may require an enrichment step. For example, many genes expressed on the cell surface (e.g., receptors, adhesion molecules) have been cloned by "panning" techniques where the cells expressing the gene of interest are selected out either with antibody or by interaction with other cells. Complementary DNA libraries are constructed in *E. coli* and the library is transferred to eukaryotic cells. Those cells expressing the gene of interest are enriched for and the library transferred back to *E. coli*. This can be done for several rounds of expression and eventually individual clones conferring the selected phenotype will be isolated.

The most extensively used form of expression cloning involves the use of plasmid or bacteriophage vectors in *E. coli* and identification of DNA clones using antisera to the protein of interest. Here a vector such as the bacteriophage λgt11 is set up so that, when cDNA fragments are cloned into sites adjacent to the β-galactosidase gene, bacteria will express a β-galactosidase fusion protein containing epitopes present in the cDNA. Recombinant phage is detected with antisera. The cDNA insert is then sequenced and the whole gene can then be isolated in a more traditional way. The antisera used can be

monoclonal antibodies, polyclonal monospecific antisera, or even polyclonal antisera with many antibody specificities present. A variation on this method allowed the initial cloning of the malarial sporozoite surface antigen. The malaralial sporozoite stage cDNAs were introduced into the ampicillin resistance gene of the plasmid pBR322. Low levels of expression of the sporozoite surface antigen were detected by solid-phase radioimmunoassay using a monoclonal antibody specific for the protein. In this way a cDNA clone coding for the antigen was isolated and subsequently sequenced. This information was then used to design peptide vaccines which have already been tested in humans.

The λgt11 system is a more sophisticated bacteriophage version of the plasmid system described above and has been used to isolate many different antigens from various stages in the life cycle of the human malarial parasite using human immune sera as well as antigens from pathogens.

Expression of Potential Vaccine Antigens In general, in the future eukaryotic cell culture is likely to be the method of choice for the production of subunit vaccine antigens where the organism to be vaccinated against replicates in eukaryotic cells. *Eschirichia coli* is unable to posttranslationally modify some vaccine candidates; for example, bacterial systems cannot add carbohydrate, which is important in the antigenicity and structure of many protective antigens.

Since 1986, the FDA has approved 22 vaccines (Table 17.14), half of them from a genetic engineering (and all, of course, from a biotechnology source). The cells used for such genetic engineering production of vaccine can be mammalian, insect, or bacterial.

The CBER has provided broad guidelines on the evaluation and production of vaccines (CBER, 2000). In general, the center's requirements have paralleled those for other biotechnology products. Beyond establishing sterility, lack of pyrogenicity, and lack of viral contaminants, a single GLP toxicity study in an appropriate species (one that has been established, if possible, to be immune responsive to the vaccine) is required. If the vaccine is to be used in pregnant women or women of childbearing potential, a segment I style reproductive study should be performed in an appropriate animal species.

Regulatory guidance for the conduct of clinical trials on vaccines is specific. Traditional phase I trials in normal volunteers are not conducted. Rather, all trials assess not only safety but also efficacy (or at least immunogenicity). Trials may well be challenge trials; that is, after immunization subjects are purposely challenged with exposure to the infective agent of concern.

In any case, injection site responses (erythema, edema, pain, and tenderness) and systemic responses are both evaluated in subjects (Mathieu, 1997).

The FDA also has specific guidance on the tracking and reporting of adverse clinical responses to vaccines.

Any adverse events of product problems with vaccines should be sent not to MedWatch but to the Vaccine Adverse Event Reporting System (VAERS), operated jointly by FDA and the Centers for Disease Control and Prevention.

TABLE 17.14 Vaccines Approved by FDA since 1986

Vaccine	Indication	Date Approved	Company
Recombivax HB	Hepatitis B	June 23, 1986	Merck, Chiron
ProHIBIT	*Haemophilus influenza* B	1988	Connaught
Pedvax	HIB	1989	Merck
Engerix-B	Hepatitis B	1989	SmithKlineBeecham
Tetramune, Hib TITR Haemophilis B, diphtheria CRM 197 protein conjugate	Bacterial meningitis	Jan. 1991	Lederle-Praxis Biologics/American Cyanamid
IPOL	Poliovirus vaccine inactivated-injected	1991	Inst. Merieux
Acel-Imune	Diphtheria, tetanus toxoids and acellular pertussis vaccine	Jan. 6, 1992	Takeda Chemical Industries/Amercan Cyanamid
Tripedia	Diphtheria, tetanus toxoids and acellular pertussis	Aug. 20, 1992	Connaught Laboratories, Inc.
JE-VAX	Japanese encephalitis	Dec. 18, 1992	Connaught/Biken
Enzon	Bubonic plague	1994	Green Labs
Typhim Vi	Typhoid	1994	Laboratories, Inc.
Havrix	HAV	Mar. 1995	SmithKlineBeecham
Varivax, Varicella Virus Vaccine Live	Chicken pox	Apr. 10, 1995	Merck
VAQTA	Hepatitis A	Mar. 29, 1996	Merck
COMVAX	Haemophilis B and hepatitis B	Oct. 2, 1996	Merck
Infanivir	Diphtheria, tetanus, and pertussis (DTP)	Jan. 27, 1997	SmithKlineBeecham
Rabovert	Rabies (pre- and postexposure)	Oct. 27, 1997	Chiron/Behring
Certina	DTP	Jul. 29, 1998	North Amercan Vaccine
RotaShield[a]	Rotavirus	Aug. 31, 1998	Wyeth
LYMEriv	Lyme disease	Dec. 21, 1998	SmithKlineBeecham
Prevnar	Pneumococcal disease	Feb. 17, 2000	Lederle
TWINRIX	Hepatitis A and B	May 11, 2001	SmithKlineBeecham

[a]Subsequently withdrawn.

For a copy of the VAERS form, call 1-800-822-7967 or download the form (in PDF format) from www.fda.gov/cber/vaers/vaers1.pdf.

17.9 SPECIFIC CHALLENGES

The problem with using a classical toxicological approach for evaluating an rDNA product or species-specific protein is that standard protocols are probably inappropriate and nonrelevant in most cases. In the traditional approach

to toxicology, a standard protocol or battery of tests is performed followed by an estimation of the types and degree of hazard or risk to humans. For example, conventional toxicity testing of a new rDNA product might lead to evaluation at excessively high doses in two rodent species. The production of antibody in the test species during preclinical toxicology testing may inactivate the test compound and thus invalidate the toxicity evaluation.

This approach appears somewhat irrational and without much scientific merit since many of these new molecules are minimally toxic or nontoxic by this sort of acute evaluation. As in the case of IFNs or monoclonal antibodies, the toxic effects observed in humans might not be predicted from safety assessments in rodents. An appropriate test species should be selected. Is the rat or mouse the appropriate species to evaluate a species-specific rDNA protein such as human growth hormone or IFNs or would nonhuman primates be more suitable? Does the nonhuman primate really offer any advantages? There is some consensus that the nonhuman primate may be a more appropriate species for testing some rDNA human proteins.

In contrast, in the "pharmacological approach" to toxicology, the potential targets of toxicity are first identified (Zbinden, 1986). The criteria for relevant effects are established, usually based on experience with reference substances, and appropriate in vivo or in vitro experimental models are selected to assess the pertinent toxicological responses.

Doses should be selected that are reasonable multiples of the proposed therapeutic dose to be employed, especially since in many cases the amount of material available for testing may be limited and not available in kilogram amounts. Preclinical rodent or primate studies should merely provide the flags to monitor during phase I clinical trials. Reason should prevail, not only in the selection of methods and models for assessing the potential toxicity of the new agents, but also in the use of these data for extrapolation to humans. Whether U.S. industry succeeds or fails in the biotechnology arena will depend on the quick resolution of issues such as selection of appropriate toxicological tests, fermentation scale-up of the rDNA microbe, product purity, and expedition of regulatory pathways.

17.9.1 Purity and Homology

Major concerns in the production of a species-specific protein by rDNA technology are the purity of the product, the amount and type of contaminants present, and the homology of the product to the native molecule (Table 17.15). The toxicologist should be concerned about the acceptability and toxicity of intentional or inadvertent contaminants introduced during fermentation or isolation of the product (e.g., DNA, chemicals, *E. coli* proteins). Other issues concern the introduction of amino acid residues that might alter the three-dimensional structure or antigenicity of the molecule, partial denaturation of the product during isolation and recovery, genetic stability of the rDNA clone during production (mutation could result in altered amino acid sequence), and

TABLE 17.15 Issues in Safety Evaluation of Species-Specific rDNA Products

Purity
Homology to native molecule (amino acid sequence, extra amino acids, three-dimensional structure)
Type and amounts of contaminants (chemicals, *E. coli* proteins, fermentation products, foreign DNA)
Stability of clone
Immunogenicity
Toxicities (direct or secondary to therapeutic effect)

the level of foreign DNA present. Although these are issues of analytical biochemistry, their impact on the potential toxicity and overall safety of the finished product is of some toxicological concern.

17.9.2 Immunogenicity

The problem of the immunogenic nature of many human recombinant DNA proteins and the potential to generate antibodies to a normal human protein are of special interest to the immunotoxicologist. For example, 3 of 16 patients administered the rDNA-derived interferon-α (IFN-α) (clone A) developed antibodies of the immunoglobulin G (IgG) class that were undetectable prior to or during therapy (Gutterman et al., 1982). These antibodies were capable of in vitro neutralization of IFN activity, although in vivo neutralization of IFN was not documented, since there are several different subtypes of IFN-α-containing epitopes not present on their own IFN subtype. Similarly, two patients treated with IFN-β for many months developed high-titered antibody, which in one case was correlated with an inability of the patient's fibroblasts to produce IFN (Vallbracht et al., 1982).

Virtually all patients treated with conventional porcine insulin develop circulating anti-insulin antibodies (Klaff et al., 1978) that are less frequent and in lower titer in individuals treated with more highly purified porcine (Falholt, 1982) or rDNA human insulin (Fineberg et al., 1983). In the study by Fineberg and associates, 44% of the patients developed antibodies to rDNA human insulin over a 12-month period compared to a 60% antibody frequency with porcine insulin. Human growth hormone (HGH; Genentech) prepared by rDNA technology was observed to produce a frequency of immunogenicity similar to that seen with human insulin (approximately 40% of the children developed antibody, according to the product insert). The ultimate goal is to develop rDNA products that will be less immunogenic than purified animal sources of these therapeutic agents.

The exact mechanism of the immunogenicity of species-specific rDNA proteins is unknown but is believed to be attributable to (1) the addition of extra amino acid residues during synthesis, which the host reads as foreign;

(2) denaturing of the native molecules; or (3) contamination by *E. coli* polypeptides or lipopolysaccharides.

A second unanswered concern is whether the antibody induced by the recombinant protein has any discernible health effect. Other than some reports of neutralization of biological activity, little pathology has been attributed to the presence of antibodies in patients given recombinant protein therapy. It should also be noted that the question of antibody specificity has not been well studied, so that it is entirely conceivable that autoimmune pathology or even an anaphylaxis response could be induced. Equally important is the concern that induced antibody might neutralize the endogenous hormone or protein that it is intended to replace or supplement.

A third consideration is that certain routes of administration may favor immunogenicity of recombinant proteins. In early trials, rDNA proteins introduced by subcutaneous or intramuscular injections (procedures known to improve the immunogenicity of proteins) resulted in a higher frequency of antibody responses than in the intravenous route.

In summary, these are the clinically relevant questions about the immunogenicity of rDNA species-specific proteins: Will antibody be induced in the recipient that will neutralize the therapeutic effect or lead to immune complex disease? What is the class (e.g., IgG or IgE) and specificity (i.e., reactivity against specific protein or contaminant) of the antibody induced? The former antibody type could potentially neutralize the product and produce immune complex disease, while the latter could result in an anaphylaxis response. It is possible that the antibody induced is of insignificant health consequence, and its presence is known only because of improvements made in the sensitivity of detection methods with the introduction of ELISA.

17.10 PLANNING SAFETY EVALUATION PROGRAM

Safety evaluation of a candidate product should start with a consideration of the specific nature and consequential hazards of the three P's:

- Producing system
- Process
- Product

The need under each heading is to decide what data are required, then how to obtain them with the greatest efficiency and economy, and last whether the toxicologist is necessarily the person with the appropriate skills and experimental techniques to do so. There will often be a trade-off between precise control by other means and possibly cheaper or more familiar, old-fashioned toxicological studies (Dorato and Vodicnik, 2001). The inventor of a new product or process, too, may often have to do a great deal of work to show

safety by excluding hypothetical hazards, which subsequent manufacturers can afford to ignore.

Producing System The questions of particular concern here are the nature of the system used to manufacture the desired substance and the precision with which it is controlled.

If the system consists of prokaryotic cells, then how well defined is their provenance and how is their consistency demonstrated? If mammalian cells are employed, their lineage must be considered. In both instances, it is important to ensure that extraneous virus, infections, DNA, and less well-defined factors such as "slow viruses" are excluded by the origins and history of the producer strain or because the physical (e.g., filtration) or chemical (pH, solvents, affinity separation) nature of the production process can be relied upon to exclude passage of an infectious agent.

If the degree of safety arising from these factors is weak, the toxicologist should consider appropriate studies in vivo to exclude contaminating agents, oncogenic factors, and so on, but there is no point in doing short-term or prolonged animal experiments or other types of tests unless the desired endpoint has first been clearly defined.

The aspect to which far more attention has been directed is the nature of the inserted gene(s) and promoters in rDNA products. Again, the toxicologist should ask how well the nucleotide sequence is known, whether there is only one reading frame, and how any introns are handled. Again, toxicity-type testing would appear to be an inefficient and expensive way to study molecular biology and biochemistry.

Last under this heading, for intact infectious organisms to be used directly in humans, is assessment of pathogenicity to the range of individuals that make up our populations, the possibility of reversion to a wild and more dangerous strain, the hazard of an allergenic reaction to the organism (e.g., vaccinia), and the possibility of spread from subject to subject in a naive or incompletely immune population.

There may be some role for animal experimentation here, if there is a suitable model, because it gives the chance to study the organism under intense pressure from commensals and the rising immune response. Possible hazards in the manufacturing plant also need to be evaluated.

In general, conventional toxicity procedures seem to have little to offer here, except in specific instances of helping to exclude certain infection factors, perhaps ruling out oncogenicity and examining the stability of engineered organisms for direct infection of humans.

The Process The toxicologist has the least to offer here. In fact, only his or her intellectual analysis and review of the literature should be required to asses the manufacturing process and any residues of its chemicals and so to set analytical limits on purity and residues in the final preparation.

The Product There are two distinct and probably divergent forces affecting the way in which the toxicologist regards the final product: (1) whether there is a need to learn about the biology and pharmacology of the product and (2) to define the minimum scientific concern, clinical caution, and industrial path before clinical trial or marketing.

Biology of Bioengineered Products This may not be a useful concept scientifically, but it represents the practical point that the pace of development often forces the rapid sequence—interesting biological property—identification of responsible molecule in very small amounts (e.g., tumor necrosis factor or erythropoietin)—cloning, for example, resulting in large-scale production, perhaps even before the full structure is known. The clinical interest in administering a substance to humans for investigative or therapeutic purposes must be balanced against the total lack of knowledge of its general effects on the body or of the consequences of prolonged high-level exposure to it.

An example is the history of interferon: discovered through its antiviral actions, subsequently found to modulate mitosis and certain immune functions, capable of producing fever, and probably ECG and EEG changes as evidence of membrane effects in excitable tissues. If interferon were a novel discovery, now just being produced for the first time, then investigation of its general biological effects on repeated administration to responsive animals would be important prior to study in humans. The same arguments apply to lymphokines, for example, IFN-α, IL-2.

The planning of this type of investigation as an empirical, open study of responses must be carefully related to the nature and what is known of the product:

(i) It is necessary to work in a species capable of responding to the principal activity. Interferons are notorious for their species specificity, but most other lymphokines at least are more generally active. Work in a primate may be required, but it depends on the substance to be tested. There may be no point in using more than one species in pivotal studies.

(ii) Any test should be as broad and as general as possible, that is, monitor many variables clinically, in the laboratory and by pathology, until enough is known for there to be confidence in a focused approach.

(iii) Relate any testing to the clinical circumstances of probable use.

Thus, if a new synthetic antigen or engineered antigen is for testing, for administration only a few times to humans, there would be no point in a multidose experiment. It should suffice to show that it was antigenic in the intended preparation. Unless there were a prior reason to do so, a special search for, say, autoimmune reactions seems unnecessary. Similarly, testing a monoclonal antibody for activity is likely to be difficult, if not impossible, because of species specificity and the antigenicity of the preparation.

(iv) The toxicologist should be prepared to do nothing if the material is well known, its properties are understood, and there is adequate characterization of the nature of the preparation supplied; for example, human insulin or growth hormone produced by genetic engineering should not be submitted to prolonged safety tests in animals provided that the molecular forms present are sufficiently well understood.

It may be useful, however, to consider limited animal studies to examine the pharmacokinetics and duration of action even of a well-known material made by a new route unless physicochemical analyses show that to be pointless.

17.10.1 Animal Models

Species selection is probably one of the most important considerations when designing a preclinical safety program; for a biotechnolically derived pharmaceutical, it requires an understanding of the biology of the product. Since most of these either are human proteins or target human receptors, they tend to be species specific. Studies in rodents and dogs, the species commonly used in traditional toxicity studies, may not provide scientifically meaningful data. However, nonhuman primates are not necessarily the most appropriate species either, despite their phylogenetic similarity to human beings.

Some approaches that offer guidance in selecting relevant species are a literature review, determining the extent of homology between the endogenous animal protein and the human recombinant protein, determining the activity of the protein in pharmacological models, and in vivo assays of the receptor/tissue binding.

A literature review may provide useful information about the physiological properties of the protein in animals and how they compare with those of the human protein in humans. For example, prior to recombinant DNA technology, growth factors and/or hormones were purified from biological fluids. Although the quantities obtained were limited, they were nevertheless sufficient to allow investigation of the physiological properties of these proteins. Computer programs are now available for online searching of databases which hold information not only on the sequences of various animal and human proteins but also on the extent of homology between an animal protein and its human equivalent, including common amino acid sequences. It should be remembered, however, that a protein showing a high degree of honology to the human protein may not necessarily share similar pharmacological activities. Evaluation of activity or lack of activity in pharmacological animal models, if available, certainly would aid species selection. Finally, in vitro assays which analyze receptor and/or tissue binding are commonly used to determine the appropriate species for preclinical safety evaluation.

Some biotechnogically derived pharmaceuticals will cross-react with species that can be evaluated toxicologically, while others cross-react only with nonhuman primates such as the chimpanzee—a protected species. In this case, a

TABLE 17.16 Alternative Models for Toxicity Assessment

Model	Example	Caveat
Nontraditional animal model	Transgenic mice carrying appropriate human receptor	Antibody formation would need to be monitored, as it is probable that a large human protein would produce an immune response
Homologous proteins and/or systems	Testing purified animal protein in the same species or, for monoclonal antibodies, testing an antibody directed against the receptor in the animal	Data should be interpreted with caution as the biological properties of the animal protein may differ from those of the human protein
In vitro methods	Tissue-binding assays	If no in vivo models are available, in vitro; methods combined with in vivo testing in a pharmacologically nonreactive may suffice

well-designed "safety," or "Phase 0," study at doses higher than the proposed clinical dose may provide valuable safety information. However, a lack of cross-reactivity with any nonhuman species does not necessarily make preclinical safety evaluation impossible, nor does it limit toxicity testing to species in which the protein lacks relevant pharmacological activity. Some alternative possibilities are summarized in Table 17.16.

Toxicity studies traditionally are conducted using "normal" animals. However, studies in animal disease models may provide additional safety information regarding the possibility of disease exacerbation. For example, the administration of human recombinant erythropoietin was associated with hypertension in patients with chronic renal failure and also in uremic dogs but not normal dogs.

Species differences must be considered when choosing a model and, in particular, species-specific immunological differences between the human and the test animal. For example, in humans, an anti-CD4 MAb will bind to CD4 expressed on monocytes, with subsequent fixing of complement and destruction of antigen-presenting cells. However, since CD4 molecules are not expressed on murine monocytes, these effects would not be evident in a murine model.

For highly humanized proteins, the approach to proper safety evaluation starts with identification of an appropriate nonclinical safety model.

First, evaluate for comparative tissue binding. Then if no appropriate species is identified, one can either:

- Preparing and testing a homologue
- Testing the molecule itself in a humanized mouse model

A tissue cross-reactivity study is required by the FDA points-to-consider document for monoclonal antibodies prior to the first clinical trial. Its usefulness is debated, but its purpose is to evaluate the potential for binding to

nontarget tissues. Positive and negative controls are important for interpreting results. Tissues from humans and all nonclinical species used in safety studies should be evaluated. At minimum, the evaluation should include the 32 tissues recommended by the FDA "Points to Consider in the Manufacturing and Testing of Monoclonal Anitbody Products for Human Use."

When is it appropriate to use a homologue? According to the ICH S6 (1996) guidance: "When no relevant species exists, the use of homologous proteins should be considered. While useful information may also be gained from the use of homologous proteins, it should be noted that the production process range of impurities/contaminants, pharmacokinetics, and exact pharmacological mechanism(s) may differ between the homologous form and the product intended for clinical use."

Comparability of the homologue with the clinical candidate is critical:

Characterize pharmacology

- Literature: What's known about the target in the test species compared to humans?
- In vitro binding: Similar affinity or neutralization?
- Functional assays: in vitro cells in vivo bioassays (if possible relevant).
- Similar tissue distribution (tissue cross-reactivity for MAb).

Pharmacokinetics

Is Fc activity important and similar?

Challenges of Homologues

They represent a second test article. The decision must be made early in development:

- Sometimes, it is not possible to make a homologue.
- If possible, months to years needed to develop construct, clones, manufacture material, characterize pharmacology, and/or establish bioanalytical support.
- May be immunogenic, thus limiting usefulness.

How do you interpret the data?

- No "validation" can be performed that homologue is predictive of human toxicities.
- What if findings are different from the clinical candidate in an appropriate toxicology species?
- How do we extrapolate safety margins to the clinical candidate?

Regulatory Challenges of Homologues

No common criteria for what's expected.

- How much comparison with the clinical candidates is enough?
- Expectations for analytical characterization and does this need to be comparable?

- Do all aspects of testing need to be good laboratory practices (GLPs)?

Can studies with a homologue replace studies with the clinical candidate?

- Development and reproductive testing.
- What if results are more severe than with the clinical candidate?

Are negative findings meaningful?

- It's not your clinical candidate, so do the data impact risk assessment?

Homologues have been used to support registration for infliximab (Remicade) (anti-TNF) and efalizumab (Raptiva) (anti-CD11a). In both cases, there was not an appropriate species for the clinical candidate. Chimpanzees were the only pharmacologically responsive species, but they are not acceptable for toxicity testing due to humane reasons.

"Humanizing" Mice

- Isolate CD34+ stem cells [human hematopoietic progenitor cells (hHPCs)] from human cord blood.
- Breed NOD. Cg-Prkdc^{scid}//2rg^{tmWjl}.
- PND1 pup irradiated 1 cGy.
- hHPCs (10^5) injected in 25 µL phosphate-buffered saline (PBS) into liver.
- Assay tail blood for reconstitution of human immune cells.

Humanized mice can be used to:

- Assess the in vivo influence of stressors and drugs on the development of immune cells.
- Evaluate how toxicants can modify in vivo human immune responses.
- Investigate whether hematopoietic stem cells from cord blood can be induced to develop into nonimmune cell types
- Determine the influence of mouse effects on human immune cell longevity and human immune cells on mouse longevity.

17.10.2 Study Design

It is questionable whether traditional toxicological paradigms are applicable to biological or protein agents. If they are not, then how can the clinician gain reassurance to administer the first dose to humans, move into multidose trials, and even assess the agent in combination with other established medicines or biological agents? Monoclonal antibodies, soluble cytokine receptors, and growth factors all have been used in patients for nearly a decade, providing a wealth of experience in this area from which to learn. One of the most striking lessons is that pharmacodynamic effects may appear long after dosing of the agent has been discontinued.

As a class, biotechnologically derived pharmaceuticals share certain characteristics which have influenced their preclinical development. They are proteins and therefore toxicity was expected to be minimal and limited to an exaggeration of their desired pharmacological effects, a myth which was ultimately exploded. These agents are designed to perturb specific molecular or cell-to-cell interactions, sometimes with minimal effect on the pathophysiology of the target disease. Owing to the species-specific nature of these agents, preclinical toxicology is usually limited. For example, if a primatized anti-CD4 MAb cross-reacts only with chimpanzee and human CD4, the species of choice for toxicity tests is the chimpanzee, the use of which is restricted by its limited availability.

These characteristics of protein agents give rise to problems in clinical development such that the traditional paradigm for preclinical testing may not be appropriate. The dose in animals may not be predictive of an appropriate starting dose for humans. A surrogate marker (e.g., CD4 cell counts in the preclinical chimpanzee model) may be useful in setting the initial human dose but may only serve to indicate a no-effect dose. Once in the clinic, trials conducted early in development are usually not sufficiently powered to distinguish effects due to the toxicity of the test agent from those due to, for example, the underlying disease and concomitant or previous medications. Finally, short-term (three- to six-month) preclinical studies do not necessarily predict the long-term effects of these agents.

The long-term toxicities of concern are opportunistic infections, lymphoproliferative disorders, and immunogenicity, manifesting as tachyphylaxis and/or allergic reactions. Preclinical approaches which serve to identify these as potential hazards to humans of a biological drug moiety are thus needed.

The choice of toxicity studies and the design of individual studies will depend on the proposed clinical program. Important issues to consider are:

- Frequency and route of administration, including use of novel delivery systems
- Duration of dosing
- Special toxicity testing

Frequency and Route of Administration Clinical trials for biotechnologically derived pharmaceuticals may be more complex than those for conventional pharmaceuticals and so the route and frequency of test drug administration should, if possible, mirror the proposed clinical use, even if that route employs a novel delivery system.

Duration Traditionally, the duration of a toxicity study depends on the intended clinical use and disease duration. The potential immunogenicity of the human protein is a significant issue since antibody binding can partially or completely inhibit the biological activity of that protein, affect its catabolism, or alter its distribution and clearance. Any multiple-dose study therefore should include evaluation of the impact of antibody formation, including their neutralizing capacity. However, antibody formation in itself should not be a

reason for termination of a toxicity study, particularly if the antibodies are not neutralizing or do not alter the pharmacodynamics of the protein.

Multiple-dose toxicity studies are usually conducted before single-dose administration to volunteers. Many of the clinical trials for biological agents target life-threatening illnesses, and it has therefore been suggested that single-dose toxicity studies are sufficient to support single-dose "proof-of-concept" clinical studies. While this approach promotes faster introduction into the clinic, it may be of limited use since there may be a tendency to overlook the preclinical data. Clinical development may not progress without interruption if relevant preclinical data are missing.

Special Toxicity Testing In addition to multiple-dose studies, information on potential functional changes—as obtained from safety pharmacology studies—and the potential for genotoxicity, reproductive toxicity, and carcinogenicity may be required for registration. Once again, the species specificity of recombinant proteins may preclude the use of traditional animal species such as rodents and/or rabbits for safety pharmacology, reproductive toxicity, and carcinogenicity studies. Functional evaluations of cardiovascular and pulmonary systems could be incorporated into a nonhuman primate multidose toxicity study. If appropriate, potential reproductive toxicity can be evaluated in a nonhuman primate.

There may be situations which warrant an assessment of carcinogenic potential, but immunogenicity and species specificity may preclude a two-year rodent bioassay. It may be necessary to develop in vitro assays to address a particular concern. For example, growth factors which may have the potential to support or stimulate the growth of transformed cells should be assessed for their ability to promote growth of either malignant or normal cells.

Large-molecular-weight compounds are unlikely to react with DNA or other chromosomal material and therefore a genotoxicity evaluation may be of little value. However, genotoxicity studies may provide useful information about the safety of products containing organic linkers.

Program Design Considerations The standard toxicological data package for any new drug entity typically evaluates:

- Potential toxicity following single and multiple dosing
- Genotoxic potential
- Functional changes, that is, safety pharmacology studies

In addition, depending on the proposed clinical plan, the following may need evaluation:

- Toxicity following chronic dosing
- Carcinogenic potential
- Possible reproductive toxicity

Although biotechnologically derived pharmaceuticals often need customized preclinical development programs, certain issues are common to all. These include species specificity, potential immunogenicity and its impact on the duration of dosing, and the need for special toxicity testing.

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18

Safety Assessment of Inhalant and Dermal Route Drugs

While the two most common routes for administering a drug are either orally or parenterally (by injection), there are many other routes that can be used. The two next most common are inhalation (with all its variations) and dermally.

Each route has special considerations as to the evaluation of their safety—both regulatory and technological—which this chapter will consider in turn.

18.1 INHALED THERAPEUTICS

Drugs and medicinal agents administered by the inhalation route include the gaseous and vaporous anesthetics, coronary vasodilators, the aerosols of bronchodilators, corticosteroids, mucolytics, expectorants, antibiotics, and peptides and proteins where there is significant nasal absorption (Cox et al., 1970; Williams, 1974; Paterson et al., 1979; Hodson et al., 1981; Lourenco and Cotromanes, 1982). Concerns with the environmental effects of chlorofluorocarbons has also led to renewed interest in dry-powder inhalers, which have additionally shown promise for better tolerance and absorption for some new drugs. Excessive inhalation of a drug into the pulmonary system during therapy or manufacturing may result in adverse local and/or systemic effects. Consequently, safety assessment of inhaled medicinal preparations with respect to

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pulmonary toxicity and the therapeutic-to-toxicity ratio are essential. The data generated are essential for charting the course of evaluation and development of a potential therapeutic agent. The art of evaluating their toxicity is a specialty area (Newton, 2000; Gad, 2006).

18.1.1 Pulmonary System

An average man inhales approximately 7.5, 28.6, and 42.9 L of air per minute during resting, light work, and heavy work periods, respectively, the corresponding mean tidal volumes being 750, 1673, and 2030 mL [National Academy of Sciences (NAS), 1958]. Each breath is distributed between 300 and 400 million alveoli, where gas exchange takes place. The total alveoli surface area is approximately 75 m^2, which is penetrated by approximately 200 km of capillary blood vessels (Hatch and Gross, 1964). The high vascularity and large surface area of the lung ensure rapid gas exchange and entry of an inhaled drug into the bloodstream. A drug is then quickly carried to the heart and brain before reaching the liver, where first-pass metabolism occurs. The pulmonary system is therefore a very effective portal through which gases, vapors, and aerosols can enter the body to exert desirable therapeutic effects and undesirable side effects locally and/or systemically.

Anatomically, the pulmonary system is divided into extrathoracic and thoracic regions. The extrathoracic, or head, region includes the nasal and pharyngeal passages. The thoracic region is subdivided into tracheobronchial (TB) and alveolar (AL) regions. The TB region consists of the trachea, primary and secondary bronchi, and primary through-ciliated bronchioles. The alveolar region consists of nonciliated terminal bronchioles, alveolar ducts, and alveoli (Lippmann, 1970). The anatomical structure of the pulmonary system maximizes gas exchange but minimizes the penetration of extraneous particulate matter into the lungs. The formalized anatomy (Davis, 1961; Weibel, 1963; Horsfield and Cumming, 1968; Parent, 1991) of the branchings, the dimensions of the airways, the penetrability by particles of certain sizes, and the distribution of cell types in the respiratory tract and lungs are summarized in Figure 18.1.

18.1.2 Penetration and Absorption of Inhaled Gases and Vapors

Pulmonary dynamics, the dimension and geometry of the respiratory tract and the structure of the lungs together with the solubility and chemical reactivity of the inhalants greatly influence the magnitude of penetration, retention, and absorption of inhaled gases, vapors (Dahl, 1990), and aerosols (Raabe, 1982; Phalen, 1984). The quantity of an inhalant effectively retained in the pulmonary system constitutes the inhaled "dose" that causes pharmacotoxic responses. Acute inhalation toxicity of vapors and gases is subject to concentration/time rules (Carpenter et al., 1949), but not repeat dose exposures.

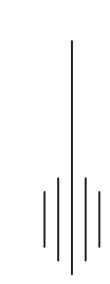
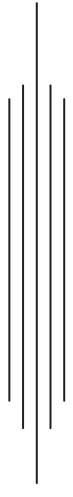
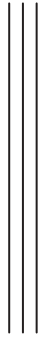
Regions			Generations	Total Cross section (cm^2)	Particle size penetration limits (μm)	Distribution			
						Cell types	Mucous glands	Smooth muscle	Parasympathetic innervation
Extrathoracic	Head	Nasopharynx			60				
Intrathoracic	Tracheo-bronchial	Trachea	0	2.54		Ciliated			
		Primary bronchi	1	2.33	20	Goblet			
		Secondary bronchi	2	2.13	10	Brush Border Squamous			
			3	2.00					
			4	2.48					
			10	13.4		Columnar			
		Bronchioles	11	19.6	6				
			15	113					
		Terminal bronchioles	16	180	4	Cuboidal Less ciliated			
	Alveolar	Respiratory bronchioles	17	300	3	Less goblet Noncilicated clara			
			18	534					
			19	944					
		Alveoli ducts	10	1.60K	<3	Type I			
			21	3.22K					
			22	5.88K		Type II			
		Alveoli	23	11.8K	<3				

Figure 18.1 *The distribution of cell types in the respiratory tract and lungs.*

Highly reactive and soluble gaseous or vaporous drugs react and dissolve readily in the mucosal membrane of the nasopharynx and the upper respiratory tract (URT), thereby exerting pharmacological effects or causing local irritation and/or adverse effects on the ciliated, goblet, brush border columnar, and squamous cells of the epithelium (Weibel, 1983). The dissolved drug is also absorbed into the bloodstream and transported to the target organ where it exerts systemic effects. Less reactive and less soluble gaseous or vaporous drugs are likely to penetrate beyond the URT and reach the bronchial and alveolar regions, causing local and systemic effects. The unabsorbed gases or vapors are then exhaled. For example, ammonia gas generated from a 10% ammonia water may be inhaled for reflex respiratory stimulation purposes (Budavari, 1989). Ammonia is extremely soluble in water at a concentration of 715 mL of ammonia per milliliter of water (Phalen, 1984) and is readily solubilized in the mucous lining, causing URT irritation. By contrast, oxygen is only sparingly soluble in water (0.031 cm^3 of oxygen per milliliter of water) and capable of penetrating deeply into the alveoli where gas exchange takes place. Oxygen that binds reversibly with the hemoglobin of erythrocytes is unloaded at the target tissues, while the unbound oxygen is exhaled. Inhalation of properly humidified oxygen is life supporting, but inhalation of unhumidified oxygen may cause a reduction in the mucociliary clearance of secretions in the trachea of animals (Pavia, 1984) and humans (Lichtiger et al., 1975; Gamsu et al., 1976). Gases or vapors of low lipid solubility are also poorly absorbed in the lungs, with much of the inhaled vapor exhaled. Other pharmacological gases and vapors, such as the anesthetics (nitrous oxide, halothane, enflurane, isoflurane, etc.) and the coronary vasodilators (amyl nitrite), likewise affect the epithelium of the respiratory tract and the lungs. The absorbed drugs exert local effects on various types of epithelial cells of the respiratory tract and on types I and II cells and the alveolar macrophages (AMs) in the alveoli. Repeated inhalation of some halogenated hydrocarbon anesthetics will result in accumulation of the vapors and systemic toxicity (Chenoweth et al., 1972). By contrast, vapors such as the fluorocarbons FC 11 and FC 12, which are used extensively as propellants for bronchodilator and corticosteroid aerosols, are absorbed rapidly but are not accumulated in the body even upon repeated inhalation (Aviado and Micozzi, 1981).

In general, dissolved gases or vapors at a nontoxic concentration are absorbed and metabolized locally by the lungs and systemically by the liver. The unchanged parent drug and its metabolites may be excreted to some extent via exhalation but mainly via the renal system. A dissolved gas or vapor at a toxic concentration, however, is likely to exert local effects such as altering the surface tension of the alveoli linings or disrupting the normal functions of the epithelial cells, the pneumocytes, and the AMs. The disrupted AMs in turn release their intracellular enzymes, causing destruction of the alveolar septa and contributing to histopathological changes of the respiratory tract and the lungs. Again, the magnitude of the adverse effects is dependent on pulmonary

dynamics and the solubilities of the inhalants in the mucous membrane of the URT and in the plasma or lipids of the erythrocytes.

18.1.3 Deposition of Inhaled Aerosols

For inhaled aerosols, particle size is the major factor affecting the penetration, deposition, and hence "dose" and site of pharmacological action (Dautrebande, 1962a,b; Agnew, 1984). Particle size is expressed in terms of *aerodynamic diameter* (AD), defined as the diameter of a spherical particle of unit density (1 g/cm^3) that has the same terminal settling velocity as the particle in question, regardless of its shape and density (Marple and Rubow, 1980). The unit for AD is the micrometer. A sample of aerosol particles having ADs within a narrow size range is considered to be a monodisperse aerosol, whereas a sample of aerosols with a wide range of ADs is a heterodisperse, or polydisperse, aerosol. The pattern of particle size distribution is usually bell shaped, with smaller and larger particles on both sides of the mean AD. An aerosol sample with a high proportion of particles of similar size has a narrow particle size distribution, or small geometric standard deviation (GSD). An aerosol sample with a GSD of less than 2 is considered to be a monodisperse aerosol. Thus, both the AD and GSD of 2 or less are considered to be optimal for pulmonary penetration and distribution in the respiratory tract and the lungs. For example, in nose breathing, aerosol particles with ADs > 15 μm are likely to be trapped in the nasopharynx (extrathoracic, or head, region) by filtration and impaction. Particles deposited in the nasopharynx are considered to be "noninhalable" (Lippmann, 1977; Miller et al., 1979).

In mouth breathing, only 10–15% of 15-μm particles penetrate through the larynx to the intrathoracic TB region. Particles reaching the TB region are considered to be "inhalable" (Lippmann, 1977; Miller et al., 1979).

In natural nose and mouth breathing, only a negligible proportion of aerosol particles of AD > 10 μm reach the lungs (Swift and Proctor, 1982). Aerosol particles of 3–4 μm in AD are considered to be optimal sizes for TB deposition. The mechanisms of deposition are by impaction along the trachea and at bronchial branchings where the direction of airflow changes and by gravity settlement in the fine airways in amounts proportional to the particle-settling velocity and the time available for settlement (Hatch and Gross, 1964; Heyder et al., 1980). Aerosol particles of 1–2 μm in AD, however, decrease in TB deposition because the particles are too small for effective impaction and sedimentation (Lippmann, 1977; Chan and Lippmann, 1980; Stahlhofen et al., 1980). Consequently, the majority of the very fine particles are exhaled. However, the deposition of the ultrafine particles of approximately 0.5 μm in AD on the walls of the finest bronchioles and the alveoli increases again due to molecular diffusion processes. Even so, some 90% of the inhaled 0.5-μm particles will still be exhaled during quiet tidal breathing and much more under forced exhalation (Davis et al., 1972; Taulbee et al., 1978). Those fine particles reach-

ing the finest bronchioles and alveoli are considered to be "respirable" (Lippmann, 1970).

In general, particles of AD > 10 μm deposit mainly in the URT (upper respiratory tract), whereas particles of 1–5 μm AD, with a GSD of less than 2, are likely to reach the lower respiratory tract, which includes the TB region and the alveoli, with small oropharyngeal loss.

The proportion of an aerosol sample suitable for inhalation can also be determined on the basis of mass median aerodynamic diameter (MMAD), which is defined as the percentage (50%) by weight of an aerosol sample having ADs equal to or less than the stated median AD. For example, a sample with an MMAD of 5 μm means that 50% by weight of that sample has ADs of 5 μm and smaller. The MMAD is therefore a good index for determining the proportion of an aerosol sample that is "noninhalable," "inhalable," or "respirable." An aerosol sample with an MMAD of 5 μm and a GSD of less than 2 is considered to be optimal for pulmonary deposition and retention (Task Group on Lung Dynamics, 1966).

In addition to AD and GSD, the pulmonary dynamics of a subject also greatly influence the distribution of aerosol particles in various regions of the respiratory tract (Agnew, 1984). For example, the velocity of airflow in the respiratory tract significantly influences the pattern of TB deposition. An increase in airflow velocity in the airways increases the effectiveness of particle impaction at the bifurcations of the large airways (Dennis, 1961; Hatch and Gross, 1964; Parent, 1991). As a result, spots impacted with a high concentration of particles (hot spots) are frequently present at the carina and the bifurcations of the airways (Lee and Wang, 1977; Bell, 1978; Stahlhofen et al., 1981). Furthermore, the depth of each breath (tidal volume) also influences the distribution of aerosols. A small tidal volume permits greater impaction in the proximal conducting airways and less sedimentation in the distal airways.

In general, slow, deep inhalation followed by a period of breath holding increases the deposition of aerosols in the peripheral parts of the lungs, whereas rapid inhalation increases the deposition in the oropharynx and in the large central airways. Thus, the frequency of respiration (the flow velocity) and the depth of breath (tidal volume) influence the pattern of pulmonary penetration and deposition of inhaled aerosols. Therefore, an aerosol of ideal size will penetrate deeply into the respiratory tract and the lungs only when the aerosols are inhaled in the correct manner.

18.1.4 Absorption and Clearance of Inhaled Aerosols

Soluble aerosols deposited on the epithelial linings of the respiratory tract are absorbed and metabolized in the same way as soluble gases and vapors.

Insoluble medicinal aerosols are few in number. Sodium cromoglycate (SCG) is probably the only insoluble powder to be administered as a prophylactic antiasthmatic (Wanner, 1979). Insoluble particles deposited on the ciliated linings of the URT are removed by a mucociliary clearance mechanism.

Particles deposited on a terminal airway devoid of ciliated cells may be endocytosed into the epithelial cells (Jones, 1984; Newhouse et al., 2000). At a toxic concentration, the cells die and the debris is then phagocytosed and transported into the interstitial space for removal via the lymph or vascular drainages or reenters the ciliated zone of the airway. Particles deposited in the alveolar walls are phagocytosed by the AMs and transported from the low-surface-tension surfactant in the alveolar lining to the high-surface-tension bronchial fluid of the ciliate airways for elimination by the mucociliary clearance mechanism (Lauweryns and Baert, 1977). The particle sizes optimal for phagocytosis are 2–3 μm, while particles smaller than 0.26 μm are less effective in activating the macrophages (Holma, 1967). In any case, AMs can phagocytose only a small fraction of a large number of deposited particles. The nonphagocytosed particles are translocated to the lymphatic system for elimination (Ferin, 1977).

Like the inhaled gases or vapors, soluble and insoluble aerosol particles can directly exert desirable and undesirable local effects at the site of deposition and/or systemic effects after solubilization, absorption, and metabolization (Sackner et al., 1975; Sackner, 1978).

18.1.5 Pharmacotoxicity of Inhaled Aerosols

The inhalation route for administering drugs into the pulmonary system for treatment of respiratory diseases eliminates many bioavailability problems such as plasma binding and "first-pass" metabolism, which are encountered in parenteral or oral administration. Consequently, a small inhalation dose is adequate for achieving the desirable therapeutic response without inducing many undesirable side effects. Furthermore, the direct contact of the drug with the target site ensures rapid action. Nevertheless, the effects from inhaled drug aerosols also depend on the pharmacological properties of the aerosols and the location of their deposition in the respiratory system. For example, the classic experiments on bronchodilation drugs (Dautrebande, 1962a,b) showed that fine aerosol particles of isoproterenol penetrate deeply in to the lower respiratory airways (LRA). In this way, a high concentration of the drug aerosol can reach the beta-adrenergic receptors of the bronchial smooth muscles. Stimulation of the receptors causes relaxation of the smooth muscle fibers and results in brochodilation (Weiner, 1984; McFadden, 1986). Such rapid bronchial responses can be produced in healthy and asthmatic subjects without inducing any cardiac effects. By contrast, the same dose of isoproterenol of large particle sizes deposits mainly along the URT, with a minimal amount reaching the smooth muscles of the LRA. The drug is quickly absorbed into the tracheal and bronchial veins and delivered immediately to the left ventricle of the heart. A high plasma concentration of the drug in the heart causes prominent cardiovascular effects such as tachycardia and hypertension. Other aerosols of beta-adrenergic drugs, such as epinephrine, isoprenaline, terbutaline, and salbutamol, induce bronchodilation effects in animals and

humans (Pavia, 1984) via inhalation and stimulate ciliary beat frequency and mucous production at the site of deposition in the trachea (Wanner, 1981). Thus the tracheobronchial mucociliary clearance mechanism is also stimulated. By contrast, anticholinergic bronchodilators, such as atropine and ipratropium bromide, cause mucous retention in the lungs (Pavia et al., 1983a,b). Therefore, in pharmacological or safety assessments of inhalant beta-adrenergic bronchial dilatation drugs, aerosols should be of small particle sizes suitable for deposition in the peripheral airways to minimize side effects. However, anticholinergic agents should be of larger particle sizes suitable for deposition in the large airways (Ingram et al., 1977; Hensley et al., 1978).

Other therapeutic aerosols—such as beclomethasone dipropionate, betamethasone valerate, and budesonide corticosteroid (Williams, 1974); the carbenicillin and gentamicin antibiotics (Hodson et al., 1981); the 2-mercaptoethane-sulfonate (Pavia et al., 1983b) and *N*-acetylcysteine (Hollinger, 1985) mucolytics; and even vaccines for the prevention of influenza and tuberculosis (Lourenco and Cotromanes, 1982)—are active by inhalation and/or oral administration. When these drugs are administered as aerosols, certain particle sizes may be targeted to a specific region or to multiple regions of the pulmonary system depending on the therapeutic target site(s). In any case, when aerosols are delivered as fine particles, the rate of absorption is increased because of an increase in the distribution area per unit mass of the drug. Thus, an effective aerosol dose of corticosteroid for treatment of asthma and bronchitis is merely a fraction of an oral dose (Williams, 1974). An aerosol of SCG dry powder, a prophylactic for preventing the onset of bronchoconstriction in asthmatic attacks (Cox et al., 1970), is effective mainly by local inhibition of the release of chemical mediators from mast cells in bronchial smooth muscle. Therefore, SCG particle sizes should be approximately 2 μm in AD for the most effective penetration into the bronchial regions (Godfrey et al., 1974; Curry et al., 1975). Likewise, therapeutic aerosols of local anesthetics and surfactants may require appropriate particle sizes to be targeted to a specific region of the pulmonary system.

Other than undesirable pharmacological effects, toxic concentrations of soluble or insoluble aerosol particles may lead to adverse physiological and/or histophathological responses. For example, irritating aerosols cause dose-related reflex depression of the respiratory rate (Alarie, 1966, 1981a), while phagocytosed particles cause chemotaxis of AMs and neutrophils to the site of deposition (Brain, 1971). The maximum response usually occurs at 24 h and returns to normal in approximately three days postexposure (Kavet et al., 1978). Furthermore, a toxic quantity of phagocytosed particles may interact with the lysosomal membrane within a macrophage, releasing cytotoxic lysosomal enzymes, proteases, and free radicals that in turn damage the adjacent lung tissue (Hocking and Golde, 1979).

In general, a specific category of drug delivered to a specific site of the pulmonary system will exert a specific pharmacological or toxicological action locally or systemically. Therefore, in safety assessments of inhalants, a drug

should be delivered to the target sites of the pulmonary system according to the toxicological information required.

Finally, there are many drugs in the categories of amphetamines, anorectics, antihistamines, antipsychotics, tricyclic antidepressants, analgesics and narcotics, and beta-adrenergic blocking agents that are known to accumulate in the lung (Wilson, 1982; Hollinger, 1985) even though these drugs are not administered via the inhalation route. Therefore, in safety assessments of these drugs, their pulmonary toxicity should also be evaluated.

18.1.6 Methods for Safety Assessment of Inhaled Therapeutics

Methods for evaluation of inhalation toxicity should be selected according to the pharmacological and/or toxicological questions asked, and the design of experiments should specify the delivery route of a drug to the target sites in the pulmonary system (Gad, 2006). For example, if an immunological response of the lungs to a drug is in question, then the lymphoid tissues of the lungs should be the major target of evaluation. The following are some of the physiological, biochemical, and pharmacological tests that are applicable for safety assessment of inhaled medicinal gases, vapors, or aerosols.

Upper respiratory tract irritation can occur from inhalation of a medicinal gas, vapor, or aerosol. For assessing the potential of an inhalant to cause URT irritation, the mouse body plethysmographic technique (Alarie, 1966, 1981a,b) has proven to be extremely useful. This technique operates on the principle that respiratory irritants stimulate the sensory nerve endings located at the surface of the respiratory tract from the nose to the alveolar region. The nerve endings in turn stimulate a variety of reflex responses (Alarie, 1973; Widdicombe, 1974) that result in characteristic changes in inspiratory and expiratory patterns and, most prominently, depression of respiratory rate. Both the potency of irritation and the concentration of the irritant are positively related to the magnitude of respiratory rate depression. The concentration response can be quantitatively expressed in terms of the RD_{50}, defined as the concentration (in logarithmic scale) of the drug in the air that causes a 50% decrease in respiratory rate. The criteria for positive URT irritation in intact mice exposed to the drug atmosphere are depression in breathing frequency and a qualitative alteration of the expiratory patterns. Numerous experimental results have shown that the responses of mice correlated almost perfectly with those of humans (Alarie et al., 1980; Alarie and Luo, 1986). Thus, this technique is useful for predicting the irritancy of airborne medicinal compounds in humans. From the drug-formulating point of view, an inhalant drug with URT-irritating properties indicates the need for an alternate route of administration. From the industrial hygiene point of view, the recognition of the irritant properties is very important. If a chemical gas, vapor, or aerosol irritates, it has a "warning property." With an adequate warning property a worker will avoid inhaling damaging amounts of the airborne toxicant; without

a warning property a worker may unknowingly inhale an injurious amount of the toxicant.

Inhalation of a cardiovascular drug, such as an aerosol of propranolol (a beta-adrenergic receptor agonist), may affect the respiratory cycle of a subject. For evaluating the cardiopulmonary effects of an inhalant, the plethysmograph technique using a mouse or a guinea pig model is useful. The criteria for a positive response in intact mice or guinea pigs are changes in the duration of inspiration and expiration and the interval between breaths (Schaper et al., 1989).

Pulmonary sensitization may occur from inhalation of drug vapors such as enflurane (Schwettmann and Casterline, 1976) and antibiotics such as spiramycin (Davies and Pepys, 1975) and tetracycline (Menon and Das, 1977). To detect pulmonary sensitization from inhalation of drug and chemical aerosols, the body plethysmographic technique using a guinea pig model has been shown to be useful (Patterson and Kelly, 1974; Paterson, 1977; Karol, 1988; Karol and Thorne, 1988; Karol et al., 1989; Thorne and Karol, 1989). The criteria for positive pulmonary sensitization in intact guinea pigs are changes in breathing frequency and their extent and the time of onset of an airway constrictive response after induction and after a challenge dose of the test drug (Karol et al., 1989).

The mucociliary transport system of the airways can be impaired by respiratory irritants, local analgenesics and anesthetics, and parasympathetic stimulants (Pavia, 1984). Any one of the above agents will retard the beating frequency of the cilia and the secretion of the serous fluid of the mucous membranes. As a result, the propulsion of the inhaled particles, bacteria, or endogenous debris toward the oral pharynx for expectoration or swallowing will be retarded. Conversely, inhalation of adrenergic agonists increases the activity of the mucociliary transport system and facilitates the elimination of noxious material from the pulmonary system. Laboratory evaluation of the adverse drug effects on mucociliary transport in animal models can be achieved by measuring the velocity of the linear flow of mucus in the trachea of surgically prepared animals (Rylander, 1966; Oyarzun and Clements, 1977, 1978). Clinically, the transportation of markers placed on the tracheal epithelium of normal human subjects can also be observed using a fiber-optic bronchoscopic technique (Pavia et al., 1980; Mussatto et al., 1988). The criteria of a positive response are changes in the transport time over a given distance of markers placed on the mucus or changes in the rate of mucous secretion (Davis et al., 1976; Johnson et al., 1983, 1987; Webber and Widdicombe, 1987). More comprehensive discussion on mucociliary clearance can be found in several reviews (Last, 1982; Pavia, 1984).

Cytological studies on the bronchial alveolar lavage fluid (BALF) permit the evaluation of the effects of an inhaled drug on the epithelial lining of the respiratory tract. This fluid can be obtained form intact animals or from excised lungs (Henderson, 1984, 1988, 1989). Quantitative analyses of fluid constituents such as neutrophils, antibody-forming lymphocytes, and antigen-specific IgG provide information on the cellular and biochemical responses of the

lungs to the inhaled agent (Henderson, 1984; Henderson et al., 1985, 1987). For example, BALF parameters were found to be unperturbed by the inhalation of halothane (Henderson and Lowrey, 1983). The criteria of a positive response are increase in protein content, increase in number of neutrophils and macrophages for inflammation, increase in number of lymphocytes and alteration of lymphocyte profiles for immune response, increase in cytoplasmic enzymes (lactate dehydrogenease) for cell lysis (Henderson, 1989), and presence of antigen-specific antibodies for specific immune responses (Bice, 1985).

Morphological examination of the cellular structure of the pulmonary system is the foundation of most inhalation toxicity studies. Inhalation of airborne drug vapors or aerosols at harmful concentrations results mainly in local histopathological changes in the epithelial cells of the airways, of which there are two types: nonciliated and ciliated cells. The nonciliated cells are the Clara cells, which contain secretory granules and smooth endoplasmic reticulum (SER); the ciliated cells are the brush cells, which have secretory granules and stubby microvilli and numerous cytoplasmic fibers on their free surfaces but lack SER. If the concentration gradient of the drug in the lung is high enough to reach the alveoli, the type I alveoli cells will also be affected (Evans, 1982). Drugs that affect the lungs via the bloodstream, such as bleomycin (Aso et al., 1976), cause changes to the endothelial cells of the vascular system that result in diffuse damage to the alveoli. The criteria of cellular damage are loss of cilia, swelling, and necrosis and sloughing of cell debris into the airway lumina. Tissues recovering from injuries are characterized by increases in the number of dividing progenitor cells followed by increases in intermediate cells that eventually differentiate into normal surface epithelium.

Pulmonary drug disposition studies are essential in the research and development of new inhalant drugs. Inhaled drugs are usually absorbed and metabolized to some extent in the lungs because the lungs, like the liver, contain active enzyme systems. A drug may be metabolized to an inactive compound for excretion or to a highly reactive toxic metabolite(s) that causes pulmonary damage. In most pulmonary disposition studies, a gas or vapor is delivered via whole-body exposure (Paustenbach et al., 1983) or head-only exposure (Hafner et al., 1975). For aerosols, over 90% of a dose administered by mouth breathing is deposited in the oropharynx and swallowed. Consequently, the disposition pattern reflects that of ingestion in combination with a small contribution from pulmonary metabolism. For determining the disposition of inhaled drugs by the pulmonary system alone, a dosimetric endotracheal nebulization technique (Leong et al., 1988) is useful. In this technique, microliter quantities of a radiolabeled drug solution can be nebulized within the trachea using a miniature air–liquid nebulizing nozzle. Alternatively, a small volume of liquid can be dispersed endotrachially using a microsyringe. In either technique, an accurate dose of a labeled drug solution is delivered entirely into the respiratory tract and lungs. Subsequent radioassay of the excreta thus reflects only the pulmo-

nary disposition of the drug without complication from aerosols deposited in the oropharyngeal regions if the drug had been delivered by mouth inhalation. For example, in a study of the antiasthmatic drug lodoximide tromethamine, the urinary metabolites produced by beagle dogs after receiving a dose of the radiolabeled drug via endotracheal nebulization showed a high percentage of the intact drug. However, metabolites produced after oral administration were mainly nonactive conjugates. The differences were due to the drug's escape from first-pass metabolism in the liver when it was administered through the pulmonary system. The results thus indicated that the drug had to be administered by inhalation to be effective. This crucial information was extremely important in the selection of the most effective route of administration and formulation of this antiasthmatic drug (Leong et al., 1988).

Cardiotoxicity of inhalant drugs should also be evaluated. For example, adverse cardiac effects may be induced by inhaling vapors of fluorocarbons, which are used extensively as propellants in drug aerosols. Inhalation of vapors of anesthetics also has been shown to cause depression of the heart rate and alteration of the rhythm and blood pressure (Merin, 1981; Leong and Rop, 1989). More important, inhalation of antiasthmatic aerosols of beta-receptor agonists delivered in a fluorocarbon propellant has been shown to cause marked tachycardia, electrocardiogram (ECG) changes, and sensitization of the heart to arrhythmia (Aviado, 1981; Balazs, 1981). Chronic inhalation of drug aerosols can also result in cardiomyopathy (Balazs, 1981). For detection of cardiotoxicity, standard methods of monitoring arterial pressures, heart rate, and ECGs of animals during inhalation of a drug or at frequent intervals during a prolonged treatment period should be useful in safety assessments of inhalant drugs.

Since the inhalation route is just a method for administering drugs, other nonpulmonary effects, such as behavioral effects (Ts'o et al., 1975) and renal and liver toxicity, should also be evaluated. In addition, attention should also be given to drugs that are not administered via the inhalation route but that accumulate in the lungs where they cause pulmonary damage (Wilson, 1982; Hollinger, 1985). Such inhaled organics can have a wide range of target organ effects—even on the eyes (Leong et al., 1987).

18.1.7 Parameters of Toxicity Evaluation

Over 400 years ago Paracelsus stated: "All substances are poison. The right dose differentiates a poison and a remedy." Thus, in safety assessments of inhaled drugs, the "dose," or magnitude of inhalation exposure, in relation to the physiological, biochemical, cytological, or morphological response(s) must be determined. Toxicity information is essential to establishing guidelines to prevent the health hazards of acute or chronic overdosage during therapy or of unintentional exposure to the bulk drugs and their formulated products during manufacturing and industrial handling.

Inhaled "Dose" Most drugs are designed for oral or parenteral administration in which the dose is calculated in terms of drug weight in milligrams divided by the body weight in kilograms:

$$\text{Dose} = \frac{\text{drug weight (mg)}}{\text{body weight (kg)}} = \frac{\text{mg}}{\text{kg}}$$

For inhalant drugs, the inhaled dose has been expressed in many mathematical models (Dahl, 1990). However, the practical approach is based on exposure concentration and duration rather than on theoretic concepts. Thus, an inhaled dose is expressed in terms of the exposure concentration (C) in milligrams per liter or milligrams per cubic meter or parts per million (ppm) of air, the duration of exposure (t) in minutes, the ventilatory parameters including the respiratory rate (R) in number of breaths per minute and the tidal volume (Tv) in liters per breath, and a retention factor α which is related to the reactivity and the solubility of the drug. The product of these parameters divided by the body weight in kilograms gives the dose:

$$\text{Dose} = \frac{CtR\text{Tv}\alpha}{\text{body weight}} = \frac{\text{mg}}{\text{kg}}$$

In critical evaluation of the effect of a gas, vapor, or aerosol inhaled into the respiratory tract of an animal, the dosimetric method has been recommended (Oberst, 1961). However, due to the complexity of measuring the various parameters simultaneously, only a few studies on gaseous drugs or chemicals have employed the dosimetric method (Weston and Karel, 1946; Leong and MacFarland, 1965; Landy et al., 1983; Stott and McKenna, 1984; Dallas et al., 1986, 1989). For studies on liquid or powdery aerosols, modified techniques such as intratracheal instillation (Brain et al., 1976) or endotracheal nebulization (Leong et al., 1988) were used to deliver an exact dose of the test material into the lower respiratory tract (LRT) while bypassing the URT and ignoring the ventilatory parameters.

In routine inhalation studies, it is generally accepted that the respiratory parameters are relatively constant when the animals are similar in age, sex, and body weight. This leaves only C and t to be the major variables for dose consideration:

$$\text{Dose} = Ct = \text{mg} \cdot \text{min/L}$$

The product Ct is not a true dose because its unit is milligrams per minute per liter rather than milligrams per kilogram. Nevertheless, Ct can be manipulated as though it were a dose, an approximated dose (MacFarland, 1976).

TABLE 18.1 Respiratory Parameters for Common Experimental Species and Humans

Species	Body Weight (kg)	Lung Volume (mL)	Minute Volume ($mL\,min^{-1}$)	Alveolar Surface Area (m^2)	Lung Volume % Surface Area	Minute Volume % Lung Volume	Minute Volume % Surface Area
Mouse	0.023	0.74	24	0.068	10.9	32.4	353
Rat	0.14	6.3	84	0.39	16.2	13.3	215
Monkey	3.7	184	694	13	14.2	3.77	53
Dog	22.8	1501	2923	90	16.7	1.95	33
Human	75	7000	6000	82	85.4	0.86	73

Source: Altman and Dittmar, 1974.

The respiratory parameters of an animal will dictate the volume of air inhaled and hence the quantity of test material entering the respiratory system. Commonly used parameters for a number of experimental species and humans are given in Table 18.1 to illustrate this point and include the alveolar surface area because this represents the target tissue for most inhaled materials. It can be seen that, by taking the ratios of these parameters and comparing the two extremes, that is, the mouse and human, (1) a mouse inhales approximately 30 times its lung volume in 1 min whereas a human at rest inhales approximately the same volume as that of his or her lung. This can increase with heavy work up to the same ratio as the mouse but is not sustained for long periods. This means that the dose per unit lung volume is up to 30 times higher in the mouse than in humans at the same inhaled atmospheric concentration. (2) The minute volume of the mouse is in contact with five times less alveolar surface area than humans and hence the dose per unit area is up to five times greater in the mouse. (3) The lung volume in comparison with the alveolar surface area in experimental animals is less than in humans, meaning that the extent of contact of inhaled gases with the alveolar surface is greater in experimental animals.

While it is possible, and common, to refer to standard respiratory parameters for different species in order to calculate inhaled dose and deposited dose with time, it is usually the case that inhaled materials influence the breathing patterns of test animals. The most common examples of this are irritant vapors, which can reduce the respiratory rate by up to 80%. This phenomenon results from a reflexive pause during the breathing cycle due to stimulation by the inhaled material of the trigeminal nerve endings situated in the nasal passages. The duration of the pause and hence the reduction in the respiratory rate are concentration related, permitting concentration–response relationships to be plotted. This has been investigated extensively by Alarie (1981a) and forms the basis of a test screen for comparing quantitatively the irritancy of different materials and has found application in assessing appropriate exposure limits for human exposure when respiratory irritancy is the predominant cause for concern.

While irritancy resulting from the above reflex reaction is one cause of altered respiratory parameters during exposure, there are many others. These include other types of reflex response, such as bronchoconstriction, the narcotic effects of many solvents, the development of toxic signs as exposure progresses, or simply a voluntary reduction in respiratory rate by the test animal due to the unpleasant nature of the inhaled atmosphere. The extent to which these affect breathing patterns and hence inhaled dose can only be assessed by actual measurement.

By simultaneous monitoring of tidal volume and respiratory rate, or minute volume, and the concentration of an inhaled vapor in the bloodstream and the vapor in the exposure atmosphere, pharmacokinetic studies on the *Ct* relationship have shown that the effective dose was nearly proportional to the exposure concentration for vapors such as 1,1,1-trichloroethane (Dallas et al., 1989), which has a saturable metabolism. However, it was also found that the steady-state plasma concentrations were disproportionally greater at higher exposure concentrations.

Acknowledging the possible existence of deviations, this simplified approach of using *C* and *t* for dose determination provides the basis for dose–response assessments in practically all inhalation toxicological studies.

Dose–Response Relationship The first principle of dose–response determination in inhalation toxicology is based on Haber's rule, which states that responses to an inhaled toxicant will be the same under conditions where *C* varies in complementary manner to *t* (Haber, 1924). For example, if *Ct* elicits a specific magnitude of the same response, that is, $Ct = K$, where *K* is a constant for the stated magnitude of response.

This rule holds reasonably well when *C* or *t* varies within a narrow range for acute exposure to a gaseous compound (Rinehart and Hatch, 1964) and for chronic exposure to an inert particle (Henderson et al., 1991). Excursion of *C* or *t* beyond these limits will cause the assumption $Ct = K$ to be incorrect (Adams et al., 1950, 1952; Sidorenko and Pinigin, 1976; Andersen et al., 1979; Uemitsu et al., 1985). For example, an animal may be exposed to 1000 ppm of diethyl ether for 420 min or 1400 ppm for 300 min without incurring any anesthesia. However, exposure to 420,000 ppm for 1 min will surely cause anesthesia or even death of the animal. Furthermore, toxicokinetic study of liver enzymes affected by inhalation of carbon tetrachloride (Uemitsu et al., 1985), which has a saturable metabolism in rats, showed that $Ct = K$ does not correctly reflect the "toxicity value" of this compound. Therefore, the limitations of Haber's rule must be recognized when it is used in interpolation or extrapolation of inhalation toxicity data.

Exposure Concentration versus Response In certain medical situations (e.g., a patient's variable exposure duration to a surgical concentration of an inhalant anesthetic or the repeated exposures of surgeons and nurses to subanesthetic concentrations of an anesthetic in the operating theater), it is neces-

sary to know the duration of safe exposure to a drug. Duration safety can be assessed by determining a drug's median effective time (ET_{50}) or median lethal time (LT_{50}). These statistically derived quantities represent the duration of exposure required to affect or kill 50% of a group of animals exposed to a specified concentration of an airborne drug or chemical in the atmosphere.

The graph in Figure 18.2 is the probit plot of percent response to exposure concentrations. The log/probit transformation(Litchfield and Wilcoxon, 1949) is used to linearize the dose response data for graphing data. Figure 18.3,

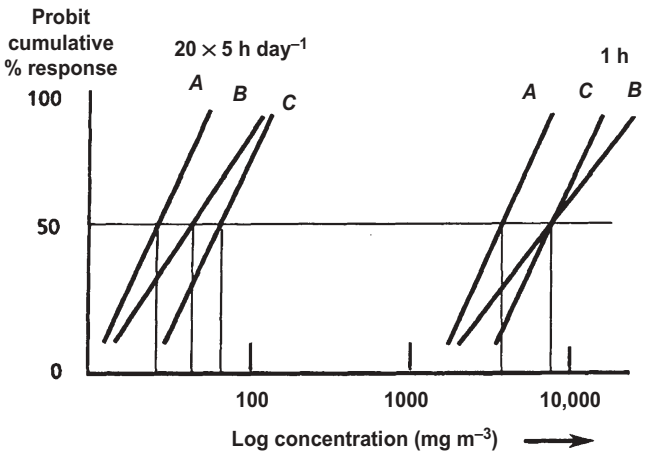


Figure 18.2 *Dose–response plot in terms of probit of cumulative percentage response to logarithm of exposure concentrations.*

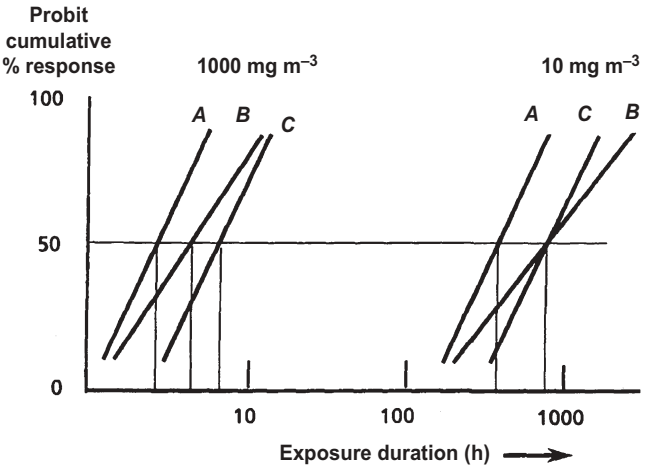


Figure 18.3 *Dose–response plot in terms of probit of cumulative percentage response to logarithm of exposure duration.*

meanwhile, is the probit plot of cumulative percentage response to logarithm of exposure duration. It shows the 1000 mg m^{-3} for 10 h or to 10 mg m^{-3} for 1000 h, each with a *Ct* (an approximated dose) of ~ 10,000 h mg m^{-3}. Similar to concentration–response graphs, the slopes indicate the differences in the mechanism of action and the margins of safe exposure of the three drugs. The ratio of the ET_{50} or LT_{50} of two drugs indicates their relative toxicity, and the ratio of ET_{50} over LT_{50} of the same drug is the therapeutic ratio.

Product of Concentration and Duration (Ct) versus Responses To evaluate inhalation toxicity in situations where workers are exposed to various concentrations and durations of a drug vapor, aerosol, or powder in the work environment during manufacturing or packaging, a more comprehensive determination of $E(Ct)_{50}$ or $L(Ct)_{50}$ values are used. The $E(Ct)_{50}$ or $L(Ct)_{50}$ values are statistically derived values that represent the magnitude of exposure, expressed as a function of the product of *C* and *t*, that is expected to affect or kill <50% and >50% of the animals. The other curve represents exposures that kill 50% or >50% of each group of animals (Irish and Adams, 1940).

The graph in Figure 18.4 illustrates inhalation exposures to a drug using various combinations of *C* and *t* that kill 50% of the animals. For example, a 50% mortality occurs when a group of animals is exposed to drug A at a concentration of 1000 mg m^{-3} for a duration of approximately 2 h or at a

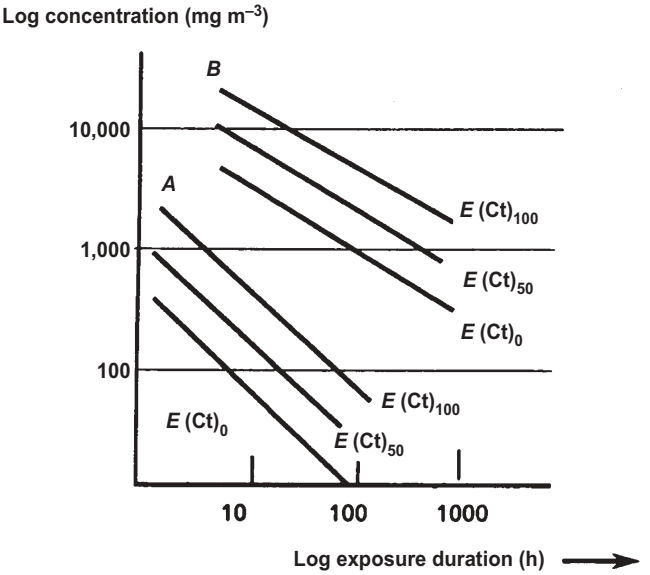


Figure 18.4 *Dose–response plot in terms logarithms of exposure concentration and durations.*

concentration of 100 mg m^{-3} for a duration of approximately 20 h. Furthermore, the graph also illustrates that the inhalation toxicity of drug A is more than one order of magnitude higher than that of drug B. For example, an exposure to drug A at the concentration of 100 mg m^{-3} for 100 h kills 100% of the animals whereas an exposure to drug B at the concentration of 1000 mg m^{-3} for 100 h does not kill any animals.

Units for Exposure Concentration For gases and vapors, exposure concentrations are traditionally expressed in parts per million (ppm). The calculation for the ppm of a gas or vapor in an air sample is based on Avogadro's law, which states that equal volumes contain equal numbers of molecules under the same temperature and pressure. In other words, under standard temperature and pressure (STP), one gram-molecular weight (mole) of any gas under a pressure of one atmosphere (equivalent to the height of 760 mm mercury) and a temperature of 273 K has the same number of molecules and occupies the same volume of 22.4 L. However, under ambient conditions, the volume of 22.4 L has to be corrected to a larger volume based on Charles's law, which states that at constant pressure the volume of gas varies directly with the absolute temperature. Thus, at a room temperature of 25 °C, 1 mol of a gas occupies a volume of 24.5 L:

$$22.4\,\text{L} \times \frac{298\,\text{K}}{273\,\text{K}} = 24.5\,\text{L}$$

Further correction of volume for an atmospheric pressure deviation from one atmosphere may be done by applying Boyle's law, which states that the volume of a gas without change of temperature varies inversely with the pressure applied to it:

$$24.5\,\text{L} \times \frac{758\,\text{mm Hg}}{760\,\text{mm Hg}} = 24.4\,\text{L}$$

In practice, atmospheric pressure in most animal experimental environments usually varies only a few millimeters of mercury, so little or no correction is required.

Using the aforementioned principles, the volume of a vapor generated from a given weight of a liquid can be calculated. For example, 1 mol of water weighs 18 g, while 1 mol of ethanol weighs 46 g. When 1 mol of each liquid is totally vaporized, each will occupy the same volume of 24.5 L at room temperature (25 °C) and pressure (760 mm Hg). In an inhalation experiment, if the volume of test liquid and the rate of airflow being mixed in the animal exposure chamber are known, the vapor concentration in the chamber atmosphere can be calculated in parts per million or milligrams per liter. A conversion table published by the U.S. Bureau of Mines enables quick conversion between parts

per million and milligrams per liter for compounds with molecular weights up to 300 g (Fieldner et al., 1921; Patty, 1958).

For aerosols of nonvolatile liquid and powdery compounds, the concentration of the mist or dust atmosphere must be expressed in terms of milligrams per liter or milligrams per cubic meter of air. With advances in biotechnology, Many pharmacological testing techniques are based on specific receptor bindings, in which the ratio of the number of molecules to those of the receptors are considered, in which case the exposure concentration may be more appropriately expressed in micromoles per unit volume of air.

18.1.8 Inhalation Exposure Techniques

Many inhalation exposure techniques, such as the whole-body, nose-only, mouth-only, or head-only technique (Drew and Laskin, 1973; MacFarland, 1976; Leong et al., 1981; Smith et al., 1981; Phalen, 1984), the intranasal exposure technique (Elliot and DeYoung, 1970), the endotracheal nebulization technique (Leong et al., 1985, 1988; Schreck et al., 1986), and the body plethysmographic techniques (Alarie, 1966; Thorne and Karol, 1989), have been developed for inhalation toxicity studies. Table 18.2 provides a summary of the advantages and disadvantages of each of the major inhalation exposure methodologies.

The main criteria for the design and operation of any dynamic (as opposed to static) inhalation exposure system are as follows:

- The concentration of the test atmosphere must be reasonably uniform throughout the chamber and should increase and decrease at a rate close to theoretical at the start or end of the exposure. Silver (1946) showed that the time taken for a chamber to reach a point of equilibrium was proportional to the flow rate of atmosphere passing through the chamber and the chamber volume. From this, the concentration–time relationship during the "run-up" and "run-down" phase could be expressed by the equation

$$t_x = k\frac{V}{F}$$

where t_x = time required to reach x percent of the equilibrium concentration, k = a constant determined by the value of x, V = chamber volume, and F = chamber flow rate. The t_{99} value is frequently quoted for exposure chambers, representing the time required to reach 99% of the equilibrium concentration and providing an estimate of chamber efficiency. Thus, at maximum efficiency, the theoretical value of k at t_{99} is 4.605, and the closer to this that the results of evaluation of actual chamber performance fall, the greater the efficiency and the better the design of the chamber.

TABLE 18.2 Advantages, Disadvantages, and Considerations Associated with Patterns of Inhalation Exposure

Mode of Exposure	Advantages	Disadvantages	Design Considerations
Whole body	Variety and number of animals; chronic studies possible; minimum restraint; large historical database; controllable environment; minimum stress; minimum labor	Messy; multiple routes of exposure: skin, eyes, oral; variability of "dose"; cannot pulse exposure easily; poor contact between animals and investigators; capital intensive; inefficient compound usage; difficult to monitor animals during exposure	Cleaning effluent air; inert materials; losses of test material; even distribution in space; sampling; animal care; observation; noise, vibration, humidity; air temperature; safe exhaust; loading; reliability
Head only	Good for repeated exposure; limited routes of entry into animal; more efficient dose delivery	Stress to animal; losses can be large; seal around neck; labor in loading/unloading	Even distribution; pressure fluctuations; sampling and losses; air temperature, humidity; animal comfort; animal restraint
Nose/mouth only	Exposure limited to mouth and respiratory tract; uses less material (efficient); containment of material; can pulse exposure	Stress to animal; seal about face; effort to expose large number of animals	Pressure fluctuations; body temperature; sampling; airlocking; animals' comfort; losses in plumbing/ masks
Lung only (tracheal administration)	Precision of dose; one route of exposure; uses less material (efficient); can pulse exposure	Technically difficult; anesthesia or tracheostomy; limited to small numbers; bypasses nose; artifacts in deposition and response; technically more difficult	Air humidity/ temperature; stress to animal; physiological support
Partial lung	Precision of total dose; localization of dose; can achieve very high local doses; unexposed control tissue from same animal	Anesthesia; placement of dose; difficulty in interpretation of results; technically difficult; possible redistribution of material within lung	Stress to animal; physiological support

Source: Gad and Chengelis, 1998.

- Flow rates must be controlled in such a way that they are not excessive, which might cause streaming effects within the chamber, but must be adequate to maintain normal oxygen levels, temperature, and humidity in relation to the number of animals being exposed. A minimum of 10 air changes per hour is frequently advocated and is appropriate in most cases. However, the chamber design and housing density also need to be taken into account and some designs, such as that of Doe and Tinston (1981), function effectively at lower air change rates.
- The chamber or exposure manifold materials should not affect the chemical or physical nature of the test atmosphere.

The whole-body exposure technique is useful for acute and chronic toxicity studies of gases and vapors. For acute whole-body exposure, a few animals are exposed for 1–4 h to a gas, vapor, or aerosol of a drug or chemical in a simple glass jar. The gaseous drug is metered with a precision flow meter into the stream of filtered room air being drawn through the glass jar or chamber. For vapor generation from a volatile liquid, a stream of clean air is bubbled at a constant rate onto the walls of a temperature-regulated flask, which vaporizes the liquid droplets rapidly and continuously. In either method, the vapor emerging from the vaporizer is directed into the filtered air stream being drawn through the glass jar or chamber. For the generation of drug aerosols from liquids or powders, various types of atomizers or nebulizers and dust generators are available (Drew and Laskin, 1973; Drew and Lippmann, 1978; Leong et al., 1981; Phalen, 1984). For more critical and precision studies, an adequate number of animals per group is calculated by an appropriate statistical method (Gad, 2007) and the exposure is carried out in an elaborate dynamic airflow chamber with precision control of the chamber airflow, temperature, and humidity.

Regardless of the exposure apparatus used, the most important aspect of an exposure study is the generation of a constant concentration of the airborne drug vapor or aerosol in the chamber atmosphere has to be sampled (Drew and Lippmann, 1978) and analyzed using an appropriate analytical instrument, such as an infrared spectrophotometer for halogenated propellants or a gas chromatograph for other gases and vapors. The concentration of the drug as detected by the analyzer is the "analytical concentration." For characterizing the aerosol atmosphere, particle sizing, in addition to concentration analysis, is essential. Because the breathing patterns of the experimental animals cannot be regulated, it is extremely important to generate aerosols of the appropriate size for bioavailability.

For critical laboratory studies on inhaled drugs, a monodisperse aerosol of a specified range of MMAD should be used to increase the probability that the aerosol reaches the specified target area of the lungs. The Dautrebande aerosol generators (Dautrebande, 1962c) and the DeVilbiss nebulizer (Drew and Lippmann, 1978) are the classic single-reservoir generators for short-duration inhalation studies. For long-duration inhalation studies, the

multiple-reservoir nebulizer (Miller et al., 1981) or the continuous syringe metering and elutriating atomizer (Leong et al., 1981) are frequently used. The nebulizers generate a polydisperse droplet aerosol either by the shearing force of a jet of air over a fine stream of liquid or by ultrasonic disintegration of the surface liquid in a reservoir (Drew and Lippmann, 1978). The aerosols emerging from a jet nebulizer generally have MMADs ranging between 1.2 and 6.9 μm with GSDs of 1.7–2.2, and aerosols from an ultrasonic nebulizer have MMADs ranging between 3.7 and 18.5 μm with GSDs of 1.4–2.0 (Mercer, 1981).

For testing therapeutic formulations, the liquid aerosols are usually generated by the pressurized metered-dose inhaler (Newman, 1984; Newton, 2000; Gad and Chengelis, 1998). The pressurized metered-dose inhaler (MDI) generates a bolus of aerosols by atomizing a well-defined quantity of a drug that is solubilized in a fluorocarbon propellant. The aerosols thus consist of the drug particles with a coating of the propellant. As the aerosols emerge from the orifice, the mean particle size may be as large as 30 μm (Moren, 1981). After traveling through a tubular or cone-shaped spacer, the propellant may evaporate, reducing the MMADs to a range of 2.8–5.5 mm with GSDs of 1.5–2.2 (Hiller et al., 1978; Sackner et al., 1981; Newman, 1984) and making the aerosols more stable for inhalation studies. In a prolonged animal exposure study, multiple metered-dose inhalers have to be actuated sequentially with an electromechanical gadget (Ulrich et al., 1984) to maintain a slightly pulsatile but relatively consistent chamber concentration.

For generating an aerosol from dry powders, various dust generators, such as the Wright dust feed, air elutriator or fluidized-bed dust generator, and air impact pulverizer, have been developed for acute and chronic animal inhalation studies and described in many articles (Hinds, 1980; Leong et al., 1981; Phalen, 1984; Gad and Chengelis, 1998; Valentine and Kennedy, 2001; Hext, 2000). For generating powdery therapeutic agents, a metered-dose dry-powder inhaler, spinhaler, or rotahaler is used (Newman, 1984). The particle size of the drug powder is micronized to a specific size range during manufacture and the spinhaler or the rotahaler only disperses the powders.

More recently, another approach for administering dry powders to both humans and test animals has arisen. Dry powders, while less frequently used in nasal drug delivery, are becoming more popular. Powders can be administered from several devices, the most common being the insufflator. Many insufflators work with predosed powder in gelatin capsules. To improve patient compliance, a multidose powder inhaler has been developed which has been used to deliver budesonide. These devices can also be used for administration to test animals delivery, in terms of both amount and aerodynamic size of the particles. While early dry-powder inhalers such as the Rotohaler used individual capsules of micronized drug which were difficult to handle, modern devices use blister packs (e.g., Diskus) or reservoirs (e.g., Turbuhaler). The dry-powder inhalers rely on inspiration to withdraw drug from the inhaler to the lung, and hence the effect of inhalation flow rate through various devices

has been extensively studied. The major problem to be overcome with these devices is to ensure that the finely micronized drug is thoroughly dispersed in the airstream. It has been recommended that patients inhale as rapidly as possible from these devices in order to provide the maximum force to disperse the powder. The quantity of drug and deposition pattern vary enormously depending on the device, for example, the Turbuhaler produces significantly greater lung delivery of salbutamol than the Diskus. Vidgren and co-workers (1987) demonstrated by gamma scintigraphy that a typical dry-powder formulation of sodium cromoglycate suffers losses of 44% in the mouth and 40% in the actuator nozzle itself.

It must also be emphasized that the major mass of a heterodispersed aerosol may be contained in a few relatively large particles since the mass of a particle is proportional to the cube of its diameter. Therefore, the particle size distribution and the concentration of the drug particles in the exposure atmosphere should be sampled using a cascade impactor or membrane filter sampling technique, monitored using an optical or laser particle size analyzer, and analyzed using optical or electron microscopy techniques.

In summary, many techniques have been developed for generating gas, vapor, and aerosol atmospheres for inhalation toxicology studies. By proper regulation of the operating conditions of the nebulizers and the formulation of metered-dose inhalers, together with the use of spacer or reservoir attachments to MDIs, more particles within the respirable range can be generated for inhalation. An accurately controlled exposure concentration is essential to an accurate determination of the dose–response relationship in a safety assessment of an inhalant drug.

Finally, comparisons of various techniques for animal exposures indicate that the whole-body exposure technique is the most suitable for safety assessment of gases and vapors and permit simultaneous exposure of a large number of animals to the same concentration of a drug; however, this technique is not suitable for aerosol and powder exposures because the exposure condition represents the resultant effects from inhalation, ingestion, and dermal absorption of the drug (Phalen, 1984; Gad and Chengelis, 1998).

18.1.9 Utility of Toxicity Data

Regardless of the type of test and the parameters to be monitored, the ultimate goal is to interpolate or extrapolate from the dose–response data to find a no-observable-adverse-effect level (NOAEL) or a no-observable-effect level (NOEL). By applying a safety factor of 1–10 to the NOAEL, a safe single-exposure dose for a phase I clinical trial may be obtained. By applying a more stringent safety factor, a multiple-exposure dose for a clinical trial may also be obtained. After the drug candidate has successfully passed all the drug safety evaluations and entered in the production stage, more toxicity tests may be needed for the establishment of a threshold limit value–time-weighted

average (TLV–TWA). A TLV–TWA is defined as "the time weighted average concentration for a normal 8-hour workday and a 40-hour workweek, to which nearly all workers may be repeatedly exposed, day after day, without adverse effect" [American Conference of Governmental Industrial Hygienists (ACGIH), 1991]. Using TLVs as guides, long-term safe occupational exposures during production and industrial handling of a drug may be achieved. Appropriate safety assessments of pharmaceutical chemicals and drugs will ensure the creation and production of a safe drug for the benefit of humans and animals. Further, inhalation toxicity data are needed for compliance with many regulatory requirements of the U.S. Food and Drug Administration (FDA), the Occupational Health and Safety Administration, and the Environmental Protection Agency (Gad and Chengelis, 1998).

More comprehensive descriptions and discussions on inhalation toxicology and technology may be found in several recent monographs, reviews, and textbooks (Willeke, 1980; Leong et al., 1981; Witschi and Nettesheim, 1982; Clarke and Pavia, 1984; Phalen, 1984; Witschi and Brain, 1985; Barrow, 1986; McFadden, 1986; Menzel and Amdur, 1986; Salem, 1986; Gardner et al., 1988; Gad and Chengelis, 1998; Valentine and Kennedy, 2001; McClellan and Henderson, 1989; Hext, 2000).

18.2 THERAPEUTIC DRUG DELIVERY BY DERMAL ROUTE

The dermal route has both some significant attractions and some significant drawbacks for use in drug administration. The ability to utilize the former and avoid (at least the relevance of) the latter is the key to successful development of new drugs administered by this route (Ozhai et al., 2008).

On the advantage side, dermal administration:

- Avoids first-pass metabolism
- Avoids acidic environment of the stomach
- Can be designed to deliver controlled amounds of drug over a prolonged period outside of a clinical setting
- Can be designed to achieve desired local tissue exposure to the therapeutic area while avoiding (or minimizing) systemic exposure

On the disadvantage side, dermal administration historically:

- Cannot be used for systemic delivery of large molecules (usually with molecular weights in excess of 1000)
- Achieves a lower systemic bioavailability than oral routes
- Uses formulation components which may irritate or damage the application site (Zhai et al., 2008) and are perceived as "messy" or aesthetically undesirable

New approaches to formulation or delivery systems (see Touitou and Barry, 2007) have overcome (or minimized) a number of the disadvantages and continue to evolve. In addition to the traditional gels, lotions, and creams (for which new chemical permeation enhancers and vesicular carriers such as liposomes have and are being added), new technology such as iontophoresis, electrophoresis, and ultrasound is also evolving. Additionally, in some cases, the barrier layer in the skin (stratum corneum) can be bypassed (using such approaches as microneedles) or removed by tape-stripping laser, adhesion, and ballistic methods (such as used for mass vaccine administration in the military).

Bioavailability is typically defined as the rate and extent at which a drug reaches the general circulation from an administered dosage form. Dermatological drug products include preparations which are designed to exert a local effect in diseased skin following topical application on the skin surface. The objective is to maximize drug concentration at the site of action within the skin with, ideally, a minimal systemic uptake. Thus, systemic availability may not properly reflect local cutaneous bioavailability (as it does for transdermal products which are designed to deliver drug into the systemic circulation). Moreover, topical doses tend to be so small (typically 2–5 mg of product per square centimeter) that serum and/or urine concentrations are often undetectable using conventional assay techniques. Further complicating this is the lack of knowledge of the drug concentration needed at the skin target site (with the exception of antifungal and antibacterial agents whose target site is the SC surface). Topical bioavailability has been more properly defined as the temporal pattern of free drug, but this approach remains largely theoretical due to the difficulty of quantifying drug within the skin.

Available options include estimating a drug's permeability coefficient through human skin from the molecular weight and octanol–water partition coefficient. This information is not really sufficient to estimate topical bioavailability. The algorithms available are only able to approximate values for drug bioavailability.

Alternatives include collecting samples by tape stripping or biopsy and then measuring the actual drug (and metabolites) present in the tissue samples. There are numerous complications to this and approaches to solving them (see Herkenne et al., 2008).

There are no direct guidance documents focused on nonclinical safety assessment for topical route drugs or on what must be done before taking such a drug into clinical trials. Rather, the FDA regulatory expectations come from ICH/M4, the Center for Drug Evaluation and Research (CDER, 2008) reformulation document, and current practice. The results are summarized in Table 18.3.

The CDER (2008) document presents the following dermal route specific expectations:

- In the United States delayed hypersensitivity of any previously not evaluated topical drug or new formulation means performing the LLNA (local

TABLE 18.3 Test Requirement Matrix for Topical Agents

Test Requirement	Species
Initial Clinical Trial/IND Requirements	
1. Acute toxicity in rodents (IV[a])	R/M
2. Acute toxicity in nonrodents	D/S/P
3. Seven-day DRF toxicity in rodents	R/M
4. Seven-day DRF toxicity in nonrodents	D/S/P
5. Genotoxicity: bacterial mutagenicity (Ames)[b]	In vitro
6. Genotoxicity: in vitro clastogenicity (CHO chromosome aberration)[b]	In vitro
7. Genotoxicity: in vivo (mouse or rat micronucleus)[b]	R/M
8. Safety pharmacology: CV-Herg[a]	In vitro
9. Safety pharmacology: CV in vivo[b]	D/P/S
10. Safety pharmacology: FOB/Irwin[b]	R/M
11. Safety pharmacology: respiratory—Rodent[b]	R
12. Pivotal/repeat dose in rodents (14–28 days intended route[c])	R/M
13. Pivotal/repeat dose in nonrodents (14–28 days intended route[c])	D/P/S
14. CYP induction/inhibition[a]	In vitro
15. Five species microsome metabolic panel[a]	In vitro
16. Develop bioanalytical for three species (human/rodent/nonrodent)	NA
17. Local irritation[c] (clinical formulation)	R
18. Of dermal—sensitization	G
To Support Continued Clinical Development	
19. Developmental toxicity (segment II)—rat and rabbit pilots and rat and rabbit studies	
20. Immunotoxicity[c] (if immune modulatory claim or there are findings in 14/28-dog studies	
21. Pivotal/repeat dose in rodents (3/6 months oral)	
22. Pivotal/repeat dose in nonrodents (3/9–12 months oral)	
To Support Marketing Approval	
23. Reproductive toxicity—segment I	
24. Reproductive toxicity—segment III	
25. Tumorgenicity/carcinogenicity—rat	
26. Tumorgenicity/carcinogenicity—mouse	

Note: Species: R = rat, M = mouse, D = dog, S = pig, P = primate, B = rabbit, G = guinea pig, TBD = to be determined. All studies described must be performed according to good labor practices.
[a]Recommended.
[b]May be required.
[c]Dermal/opthalmic/vaginal/rectal.

lymph node assay), guinea pig maximization test (GPMT), or Buehler. As the Buehler is not accepted in Europe, use of one of the other two (see Chapter 9 on immunotoxicology for details on these and their limitations) should be used.

- Photoirritation should be evaluated if the new formulation absorbs ultraviolet or visible radiation (290–700 nm) and if the product is applied to

sun-exposed skin. If the new formulation is a patch, then photoirritation should be considered if the patch is permeable to light and is applied to sun-exposed skin (see details in local tissue tolerance). A screen using three T cells is acceptable in place of the traditional rabbit study.

- If the new formulation contains an active ingredient that has not been used by the dermal route, the repeat-dose local toxicity study mentioned earlier should be conducted in a nonrodent species (preferably the pig). This study should be of at least the same duration as clinical use (up to nine months) and include both local and systemic evaluation. For NCEs (new chemical entities), repeat-dose studies in both rodents and nonrodents (preferably the pig) are required. In one of these (typically the rodent), it should be ensured that sufficient systemic exposure of the NCE is achieved usually by conducting such a study by the oral or parenteral route.
- The skin dose from topically applied drug products can be orders of magnitude larger than the skin dose after systemic administration. Therefore, a dermal carcinogenicity study might be recommended for drugs with a chronic indication even if systemic carcinogenicity studies are available.
- The photocarcinogenic potential should be evaluated if the new formulation is used chronically on sun-exposed skin. Evaluation of photocarcinogenicity generally is not recommended for path products (see the guidance for industry on photosafety testing) (FDA, 2006).
- Nonclinical dermal studies generally should be conducted with untreated control, vehicle control, and complete formulation groups.

A key point is if significant systemic exposure is or is not achieved. If it is not, then genotoxicity and safety pharmacology testing is not required. Such testing is required if it cannot be shown that systemic absorption is minimal or not detectable.

For the repeat-dose systemic toxicity studies, added to the usual details of study design is the need to evaluate local tissue effects of application sites and to collect tissue and histopathologically evaluate such sites.

As a last point, it should be added that topical administration is not limited to the dermal route. It also includes the body surfaces (vaginal and rectal) for which evaluation of irritation and hypersensitivity is required and for which systemic toxicology repeat-dose studies are performed by administration to these sites (but there is no phototoxicity concern) and ocular for which it is expected that:

- The dermal irritation and delayed contact hypersensitivity potential of the new formulation should be evaluated (because dermal exposure will occur with such drugs).
- The local tissue (eye) irritation must be evaluated.

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19

Special-Case Products: Imaging Agents and Oncology Drugs

19.1 INTRODUCTION

Special cases in the assessment of nonclinical safety arise from a variety of situations or for a number of reasons. We have already explored the major cases that arise as a result of different routes of administration—inhalation and topical routes with their variations. These are the most common variations from the general cases of oral and parenteral administration.

Likewise, the major cases associated with different physical forms (small molecules, being the general case, the major variation being biotechnology sourced materials) have been discussed.

In this chapter, two special cases which arise from the nature of the therapeutic use are considered: imaging agents (in vivo diagnostic agents) and oncology drugs.

19.2 IMAGING AGENTS

Medical imaging agents used for diagnosis or monitoring serve a variety of different modes of activity such as radiography, computed tomography (CT), ultrasonography, magnetic reasonance imaging (MRI), and radionuclide

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imaging (Szabo, 2004). These imaging agents can be classified into at least two general categories—contrast agents and diagnostic radiopharmaceuticals (Van Brocklin, 2008).

Historically, imaging agents (and the subset of radiopharmaceuticals) start as a real exception to the general case in that they do not require an investigational new drug (IND) before an initial clinical evaluation. Rather, under the RDRC [Radioactive Drug Research Committee, a special type of institutional review board (IRB)] process as specified in Code of Federal Regulations (CFR), Title 21, Part 36.1, single-dose studies may be conducted in one of the few academic medical centers with only RDRC review and with use limited to a small number of individuals (up to 30) (CDER, 2000).

The RDRC is an institutional body that reviews research protocols for scientific and technical merit. An overview of the RDRC program from 1975 to 2004 has been recently reported (Van Brocklin, 2008). As of 2003, there were 84 active RDRCs in the United States.

In order to be GRAS (generally regarded as safe), a radiopharmaceutical is limited in terms of pharmacological and radiation dose. The mass associated with the radiopharmaceutical must "be known not to cause any clinically detectable pharmacological effect in human beings" (CDER, 2000). As a result of this limitation, FIH (first in human) studies must be limited to "microdosing" under an RDRC protocol. Typically, RDRCs require published human studies involving the tracer to be evaluated before approving a protocol at near-therapeutic dose. The dose limitation requires that the smallest radiation dose needed to obtain meaningful data from the study be administered to the study subject. The maximum allowable single dose to the whole body, blood-forming organs, lens of the eye, and gonads is 30 mSv (3 rem) with a maximum annual or total dose to all other organs of 50 mSv (5 rem). The maximum single dose and total annual dose to all other organs is 50 mSv (5 rem) and 150 mSv (15 rem), respectively. There is also a significant radiation dose limit on studies involving research subjects that are less than 18 years of age. The dose may not exceed 10% of the adult doses reported above. Additionally, all radiation doses associated with the study must be included in the total study dose. This means that the CT dose from a positron emission tomography (PET)/CT study must be included in the total and this total may not exceed the maximum limits set forth in the regulations.

The types of studies that may be conducted under an RDRC-approved protocol are also regulated. The research must be basic in nature and may include the evaluation of the radiopharmaceutical pharmacoKinetic (PK), metabolism, and excretion. The distribution of a radiopharmaceutical to evaluate human physiology, pathophysiology, or biochemistry is permitted as long as the studies are not for diagnostic or therapeutic benefit. Safety and efficacy studies are not permitted under these regulations. An example of a study that is permissible under RDRC would be the brain distribution of [^{18}F] fluoror DOPA relative to subject age or neurodegenerative disease.

The RDRC may not approve protocols that require more than 30 subjects. If more than 30 subjects need to be studied and may be justified by the

researcher then a special summary form is submitted to the U.S. Food and Drug Administration (FDA) for review and a formal IND must be opened. A pediatric consultant to the RDRC must review studies involving minors under 18 years old and a special summary must be submitted to the FDA. In addition, all adverse reactions "attributable to the use of the radioactive drug" must be reported immediately to the FDA (CDER, 2000). It is interesting to note, however, that over 30 years since the inception of the RDRC regulations with an estimated 60,000 subjects enrolled in the studies not one adverse event has been reported.

The regulations also stipulate the constitution of the RDRC with appropriate expertise to review the protocol applications. The committee must have at least five members. Three of those members must be a nuclear medicine physician, a qualified individual with radiopharmaceutical preparation experience, and a radiation dosimetry/radiation safety expert. The remaining members must have experience and qualifications in disciplines related to nuclear medicine.

19.2.1 Contrast Agents

As used in the guidance, a contrast agent is a medical imaging agent used to improve the visualization of tissues, organs, and physiological processes by increasing the relative difference of imaging signal intensities in adjacent regions of the body. Types of contrast agents include but are not limited to (1) iodinated compounds used in radiography and CT; (2) paramagnetic metallic ions (such as ions of gadolinium, iron, and manganese) linked to a variety of molecules and microparticles (such as superparamagnetic iron oxide) used in MRI; and (3) microbubbles, microaerosomes, and related microparticles used in diagnostic ultrasonography.

19.2.2 Diagnostic Radiopharmaceuticals

As used in the guidance, a diagnostic radiopharmaceutical is (1) an article that is intended for use in the diagnosis or monitoring of a disease or a manifestation of a disease in humans and that exhibits spontaneous disintegration of unstable nuclei with the emission of nuclear particles or photons or (2) any nonradioactive reagent kit or nuclide generator that is intended to be used in the preparation of such an article. As stated in the preamble to the FDA's proposed rules on regulations for in vivo radiopharmaceuticals used for diagnosis and monitoring, the agency interprets this definition to include articles that exhibit spontaneous disintegration leading to the reconstruction of unstable nuclei and subsequent emission of nuclear particles or photons (*Federal Register*, Vol. 63, 28301 to 28303, May 22, 1998).

Diagnostic radiopharmaceuticals are generally radioactive drugs or biological products that contain a radionuclide that typically is linked to a ligand or carrier. These products are used in nuclear medicine procedures, including

planar imaging, single-photon emission computed tomography (SPECT), PET, or in combination with other radiation detection probes.

Diagnostic radiopharmaceuticals used for imaging typically have two distinct components:

- A radionuclide that can be detected in vivo (e.g., technetium-99m, iodine-123, indium-111): The radionuclide typically is a radioactive atom with a relatively short physical half-life that emits radioactive decay photons having sufficient energy to penetrate the tissue mass of the patient. The photons can then be detected with imaging devices or other detectors.
- A nonradioactive component to which the radionuclide is bound that delivers that radionuclide to specific areas within the body: This nonradionuclide portion of the diagnostic radiopharmaceutical often is an organic molecule such as a carbohydrate, lipid, nucleic acid, peptide, small protein, or antibody.

As technology advances, new products may emerge that do not fit into these traditional categories (e.g., agents for optical imaging, magnetic resonance spectroscopy, combined contrast and functional imaging). It is anticipated that the general principles discussed here could apply to these new diagnostic products. Developers of these products should contact the appropriate reviewing FDA division for advice on product development.

19.2.3 Medical Imaging Agent Characterisitics Relevant to Safety

The following sections discuss the special characteristics of a medical imaging agent that can lead to a more focused safety evaluation. Characteristics include its radiation absorbed dose, mass dose, route of administration, frequency of use, biodistribution, and biological, physical, and effective half-lives in the serum, the whole body, and critical organs.

Mass Dose Some medical imaging agents can be administered at low mass doses. For example, the mass dose of a single administration of a diagnostic radiopharmaceutical can be small because device technologies can typically detect relatively small amounts of radionuclide (e.g., radiopharmaceuticals for myocardial perfusion imaging). When a medical imaging agent is administered at a mass dose that is at the low end of the dose–response curve, safety concerns are minimal.

Route of Administration Some medical imaging agents are administered by routes that decrease the likelihood of systemic adverse events. For example, medical imaging agents that are administered as contrast media for radiographic examination of the gastrointestinal tract (e.g., barium sulfate) can be administered orally, through an oral tube, or rectally. In patients with normal gastrointestinal tracts, many of these products are not absorbed, so systemic

adverse events are less likely to occur. In general, nonradiolabeled contrast agents pose safety issues similar to therapeutic drugs and generally should be treated as therapeutic agents for the purpose of conducting clinical safety assessments.

Frequency of Use Many medical imaging agents, including both contrast and diagnostic radiopharmaceuticals, are administered infrequently or as single doses. Accordingly, adverse events that are related to long-term use or to accumulation are less likely to occur with these agents than with agents that are administered repeatedly to the same patient. Therefore, the nonclinical development programs for such single-use products usually can omit long-term (i.e., 3 months duration or longer), repeat-dose safety studies. In clinical settings where it is possible that the medical imaging agent will be administered to a single patient repeatedly (e.g., to monitor disease progression), we recommend that repeat-dose studies (of 14–28 days duration) be performed to assess safety.

Biological medical imaging agents are frequently immunogenic, and the development of antibodies after intermittent, repeated administration can alter the pharmacokinetics, biodistribution, safety, and/or imaging properties of such agents and, potentially, of immunologically related agents. We recommend studies in which repeat dosing of a biological imaging agent is planned to incorporate pharmacokinetic data, human antimouse antibody (HAMA), human antihumanized antibody (HAHA), or human antichimeric antibody (HACA) levels as well as whole-body biodistribution imaging to assess for alterations in the biodistribution of the imaging agent following repeat dosing. Studies of immunogenicity in animal models are generally of little value. Therefore, we recommend that human clinical data assessing the repeat use of a biological imaging agent be obtained prior to application to licensure of such an agent.

Biological, Physical, and Effective Half-Lives Diagnostic radiopharmaceuticals often use radionuclides with short physical half-lives or that are excreted rapidly. The biological, physical, and effective half-lives of diagnostic radiopharmaceuticals are incorporated into radiation dosimetry evaluations that require an understanding of the kinetics of distribution and excretion of the radionuclide and its mode of decay. We recommend that biological, physical, and effective half-lives be considered in planning appropriate safety and dosimetry evaluations of diagnostic radiopharmaceuticals.

19.2.4 Performance of Nonclinical Safety Assessments

The FDA recommends that the nonclinical development strategy for an agent be based on sound scientific principles, the agent's unique chemistry (including, e.g., those of its components, metabolites, and impurities), and the agent's intended use. Because each product is unique, we encourage sponsors to

consult with us before submitting an IND application and during product development. The number and types of nonclinical studies recommended would depend in part on the phase of development, what is known about the agent or its pharmacological class, its proposed use, and the indicated patient population. If it is determined that nonclinical pharmacology or toxicology studies are not needed, the FDA is prepared to grant a waiver under 21 CFR 312.0 if adequate justification is provided.

In the discussion that follows, a distinction is made between drug products and biological products. The existing specific guidance for biological products is referenced but not repeated here (ICH S8).

Nonclinical Safety Assessments for Nonbiological Drug Products

Timing of Nonclinical Studies Submitted to IND application

- The FDA recommends that nonclinical studies be timed so that they help facilitate the timely conduct of clinical trials (including appropriate safety monitoring based on findings in nonclinical studies) and reduce the unnecessary use of animals and other resources. The recommended timing of nonclinical studies for medical imaging drugs is summarized in Table 19.1.

Contrast Agents

- Because of the characteristics of contrast drug products (e.g., variable biological half-life) and the way they are used, the FDA recommends that nonclinical safety evaluations of such drug products be made more efficient with the following modifications:
  (a) Long-term (i.e., greater than 3 months), repeat-dose toxicity studies in animals usually can be omitted. (Exceptions are products with long residence time, e.g., >90 days.)
  (b) Long-term rodent carcinogenicity studies usually can be omitted.
  (c) Reproductive toxicology studies required under CFR 312.23(a)(8)(ii)(a) often can be limited to an evaluation of embryonic and fetal toxicities in rats and rabbits and to evaluations of reproductive organs in other short-term toxicity studies. If you determine that such reproductive studies are not needed, FDA is prepared to grant a waiver under CFR 312.10 if adequate justification is provided.

The FDA recommends that studies be conducted to address the effects of large mass dose and volume (especially for iodinated contrast materials administered intravenously); osmolality effects; potential transmetallation of complexes of gadolinium, manganese, or iron (generally MRI drugs); potential effects of tissue or cellular accumulation on organ function (particularly if the drug is intended to image a diseased organ system); and the chemical, physiological, and physical effects of ultrasound microbubble drugs (e.g., coalescence, aggregation, margination, and cavitation).

TABLE 19.1 Timing of Nonclinical Studies for Nonbiological Products Submitted to IND

Study Type	Before Phase 1	Before Phase 2	Before Phase 3	Before NDA
Safety pharmacology	Major organs[a] and organ systems the drug is intended to visualize			
Toxicokinetic pharmacokinetic	See ICH guidances			
Expanded single-dose toxicity	Expanded acute single dose[b]			
Short-term (2–4 weeks) multiple-dose toxicity	—	Repeat-dose toxicity[c]		
Special toxicology	Conduct as necessary based on route irritancy, blood compatibility, protein flocculation, misadministration, extravasation			
Radiation dosimetry	If applicable			
Genotoxicity	In vitro[d]	Complete standard battery		
Immunotoxicity	—	—	May be needed based on molecular structure, biodistribution pattern, class concern, or clinical or nonclinical signal	
Reproductive and developmental toxicity	—	—	Needed or waiver obtained[d]	
Drug interaction	—	—	—	As needed
Other based on data results	—	—	—	As needed

[a]See guidances S7: A safety pharmacology studies for human pharmaceutical and S7B: Safety pharmacology studies for assessing the potential for delayed ventricular repolarization (QT interval prolongation) by human pharmaceuticals (note that S7B allows for phase evaluation of required studies).
[b]See the guidance on single-dose acute testing for pharmaceuticals.
[c]When repeat-dose toxicity studies have been performed but single-dose toxicology studies have not, dose selection for initial human studies will likely be based on the results of the no-adverse-effect level (NOAEL) obtained in repeat-dose study. The likely result will be a mass dose selection for initial human administration that is lower than if the dose selection had been based on the results of acute, single-dose toxicity studies.
[d]See radiopharmaceutical discussion in Section III.B.1.c of CDER, 2000.

Diagnostic Radiopharmaceuticals (Nonbiological Products) Because of the characteristics of diagnostic radiopharmaceuticals and the way they are used, we recommend that nonclinical safety evaluations of these drugs be made more efficient by the following modifications:

- Long-term, repeat-dose toxicity studies in animals typically can be omitted.
- Long-term rodent carcinogenicity studies typically can be omitted.
- Reproductive toxicology studies can be waived when adequate scientific justification is provided.
- Genotoxicity studies should be conducted on the nonradioactive component because the genotoxicity of the nonradioactive component should be identified separately from that of the radionuclide. Genotoxicity studies can be waived if adequate scientific justification is provided.

The FDA recommends that special safety considerations for diagnostic radiopharmaceuticals include verification of the mass dose of the radiolabeled and unlabeled moiety; assessment of the mass, toxic potency, and receptor interactions for any unlabeled moiety; assessment of potential pharmacological or physiological effects due to molecules that bind with receptors or enzymes; and evaluation of all components in the final formulation for toxicity (e.g., excipients, reducing drugs, stabilizers, antioxidants, chelators, impurities, and residual solvents). It is recommended that the special safety considerations include an analysis of particle size (for products containing particles) and an assessment of instability manifested by aggregation or precipitation. It is also recommended that an individual component be tested if specific toxicological concerns are identified or if toxicological data for that component are lacking. However, if toxicological studies are performed on the combined components of a radiopharmaceutical and no significant toxicity is found, toxicological studies of individual components are seldom required.

Nonclinical Safety Assessments for Biological Products Many biological products raise relatively distinct nonclinical issues such as immunogenicity and species specificity. We recommend the following agency documents be reviewed for guidance on the preclinical evaluation of biological medical imaging agents:

- S6 Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals
- Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use

Table 19.2 seeks to integrate these nonclinical safety testing requirements into a test matrix.

Unfortunately, most recently it has come to light that imaging agents used for cardiovascular effects (Definity) are associated with patient deaths (*Health Imaging News*, 2008). The Optison agent also has come under suspicion.

TABLE 19.2 Special Case: Imaging Agents

Test Requirment	Species[a]
Initial clinical trial/IND Requirements[b]	
1. Acute toxicity in rodents (IV), expanded	R/M
2. Acute toxicity in nonrodents (IV), expanded	D/S/P
3. Genotoxicity: bacterial mutagenicity (Ames)	In vitro
4. Genotoxicity: in vitro clastogenicity (CHO chromosome aberration)	In vitro
5. Genotoxicity: in vivo (mouse or rat micronucleus)	R/M
6. Safety pharmacology: CV in vivo	D/P/S
7. Safety pharmacology: respiratory, rodent	R
8. Pivotal/repeat dose in nonrodents (14–28 days oral)	D/P/S
9. Develop bioanalytical for 2 species (human/nonrodent)	NA
10. Hemolysis	In vitro

[a]R = rat, M = mouse, D = dog, S = pig, P = primate.
[b]Initial single-dose clinical study at single academic center may not require IND.
Note: All studies described must be performed according to good laboratory practices.
Source: Center for Biologics Evaluation and Research (CBER), 2004.

19.3 ONCOLOGY DRUGS

Traditional oncology drugs now have a specific proposed International Conference on Harmonisation (ICH) guidance for their nonclinical safety assessment requirements (ICH, 2008). This chapter reflects that guidance.

These drugs are intended for use in individuals with a serious and life-threatening disease. Initial clinical trials are conducted in patients who have already failed other forms of therapy. Because of this, as summarized in Tables 19.3 and 19.4, the requirements are not as strenuous as the general case. Indeed, genotoxicity and safety pharmacology are usually not required (Baguley and Kerr, 2002).

The other unusual feature in such trials, whether the drug is a small or large (protein) molecule, is that clinical dosing regiments are not daily but rather in accordance with a schedule set as much by tradition and clinical operations as by drug pharmacokinetics.

Administrations are usually two or three times a week on a three- or four-week sequence of dosings followed by a period without dosing. It is normal practice for an initial clinical tolerance trial to be conducted with the dose level being escalated with each three- or four-week dosing series. The 28-day two-species nonclinical safety assessment studies are taken to serve for the entire multiseries set of administrations.

Dose scaling as we now practice it across the range of pharmaceuticals arose from the practice of oncology. Clinicians in this therapeutic area think of dosing in terms of milligrams per square meter, which leads to the need to perform conversions of the expression of dose in safety studies (milligrams per kilogram) to the dose for body surface area form.

TABLE 19.3 Special Case: Oncology Agents (Cytotoxic)

Test Requirment	Species[a]
Initial clinical trial/IND Requirements	
1. Acute toxicity in rodents (IV)	R/M
2. Acute toxicity in nonrodents (IV)	D/S/P
3. 7-day DRF toxicity in rodents (IV)	R/M
4. 7-day DRF toxicity in nonrodents (IV)	D/S/P
5. Safety pharmacology: CV-hERG[b]	In vitro
6. Pivotal/repeat dose in rodents (14–28 days IV)	R/M
7. Pivotal/repeat dose in nonrodents (14–28 days IV)	D/P/S
8. CYP induction/inhibition[b]	In vitro
9. Five species microsome metabolic panel[b]	In vitro
10. Develop bioanalytical for 3 species (human/rodent/nonrodent)	NA
To support continued clinical development	
10. Pivotal/repeat dose in rodents (3/6 months oral)	R/M
11. Pivotal/repeat dose in nonrodents (3/9–12 months oral)	D/P/S

[a]R = rat, M = mouse, D = dog, S = pig, P = primate.
[b]Recommended.
Note: All studies described must be performed according to good laboratory practices.

TABLE 19.4 Special Case: Oncology Agents (Protein-Targeted Molcules)

Test Requirment	Species
Initial clinical trial/IND Requirements	
1. Acute toxicity in rodents (IV)	R/M
2. Acute toxicity in nonrodents (IV)	D/S/P
3. 7-day DRF toxicity in rodents (IV)	R/M
4. 7-day DRF toxicity in nonrodents (IV)	D/S/P
5. Safety pharmacology: CV in vivo	D/P/S
6. Safety pharmacology: FOB/Irwin	R/M
7. Safety pharmacology: respiratory, rodent	R
8. Pivotal/repeat dose in rodents (14–28 days IV)	R/M
9. Pivotal/repeat dose in nonrodents (14–28 days IV)	D/P/S
10. CYP induction/inhibition[b]	In vitro
11. Five species microsome metabolic panel[b]	In vitro
12. Develop Bioanalytical for 3 species (human/rodent/nonrodent)	NA
To support continued clinical development	
13. Developmental toxicity (segment II), rat and rabbit pilots and rat and rabbit studies	R/B
14. Immunotoxicity	TBD
15. Pivotal/repeat dose in nonrodents (3/6 months oral)	D/P/S
To support marketing approval	
16. Reproductive toxicity, segment I	R
17. Reproductive toxicity, segment III	R

[a]R = rat, M = mouse, D = dog, S = pig, P = primate, B = rabbit, TBD = To be determined.
[b]Recommended.
[c]May be required.
Note: All studies described must be performed according to good laboratory practices.

The toxicity of cytotoxic anticancer drugs correlates more closely with body surface area (BSA) than with body weight (BW). Thus, it is standard practice to administer and compare such drugs on the basis of BSA. To convert between milligrams per kilogram and milligrams per square meter, it is necessary to know either BSA or BW. Accurate values for BW are easy to obtain. In contrast, BSA is difficult to measure and therefore is usually calculated from a known BW. The relationship between BW and BSA is described by the formula: $BSA(m^2) = b[BW/(kg)]^{2/3}$, where b is a species-specific factor derived from empirical data. In practice, the values for b vary between laboratories (e.g., values for b for the mouse have been found to vary from 0.090 to 0.096). Confusion and miscommunication can occur as a result of the use of different species-specific factors. For example, difficulties have arisen during the dose selection process for a novel cytotoxic anticancer agent with a sleep dose–response curve. In this case, if different investigators use b values of 0.099, 0.101, and 0.111, then animal BSA values for a 10-kg dog will likewise vary, depending on the choice of b (0.926, 0.945, and 1.038 mg, respectively, for a 2-mg/m^2 dose). The use of a single set of species-specific factors by all investigators is proposed as it would be beneficial in removing confusion from discussions of study designs and results. Such a proposal has an even more immediate benefit in the case of compounds with very steep dose–response curves, where even small variations in dosing can profoundly influence the results obtained and their interpretation.

19.3.1 Dose Conversions: Perspective

Dosages of pharmaceuticals are typically calculated on the basis of BW. Interspecies comparisons of toxicological effects of drugs and chemicals are commonly based on exposures normalized with respect to body weight or systemic exposure [i.e., area under the curve (AUC) and C_{max} values].

19.3.2 Use of mg/m^2 Dose Unit

Calculations of Drug Dosages for Treatment Cytotoxic anticancer drugs are typically administered in dose units of milligrams per square meter. However, BSA is difficult to measure. In contrast, values for height and body weight are easy to obtain. Two solutions present themselves:

1. In a clinic, estimates of BSA can be accomplished from height and weight data using established nomograms.
2. In the toxicology laboratory, BSA can be calculated from animal body weight data *using the appropriate formula.*

Conversion of mg/kg Body Weight Doses to Units of mg/m^2 Conversions from milligrams per kilogram body weight to milligrams per square meter occur often in the design of toxicity studies, in the interpretation of data

TABLE 19.5 Factors Used for Milligram-per-Kilogram to Milligram-per-Square-Meter Conversions

Species	Conversion Factor
Mouse	3
Rat	6
Guinea pig	7.7
Hamster	4.1
Rabbit	11.8
Dog	20
Monkey	12
Human (60 kg)	37

from a number of different studies, including old studies, and in comparisons of animal and human data where dose units are not similar. Table 19.5 provides conversion factors for this.

Another unusual feature in oncology is that the maximum utilized clinical dose is the toxic-limited dose (TLD). Patients are typically titrated to toxicity and actual clinical treatment practice doses patients up to a frankly tolerated dose.

Somewhat in parallel, what constitutes a dose-limiting adverse effect in a nonclinical study [a no-observable-adverse-effect level (NOAEL)] is usually taken to be a frank toxicity and not a simple intolerance (such as sporadic emesis).

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20

Occupational Toxicology in Pharmaceutical Industry

20.1 INTRODUCTION

Most of the assessment of toxicology and safety of therapeutics is focused on the patients who are to benefit from the new medicine. However, there are two other groups of individuals (each of which has different exposure profiles) that one must be concerned about: health care providers (nurses, pharmacists, and physicians) who provide and/or administer the drugs and individuals involved in manufacturing them. The concerns here are in the realm of occupational toxicology.

Modern toxicology has its roots in the occupational environment. The earliest recorded observations relating exposure to chemical substances and toxic manifestations were made about workers. These include Agricola's identification of the diseases of miners and Pott's investigation of scrotal cancer incidence among chimney sweeps. Occupational toxicology, as its name implies, concerns itself with the toxicological implications of exposure to chemicals in the work environment.

In this chapter we will examine occupational toxicology as it applies to and is currently practiced in the pharmaceutical industry. This industry, which by definition involves biologically active compounds, has been a driving force in the development of the science of toxicology. The need for a thorough safety evaluation of potential therapeutics prior to marketing approval has driven

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the continued evolution of toxicological testing methods and the identification of mechanisms of toxic action. The area of occupational toxicology gained great momentum in the pharmaceutical industry from the early 1980s through the 1990s. This is made clear by the increased number of companies that have implemented occupational toxicology programs during this period. Yet, occupational-related activities generally represent only a small fraction of activity in safety assessment in the pharmaceutical and biotechnology industries. It is difficult to gauge the level of activity in this occupational areadue to the paucity of publications on the subject (Teichman et al., 1988). This is probably the result of the fact that most occupational toxicologists function in an administrative environment and thus experience less pressure to publish and that they in general deal with information relating to new chemical processes that may not be protected by patents. The lack of general knowledge about the function of the occupational toxicologist that has resulted could lead one to conclude that (1) the thorough evaluation of drugs to obtain marketing approval makes an investigation of their potential hazards to manufacturing employees unnecessary and (2) because they are used therapeutically, pharmaceutical agents are safe under any circumstances in occupational settings.

20.2 OCCUPATIONAL TOXICOLOGY VERSUS DRUG SAFETY EVALUATION

While pharmaceutical products are indeed created to treat disease, they cannot always be considered nonhazardous. The clinician must evaluate the benefits to the patient in light of any side effects or adverse reactions that may be associated with drug usage and any other toxicological properties uncovered in animal studies. Examples range from antibiotics, which have clastogenic properties in mice (for which, to the patient, its activity in suppressing life-threatening infections presents a clear and overriding benefit), to an antineoplastic that has extreme renal toxicity but which effectively kills established tumors. The occupational toxicologist must look at pharmaceutical agents in a completely different light. These same risk–benefit analyses do not apply in an occupational setting. Even agents with minimal clinical adverse reactions and whose pharmacological activity could be considered generally beneficial in the clinical setting may present certain employees with health hazards in manufacturing or health care provider settings.

Table 20.1 presents some of the basic differences in the way preclinical and occupational toxicologists must approach their work. Preclinical development of a pharmaceutical product requires exhaustive testing of drug candidates under the requirements of the U.S. Food and Drug Administration (FDA) or equivalent national agency to help predict and evaluate clinical findings and to preclude serious or chronic hazards that would not ordinarily be observed in clinical studies limited in duration and population (FDA, 1990). By contrast, the occupational toxicologist must evaluate the potential of a compound to

TABLE 20.1 Comparison of Occupational and Preclinical Toxicology

Parameter	Occupational	Preclinical
Purpose	Potential for effects from unintended exposure	Predict and evaluate clinical findings and preclude serious hazards from clinical use
Routes of administration	Inhalation, direct contact with skin or eyes	Oral and/or parenteral most likely
Dose level	Not really predictable	Relative to estimated therapeutic dose or maximum tolerated dose
Duration of exposure	Extremely variable; depends on campaign/batch, procedure, etc.; may be short daily exposure for working lifetime	Dependent on therapeutic use and test model

cause toxicity from unintended exposures via a variety of routes of administration and a wide range of exposure levels of varying lengths. In drug safety evaluations, studies are designed to approximate the clinical setting, particularly in terms of routes of administration and dosage. Thus, most preclinical studies generally focus on oral and/or parenteral administration with dosages that either are comparable to or exceed therapeutic levels. However, neither oral nor parenteral administration is a likely route of exposure among employees. Rather, during manufacturing operations employees are more likely to be exposed via inhalation or direct contact with the skin or eyes. In addition to the route of exposure itself, the effects occurring following direct contact or inhalation exposure may be of a nature not predictable by the studies undertaken for preclinical safety evaluation. Most important among these effects are irritation and sensitization. Dermal, ocular, and respiratory irritation potential generally cannot be predicted from preclinical studies utilizing oral or parenteral administration. Similarly, sensitization, which has the potential to significantly add to the difficulty of conducting manufacturing operations safely, is difficult to evaluate even with current specific methods and models. For most pharmaceutical agents, testing to ascertain the potential to induce dermal sensitization reactions is not conducted during a typical preclinical development program.

Other important differences lie in the length of treatment and the dosages involved. Therapeutic use of pharmacological agents may be of acute or limited duration, such as in the administration of anti-infectives, or chronic, as with antihypertensive agents. Occupational exposure may also be of varying length, limited by shift or batch manufacturing methods. It is possible, however, that the manufacture of certain high-volume products such as antibiotics may result in daily exposure, if only in limited doses, over a significant portion of a working lifetime. The levels to which employees may be exposed are, in

general, potentially lower than those that are used therapeutically, although exposures will vary with the type of operations performed.

The area of occupational toxicology has received a great deal of attention in the chemical industry. Historically, the chemical industry has focused on the occupational environment and developed many of our current toxicological methods to address health and safety concerns. However, since the mid-1970s the chemical industry has increasingly become subject to testing requirements relevant to the protection of the environment and the public at large, as mandated by U.S. Environmental Protection Agency regulations (EPA, 1976, 1979). Data development for occupational health hazard evaluation has seldom been sought by the Occupational Safety and Health Administration (OSHA). Consequently, few new test methods have been developed and those in current use are generally modifications of methods introduced in the 1930s and 1940s. Some of the differences in the issues addressed by occupational toxicologists in the pharmaceutical and chemical industries are highlighted in Table 20.2. Among these, a major difference lies in the physical and functional nature of the substances involved. Pharmaceutical agents are generally handled as solids, while chemical industry products are generally processed as liquids or vapors. Although there is a greater focus on the consequences of occupational exposure, the occupational toxicologist in the chemical industry rarely has available the wealth of information that exists in the

TABLE 20.2 Toxicological Testing Requirements under EC Seventh Amendment (Directive 92/32/EC—Notification of New Substances)

Quantity Imported to or Manufactured in EC	Test Results to be Submitted
<1000 kg yr^{-1} or <5000 kg total	None needed unless compound is considered toxic (oral LD_{50} 25–200 mg kg^{-1}) or very toxic (oral LD_{50}<25 mg kg^{-1})
>1000 kg yr^{-1} or 5000 kg total	Acute oral/dermal LD_{50} Acute inhalation LC_{50} Skin irritation Eye irritation Skin sensitization 28-day subacute toxicity Mutagenicity (bacteriological and nonbacteriological tests) Acute toxicity to fish (LC_{50}) Acute toxicity to *Daphnia*
>10,000 kg yr^{-1} or 50,000 kg total	Additional tests may be required depending on results, including: • Fertility—1 or 2 generations (males or females) • Teratology—additional species • Subchronic or chronic—90 days to 2 years • Carcinogenicity • Acute and subacute on additional species

Source: EEC, 1992.

pharmaceutical industry. New drug dossiers include toxicological information as well as data on the pharmacology, pharmacokinetics, and mechanism of action, and, most important, much of this information has been gathered from clinical trials on human beings. Even though the data are not developed for the purpose of evaluating the occupational environment, they can be invaluable for this purpose. Clinical studies, even if the doses and routes of administration may be different from those used in clinical trials/therapeutically, provide insight into the unique responses of the human body. Another important difference in the parameters involved in the chemical and pharmaceutical industries concerns biological activity. The chemical industry strives to minimize it, while pharmaceutical agents are specifically designed to be biologically active. Recent advances in our understanding of molecular and cellular processes have led to the development of agents with improved specificity for unique receptor or molecular targets. These potent agents may present increased hazards for employees and a great challenge to the occupational toxicologist in the pharmaceutical industry.

At the same time, several types of data necessary to ensure proper management of occupational risks associated with a drug substance are not generally useful in evaluating potential patient risks. So the necessary tests—eye and skin irritation, sensitization and inhalation toxicity, as well as assessment of the hazards of by-products and impurities that do not get incorporated into the final therapeutic product—are not performed in the normal course of development.

20.3 REGULATORY PRESSURES IN UNITED STATES AND EUROPEAN COMMUNITY

The safety and health of workers in the United States is regulated under the Occupational Health and Safety Act of 1970, which established OSHA. Since its inception, OSHA has promulgated a variety of health standards, including compound-specific regulations, permissible exposure limits (PELs), and rules for providing access to medical records (OSHA, 1986) and for communicating to employees the hazards of the materials they handle. This last regulation, the hazard communication standard (OSHA, 1987), is a standard that specifically requires manufacturers or importers to carry out an evaluation of the toxicological properties of chemicals. This standard outlines specific criteria for evaluating substances as hazardous or nonhazardous. However, there still is no U.S. regulatory requirement for testing a compound of unknown toxicity (Gad, 2001). Rather, such a compound could be classified as nonhazardous based on the unavailability of data. Pharmaceutical agents are generally considered hazardous under the standard since they meet the criterion of having a biological effect on humans. Any adverse reaction observed during clinical use will be construed as toxicity, however irrelevant to the occupational environment. The main result of classification as hazardous is a requirement

to develop a material safety data sheet (MSDS) as the main vehicle for providing to employees. The model MSDS suggested for use in complying with the OSHA hazard communication standard contains a great deal of information about the physical properties and hazards and the procedures necessary to deal with the accidental spill, fire, explosion, or accidental contact with hazardous material. The standard requires that all information regarding adverse effects in human beings and most animal toxicity data be included in the MSDS, however irrelevant this information may be to the work environment. The resulting MSDS can be a highly technical document that may not be the optimal vehicle for conveying this type of information to manufacturing employees handling pharmaceutical agents.

In the environmental area, the EPA's TSCA (Toxic Substances Control Act) (EPA, 1976) regulations for filing of premanufacture notification (PMN) (EPA, 1979) have resulted in the development of toxicological information on many new industrial chemicals. New chemical entities generated for use as pharmaceutical agents are exempted from PMN requirements. This exemption may also extend to all intermediates generated during chemical synthesis. Many pharmaceutical companies have instituted toxicological testing of these compounds even though there is no specific U.S. regulatory impetus to develop such information. The European Community (EC) has implemented several directives that parallel and exceed the OSHA hazard communication standard and TSCA regulations. European Community Directive 80/1107 [European Economic Community (EEC), 1979] requires employee communication of hazards of chemical substances as well as biological materials. Another EEC directive, which was the impetus for the development of notification of new substances regulations in several member nations, requires the development of toxicological data on new compounds and does not exempt pharmaceutical agents or isolated intermediates (EEC, 1979). Notification and testing must be conducted in accordance with the amount of the substance manufactured in or imported into Europe yearly (Table 20.2). These regulations will support the development of toxicological data on entities manufactured or processed in Europe that can in turn be applied to occupational health hazard evaluations. Pharmaceutical companies are, as demonstrated in Table 20.3, very different from chemical companies in their handling of occupational toxicology.

20.4 ORGANIZATIONAL STRUCTURE

The occupational toxicology function is organized and structured in very different ways across the industry, particularly with so much of the sector now composed of small companies that are not in possession of any internal development resources. The function exists in many of the major PrMA (Pharmaceutical Manufacturing Association) member companies. In most of these companies the occupational toxicology function is located within the employee safety/industrial hygiene area, while in some it resides within research and

TABLE 20.3 Comparison of Occupational Toxicology in Pharmaceutical and Chemical Industries

Parameter	Pharmaceutical	Chemical
Compounds		
Physical state	Generally solids	Liquids, vapors, polymers, solids
Biological activity	Designed for biological activity	Strive for biological inertness
Toxicology data		
Development	Focus on preclinical evaluation	Focus on occupational and general environment
Study length	Acute to chronic for final products; acute for intermediates	Acute to chronic (depending on volume)
Human data		
ADME[a]	Generally available for oral/parenteral routes	Not generally available
Mechanism of action	Targeted during drug development	Not generally studied
Adverse effects	Extensive clinical trial studies for final products from oral or parenteral route	Generally only known as result of overexposure, accident, etc.

[a]ADME: absorption, distribution, metabolism, and excretion.

development (R&D), toxicology, or employee health/medical services areas. How the function fits into the organization greatly depends on its mission. Occupational toxicology will function well with the R&D environment if evaluation of occupational health hazards is considered an integral requirement in the development and approval process. In such an organization there would likely be a greater emphasis on developing toxicological data on novel compounds and their synthetic intermediates, rather than on existing processes, or such other activities as training. Most occupational toxicology departments are located within the employee safety/industrial hygiene areas. This organization provides great opportunity for interaction and cooperation with those disciplines that are charged with implementing the toxicologists' recommendations. Good interaction between the occupational toxicologist and the industrial hygienist can be particularly useful in developing and implementing solutions to potential health hazards. However, poor understanding of the limitations of scientific data by the more engineering-oriented safety specialists may lead to unrealistic expectations for easy solutions or answers. The last existing arrangement is for the occupational toxicologist to report into the medical services area. This arrangement provides perhaps the easiest interactions for the toxicologist, who shares a common language and understanding of biological systems with the occupational physician. However, in order to effect any changes in the work environment, it is necessary to enlist the aid of the employee safety/industrial hygiene group, an act that may incur the potential problems mentioned above. Clearly, wherever the function is located, the occupational toxicologist must be able to interact well with a

variety of disciplines, including R&D, safety, industrial hygiene, medical services, legal services, regulatory affairs, technical services, and, of course, operations management.

Staffing of industrial toxicology programs varies among the different programs, including groups with two or three full-time Ph.D.'s who spend all of their time on occupational issues and those with one or two Ph.D.'s or masters-level staff members who may have part-time responsibility for occupational-related issues along with R&D responsibilities. The level of staffing depends, of course, on the activities assigned to the occupational toxicology group and these may vary from one organization to another. It is impossible to generalize or recommend an adequate staffing level, since that will be dictated by the emphasis placed on specific activities. Whatever the mission of the occupational toxicology function, a high level of education or professional credentials is desirable. A doctoral degree and/or board certification in toxicology should be imperative to be able to interact effectively with many of the other disciplines mentioned above, particularly R&D management.

20.5 ACTIVITIES

The scope of activities of occupational toxicologists may be quite different from one organization to another, depending on its specific mission, resources available, and corporate culture. In general, their activities can be divided into four broad areas: data development, data evaluation and dissemination, hazard assessment, and employee training.

20.5.1 Data Evaluation and Dissemination

It is important to establish who will use the toxicological information provided and how this information will be applied. Unlike the preclinical toxicologist who provides information to other toxicologists, to the regulatory agencies, or to physicians for evaluation of potential therapeutic liabilities, the occupational toxicologist is providing information to a variety of individuals and functions. First, the information will be provided to the industrial hygienist or safety specialist who must evaluate the quality of the work environment and the appropriateness of personal protective equipment. Second, the information will be given to the occupational physician who must evaluate the potential causes of any symptoms reported by employees who may have been exposed to the material. Third, R&D, plant management, and/or manufacturing services must evaluate the need to implement engineering or other controls and weigh these costs against the commercial viability of the product. Last, but not least, the information must be provided to production employees who will be handling the compound and who need to know of its hazards. Clearly, there is a need to provide the necessary information in such a way

that it can be clearly understood by nonscientists. With an audience of such potentially wide-ranging educational levels and understanding, multiple communication vehicles may be necessary.

To be effective the toxicology evaluation must meet several criteria: it must (1) be thorough, (2) be clear and concise, (3) be in a form appropriate to its target audience, and (4) include a conclusion or recommendation.

Thoroughness can be achieved through an exhaustive search of the published literature using the available computerized databases. There is a risk, particularly when dealing with pharmaceutical agents, that the most relevant information to occupational toxicology can be overlooked in the great number of clinical case reports, many of which are not relevant to the work environment. In general, little information has been published on the occupational hazards of pharmaceutical agents. A thorough review does not mean a listing of every reported clinical adverse reaction. This type of information is more likely to confuse the reader and my lead him to her to ignore the important occupational hazards. The toxicologist must therefore be extremely selective in performing this evaluation. A review of the available clinical information, however, may yield data that can be used in the evaluation, particularly if the product has been tested for dermal administration. An integral part of assuring the thoroughness of the evaluation must be a process of updating the information on a regular basis. In general, it is unlikely that new clinical data will significantly change a review for an established pharmaceutical agent. However, new therapeutic entities should be reviewed more frequently since new data may be published on potential adverse reactions not identified in clinical trials, and these data may impact the occupational evaluation.

When providing information to technical personnel, it is best to use language that does not require the use of a medical dictionary. It is tempting to use medical terms, particularly when quoting from the clinical literature. However, use of these terms may result in poor understanding of the information and may also evoke unnecessary anxiety in the reader. A good rule of thumb is to think of what the reader will do with the information: If the biological effect will require more than a few words to be clearly explained in plain language and it is irrelevant or unimportant to the work environment, it is best left off any communication to the field.

It is not always possible to reach a conclusion regarding the degree of hazard of exposure to a compound, particularly if the data are not directly relevant to the work environment. There is often a temptation to provide a thorough evaluation, setting out all necessary information in plain language but leaving the formulation of a conclusion to the reader. However, if it is difficult for the trained toxicologist to reach such a conclusion, it must be even more difficult for the layperson. If an estimate of the hazard cannot be reached, then the evaluation must be concluded with a recommendation of the type of exposures that may increase hazard or the type of effect that is most likely to occur should there be an overexposure. These may at least give the industrial hygienist or physician a useful reference point. At the same time, it is

important to express to the reader the inherent limitations of such a conclusion. The audience may expect black-and-white answers; if this is not possible, they should be made to understand why.

Perhaps the most difficult part of the communication equation is the ability to match the information to the audience. This may be best illustrated using an example. Over the past 10 years one company has developed several vehicles for communicating information to various audiences. One instrument is the toxicology review. In general, this is a one- to two-page document that reviews the published literature on the compound. A reference list is prepared and maintained on file for future reference. This review is provided to safety, medical, and industrial hygiene personnel and, if appropriate, research chemists. These individuals have received training to help them understand the terms used and the effects outlined. A second method of communication involves a computerized database. This personal computer–based system, which provides only bottom-line information, is available online via a modem to safety, industrial hygiene, nursing, and research personnel (Sussman and Gáler, 1990). It includes only that information specifically relevant to the work environment and necessary for compliance with OSHA hazard communication standard or EC directive 80/1107. A third vehicle was developed jointly with an industrial hygiene department and consists of a short paragraph highlighting the specific hazards of the compound followed by safe handling recommendations. A fourth commonly used method is the MSDS. The toxicology department prepares the toxicology section of the MSDS. The appropriate other disciplines complete the remaining sections, and the completed MSDS is then reviewed and approved by a committee. Last, for certain compounds, on-site training programs, such as those described below, can be presented by the toxicologist on the hazards of the chemical. These various formats for the same information were developed to serve the informational needs and educational levels of various audiences. This is one approach to filling the need to communicate toxicological information to a variety of groups. The appropriate vehicle for each company will, of course, depend on the available resources and corporate culture. Even a large number of formats may not suffice. The occupational toxicologist should determine, through discussions and follow-up communications, how the information is received and if it is understood. The communication of toxicological information may represent approximately 50% of the occupational toxicologists' responsibilities, thus explaining the level of commitment to developing appropriate formats. The MSDS alone is often insufficient for the successful communication of health hazard information to employees.

20.5.2 Data Development

The motivation for conducting toxicological tests for pharmaceutical, chemical intermediates and impurities arises from the need to ensure the health of employees by preventing the occurrence of adverse reactions from occupa-

tional exposure. Employers thus secondarily minimize the associated potential for work interruption. Programs in place at many larger companies routinely test new drug candidates and/or isolated synthetic intermediates for the purpose of occupational health hazard evaluation.

The development of a toxicological testing program for occupational health hazard evaluation requires consideration of (1) the compounds to be tested, (2) the stage of drug development at which testing occurs, (3) the specific tests to be conducted, and (4) the means for funding.

These four issues are, of course, interdependent, and it is not always possible to deal with one without affecting the others. As indicated previously, drug candidates undergo extensive toxicological testing to ensure an adequate margin of safety for patients. Additional tests are usually required to obtain information specific to the work environment. By contrast, synthetic intermediates are generally not subject to testing for drug safety evaluation. These compounds, if they have the potential to present an exposure hazard to employees, may warrant evaluation. Clearly, it is neither feasible nor necessary to conduct the same level of testing required for drug marketing approval. However, a toxicological assessment can often be developed to determine whether these isolated intermediates have the potential to elicit toxicity from exposures that could occur during work.

Compounds should be selected for testing on the basis of an evaluation of potential exposure and likelihood of their causing adverse effects. The first evaluation is best achieved by including the research chemist, industrial hygienist, and/or safety specialist in the decision-making process. They are in the best possible position for judging potential sources of employee exposure. Including these disciplines in the pretesting stage ensures not only their commitment to the program but also that the studies will be designed with careful consideration of the work experience. The second evaluation, an estimate of toxic effects, may be obtained from a comparison of the compounds in question to known toxic agents, also known as a structure–activity relationship (SAR) evaluation. Software programs are currently available for obtaining a quantitative estimate of toxicity using SAR models. However, it is most likely that an SAR evaluation will be achieved by simple comparison to the final product, similar pharmacological agents, or raw materials that have known toxic properties. Information on potential exposures and toxic effects can thus be utilized to decide which compounds to test or to assign priorities to compounds selected for testing.

The timing of these studies depends greatly on the developmental track for the test compounds and may vary for intermediates and final products. Discovery early in the development process that an isolated intermediate poses a significant health hazard may prompt a change in the chemical synthesis or process or in the implementation of engineering controls or personal protective equipment. This it is generally useful to test intermediates at an early stage. This approach presents several practical problems. First, in a long development program, such as occurs in the pharmaceutical industry, there are many oppor-

tunities for changing the synthetic route for reasons other than toxicity. Thus, a large percentage of the intermediates tested during the early development stages may be replaced in the ultimate manufacturing process. Second, only a fraction of new drug candidates actually reach the drug approval process. Therefore, the majority of intermediates tested early in the development process will likely never reach large-scale manufacture. Conducting a toxicological assessment of intermediates at a later stage in the development process presents a comparable set of advantages and disadvantages: It is more likely that the compounds tested will be manufactured on a large scale, but the ability to make fundamental changes in the chemical process will be greatly diminished. Testing of new drug candidates for occupational health hazards can be an integral part of the drug safety evaluation process. Acute oral toxicity is frequently evaluated as the first step in the drug's safety assessment. Adding acute dermal toxicity and thus skin irritation evaluation at the same time can often be accomplished with minimum impact on the development schedule. This additional information can then be used to protect not only employees manufacturing supplies of the chemical but also laboratory employees handling test doses of the substance. Eye irritation testing requires minimal amounts of test compounds and could also be accomplished at the same time. Sensitization testing requires a greater commitment in terms of time and the quantity of compounds needed. Therefore, investigation of a compound's allergenic properties is often postponed until sufficient toxicological information is available to permit a decision as to whether the compounds will advance to the next stage in the development process.

Practical considerations of funding and the selection of the testing laboratory need to be addressed when developing an occupational toxicology testing program. As indicated above, if the activity is located within the R&D department, it may be simple to include the cost of conducting these tests within the new drug's development budget. There is a possible risk in this situation, however, that the safety and industrial hygiene communities may be inadvertently omitted from the prioritization process and the information loop. Explaining the necessity of testing programs to nonscientific management personnel may be challenging. Solutions to these barriers may be found with R&D funding of testing or designation of testing cost, thus possibly including these programs in research funds.

While there are no set regulations on what tests should be done or when these should be conducted and, indeed, no activity to preclude such testing on animal welfare grounds, there is general agreement as to the type of effects that need to be addressed: skin and eye irritation, sensitization, and acute oral and dermal toxicity. Testing for these effects generally involves studies of short duration. Thus, results can often be obtained relatively quickly. Additional tests for inhalation toxicity and/or sensory irritation are conducted by several companies.

Although there is general agreement on the effects to be investigated, the methods used have not necessarily been consistent. Several companies have

developed protocols uniquely tailored to the needs of their workplace health hazard evaluation and their in-house testing resources. The most common protocols utilized for occupational health hazard evaluation are briefly described in Table 20.4 (Gad and Chengelis, 1998).

These modifications included a combined protocol to assess acute dermal toxicity as well as skin irritation in rabbits and a stepwise approach to acute oral toxicity determination rather than a classic LD_{50} (Gáler, 1989). Doses are selected based on regulatory criteria, such as those that are required for classification as a toxic under the OSHA hazard communication standard and/or EEC 80/1107. Testing for eye irritation involves a modification of current methods using rabbits. While in some views there is no justification for testing cosmetic produces in live animals, eye irritation information pertaining to unique pharmacological chemicals is important to protect employees from accidental exposures. There are currently no acceptable alternatives to the rabbit eye irritation test (Society of Toxicology, 1989); therefore, the rabbit eye irritation test is used by the occupational toxicologist. Current protocols include refinements to the original method, including a reduction in the number of animals used, the application of topical anesthetics to decrease animals' sensation, and rinsing with distilled water following the instillation of the test compound to allow evaluation of the benefits of washing the eye as a first aid measure. Another refinement that may be utilized is a reduction in the amount of material instilled into the eye (Griffith and Yam, 1989). In general, modifications of this type have not affected the reliability of this test (Hatoum et al., 1990) and may, in fact, better simulate possible workplace accidents and provide additional information.

The battery of tests shown in Table 20.4 can provide useful information to complete a workplace hazard assessment. However, they are not the only tools that may be used to determine the toxic potential of a workplace contaminant. Additional tests may be required to provide more rigorous recommendations. Depending on the results of initial tests, a second stage of testing may be initiated to address specific needs. For example, sensory irritation tests may be conducted for compounds that are found to have irritant properties. The sensory irritation test, developed by Alarie (1966), is used to develop a parameter—the RD_{50}—that has been directly correlated with threshold limit values (TLVs) for a certain class of compounds (Kane et al., 1979; Alarie, 1981). However, the usefulness of this test for solid compounds, which include most pharmaceutical agents, has not been determined. Results of genotoxicity tests may present a need for testing in additional systems to assess genotoxic potential. Mechanistic studies may also be appropriate for certain compounds, such as intermediates, in the synthesis of inhibitors of specific enzymes or receptor agonists/antagonists. The information available from clinical, pharmacology, or pharmacokinetic studies on the final drug can be useful in determining possible avenues for investigation. A particularly interesting type of study, yet to be developed, might involve determination of the absorption and bioavailability of compounds from occupational exposures that could then be related

TABLE 20.4 Summary of Protocols used for Current Test Methods

Test	Species	Method	Dose	Data Application	Additional Data
Acute toxicity					
Oral	Rat or mouse	N = 5/sex/dose; 14-day observation period; necropsy with/without histopathology	Chosen as limit (0.5 or 5 g kg^{-1}) or for LD_{50}	Classify compounds as harmful/toxic/highly toxic	
Dermal	Rabbit	N = 5/sex/dose; 24-h dermal application under occlusion; 14-day observation period; necropsy with/without histopathology; dermal irritation scores	Chosen as limit (2 g kg^{-1}) or for LD_{50}	Classify compounds as harmful/toxic/highly toxic; selection of protective equipment	Irritation potential class; target organ information
Inhalation	Rat or mouse	N = 5/sex/dose; 4-h nose only or whole-body exposure; 14-day observation period; necropsy with/without histopathology	Chosen as limit (20 g m^{-3}) or for LD_{50}	Classify compounds as harmful/toxic/highly toxic; selection of protective equipment	Respiratory irritation potential; target organ information
Skin irritation	Rabbit	N = 3; 4-h application under semiocclusive binder to abraded and nonabraded skin; irritation scores at ½, 1, 24, 48, and 72 h	500 mg/site	Classify irritation potential	
Eye irritation	Rabbit	N = 3/group; into right eye, compare to untreated eye; test only compounds with pH <12 or >2; score at 1, 24, 48, and 72 h and up to 21 days for corneal opacity, conjunctivitis, iritis: may use a rinse with some animals	100 mg or 0.1 mL in standard protocol or 10 mg or 0.01 mL in low-volume protocol	Classify eye irritation potential; selective equipment	Evaluate first aid methods; ocular toxicity
Sensitization					
Buehler method	Guinea pigs	N = 10–15; topical applications 1–3 times per week for 3 weeks, 2-week rest, then challenge at a naive site; concurrent negative/vehicle and positive controls	Up to 500 mg or 0.5 mL per dose	Classify as sensitizer—most sensitive for moderate to strong sensitizers; selection of protective equipment; evaluation of allergic reactions	Repeated dermal dosing; additional data on skin irritation and dermal absorption
Maximization	Guinea pigs	N = 10–15; combines 2 intradermal ± adjuvant and 1 topical occlusive administration for induction, 2-week rest, then topical application for challenge at naive site; concurrent positive and negative/vehicle controls	0.1 mL of compound in solution for induction; nonirritating concentration for challenge	May be sensitive to mild sensitizing agents but may also overpredict severity	
Local lymph node	Mouse (female)	N = 5; combines dermal exposure on ear with IV challenge by tail vein on day 6 with tritiated methyl thymidine	25 μL of compound in solvent on each ear for 3 days	Does produce some false positives	No rechallenge possible

to similar parameters developed in clinical or preclinical pharmacokinetic studies. The need to conduct additional testing will depend on the application of the information by the individual toxicologist and the resources available. The cost of additional tests should be weighed against the cost of applying the most conservative interpretation of the data to the work environment. In some cases implementing stricter controls based on preliminary tests may be less costly than conducting more extensive confirmatory testing.

There has recently been increasing pressure from governmental agencies and animal rights advocates to reduce the number of animals used in toxicological testing. As alternative toxicological methods become more accurate and sophisticated, they should be considered for incorporation into the occupational toxicology battery. Additional tools such as computer-aided quantitative structure–activity relationship (QSAR) evaluations may also be considered as additions or alternatives to animal tests (Jurs et al., 1985; Klopman, 1985; Frierson et al., 1986; Enslein, 1988). As indicated previously, QSAR methods may be particularly well suited to aid in the selection and/or prioritization of chemicals for testing, particularly in the case of intermediates. Alternative test methods currently under investigation, such as those being proposed for replacement of the Draize eye irritation test, do not appear to be well suited to the testing of pharmaceuticals or their synthetic intermediates (Booman et al., 1988, 1989). An intensive program of testing the available alternative models with compounds in this class is required to determine the ultimate usefulness of these alternative testing methods.

Occupational toxicologists from several companies supported a program to evaluate several experimental models as alternatives to the rabbit eye irritation test (Gáler et al., 1993; Sina et al., 1994). As a result of this cooperative study, several of the participating companies have implemented the routine use of several of these alternative models in their test batteries (R. G. Sussman and J. Sina, personal communications), thus effectively increasing the number and classes of compounds evaluated in alternative models.

20.5.3 Hazard Assessment

This is quite possibly the most difficult and controversial activity for the occupational toxicologist. Just as there is no blueprint for conducting toxicological testing, there is no formula for performing an occupational risk assessment. I have indicated above the need to include an indication of the degree of hazard from occupational exposure in informational communications. A more specific form of hazard assessment is the development of occupational exposure limits (OELs). Occupational exposure limits, such as the American Conference of Governmental Industrial Hygienists (ACGIH) TLVs, have been available for approximately 50 years (Cook, 1987). Similar workplace limits developed by OSHA are known as PELs (OSHA, 1989). The majority of the substances for which TLVs or PELs have been developed are large-volume industrial chemicals, often encountered in the workplace as vapors. The process of developing

TLVs and PELs has been documented by ACGIH and OSHA, respectively (OSHA, 1987; ACGIH, 1995). Unfortunately, the process is not always consistent or straightforward. Some TLVs or PELs are intended to prevent chronic effects yet are developed using acute reactions as reference points. Others are based on an existing TLV developed for a third compound. Clearly, these are not examples that can be easily followed by the pharmaceutical industry.

Individual companies have, nonetheless, developed methodologies for developing OELs based on the type of data and other resources available. One method involves a formula for extrapolating to an 8-h time-weighted average from the therapeutic dose of the drug using safety factors (Sargent and Kirk, 1988). A group composed of occupational toxicologists from several companies presented a monograph at the second annual Occupational Toxicology Roundtable, held in November 1989, regarding the development of OELs (Gáler et al., 1989, 1992). Several methods are available for developing OELs: analogy, correlation, safety and uncertainty factors, and low-dose extrapolation (Table 20.5). The appropriate method must be selected on the basis of the appropriateness of the available data. For example, low-dose extrapolation may be appropriate only if sufficient pharmacokinetic data are available to build a suitable physiologically based pharmacokinetic (PBPK) model. Analogy is a method by which the OEL for one compound is adopted for a second compound, based on the two compounds' structural and functional similarity. This method is suitable only if the two compounds are similar in every aspect, including therapeutic or toxic dose and physical properties. Correlation is similar to analogy in that it compares similar compounds. However, the OEL is chosen based on a key property of the chemical that influences its toxicological properties. An example would be the use of the relative potency of two drugs as the key property used to adjust the reference OEL. The most commonly used method is that of applying safety and/or uncertainty factors to a reference dose, which may be the lowest therapeutic dose. In using this method, it is important to choose the reference dose and endpoint with great care. In general, the most sensitive endpoint should be chosen. Uncertainty factors are selected to approximate levels from effective doses and to account

TABLE 20.5 Methods for Setting Occupational Exposure Limits

Method	Formula[a]
Analogy	$OEL_i = OEL_j$
Correlation	$OEL_j = (PP_i/PP_j) \times OEL_j$
Safety factors	$OEL = \text{reference dose}/UF_1 \times UF_2 \times SF \times BR$
Low-dose extrapolation	$OEL = [\text{rodent RSD} \times (BW_H/BW_R)^{-1/3}]/BR$ Or, if PBPK available, OEL = (human reference dose)/BR

[a]OEL, occupational exposure limit; PP, physical property; UF_1, uncertainty in extrapolation to NOEL; UF_2, uncertainty from interspecies extrapolation; SF, safety factor; BR, breathing rate for 8-h workday; PBPK, physiologically based pharmacokinetic model; RSD, risk-specific dose; BW, body weight.

Source: Adapted from Gáler et al., 1989.

for interspecies differences. A safety factor is selected based on the overall toxicological evaluation of the compound. Because it is necessary to look at the complete toxicological profile, it is generally inappropriate to assign specific values to each type of toxic effect.

Most commonly employed now is the approach suggested initially by Sargent and Kirks in 1988, where the OEL for airborne pharmaceutical materials is calculated as

$$\text{OEL} = \frac{\text{NOAEL (mg/kg)(BW in kg)}}{(\text{UF})(V \text{ in nm}^3)(\propto)(s)}$$

where NOAEL is the no effect level in the most sensitive species, BW is body weight, UF is an uncertainty factor (usually 10), V is the volume of air breathed by a worker in 8 h (usually 10 m is used), $\propto$ is an adjustment for bioavailability between routes (if the animal data are from a route other than inhalation), and s is for already known plasma levels if known (Binks, 2003). The FDA HED (human equivalent dose) factor is commonly used in place of plasma level if the steady state is unknown.

The OEL process has essentially become an industry standard. While U.S. OSHA regulations do not require that manufacturers establish OELs, European governmental agencies do. The first country to require this activity is the United Kingdom, under its Control of Substances Hazardous to Health (COSHH) regulations (Health and Safety Executive, 1988; Agius, 1989). A similar requirement is included in the EEC directive 80/1107, which was promulgated into law by other EC member nations.

In creating a program for establishing OELs, several disciplines will generally be included in the development or approval process. In those programs that are currently in place, safety, industrial hygiene, manufacturing or technical services, medical services, legal services, R&D, and, of course, occupational toxicology may take part in the process. While the ability to make the OEL level in the workplace does not drive the process, the OEL may often be issued as an interim guideline to provide manufacturing locations an opportunity to bring their operations into compliance and for the development of a suitable industrial hygiene sampling and analysis method.

20.5.4 Employee Training

The ultimate client for the services of the occupational toxicologist is the manufacturing or research employee who must be informed of and protected from the potential hazards of chemicals present in the work environment. Providing employees with health hazard information directly through presentations or training programs accomplishes this task better than most written communications and also provides an excellent way to build confidence in the organization and its safety and health programs. The trust gained in this manner can be an invaluable asset when a company is challenged with the

manufacture of especially toxic or potent compounds. Face-to-face communication will also promote discussions with line employees and give the occupational toxicologist the opportunity to learn of those adverse health effects that might otherwise go unnoticed or uninvestigated.

There are several areas for which it may be useful to consider developing specific training programs. The OSHA hazard communication standard requires that employees be trained to understand the hazards of chemicals as they are outlined in the MSDS. There is an obvious need for the occupational toxicologist to be involved in the development of an internal training program or the selection of a commercial program to address this need. In addition to this required training, it may be useful to consider a more in-depth program on basic concepts involved in health hazard evaluation, particularly the dose–response relationship and the different types of chronic health hazards. It may be particularly important to promote an understanding of health hazard information obtained at work as well as through the news media. There are several commercial training programs available that may be useful for this purpose, including computer-based self-training programs and videos.

Table 20.6 presents the currently most commonly used classification system for pharmaceutical occupational risks. Based on the assigned categorization from this risk, appropriate worker protection methods are selected (Olsen et al., 2002; Binks, 2003).

As most pharmaceutical manufacturing is now performed on contract (in "toll" manufacturers), categorization is usually performed on contract (by a firm such as a safe bridge) or by a consultant.

Specific training on compounds of interest can also be useful, particularly before the beginning of a manufacturing campaign, and is particularly effective if coupled with industrial hygiene training on appropriate safe handling techniques. If a testing program is in place, it is good policy to present an evaluation of the information gained in the compound's testing program to the research or manufacturing chemists involved in its manufacture.

Issues continuing to gain in importance for pharmaceutical industry occupational toxicologists are those relating to the new, more potent drugs currently being designed. Current drugs have therapeutic dose levels ranging down to the submicrogram (and in some cases even nanogram) levels. Hazard assessment and OEL development, already difficult, may be nearly impossible or inappropriate when dealing with drugs active at pico- or femtogram levels. Alternative methods of evaluating occupational exposures and assuring a safe work environment may need to be developed. Then there are biotechnology products and their associated issues.

A third of all new drugs come from the the biotechnology pipeline, most commonly peptides and proteins with significant allergenic potential in an occupational setting. The potential occupational health hazards of this class of potent but high-molecular-weight products have not been fully evaluated. Because of the inherent functional and structural differences, the extrapolation of testing methods from traditional pharmaceutical products to

TABLE 20.6 Categorization Code Enrollment Criteria

	Categorization Code				
Criteria	1	2	3	4	5
OEL	>1 mg m^{-3}	0.10 to <1 mg m^{-3}	10 to <100 µg m^{-3}	1 to <10 µg m^{-3}	<1 µg m^{-3}
Potency (mg day^{-1})	>100 low	100–10 low/moderate	10–0.01 moderate	0.1–0.01 moderate/ high	<0.01 high
Acute toxicity	Slightly toxic	Moderately toxic	Highly toxic	Extremely toxic	Supertoxic
Severity of acute effects	Low	Low/moderate	Moderate	Moderate high	High
Chronic toxicity	None	None	Slight to moderate	Moderate	Severe
Absorption by inhalation/skin	Minimal inhalation or skin adsorption	Moderate inhalation or skin absorption	Significant inhalation or skin absorption	Significant inhalation or skin absorption	Significant inhalation or skin absorption
Irritation potential					
Skin	None	Mild to moderate	Moderate to severe	Severe to extreme	Extreme
Eye	Mild to moderate	Mild to moderate	Severe	Severe to extreme	Extreme
Sensitization	Not a sensitizer	Mild sensitizer	Moderate sensitizer	Strong sensitizer	Extreme sensitizer
Mutagenicity	None	Yes	Yes	Severe	Severe
Carcinogenicity	Negative	Suspected	Suspected–confirmed animal	Defined medical case studies	Defined medical case studies
Reproductive disorders	None	None	Slight	Moderate	Known
Teratogenicity	None	None	Yes	Severe	Severe
Neurotoxicity	Minimal	Minimal to moderate	Moderate to immediate and delayed	Life threatening	Life threatening
Epidemiology	Confirmed, no effect	Case studies/ ongoing definitive data	Defined medical case studies	Defined medical case studies	Defined medical case studies
Genotoxicity	None	None –(+) Ames test	(+) In battery of genotox studies	(+) In a battery of genotox studies	(+) In a battery of genotox studies
Warning properties	Good	Fair	Fair/poor	Poor	None

biotechnology-derived compounds may be fraught with many difficulties. Hypersensitivity and other immunologically based toxicities are particularly of concern for protein- and peptide-based therapeutics.

20.6 CONCLUSION

The field of occupational toxicology in the pharmaceutical industry presents continuing challenges to the industry, particularly as it shifts increasingly to an outsourced function in companies. The occupational toxicologist finds that he or she must become an "expert" in several fields and not be limited to the scientific area. Unlike the preclinical toxicologist, the occupational practitioner functions under less stringent regulatory requirements and minimal precedents. Additionally, as new classes of therapeutic agents enter development and commerce, new concerns and challenges will accompany them.

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21

Strategy and Phasing for Nonclinical Drug Safety Evaluation in Discovery and Development of Pharmaceuticals

21.1 INTRODUCTION

The preclinical assessment of the safety of potential new pharmaceuticals represents a special case of the general practice of nonclinical safety assessment (Gad, 1996, 2000; Meyer, 1989) possessing its own peculiarities and special considerations and differing in several ways from the practice of toxicology in other fields—for some significant reasons. Because of the economics involved and the essential close interactions with other activities (e.g., clinical trials, chemical process optimization, formulation development, regulatory reviews), the development and execution of a crisp, timely, and flexible, yet scientifically sound, program are prerequisites for success. The ultimate aim of preclinical assessment also makes it different. A good pharmaceutical safety assessment program seeks to efficiently and effectively move safe, potential therapeutic agents into, and support them through, the clinical evaluation, then to registration, and, finally, to market. This requires the quick identification of those agents that are not safe. At the same time, the very biological activity which makes a drug efficacious also acts to complicate the design and interpretation of safety studies.

Drug Safety Evaluation, Second Edition, by Shayne Cox Gad

Such evaluations occur on different time scales because of different objectives. There is the traditional big pharma case and different versions used predominantly by the much more numerous small pharmaceutical cases. These small pharma cases may be either the short case (do only what is required to initiate and support initial clinical trials) or the midgame (a variety where studies to support further clinical trials are performed but spread out).

Pharmaceuticals, unlike industrial chemicals, agricultural chemicals, and environmental agents, are intended to have human exposure and biological activity. And, unlike these materials and food additives, pharmaceuticals are intended to have biological effects on the people that receive them. Frequently, the interpretation of results and the formulation of decisions about the continued development and eventual use of a drug are based on an understanding of both the potential adverse effects of the agent (its safety) and its likely benefits as well as the dose separation between these two (the "therapeutic index"). This makes a clear understanding of dose–response relationships critical, so that the actual risk–benefit ratio can be identified. It is also essential that the pharmacokinetics be understood and that "doses" (plasma tissue levels) at target organ sites are known (Scheuplein et al., 1990). Integral evaluation of pharmacokinetics is essential to any effective safety evaluation program.

The development and safety evaluation of pharmaceuticals have many aspects specified by regulatory agencies, and this has also tended to make the process more complex [until recently, as the International Conference on Harmonisation (ICH) has tended to take hold] as markets have truly become global. An extensive set of safety evaluations is absolutely required before a product is ever approved for market. There are even novels on the subject (see Zbinden, 1992). Regulatory agencies have increasingly come to require not only the establishment of a "clean dose" in two species with adequate safety factors to cover potential differences between species but also an elucidation of the mechanisms underlying such adverse effects as are seen at higher doses and are not well understood. These regulatory requirements are compelling for the pharmaceutical toxicologist (Traina, 1983; Guarino, 1987; Smith, 1992). There was not, however, a set menu of what must be done. Rather, much (particularly in terms of the timing of testing) is open to professional judgment and is tailored for the specific agent involved and its therapeutic claim. ICH M3(R2) guidance (ICH, 2009) has acted to improve this situation significantly, but significant "regulatory drift" leading to the need for experience, judgment, and interaction with FDA remains.

The discovery, development, and registration of a pharmaceutical is an immensely expensive operation and represents a rather unique challenge. For every 9000–10,000 compounds specifically synthesized or isolated as potential therapeutics, one (on average) will actually reach the market. This process is illustrated diagrammatically in Figure 21.1. Each successive stage in the process is more expensive, making it of great interest to identify as early as possible those agents that are likely to not go the entire distance, allowing a concentration of effort on the compounds that have the highest probability of

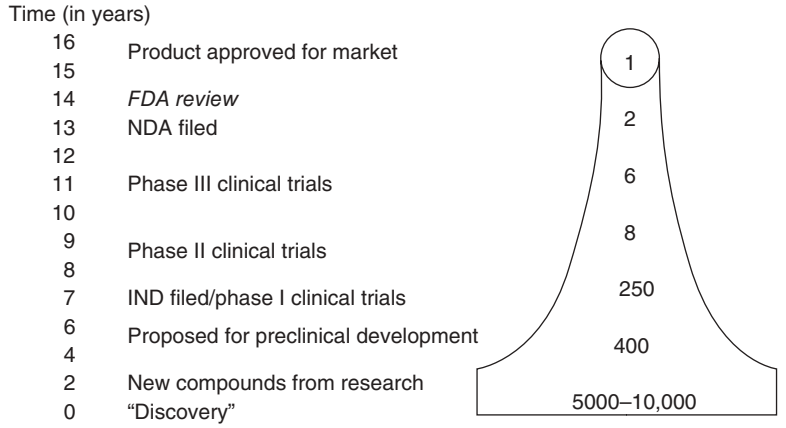

Figure 21.1 *Attrition during development of new molecules with promise of therapeutic potential. Over the course of taking a new molecular entity through scaleup, safety and efficacy testing, and, finally, to market typically only one out of every 9000–10,000 will go to the marketplace.*

reaching the market. Compounds "drop out" of the process primarily for three reasons:

1. Toxicity (or lack of toxicity tolerance)
2. (Lack of) efficacy
3. (Lack of) bioavailability of the active moiety in humans.

Early identification of poor or noncompetitive candidates in each of these three categories is thus extremely important (Fishlock, 1990), forming the basis for the use of screening in pharmaceutical discovery and development. How much and which resources to invest in screening and each successive step in support of the development of a potential drug are matters of strategy and phasing that are detailed in Section 21.5 of this chapter. In vitro methods are increasingly providing new tools for use in both early screening and the understanding of mechanisms of observed toxicity in preclinical and clinical studies (Gad, 1989b, 2001), particularly with the growing capabilities and influence of genomic and proteomic technologies. This is increasingly important as the societal concern over drug prices has grown (Littlehales, 1999). Additionally, the marketplace for new drugs is exceedingly competitive. The rewards for either being introduced early (first or second) into the marketplace ("first movers advantage") or achieving a significant therapeutic advantage are enormous in terms of eventual market share. Additionally, the first drug approved sets agency expectations for those drugs which follow. In mid-2008, there were 254 pharmaceutical products awaiting approval (86 of these biotechnological products)—the "oldest" having been in review nine years) and some 16,000 additional agents in the investigational new drug (IND) stage [U.S. Food and

Drug Administration (FDA) website]. Not all of these (particularly the oldest) will be economically successful.

The successful operation of a safety assessment program in the pharmaceutical industry requires that four different phases of the product-related operation be simultaneously supported. These four phases of pharmaceutical product support (discovery support, IND support, clinical and registration support, and product support) constitute the vast majority of what is done by the safety assessment groups in the pharmaceutical industry. The constant adjustment of balance of resources between these four areas is the greatest management challenge in pharmaceutical safety assessment. An additional area, occupational toxicology, is conducted in a manner similar to that for industrial environments and is the subject of Chapter 14 of this volume. In most companies, occupational toxicology is the responsibility of a separate group.

The usual way in which transition (or "flow") between the different phases is handled in safety assessment is to use a tiered testing approach. Each tier generates more specific data (and costs more to do so) and draws on the information generated in earlier tiers to refine the design of new studies. Different tiers are keyed to the support of successive decision points (go/no-go points) in the development process, with the intent of reducing risks as early as possible.

The first real critical decisions concerning the potential use of a compound in humans are the most difficult. They require an understanding of how well particular animal models work in predicting adverse effects in humans (usually very well, but there are notable lapses; e.g., giving false positives and false negatives) and an understanding of what initial clinical trials are intended to do. Though an approved IND grants one entry into limited evaluations of drug effects in humans, flexibility in the execution and analysis of these studies offers a significant opportunity to also investigate efficacy (O'Grady and Linet, 1990).

Once past the discovery and initial development stages, the safety assessment aspects of the process become extremely tightly connected with the other aspects of the development of a compound, particularly the clinical aspects. These interconnections are coordinated by project management systems. Many times during the early years of the development process, safety assessment constitutes the rate-limiting step—it is, in the language of project management, on the critical path.

Another way in which pharmaceutical safety assessment varies from toxicology as practiced in other in industries is that it is a much more multidisciplinary and integrated process. This particularly stands out in the incorporation of the evaluation of ADME (absorption, distribution, metabolism, and excretion) aspects in the safety evaluation process. These pharmacokinetic/metabolism (PKM) aspects are evaluated for each of the animal model species (most commonly the rat and dog or primate) utilized to evaluate the preclinical systemic toxicity of a potential drug prior to evaluation in humans. Frequently, in vitro characterizations of metabolism for model (or potential model) species and humans are performed to allow optimal model selection and understand-

ing of findings. This allows for an early appreciation of both the potential bioavailability of active drug moieties and the relative predictive values of the various animal models. Such data early on are also very useful (in fact, sometimes essential) in setting dose levels for later animal studies and in projecting safe dose levels for clinical use. Unlike the case in most other areas of industrial toxicology, one is not limited to extrapolating the relationships between administered dose and systemic effects. Rather, one has significant information on systemic levels of the therapeutic moiety—typically, total area under the curve (AUC), peak plasma levels (C_{max}), and plasma half-lives at a minimum. Chapter 18 looks at these aspects in detail.

The state of the art for preclinical safety assessment has now developed to the point where the resulting products of the effort [reports, IND/new drug application (NDA) summaries, and the overall professional assessment of them] are expected to reflect and integrate the best effort of all the available scientific disciplines. Actual data and discussion should thus come from toxicology, pharmacology, pathology, and metabolism at a minimum. The success of current premarket efforts to develop and ensure that only safe drugs make it to market are generally good but clearly not perfect. This is reflected in popular (Arnst, 1998; Raeburn, 1999) and professional (Moore et al., 1998; Lazaron et al., 1998) articles looking at both the number of recent marketed drug withdrawals for safety (summarized in Table 21.1) and rates of drug-related adverse drug events and deaths in hospital patients. It is hoped that this system can be improved, and there are continuing efforts to improve or optimize drug candidate selection and development (Lesko et al., 2000). Indeed, the entire pharmaceutical development paradigm is clearly in need of a complete change—a synthesis, as opposed to a continual application of small corrections.

21.2 REGULATORY REQUIREMENTS

Minimum standards and requirements for safety assessment of new pharmaceuticals are established by the need to meet regulatory requirements for developing and eventually gaining approval to market the agent. Determining what these requirements are is complicated by (1) the need to compete in a global market, which means gaining regulatory approval in multiple countries that do not have the same standards or requirements, and (2) the fact that the requirements are documented as guidelines, the interpretation of which is subject to change as experience alters judgments. The ICH process has much improved this situation, as detailed in Chapter 2. The ICH (M3) (2000) clearly denotes what nonclinical studies are required to support clinical drug development. Unfortunately, since then additional requirements (safety pharmacology and immunotoxicology) have been promulgated by additional guidelines. Accordingly, M3 is not all current or inclusive in its guidance.

Standards for the performance of studies (which is one part of regulatory requirements) have as their most important component good laboratory practices (GLPs). Good laboratory practices largely dictate the logistics of

TABLE 21.1 Post-approval Adverse Side Effects and Related Drug Withdrawals Since 1990

Year	Drug	Indication/Class	Causative Side Effect
1991	Enkaid (4 years on market)	Antiarrhythmic	Cardiovascular (sudden cardiac death)
1992	Temafloxacin	Antibiotic	Blood and kidney
1997	Fenfluramine[a]/ dexafluramine (combo used since 1984)	Diet pill	Heart valve abnormalities
1998	Posicor (Midefradil) (1 year on market)	Ca^{2+} channel blocker	Lethal drug interactions (inhibited liver enzymes)
	Duract (Bronfemic Sodium) (early preapproval warnings of elevated liver enzymes)	Pain relief	Liver damage
1999	Tronan (use severely restricted)	Antibiotic	Liver/kidney damage
	Raxar	Quinolone antibiotic	QT internal prolongation/ ventricular arrhythmias (deaths)
	Hismanal	Antihistamine	Drug–drug interactions
	Rotashield	Rotavirus vaccine	Bowel obstruction
2000	Renzulin (approved Dec. 1996)	Type II diabetes	Liver damage
	Propulsid	Heartburn	Cardiovascular irregularities/death
	Lotonex	Irritable bowel syndrome	Ischemic colitis/death
2001	Phenylpropanolamine (PPA)	OTC ingredient	Hemorrhagic stroke
	Baychlor	Cholesterol reducing (statin)	Rhabdomyolysis (muscle weakening) (deaths)
2002/2003	None		
2004	Serazone	Antidepressant	Liver failure and injury
	Vioxx	Arthritis (CoOX-2 inhibitor)	Heart attack/ cardiovascular (thrombosis)
2005	Tysabri	Multiple sclerosis (MS)	Progressive multifocal leukoencephalopathy (PML)
	Bextra	Arthritis (CoOX-2 inhibitor)	Skin reaction (sometimes fatal)
2006	Dolophine (methadone hydrochloride)	Treatment of moderate to severe pain	Respiratory depression and cardiac arrhythmias
2007	Zelnorm	Constipation	Cardiovascular safety
	Permax	Parkinson's disease	Heart valve damage

Note: 51% of approval drugs had serious postapproval identified side effects. FDAMA (Food and Drug Administration Modernization Act) passed in 1997.

[a]Twenty four years on market.

safety assessment—training, adherence to other regulations (such as those governing the requirements for animal care), and (most of all) the documentation and recordkeeping that are involved in the process. There are multiple sets of GLP regulations [in the United States alone, agencies such as the FDA and the Environmental Protection Agency (EPA) each have their own] that are not identical; however, adherence to FDA (1987a) GLPs will rarely lead one astray.

Not all studies that are done to assess the preclinical safety of a new pharmaceutical need be done in strict adherence to GLPs. Those studies that are "meant to support the safety of a new agent" (FDA, 1987a) (i.e., are *required* by regulatory guidelines) must be so conducted or run a significant risk of rejection. However, these are also many other studies of an exploratory nature (such as range finders and studies done to understand the mechanisms of toxicity) that are not required by the FDA and which may be done without strict adherence to GLPs. A common example is those studies performed early on to support research in selecting candidate agents. Such studies do not meet the requirements for having a validated analytical method to verify the identity, composition, and stability of materials being assayed, yet they are essential to the processes of discovery and development of new drugs. All such studies must eventually be reported to the FDA if an IND application is filed, but the FDA does not in practice "reject" such studies (and therefore the IND) because they are "non-GLP."

There is a second set of "standards" of study conduct that are less well defined. These are "generally accepted practice" and, though not written down in a regulation, are just as important as GLPs for studies to be accepted by the FDA and the scientific community. These standards, which are set by what is generally accepted as good science by the scientific community, include techniques, instruments utilized, and interpretation of results. Most of the chapters in this book will reflect these generally accepted practices in one form or another.

Guidelines establish which studies must be done for each step in the process of development. Though guidelines supposedly are suggestion (and not requirements), they are in fact generally treated as minimums by the promulgating agency. The exceptions to this are special cases where a drug is to meet some significant need [a life-threatening disease such as AIDS or amyotrophic lateral sclerosis (ALS)] or where there are real technological limitations as to what can be done [as with many of the new biologically derived (or biotechnology) agents, where limitations on compound availability and biological responses make traditional approaches inappropriate].

There are some significant differences in guideline requirements between the major countries [see Alder and Zbinden (1988) for an excellent country-by-country review of requirements], though this source is now becoming dated. The core of what studies are generally done are those studies conducted to meet FDA requirements. These are presented in Table 21.2. As will be discussed in Chapter 2, these guidelines are giving way to the ICH guidelines. However, while the length and details of studies have changed, the nature and order of studies remain the same.

TABLE 21.2 Synopsis of General Guidelines for Animal Toxicity Studies (U.S. FDA, *Total Drug Quality*)

Category	Duration of Human Administration[a]	Phase[b]	Subacute or Chronic Toxicity[c]	Special Studies
Oral or parenteral	Several days (up to 3)	I, II, III, NDA	2 species: 2 weeks	For parenterally administered drugs; compatibility with blood and local tolerance at injection site where applicable
	Up to 2 weeks	I	2 species: 4 weeks	
		II	2 species: up to 4 weeks	
		III, NDA	2 species: up to 3 months	
	Up to 3 months	I, II	2 species: 4 weeks	
		III	2 species: 3 months	
		NDA	2 species: up to 6 months	
	6 months to unlimited	I, II	2 species: 3 months	
		III	2 species: 6 months or longer	
		NDA	2 species: 12 months in rodents, 9 months in nonrodents + 2 rodent species for CA; 18 months (mouse)—may be met by use of transgenic model 24 months (rat)	
Inhalation (general anesthetics)	Single administration	I, II, III, NDA	4 species: 5 days (3 h/day)	
Dermal	Single application	I	1 species: single 24-h exposure followed by 2-week observation	Sensitization
	Single or short-term application	II	1 species: 20-day repeated exposure (intact and abraded skin)	
	Short-term application	III	As above	
	Unlimited application	NDA	As above, but intact skin study extended up to 6 months	
Ophthalmic	Single application	I		Eye irritation tests with graded doses
	Multiple application	I, II, III	1 species: 3 weeks, daily applications, as in clinical use	
		NDA	1 species: duration commensurate with period of drug administration	

TABLE 21.2 ***Continued***

Category	Duration of Human Administration[a]	Phase[b]	Subacute or Chronic Toxicity[c]	Special Studies
Vaginal or rectal	Single application	I		Local and systematic toxicity after vaginal or rectal application in 2 species
	Multiple application	I, II, III, NDA	2 species: duration and no. of applications determined by proposed use	Lethality by appropriate route, compared to components run concurrently in 1 species
Drug combinations[d]	I, II, III, NDA	2 species: up to 3 months		

[a]Phases I dosing of females if childbearing potential requires a Segment II study in at least one species; Phase III dosing of this population requires a Segment I study and both Segment II studies.
[b]Phases I, II, and III are defined in Section 130.0 of the New Drug Regulations.
[c]Acute toxicity should be determined in three species; subacute or chronic studies should be by the clinical route to be used. Suitable mutagenicity studies should also be performed.
Observations:
Body weights
Metabolic studies
Gross and microscopic examination
Coagulation tests
Food consumption
Ophthalmological examination
Hemogram
Others as appropriate
Behavior
Fasting blood sugar
Liver and kidney function tests
[d]Where toxicity data are available on each drug individually.
Source: FDA, *Total Drug Quality*, 1971.

The major variations in requirements for other countries still tend to be in the area of special studies. The United States does not formally require any genotoxicity studies, but common practice for U.S. drug registration is to perform at least a bacterial gene mutation assay (Ames test), a mammalian cell mutation assay, and a clastogenicity assay, while Japan requires specific tests, including a gene mutation assay in *Escherichia coli*. Likewise, the European Economic Community (EEC) has a specified set of requirements, while individual countries have additional special requirements (Italy, for example, requires a mutagenicity assay in yeast). As detailed in Chapter 6, the new ICH genotoxicity guidelines have come to meet multinational requirements. Japan maintains a special requirement for an antigenicity assay in guinea pigs. The

new safety pharmacology requirements are likely to be adopted over a period of time by different adherents.

It is possible to interact with the various regulatory agencies (particularly the FDA) when peculiarities of science or technology leave one with an unclear understanding of what testing is required. It is best if such discussions directly involve the scientists who understand the problems, and it is essential that the scientists at the FDA be approached with a course of action (along with its rationale) that has been proposed to the agency in advance.

The actual submissions to a regulatory agency that request permission either to initiate (or advance) clinical trials of a drug or to market a drug are not just bundles of reports on studies. Rather, they take the form of summaries that meet mandated requirements for format accompanied by the reports discussed in these summaries. In the United States, these summaries are the appropriate section of the IND and NDA. The formats for these documents have recently been revised (FDA, 1987b). The EEC equivalent is the expert report, as presented in EEC Directive 75/319. Similar approaches are required by other countries. In each of these cases, textual summaries are accompanied by tables that also serve to summarize significant points study design and study findings.

All of these approaches have in common that they are to present integrated evaluations of the preclinical safety data that are available on a potential new drug. The individual studies and reports are to be tied together to present a single, cohesive overview of what is known about the safety of a drug.

Leber (1987) presents an excellent overview of the regulatory process involved in FDA oversight of drug development and gives the historical perspective for the evolution of the conservative process that is designed to ensure that any new pharmaceutical is both safe and efficacious.

There are other regulatory, legal, and ethical safety assessment requirements beyond those involved in the selection and marketing of a drug as a product entity. The actual drug product must be manufactured and transported in a safe manner and any waste associated with this manufacture disposed of properly. Chapter 14 of this volume specifically addresses this often overlooked aspect of safety assessment programs.

21.3 ESSENTIAL ELEMENTS OF PROJECT MANAGEMENT

It is important to keep in mind that safety assessment is only one of many components involved in the discovery and development of new pharmaceuticals. The entire process has become enormously expensive, and completing the transit of a new drug from discovery to market has to be as efficient an expedition process as possible. Even the narrow part of this process (safety assessment) is dependent on many separate efforts. Compounds must be made, analytical and bioanalytical methods developed, and dosage formulations developed, to name a few. One needs only to refer to Beyer (1978), Hamner

(1982), Matoren (1984), Sneader (1986) (a good short overview), or Spilker (1994) for more details on this entire process and all of its components.

The coordination of this complex process is the province of project management, the objective of which is to ensure that all the necessary parts and components of a project match up. This discipline in its modern form was first developed for the Polaris missile project in the 1960s. Its major tool, which is familiar to pharmaceutical scientists, is the "network" or PERT (program evaluation review technique) chart, as illustrated in Figure 21.2. This chart is a tool that allows one to see and coordinate the relationships between the different components of a project. One outcome of the development of such a network is identification of the rate-limiting steps, which in aggregate comprise the critical path (see Table 21.3 for a lexicon of the terms used in project management).

A second graphic tool from project management is the Gantt chart, as illustrated in Figure 21.3. This chart allows one to visualize the efforts underway in any one area, such as safety assessment, for all projects that are currently being worked on.

Figure 21.4 is a hybrid from of the PERT and Gantt charts, designed to allow one to visualize all the resources involved in any one project.

An understanding of the key concepts of project management and their implications is critical in strategic planning and thinking for safety assessment. Kliem (1986) and Knutson (1980) offer excellent further reading in the area of project management.

21.4 SCREENS: THEIR USE AND INTERPRETATION IN SAFETY ASSESSMENT

Much (perhaps even most) of what is performed in safety assessment can be considered screening—trying to determine if some effect is or is not (to an acceptable level of confidence) present (Zbinden et al., 1984). The general concepts of such screens are familiar to toxicologists in the pharmaceutical industry because the approach is a major part of the activities of the pharmacologists involved in the discovery of new compounds. But the principles underlying screening are not generally well recognized or understood. And such understanding is essential to the proper use, design, and analysis of screens (Gad, 1988, 1989a). Screens are the biological equivalent of exploratory data analysis (EDA) (Tukey, 1977).

Each test or assay has an associated activity criterion, that is, a level above which the activity of interest is judged to be present. If the result for a particular test compound meets this criterion, the compound may pass to the next stage. This criterion could be based on statistical significance (e.g., all compounds with observed activities significantly greater than the control at the 5% level could be tagged). However, for early screens, such a formal criterion may be too strict, resulting in a few compounds begin identified as "active."

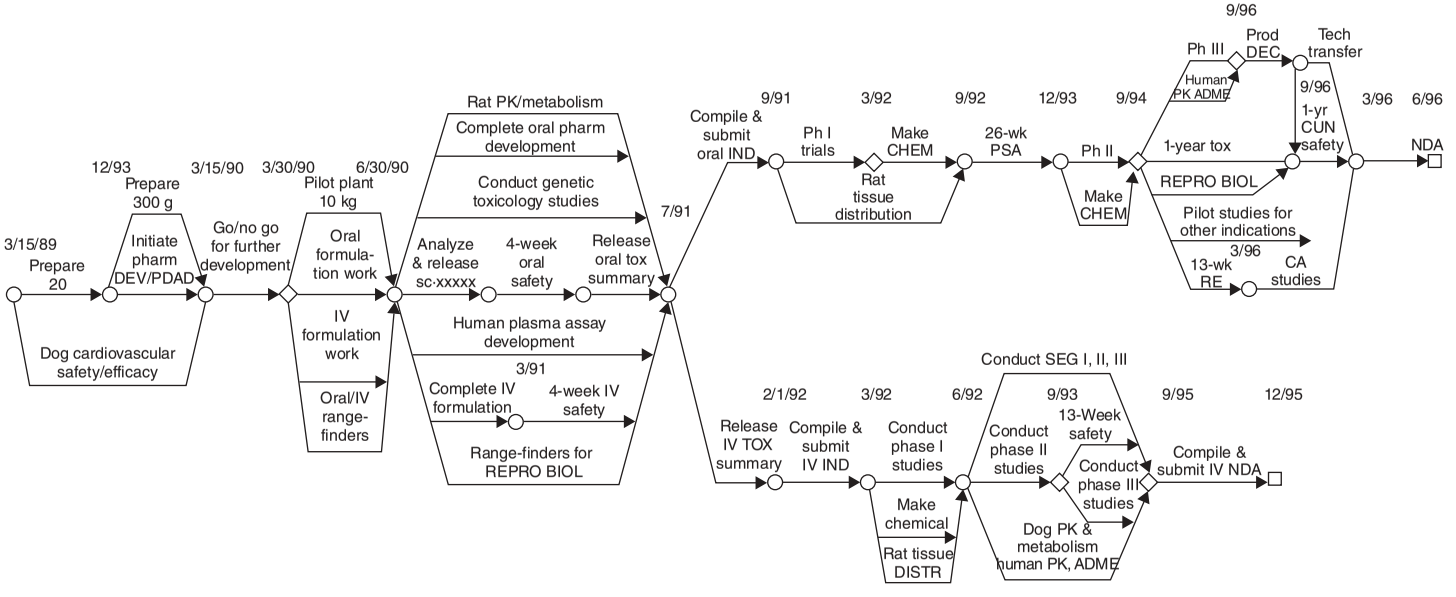

Figure 21.2 *An example PERT (program evaluation review technique) chart of the development of a new pharmaceutical through to the filing of an NDA (new drug application). Circles are "nodes" indicating completion of activities. Diamonds are initiation points for tasks that have starting points independent of others. This "network" serves to illustrate the relationships between different activities and to evaluate effects of changes on project timing.*

TABLE 21.3 Glossary of Project Management Terms

Activity	The work or effort needed to complete a particular event. It consumes time and resources.
Average daily resource requirement	The likely amount of resources required to complete an activity or several activities on any workday during a project. The average daily labor requirement is one example.
CPM	Acronym for critical path method. A network diagramming technique that places emphasis on time, cost, and the completion of events.
Critical path	The longest route through a network that contains activities absolutely crucial to the completion of the project.
Dummy arrow	A dashed line indicating an activity that uses no time or resources.
Duration	The time it takes to complete an activity.
Earliest finish	The earliest time an activity can be completed.
Earliest start	The earliest an activity can begin if all activities before it are finished. It is the earliest time that an activity leaves its initiation node.
Event	A synonym for node. A point in time that indicates the accomplishment of a milestone. It consumes neither time nor resources and is indicated whenever two or more arrows intersect.
Free float	The amount of time that an activity can be delayed without affecting succeeding activities.
Gannt chart	A bar chart indicating the time interval for each of the major phases of a project.
Histogram	A synonym for bar chart.
Latest finish	The latest time an activity can be completed without extending the length of a project.
Latest start	The latest time an activity can begin without lengthening a project.
Leveling	The process of “smoothing” out labor, material, and equipment requirements to facilitate resource allocation. The project manager accomplishes this by “rescheduling” noncritical activities so that the total resource requirements for a particular day match the average daily resource requirements.
Most likely time	Used in PERT diagramming. The most realistic time estimate for completing an activity or project under normal conditions.
Node	A synonym for event.
Optimistic time	Used in PERT diagramming. The time the firm can complete an activity or project under the most ideal conditions.
PERT	Acronym for program evaluation and review technique. A network diagramming technique that places emphasis on the completion of events rather than cost or time.
Pessimistic time	Used in PERT diagramming. The time the firm can complete an activity or project under the worst conditions.
Project	The overall work or effort begin planned. It has only one beginning node and ending node. Between those nodes are countless activities and their respective nodes.
Project phase	A major component, or segment, of a project. It is determined by the process known as project breakdown structuring.
Total float	The total amount of flexibility in scheduling activities on a noncritical path. Hence, it provides the time an activity could be prolonged without extending a project’s final completion date.

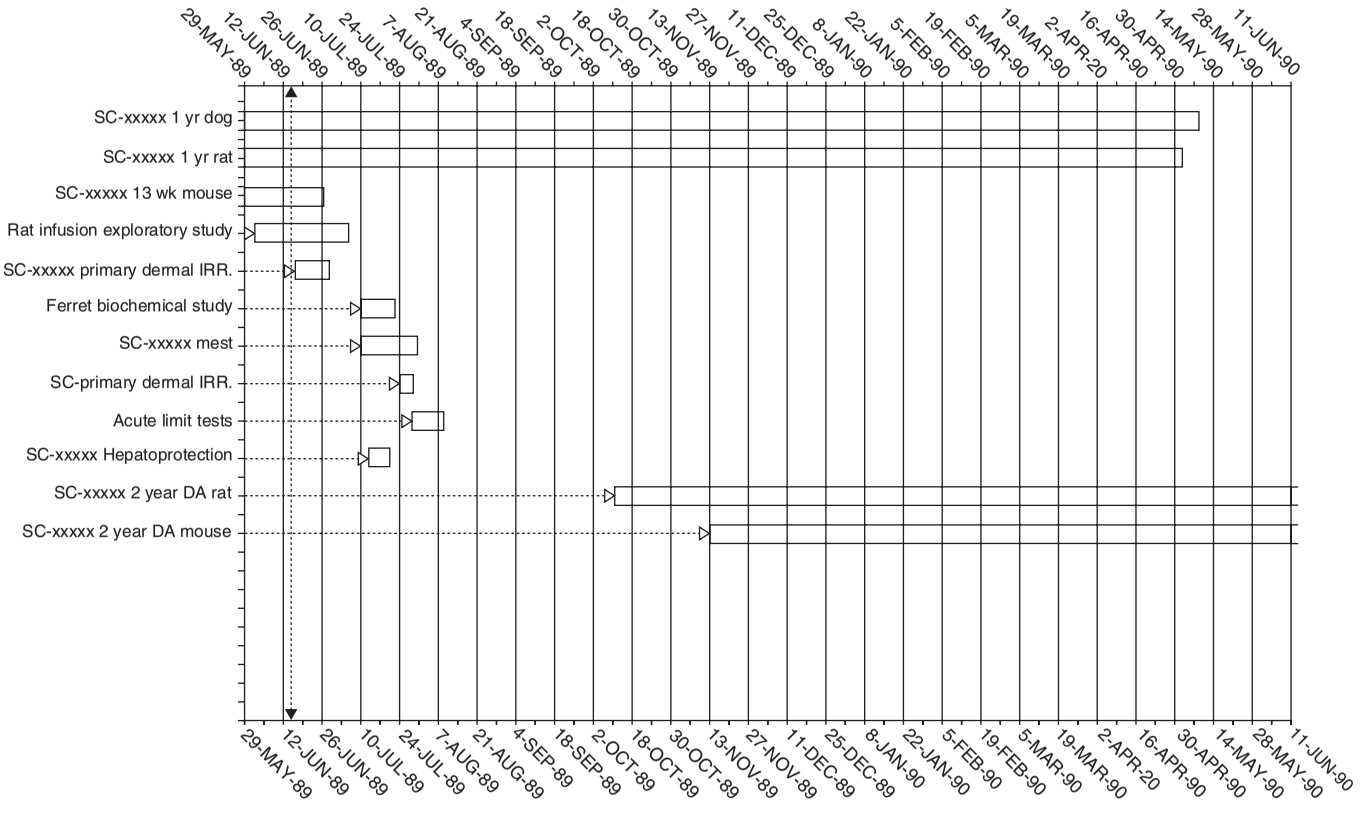

Figure 21.3 *Gantt, or bar, chart showing scheduling of major safety assessment activities (studies) involved in pharmaceutical development project.*

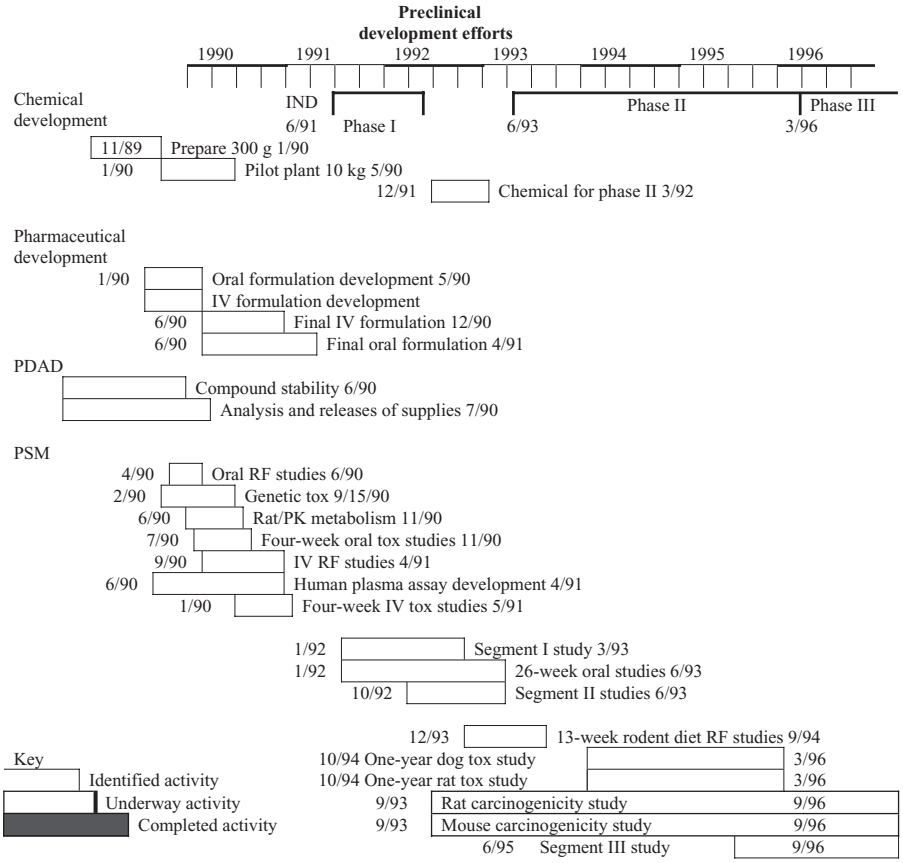

Figure 21.4 *Hybrid project Gantt chart which identifies work of each development function ("line operation") in development of new compound and how it matches phase of development.*

A useful indicator of the efficacy of an assay series is the frequency of discovery of truly active compounds. The frequency is related to the probability of discovery and to the degree of risk (hazard to health) associated with an active compound passing a screen undetected. These two factors in turn depend on the distribution of activities in the series of compounds being tested and the chances of rejecting or accepting compounds with given activities at each stage.

Statistical modeling of the assay system may lead to the improvement of the design of the system by reducing the interval between discoveries of active compounds. The objectives behind a screen and considerations of (1) costs for producing compounds and testing and (2) the degree of uncertainly about test performance will determine the desired performance characteristics of specific cases. In the most common case of early toxicity screens performed to remove possible problem compounds, preliminary results suggest that it may be beneficial to increase the number of compounds tested, decrease the number of animals per group, and increase the range and number of doses. The result

will be less information on more structure, but there will be an overall increase in the frequency of discovery of active compounds (assuming that truly active compounds are entering the system at a steady rate).

The methods described here are well suited to analyzing screening data when the interest is truly in detecting the absence of an effect with little chance of false negatives. There are many forms of graphical analysis methods available, including some newer forms that are particularly well suited to multivariate data (the type that are common in more complicated screening test designs). It is intended that these aspects of analysis will be focused on in a later publication.

The design of each assay and the choice of the activity criterion should therefore be adjusted, bearing in mind the relative costs of retaining false positives and rejecting false negatives. Decreasing the group sizes in the early assays reduces the chance of obtaining significance at any particular level (such as 5%), so that the activity criterion must be relaxed, in a statistical sense, to allow more compounds through. At some stage, however, it becomes too expensive to continue screening many false positives, and the criteria must be tightened accordingly. Where the criteria are set depends on the acceptable noise levels in a screening system.

21.4.1 Characteristics of Screens

An excellent introduction to the characteristics of screens is Redman's (1981) interesting approach, which identifies four characteristics of an assay. Redman assumes that a compound is either active or inactive and the proportion of activities in a compound can be estimated from past experience. After testing, a compound will be classified as positive or negative (i.e., possessing or lacking activity). It is then possible to design the assay so as to optimize the following characteristics:

1. Sensitivity: the ratio of true positives to total activities.
2. Specificity: the ratio of true negatives to total inactives.
3. Positive accuracy: the ratio of true to observed positives.
4. Negative accuracy: the ratio of true to observed negatives.
5. Capacity: the number of compounds that can be evaluated.
6. Reproducibility: the probability that a screen will produce the same result at another time (and, perhaps, in some other lab).

An advantage of testing many compounds is that it gives the opportunity to average activity evidence over structural classes or to study quantitative structure–activity relationships (QSARs). Quantitative structure–activity relationships can be used to predict the activity of new compounds and thus reduce the chance of in vivo testing on negative compounds. The use of QSARs can increase the proportion of truly active compounds passing through the system.

To simplify this presentation, data sets drawn only from neuromuscular screening activity were used. However, the evaluation and approaches should be valid for all similar screening data sets, regardless of source. The methods are not sensitive to the biases introduced by the degree of interdependence found in many screening batteries that use multiple measures (such as the neurobehavioral screen):

1. Screens almost always focus on detecting a single endpoint of effect (such as mutagenicity, lethality, neurotoxicity, or development toxicity) and have a particular set of operating characteristics in common.
2. A large number of compounds are evaluated, so ease and speed of performance (which may also be considered efficiency) are very desirable characteristics.
3. The screen must be very sensitive in its detection of potential effective agents. An absolute minimum of active agents should escape detection; that is, there should be very few false negatives (in other words, the type II error rate or beta level should be low). Stated yet another way, the signal gain should be way up.
4. It is desirable that the number of false positives be small (i.e., there should be a low type I error rate or alpha level).
5. Items 2–4, which are all to some degree contradictory, require the involved researchers to agree on a set of compromises, starting with the acceptance of a relatively high alpha level (0.10 or more), that is, an increased noise level.
6. In an effort to better serve item 2, safety assessment screens are frequently performed in batteries so that multiple endpoints are measured in the same operation. Additionally, such measurements may be repeated over a period of time in each model as a means of supporting item 3.
7. This screen should use small amounts of compound to make item 1 possible and should allow evaluation of materials that have limited availability (such as novel compounds) early on in development.
8. Any screening system should be validated initially using a set of blind (positive and negative) controls. These blind controls should also be evaluated in the screening system on a regular basis to ensure continuing proper operation of the screen. As such, the analysis techniques used here can then be used to ensure the quality or modify performance of a screening system.
9. The more that is known about the activity of interest, the more specific the form of screen that can be employed. As specificity increases, so should sensitivity.
10. Sample (group) sizes are generally small.
11. The data tend to be imprecisely gathered (often because researchers are unsure of what they are looking for) and therefore possess extreme

within-group variability. Control and historical data are not used to adjust for variability or modify test performance.

12. Proper dose selection is essential for effective and efficient screen design and conduct. If insufficient data are available, a suitably broad range of doses must be evaluated (however, this technique is undesirable on multiple grounds, as has already been pointed out).

The design, use, and analysis of screens is covered in detail in Chapter 4 of this volume.

21.5 STRATEGY AND PHASING

Regulatory requirements and our understanding of the pharmacology, marketing, and clinical objectives for a potential product provide a framework of requirements for the safety assessment of potential new pharmaceuticals. How one meets these requirements is not fixed, however. Rather, exactly what is done and when are reflections of the philosophy and managerial climate of the organization that is doing the discovery and development. It should be kept in mind that establishing and maintaining an excellent information base on the biological basis for a compound's expected therapeutic activity and safety are essential but often left undone. This subject is addressed in Chapter 2 of this volume.

There are multiple phases involved in the safety assessment portion of the discovery, development, and marketing process. The actual conduct of the studies in each phase forms the basis of the bulk of the chapters in this book. However, unless the pieces are coordinated well and utilized effectively (and completed at the right times), success of the safety assessment program is unlikely or very expensive.

First, support needs to be given to basic research (also called discovery, biology, or pharmacology in different organizations) so that it can efficiently produce a stream of potential new product compounds with as few overt toxicity concerns as possible. This means that there must be early and regular interaction between the individuals involved and safety assessment must provide screening services to rank the specific safety concerns of the compounds. These screens may be in vitro (both for genetic and nongenetic endpoints) or in vivo (designed on purpose for a single endpoint, such as effects on reproductive performance, promotion activity, etc.). There must also be ongoing work to elucidate the mechanisms and structure–activity relationships behind those toxicities that are identified (Gad, 1989b).

Second is the traditional core of safety assessment that is viewed as development. Development includes providing the studies to support compounds getting into the clinic (an IND application being filed and accepted), evaluating a compound to the point at which it is considered safe, able to be absorbed, and effective (clinical phase II), and, finally, registration (filing an NDA and

having it approved). Various organizations break this process up differently. Judgments are generally made on the likelihood of compounds failing ("dying") at different stages in the clinical development process, and the phasing of preclinical support is selected and/or adjusted accordingly. If an organization has a history of many compounds failing early in the clinic (such as in the initial phase I tolerance trials, where there may be only 3–10 days of human dosing), then initial "pivotal" preclinical studies are likely to be only 4-week-long studies. If compounds tend to fail only in longer efficacy trials, then it is more efficient to run longer initial preclinical trials. Figure 21.5 shows several variations on these approaches. Additionally, the degree of risk involved in study design (particularly in dose selection) is also an organizational characteristic. Pivotal studies can fail on two counts associated with dose selection. Either they cannot identify a "safe" (no-effect) dose or they can neglect to find a dose that demonstrates a toxic effect (and therefore allows identification of potential target organs). Therefore, picking the doses for such studies is an art that has been risky because, traditionally, only three different dose groups have been used, and before clinical trials are conducted, there is

Plan 1: Clinical decision point is short term tolerance or human pharmacokinetics

Range finder		**Pivotal study**		**Phase I**		**Phase II**	
"Pyramid" or "rolling acute" (see Chapter 4)	→	Two or four weeks in two species by the intended route	→	Tolerance and pharmacokinetics (PK) with up to 3–14 days human dosing	Delay*** →		→

Plan 2: Clinical decision point is an indication of efficacy in humans

Range finder		**Pivotal study**		**Phase I/II**		**Phase II/III**	
"Pyramid" or "rolling acute" and/or two- or four-week study	→	Thirteen weeks in two species	→	Tolerance, PK and efficacy with human dosing up to one month in length	Delay*** →		→

Plan 3: Plan for success or resources are not a constraint

Range finder	**Pivotal study**	**Phase I/II**	**Phase III**	
"Pyramid" or "rolling acute: and/or two week study	Four weeks in two species	**Preclinical**	**Preclinical**	
		Thirteen weeks in two species	One year in two species	Carcinogenicity in two rodents (if required)

Figure 21.5 *Three different approaches to matching preclinical safety efforts to support clinical development of new drug. Which is the best one for any specific case depends on considerations of resource availability and organizational tolerance of "risk." In plan 1, little effort will be "wasted" on projects that fail during early (phase I) clinical trials, but if phase I trials are successful, there will be major delays. In plan 3, clinical development will never be held up waiting for more safety work, but a lot of effort will go into projects that never get past phase I. Plan 2 is a compromise. Delays are to allow additional preclinical (animal safety) studies to support longer clinical trials in accordance with FDA or other applicable guidelines.*

at best a guess as to what clinical dose will need to be cleared. The use of four (or five) dose groups only marginally increases study cost and, in those cases where the uncertainty around dose selection is great, provides a low-cost alternative to repeating the study.

Pivotal studies can also be called *shotgun tests* because it is unknown in advance what the endpoints are being aimed at. Rather, the purpose of the study is to identify and quantitate all potential systemic effects resulting from a single exposure to a compound. Once known, specific target organ effects can then be studied in detail if so desired. Accordingly, the generalized design of these studies is to expose groups of animals to controlled amounts or concentrations of the material of interest and then to observe for and measure as many parameters as practical over a period past or during the exposure. Further classification of tests within this category would be the route by which test animals are exposed/dosed or by the length of dosing. "Acute," for example, implies a single exposure interval (of 24 h or less) or dose of test material. Using the second scheme (length of dosing), the objectives of the successive sets of pivotal studies could be defined as follows:

Acute or dose range-finding (DRF) studies:

1. Set maximum doses for next studies.
2. Identify very or unusually toxic agents.
3. Estimate upper limit of tolerability.
4. Identify organ system affected.

Two-week studies:

1. Set doses for next studies.
2. Identify organ toxicity.
3. Identify very or unusually toxic agents.
4. Estimate lethality potential.
5. Evaluate potential for accumulation of effects.
6. Get estimate of kinetic properties (blood sampling/urine sampling).

Four-week studies:

1. Set doses for next studies.
2. Identify organ toxicity.
3. Identify very or unusually toxic agents.
4. Estimate lethality potential.
5. Evaluate potential for accumulation of effects.
6. Get estimate of kinetic properties (blood sampling/urine sampling).
7. Elucidate nature of specific types of target organ toxicities induced by repeated exposure.

Thirteen-week studies (now commonly included as an interim necropsy and report in chronic studies):

1. Set doses for next studies.
2. Identify organ toxicity.
3. Identify very or unusually toxic agents.
4. Evaluate potential for accumulation of effects.
5. Evaluate pharmacokinetic properties.
6. Elucidate nature of specific types of target organ toxicities induced by repeated exposure.
7. Evaluate reversibility of toxic effects.

Chronic studies:

1. Elucidate nature of specific types of target organ toxicities induced by prolonged repeated exposure or
2. identify potential carcinogens.

The problems of scheduling and sequencing toxicology studies and entire testing programs have been minimally addressed in print. Though there are several books and many articles available that address the question of scheduling multiple tasks in a service organization (French, 1982) and an extremely large literature on project management (as briefly overviewed earlier in this chapter), literature specific to a research testing organization does not exist.

For all the literature on project management, however, a review will quickly establish that it does not address the rather numerous details that affect study/program scheduling and management. There is, in fact, to my knowledge, only a single article (Levy et al., 1977) in the literature that addresses scheduling, and it describes a computerized scheduling system for single studies.

There are commercial computer packages available for handling the network construction, interactions, and calculations involved in what, as will be shown below, is a complicated process. These packages are available for use on both mainframe and microcomputer systems.

Scheduling for the single-study case is relatively simple. One should begin with the length of the actual study and then factor in the time needed before the study is started to secure the following resources:

- Animals must be on hand and properly acclimated (usually for at least two weeks prior to the start of the study).
- Vivarium space, caging, and animal care support must be available.
- Technical support for any special measurements such as necropsy, hematology, urinalysis, and clinical chemistry must be available on the dates specified in the protocol.
- Necessary and sufficient test material must be on hand.
- A formal written protocol suitable to fill regulatory requirements must be on hand and signed.

The actual study (from first dosing or exposure of animals to the last observation and termination of the animals) is called the in-life phase, and many people assume the length of the in-life phase defines the length of a study. Rather, a study is not truly completed until any samples (blood, urine, and tissue) are analyzed, slides are prepared and microscopically evaluated, data are statistically analyzed, and a report is written, proofed, and signed off. Roll all of this together, and if you are conducting a single study under contract in an outside laboratory, an estimate of the least time involved in its completion should be equal to (other than in the case of an acute or single-endpoint study) no more than:

$$L + 6 \text{ weeks} + \tfrac{1}{2} L$$

where L is the length of the study. If the study is a single-endpoint study and does not involve pathology, then the least time can be shortened to $L + 6$ weeks. In general, the best that can be done is $L + 10$ weeks.

When one is scheduling an entire testing program on contract, it should be noted that, if multiple tiers of tests are to be performed (such as acute, 2-week, 13-week, and lifetime studies), then these must be conducted sequentially, as the answer from each study in the series defines the design and sets the doses for the subsequent study.

If, instead of contracting out, one is concerned with managing a testing laboratory, then the situation is considerably more complex. The factors and activities involved are outlined below. Within these steps are rate-limiting factors that are invariably due to some critical point or pathway. Identification of such critical factors is one of the first steps for a manager to take to establish effective control over either a facility or a program.

Before any study is actually initiated, a number of prestudy activities must occur (and, therefore, these activities are currently underway—to one extent or another—for the studies not yet underway but already authorized or planned for this year for any laboratory):

- Test material procurement and characterization
- Development of formulation and dosage forms for study
- If inhalation study, development of generation and analysis methodology, chamber trials, and verification of proper chamber distribution
- Development and implementation of necessary safety steps to protect involved laboratory personnel
- Arrangement for waste disposal
- Scheduling to assure availability of animal rooms, manpower, equipment, and support services (pathology and clinical)
- Preparation of protocols
- Animal procurement, health surveillance, and quarantine
- Preparation of data forms and books

- Conduct of prestudy measurements on study animals to set baseline rates of body weight gain and clinical chemistry values

After completion of the in-life phase (i.e., the period during which live animals are used) of any study, significant additional effort is still required to complete the research. This effort includes the following:

- Preparation of data forms and books, preparation of tissue slides, and microscopic evaluation of these slides
- Preparation of data tables
- Statistical analysis of data
- Preparation of reports

There are a number of devices available to a manager to help improve the performance of a laboratory involved in these activities. One such device (cross training) is generally applicable enough to be particularly attractive.

Identification of rate-limiting steps in a toxicology laboratory over a period of time usually reveals that at least some of these are variable (almost with the season). At times, there is too much work of one kind (say, inhalation studies) and too little of another (say, dietary studies). The available staff for inhalation studies cannot handle this peak load, and since the skills of these two groups are somewhat different, the dietary staff (which is now not fully occupied) cannot simply relocate down the hall and help out. However, if, early on, one identifies low- and medium-skill aspects of the work involved in inhalation studies, one could cross train the dietary staff at a convenient time so that it could be redeployed to meet peak loads.

It should be kept in mind that there are a number of common mistakes (in both the design and conduct of studies and in how information from studies is used) that have led to unfortunate results, ranging from losses in time and money and the discarding of perfectly good potential products to serious threats to people's health. Such outcomes are indeed the great disasters in product safety assessment—especially since many of them are avoidable if attention is paid to a few basic principles.

It is quite possible to design a study for failure. Common shortfalls include:

1. Using the wrong animal model.
2. Using the wrong route or dosing regimen.
3. Using the wrong vehicle or formulation of test material.
4. Using the wrong dose level. In studies where several dose levels are studied, the worst outcome is to have an effect at the lowest dose level tested (i.e., the safe dosage in animals remains unknown). The next worst outcome is to have no effect at the highest dose tested (generally meaning that the signs of toxicity remain unknown, invalidating the study in the eyes of many regulatory agencies).

5. Making leaps of faith. An example is to set dosage levels based on others' data and to then dose all test animals. At the end of the day, all animals in all dose levels are dead. The study is over; the problem remains.
6. Using the wrong concentration of test materials in a study. Many effects (including both dermal and gastrointestinal irritation, for example) are very concentration dependent.
7. Failing to include a recovery (or rebound) group. If one finds an effect in a 90-day study (say, gastric hyperplasia), how does one interpret it? How does one respond to the regulatory question, "Will it progress to cancer?" If an additional group of animals were included in dosing and, then were maintained for a month after dosing had been completed, recovery (reversibility) could be both evaluated and (if present) demonstrated.

Additionally, there are specialized studies designed to address endpoints of concern for almost all drugs (carcinogenicity, reproductive or developmental toxicity) or concerns specific to a compound or family of compounds (local irritation, neurotoxicity, or immunotoxicity, for example). When these are done (timing) also requires careful consideration. It must always be kept in mind that the intention is to ensure the safety of people in whom the drug is to be evaluated (clinical trials) or used therapeutically. An understanding of special concerns for both populations should be considered essential.

Safety evaluation does not cease being an essential element in the success of the pharmaceutical industry once a product is on the market. It is also essential to support marketed products and ensure that their use is not only effective but also safe and unclouded by unfounded perceptions of safety problems. This requires not only that clinical trials be monitored during development (Spector et al., 1988) but also that experience in the marketplace be monitored.

The design and conduct of safety assessment studies and programs also require an understanding of some basic concepts:

1. The studies are performed to establish or deny the safety of a compound, rather than to characterize the toxicity of a compound.
2. Because pharmaceuticals are intended to affect the functioning of biological systems and safety assessment characterizes the effects of higher than therapeutic doses of compounds, it is essential that one be able to differentiate between hyperpharmacology and true (undesirable) adverse effects.
3. Focus of the development process for a new pharmaceutical is an essential aspect of success but is also difficult to maintain. Clinical research units generally desire to pursue as many or as broad claims as possible for a new agent and frequently also apply pressure for the development of multiple forms for administration by different routes. These forces

must be resisted because they vastly increase the work involved in safety assessment, and they may also produce results (in one route) that cloud evaluation [and impede institutional review board (IRB) and regulatory approval] of the route of main interest.

21.6 CRITICAL CONSIDERATIONS

In general, at the beginning of a project the management of a pharmaceutical development enterprise wants to know three things: what are the risks (and how big are they), how long will it take, and how much (money and test compound) will it take?

The risk question is beyond the scope of this volume. The time question was addressed earlier in this chapter. How much money is needed is also beyond the scope of this volume. But calculating projected compound needs for studies is a fine challenge in the design and conduct of a safety evaluation program. The basic calculation is simple. The amount needed for a study is equal to

$$NWILD$$

where N = number of animals per group
W = mean weight per animal during course of study (kg)
I = total number of doses to be delivered (such as, in a 28-day study, 28 consecutive doses)
L = loss or efficiency factor (to allow for losses in formulation and dose delivery a 10% factor is commonly employed, meaning a value of 1.1 is utilized)
D = total dose factor, i.e., the sum of all dose levels; e.g., if groups are to receive 1000, 300, 100, and 30 mg kg^{-1}, then total dose factor is 1000 + 300 + 100 + 30, or 1430 mg kg^{-1}.

As an example, take a 28-day study in rats where there are 10 males and 10 females per group and the dose levels employed are 1000, 3000, 100, and 30 mg kg^{-1}. Over the course of the 28 days the average weight of the rats is likely to be 300 g (or 0.3 kg). This means our values are

$$N = 20 \quad W = 0.3\,\text{kg} \quad I = 28 \quad L = 1.1 \quad D = 1430\,\text{mg kg}^{-1}$$

and therefore our total compound needs will be

$$(20)(0.3)(28)(1.1)(1430\,\text{mg}) = 2642.64\,\text{mg or } 2.642\,\text{g}$$

This is the simplest case but shows the principles.

A governing principle of pharmaceutical safety assessment is the determination of safety factors—the ratio between the therapeutic dose (that which

achieves the desired therapeutic effect) and the highest dose which evokes no toxicity. This grows yet more complex (but has less uncertainty) if one bases these ratios on plasma levels rather than administered doses. Traditionally based on beliefs of differences of species sensitivity, it has been held that based on toxicity findings a minimum 5-fold safety factor should be observed in nonrodents and 10-fold in rodents.

The desire to achieve at least such minimal therapeutic indices and to also identify levels associated with toxicity (and the associated toxic effects) forms the basis of dose selection for systemic (and most other in vivo) toxicity studies.

21.7 SPECIAL CASES IN SAFETY ASSESSMENT

It may seem that the course of preclinical safety assessment (and of other aspects of development) of a pharmaceutical is a relatively linear and well-marked route within some limits. This is generally the case, but not always. There are a number of special cases where the pattern and phasing of development (and of what is required for safety assessment) do not fit the usual pattern. Four of these cases are:

1. When the drug is intended to treat a life-threatening disease, such as acquired immunodeficiency syndrome (AIDS)
2. When the drug is a combination of two previously existing drug entities
3. When the drug consists of two or more isomers
4. When the drug is a peptide produced by a biotechnology process

Drugs intended to treat a life-threatening disease for which there is no effective treatment are generally evaluated against less rigorous standards of safety when making decisions about advancing them into and through clinical testing. This acceptance of increased risk (moderated by the fact that the individuals involved will die if not treated at all) is balanced against the potential benefit. These changes in standards usually mean that the phasing of testing is shifted: Animal safety studies may be done in parallel or (in the case of chronic and carcinogenicity studies) after clinical trials and commercialization. But the same work must still eventually be performed.

Combination drugs, at least in terms of safety studies up to carcinogenicity studies, are considered by regulatory agencies as new drug entities and must be so evaluated. Accordingly the required safety tests must be performed on a mixture with the same ratio of components as is to be a product. Any significant change in ratios of active components means one is again evaluating a new drug entity.

Now that it is possible to produce drugs that have multiple isomers in the form of single isomers (as opposed to racemic mixtures), for good historical

reasons, regulatory agencies are requiring at least some data to support any decision to develop the mixture as opposed to a single isomer. One must, at a minimum, establish that the isomers are of generally equivalent therapeutic activity and, if there is therapeutic equivalence, that any undesirable biological activity is not present to a greater degree in one isomer or another.

21.8 SUMMARY

It is the belief of this author that the entire safety assessment process that supports pharmaceutical research and development is a multistage process of which no single element is overwhelmingly complex. These elements must be coordinated and their timing and employment carefully considered on a repeated basis. Focus on the objective of the process, including a clear definition of the questions being addressed by each study, is essential, as is the full integration of the technical talents of each of the many disciplines involved. A firm understanding of the planned clinical development of the drug is essential. To stay competitive requires that new technologies be identified and incorporated effectively into safety assessment programs as they become available. It is hoped that this volume will provide the essential knowledge of the key elements to allow these goals to be realized.

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22

Application of In Vitro Techniques in Drug Safety Assessment

22.1 INTRODUCTION

The key assumptions underlying modern toxicology are (1) that other organisms can serve as accurate predictive models of toxicity in humans, (2) that selection of an appropriate model to use is the key to accurate prediction of potential hazard in humans, and (3) that understanding the strengths and weaknesses of any particular model is essential to translating potential hazards identified in these models to assess relevant hazards in humans and in the subsequent management of actual risks. The nature of models and their selection in toxicological research became the subject of critical scientific review starting in the 1980s. Usually in toxicology, when we refer to "models," we really have meant test organisms or systems, although, in fact, the manner in which parameters are measured (and which parameters are measured to characterize an endpoint of interest) is also a critical part of the model (or, indeed, may actually constitute the model).

Although there have been accepted principles for test organism selection, these have not generally been the actual final basis for such selection. It is a fundamental hypothesis of both historical and modern toxicology that adverse effects caused by chemical entities in higher animals are generally the same as those induced by those entities in humans. There are many who point to individual exceptions to this and conclude that the general principle is false.

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Yet, as our understanding of molecular biology advances and we learn more about the similarities of structure and function of higher organisms at the molecular level, it becomes clear that the mechanisms of chemical toxicity are largely identical in all higher life forms, including humans. The target sites are molecular, and differences in responses are all about similarities in receptor populations, receptor population distribution in organ systems, and the manner and means of getting toxicophores to these sites or preventing them from reaching these sites. In this sense, it is now the age of translational toxicology. This increased understanding has caused some of the same people who question the general principle of predictive value to in turn suggest that our state of knowledge is such that mathematical models or simple cell culture systems could be used just as well as intact higher animals to predict toxicities in humans. This last suggestion has unfortunately missed the point that the final expressions of toxicity in humans or animals are frequently the summations of extensive and complex interactions on cellular and biochemical levels. Zbinden (1987) and Gad (1996a) published extensively in this area, including a very advanced defense of the value of animal models. Lijinsky (1988) has reviewed the specific issues about the predictive value and importance of animals in carcinogenicity testing and research. Although it was once widely believed, and may still be believed by many animal rights activists, that in vitro mutagenicity tests would entirely replace animal bioassays for carcinogenicity, this is clearly not the case on either scientific or regulatory grounds (despite the limitations of the current bioassay models). Although there are differences in the responses of various species (including humans) to carcinogens, the overall predictive value of such results, when tempered by judgment, is clear. At the same time, a well-reasoned use of in vitro or other alternative test model systems is essential to the continued development of a product safety assessment program that is effective, efficient, and relevant to human safety (Gad, 1990, 1996a, 1998, 2000, 2007).

The subject of intact animal models and their proper selection and use has been addressed elsewhere (Gad, 2007) and will not be further addressed here. However, alternative models which use other than intact higher organisms are seeing increasing use in toxicology for a number of reasons.

The first and most significant factor behind the interest in so-called in vitro systems has clearly been philosophical and political—an unremitting campaign by a wide spectrum of individuals concerned with the welfare and humane treatment of laboratory animals (Singer, 1975), though some are also clearly simply antiscience and antitechnology. In 1959 Russell and Burch first proposed what have come to be called the 3 R's of humane animal use in research—replacement, reduction, and refinement. These have served as the conceptual basis for reconsideration of animal use in research.

Replacement means utilizing methods that do not use intact animals in place of those that do. For examples, veterinary students may use a canine cardiopulmonary resuscitation simulator, Resusci-Dog, instead of living dogs and cell cultures may replace mice and rats that are fed new products to

discover substances poisonous to humans. In addition, using the preceding definition of animal, an invertebrate (e.g., a horseshoe crab) could replace a vertebrate (e.g., a rabbit) in a testing protocol.

Reduction refers to the use of fewer animals. For instance, changing practices allow toxicologists to estimate the lethal dose of a chemical with as few as one-tenth the number of animals used in traditional tests. In biomedical research, long-lived animals, such as primates, may be used in multiple sequential protocols, assuming that they are not deemed inhumane or scientifically conflicting. Designing experimental protocols with appropriate attention to statistical inference can lead to decreases or increases in the number of animals used. Through coordination of efforts among investigators, several tissues may be simultaneously taken from a single animal. Reduction can also refer to the minimization of any unintentionally duplicative experiments, perhaps through improvements in information resources.

Refinement entails the modification of existing procedures so that animals are subjected to less pain and distress. Refinements may include administration of anesthetics to animals undergoing otherwise painful procedures, administration of tranquilizers for distress, humane destruction prior to recovery from surgical anesthesia, and careful scrutiny of behavioral indices of pain or distress followed by cessation of the procedure or the use of appropriate analgesics. Refinements also include the enhanced use of noninvasive imaging technologies that allow earlier detection of tumors, organ deterioration or metabolic changes, and the subsequent early euthanasia of test animals.

Progress toward these first three R's has been previously reviewed (Gad, 1990, 1993; Salem, 1995; Salem and Katz, 1998; Gribaldo, 2007). However, there is fourth R—responsibility—which was not in Russell and Burch's initial proposal. To toxicologists this is the cardinal R. They may be personally committed to minimizing animal use and suffering and to doing the best possible science of which they are capable, but at the end of it all, toxicologists must stand by their responsibility to be conservative in ensuring the safety of the people using or exposed to the drugs and chemicals produced by our society.

Since 1980, issues of animal use and care in toxicological research and testing have become one of the fundamental concerns of both science and the public. Are our results predictive of what may or may not be seen in humans? Are we using too many animals, and are we using them in a manner that gets the answer we need with as little discomfort to the animals as possible? How do we balance the needs of humans against the welfare of animals?

During the same time frame, interest and progress in the development of in vitro test systems for toxicity evaluations have also progressed. Early reviews by Hooisma (1982), Neubert (1982), and Williams et al. (1983) record the proceedings of conferences on the subject, but Rofe's (1971) review was the first found by this author. Although it is hoped that in the long term some of these (or other) in vitro methods will serve as definitive tests in place of those that use intact animals, at present it appears more likely that their use in most cases will be as screens. Frazier (1992), Gad (2000), and Gad and Chengelis

(1999) give recent overviews of the general concepts and status of in vitro alternatives.

Lastly, in viewing the progression of in vitro toxicology in the pharmaceutical industry, it is relevant to note that the advent of toxicology itself as a profession is a recent development in this century. This development can be reflected by the formation of the Society of Toxicology in 1961 and the establishment of drug safety units distinct from pharmacology departments within the industry in the 1950s and 1960s. Toxicology can therefore be regarded as a relatively young profession when compared to pharmacology, whose professional society, the American Society for Pharmacology and Experimental Therapeutics (ASPET), was established in 1908. Industrial laboratories have therefore been in the forefront in the incorporation of in vitro techniques in toxicology.

22.2 INDUSTRIAL APPLICATIONS

The number and types of in vitro toxicological models utilized in the pharmaceutical industry encompass virtually every major target organ of toxicological interest. A partial listing of representative test methods is provided in Table 22.1. The breadth of the systems available is impressive and again signifies a relatively rapid progression of in vitro test development in toxicology.

The fact that many of the in vitro test systems listed in Table 22.1 are utilized in toxicology underscores the commitment of industry to the principles of reduction, refinement, and replacement of whole-animal in vivo tests whenever possible. However, industrial toxicologist must appropriately balance this commitment with the fourth R—responsibility (Figure 22.1)—recently discussed by Gad (1990). The ethical and legal responsibility of the toxicologist is to assess the safety of new products and to protect, to the best of his or her ability, the public from harm. Thus, current test procedures cannot be abandoned unless the new tools can be adopted with the assurance that adverse properties will be reliably detected. A key factor in the application of in vitro techniques in toxicology involves the degree of correlation between events occurring in vitro and those that the toxicologist evaluates in the intact animal. This correlation determines the ultimate scientific value of the techniques and the level of confidence associated with a particular test in terms of its predictability from a safety perspective.

The criteria that determine the degree of correlation or level of confidence in a given test are summarized in Table 22.2. The first of these is predictability, both qualitative and quantitative. Qualitatively, do the rankings or order of toxicities in vitro correlate with the order of toxicities in vivo? From a quantitative aspect, what are the dose–response characteristics from which potency estimates and comparisons can be made? Finally, how do drug concentrations in vitro compare with those achieved in vivo? The second criterion to examine is that of test system identity. To what degree does the in vitro

TABLE 22.1 In Vitro Toxicity Models

Organ	Models(s)	Applications
Liver	Hepatocytes	Hepatotoxicity
	Enzymes	Peroxisomal proliferation
	Isolated perfused liver	Enzyme inhibition/induction
Kidney	Tissue slices	Nephrotoxicity
	Cells	Renal transport
	Tubules	
	Membranes	
Brain	Slices	Receptor interactions
	Homogenates	Neurotoxicity
	Cells	
Heart	Myocytes	Cardiotoxicity
	Isolated atria	Receptor interactions
	Isolated perfused heart	
Muscle	Cells	Muscle irritation
	Smooth muscles	Receptor interactions and smooth muscle effects
	Skeletal muscle: phrenic nerve/diaphragm	Neuromuscular blockade
Blood	Red blood cells	Hemolytic potential
	Mast cells	Histamine release
	Platelets	Aggregation
Reproductive/endocrine	Sperm	Fertility
	Limb bud growth	Teratogenic potential
	Whole-embryo culture	Teratogenic potential
	Pituitary cultures	Prolactin, leuteinizing hormone, follicle-stimulating hormone release
	Testicular cultures	Reproductive toxicity
Immune	Mitogen assay	Immunomodulation
	Mixed-lymphocyte response	Immunomodulation
	Plaque-forming cell assay	Immunomodulation
	Macrophage phagocytosis assay	Immunomodulation
	Bone marrow colony-forming unit assay	Myelosuppression
Other	Yeast	Phototoxicity
	Bacterial/mammalian cells	DNA/chromosome damage
	Ocular cells/organ systems	Ocular irritation
	Dermal cells/organ systems	Dermal irritation

system structurally and functionally mimic the in vivo organ? Third, the area of mechanisms of cellular injury is a key criterion to consider in the utilization of in vitro models for toxicological evaluation. The variety of mechanisms that can play a central role in cell injury underscores the need to recognize specifically how the response of the in vitro test system correlates with the response in the intact organ or organism. Lastly, the topic of compensatory factors needs to be considered. How does the ability of the in vitro system to scavenge toxic

Figure 22.1 *Four R's of in vitro testing.*

TABLE 22.2 Criteria for Establishing In Vitro–In Vivo Correlations

Predictability
Qualitative: Rankings (% maximum response)
Quantitative: Dose–response characteristics, relative drug concentrations in vitro–in vivo
Identity
Structure: Morphological correlates
Function: Tissue specificity (e.g., transport characteristics, metabolic pathways
Mechanisms of injury
Membrane damage (structural, functional)
Synthetic activity (protein, RNA, DNA)
Metabolic poisoning (O_2 utilization/consumption, glycolysis, gluconeogenesis)
Compensatory factors
Biochemical scavengers (glutathione, metalloproteins)
Detoxification pathways \ oxidation/reduction/hydrolysis/conjugation
Toxification pathways/

reaction products compare with in vivo abilities? What detoxification or toxification pathways relevant to the in vivo fate of toxins or chemicals are present in the in vitro test system? These questions must be addressed prior to utilization of in vitro procedures to establish a clear understanding of the assumptions and/or limitations of the data to be generated. While in vitro systems need not fulfill all the correlative criteria outlined to be useful, the successful achievement of these criteria will be required to replace current whole-animal tests.

22.2.1 In Vitro Testing in Pharmaceutical Safety Assessment

The preclinical assessment of the safety of potential new pharmaceuticals and new devices represents a special case of the general practice of toxicology (Gad, 1999; Meyer, 1989), possessing its own peculiarities and special considerations and differing in several ways from the practice of toxicology in other fields—for some significant reasons. Because of the economics involved and

the essential close interactions with other activities (e.g., clinical trials, chemical process optimization, formulation development, regulatory reviews), the development and execution of a crisp and flexible, yet scientifically sound, program is a prerequisite for success. The ultimate aim of preclinical safety and biocompatibility assessment also makes them different. A good safety assessment program seeks to efficiently and effectively move safe potential therapeutic agents or devices into the clinical evaluation, then to registration, and finally to market and to support them through this process. This requires the quick identification of those agents that are not safe so that effort (and limited resources) are not wasted on them.

Pharmaceuticals are intended to have human exposure. Furthermore, pharmaceuticals are intended to have biological effects on the people that receive them. Frequently, the interpretation of results and the formulation of decisions about the continued development and eventual use of a drug are based on an understanding of both the potential adverse effects of the agent and its likely benefits as well as the dose separation between these two. This makes a clear understanding of the dose–response relationship critical, so that the actual risk–benefit ratio can be identified. It is also essential that the pharmacokinetics be understood and that "doses" (plasma tissue levels) at target organ sites be known (Scheuplein et al., 1990). Integral pharmacokinetics are essential to such a safety program, especially now that there is wider recognition of the existence and importance of subpopulations with different metabolic competencies. As we have come to understand that pharmacogenetics underlie many of the subpopulation effects we see in both the safety and efficacy of drugs, we have also come to recognize that in vitro methods also offer some of the best and most efficient means of understanding the basis for these differences and for identifying members of specific subpopulations.

The development and safety evaluation of pharmaceuticals and medical devices have many aspects broadly or tightly specified by regulatory agencies (Gad, 2001a). An extensive set of defined safety evaluations is required before a product is ever approved for market. For pharmaceuticals, regulatory agencies have increasingly come to require not only the establishment of a "clean dose" in two species with adequate safety factors to cover potential differences between species but also an elucidation of the mechanisms underlying those adverse effects that are seen at higher doses and are not well understood. These regulatory requirements are compelling to the pharmaceutical toxicologist (Traina, 1983). There is not, however, a set menu of what must be done. Rather, many decisions (particularly in terms of the timing of testing) are open to professional judgment. Devices have tended to be more set piece in their testing approach but are beginning to likewise require more mechanistic understanding to allow for competitive positioning in the marketplace.

The discovery, development, and registration of a pharmaceutical or biological comprise an immensely expensive operation and represent a rather unique challenge. For every 9000–10,000 compounds specifically synthesized or isolated as potential therapeutics, one (on average) will actually reach the

market. The overall cost for each successful compound is currently estimated to be between $250 and $320 million (though those figures are, of course, burdened with the cost of all the unsuccessful compounds), with each successive stage in the development process being more expensive. This dynamic makes it of great interest to identify as early as possible those agents that are likely not to go the entire distance, allowing a concentration of effort on the compounds that have the highest probability of reaching the market (and of possessing therapeutic utility) to do so.

Compounds "drop out" of the process primarily for three reasons: (1) toxicity or (lack of tolerance), (2) (lack of) efficacy, and (3) (lack of) bioavailability of the therapeutic active moiety in humans. Early identification of "losers" in each of these three categories is thus extremely important (Fishlock, 1990), forming the basis for the use of screening in pharmaceutical discovery and development. How much and which resources to invest in screening and each successive step in support of the development of a potential drug are matters of strategy and phasing that are detailed elsewhere (Gad, 2000). A range of test systems is available to be used in screening and in the definitive testing that follows for selected promising compounds. Table 22.3 presents a summary of the levels of available model systems. Those test systems that involve in vitro methods are now providing new tools for use in both early screening and understanding the mechanisms of observed toxicity in preclinical and clinical studies (Gad, 1988b, 1992). Devices are generally less complicated in design and in their testing procedures and have a much lower rate of failure in the qualification and approval stages that precede going to market. The trend in devices, however, is for regulatory authorities to require more testing, to be more critical of results, and to take longer in the review and approval process.

The entire safety assessment process that supports new product research and development is a multistage effort in which none of the individual steps is overwhelmingly complex but for which the integration of the whole process involves fitting together a large and complex pattern of pieces. This chapter proposes an approach in which integration of in vitro test systems calls for a modification of the approach to the general safety assessment problem. This modification can be addressed by starting with the current general case and progressing to a means of changing the process in an iterative fashion as new tools become available. Particularly with an understanding of mechanisms of toxicity becoming increasingly important in both candidate drug selection and the design and evaluation of the relevance of findings, the integration of in vitro methodologies, particularly into the pharmaceutical safety assessment process, has become essential. Determining what information is needed calls for an understanding of the way in which the device or pharmaceutical is to be made and used as well as an understanding of the potential health and safety risks associated with exposure of humans who will either be using the drug/device or be associated with the processes involved in making it. This is on the basis of a hazard and toxicity profile. Once such a profile is established, the available literature is searched to determine what is already known. Much

TABLE 22.3 Levels of Models for Safety Assessment and Toxicological Research

Level/Model	Advantages	Disadvantages
In vivo (intact higher organism)	Full range of organismic responses similar to target species	Cost Ethical/animal welfare concerns Species-to-species variability
Lower organisms (earthworms, fish)	Range of integrated organismic responses	Frequently lack responses typical of higher organisms Animal welfare concerns
Isolated organisms	Intact yet isolated tissue and vascular system Controlled environment and exposure conditions	Donor organism still required Time consuming and expensive No intact organismic responses Limited duration of viability
Cultured cells	No intact animals directly involved Ability to carefully manipulate system Low cost Ability to study a wide range of variables	Instability of system Limited enzymatic capabilities and viability of system No (or limited) integrated multicell and/or organismic responses
Chemical/biochemical systems	No donor organism problems Low cost Long-term stability of preparation Ability to study wide range of variables Specificity of response	No de facto correlation to in vivo systems Limited to investigation of a single defined mechanism
Genomics and proteomics	Speed and broad scope	Much effort is still required to correlate to intact organism effects
Computer simulations	No animal welfare concerns Speed and low per-evaluation cost	May not have predictive value beyond narrow range of structures Expensive to establish

of the necessary information for the support of safety claims in the registration of a new drug/device is regulatorily mandated. This is not the case at all, however, for those safety studies done (1) to select candidate products or materials for development, (2) to design pivotal safety studies to support registration, or (3) to pursue mechanistic questions about materials and products in development.

Taking into consideration this literature information and the previously defined exposure profile, investigators have traditionally used a tier approach to generate a list of tests or studies to be performed based on regulatory requirements. What goes into a tier system is determined by (1) regulatory requirements imposed by government agencies, (2) the philosophy of the parent organization, (3) economics, and (4) available technology. How such tests are actually performed is determined on one of the two bases. The first

(and most common) is the menu approach, which involves selecting a series of standard design tests as "modules" of data. This assumes that all drugs or devices are alike except for route and duration of administration. The second is an interactive/iterative approach, where strategies are developed and studies are designed based both on needs and on what has been learned to date about the product.

22.3 DEFINING TESTING OBJECTIVE

The initial and most important aspect of a product safety evaluation program is the series of steps that leads to an actual statement of the problem or of the objectives of testing and research programs. This definition of objectives is essential and, as proposed here, consists of five steps: (1) defining product or material use, (2) estimating or quantitating exposure potential, (3) identifying potential hazards, (4) gathering baseline data, and (5) designing and defining the actual research program to answer outstanding questions.

22.3.1 Objectives Behind Data Generation and Utilization

To understand how product safety and toxicity data are used and how the data generation process might be changed to better meet the product safety assessment needs of society, it is essential to understand that different regulatory organizations have different answers to these questions. The ultimate solution is in the form of a multidimensional matrix, with the three major dimension of the matrix being (1) the toxicity/biocompatibility data type (lethality, sensitization, corrosion, irritation, photosensitization, phototoxicity, etc.), (2) exposure characteristics (extent, population size, population characteristics, etc.), and (3) the stage in the research and development process we are dealing with.

What is called for is a careful zero-based consideration of what the optimum product safety assessment strategy for a particular development problem should be. Before formulating such a strategy and deciding what mix of tests should be used, it is first necessary to decide criteria for what would constitute an ideal (or at least acceptable) test system.

The ideal test should have an endpoint measurement that provides data such that dose–response relationships can be obtained where possible or necessary (and such are almost always necessary). Furthermore, any criterion of effect must be sufficiently accurate in the sense that it can be used to reliably resolve the relative toxicity of two compounds that produce distinct (in terms of hazard to humans) yet similar responses. In general, it may not be sufficient to classify compounds into generic toxicity categories, such as "intermediate" toxicity, since a candidate chemical that falls in a given category yet is borderline to the next more severe toxicity category should be treated with more concern than a second candidate that falls at the less toxic extreme of

the same category. Therefore it is useful for a test system to be able to accurately rank compounds with potentially similar uses within any common toxicity category.

The endpoint measurement of the "ideal" test system must be objective, so that a given compound will give similar results when tested using the standard test protocol in different laboratories. If it is not possible to obtain reproductive results in a given laboratory over time or between various laboratories, then the historical database against which new compounds are evaluated will be time and laboratory dependent. Along these lines, it is important for the test protocol to incorporate internal standards to serve as quality controls. Thus, test data could be represented utilizing a reference scale based on the test system response to the internal controls. Such normalization, if properly documented, could reduce intertest variability.

The test results from any given compound should be reproducible both intrinsically (within the same laboratory over time) and extrinsically (between laboratories). If these conditions are not satisfied, then there will be significant limitations on the application of the test system because it could potentially produce conflicting results at different times and places. Such a possibility would significantly reduce confidence in the outcome of any single assay or assay set. From a regulatory point of view, this possibility would be highly undesirable (and perhaps indefensible). Alternatives to current in vivo test systems basically should be designed to evaluate the subject toxic response in a manner as closely predictive of that occurring in humans as possible while also reducing animal use and avoiding inhumane treatments where possible.

From a practical point of view, several additional features of the ideal test should be satisfied. The test should be rapid so that the turnaround time for a given compound is reasonable. Obviously, the speed of the test and the ability to conduct tests on several candidate drugs or materials simultaneously will determine the overall productivity. The test should be inexpensive, so that it is economically competitive with current testing practices (in the pharmaceutical industry, any reduction in critical path time for decisions has great economic value, so speed is generally preferable to lower cost—within limits). Finally, the technology should be easily transferred from one laboratory to another without excessive capital investment specific to test implementation. Although some of these practical considerations may appear to present formidable limitations for a given test system at the present time, the possibility of future developments in testing technology could overcome these obstacles.

This brief discussion of the characteristics of the ideal test system provides a general framework for evaluation of alternative test systems in general. No test system is likely to be ideal, of course. Our current armamentarium of tests, primarily in vivo tests using mammals, has developed and been maintained because (1) the tests have generally performed well in preventing dangerous drugs and materials from reaching the marketplace (Gad, 1996b,c) and (2) we

are comfortable with them. A significant number of rationales exist for the use of current in vivo test systems:

1. They provide evaluation of actions/effects on intact animal and organ/tissue interactions.
2. Either pure chemical entities or complete formulated products (complex mixtures) can be evaluated.
3. Either concentration or diluted products can be tested.
4. They yield data on the recovery and healing processes.
5. They are required statutory tests for agencies such as the U.S. Food and Drug Administration (FDA) (for "pivotal" safety studies) and the European Economic Community (EEC).
6. They are quantitative and qualitative tests with scoring systems generally capable of ranking materials as to relative hazard.
7. They are amenable to modifications to meet the requirements of special situations (such as multiple dosing or exposure schedules).
8. They have extensive available database and cross-reference capability for evaluation of relevance to human situation.
9. They are easy to perform and relatively low in capital costs in many cases.
10. They are generally both conservative and broad in scope, providing for maximum protection by erring on the side of overprediction of hazard to humans.
11. They can be either single endpoint (such as lethality and pyrogenicity) or shotgun (also called multiple endpoint, including such test systems as a 13-week oral toxicity study).

At the same time, progress and critical examination over the last 15 years have led to the formulation of an equally impressive list of reasons for pursuing the development of in vitro test systems:

1. They avoid the complications (and potential confounding or masking findings) of animal and tissue/organ in vivo evaluation.
2. In vivo systems may assess only short-term sites of application or immediate structural alterations produced by agents. Note, however, that tests may be intended to evaluate only local effects.
3. Technician training and monitoring are critical in in vivo testing (particularly if the evaluation called for is subjective by nature).
4. If our objective is either the total exclusion of a particular type of agent or the identification of truly severe acting agents on an absolute basis (i.e., without false positives or false negatives), then in vivo tests in animals do not perfectly predict results in humans.
5. Structural and biochemical differences exist between test animals and humans that make extrapolation from one to the other difficult.

6. In vivo systems are not standardized.
7. In vitro tests provide variable correlation with human results.
8. Large biological variability exists between more complex experimental units (i.e., individual animals).
9. Large, diverse, and fragmented databases (which are not readily comparable) are generated by in vivo studies.

Therefore, it will be necessary to weigh the strengths and weaknesses of each proposed test system in order to reach a conclusion on how "good" any particular test is. The next section presents the basis for specific test evaluations.

22.4 DESIGNING TESTING PROGRAM AND BUILDING LIBRARY

The next step, given that no relevant data can be found from any literature sources or from any internal files (and that it has been determined what data are needed or most likely to allow selection of desirable candidate compounds), is to perform appropriate predictive tests. The bulk of this section addresses the specifics of performing such evaluations using in vitro models. Before considering how to design, develop the components of, and conduct such a testing program, we must first consider how the practice of safety assessment came to its current state of acceptance and utilization of such tests.

To understand how product safety and toxicity data are used and how the data generation process might be changed to better meet the safety assessment needs of both industry and society, it is essential to understand that different commercial and regulatory organizations have different questions to address and operate in different cultures. The ultimate answer as to whether a drug, biological, or device is safe requires consideration of a multidimensional matrix, whose four major dimensions are (1) the toxicity data type (lethality, sensitization, irritation, photosensitization, genetic toxicity, liver toxicity, etc.), (2) exposure characteristics (extent of use and routes of exposure, patient population size, patient population characteristics, etc.), (3) the benefit to be derived from the marketing and use of the drug, device, or biological, and (4) the type of commercial organization (what do they make and who regulates them, i.e., what is the community of interest?).

Medical devices and pharmaceuticals are two closely related communities. Their materials of concern are agents intended as therapeutics or as components of devices to be used in health care, where the production worker or health care provider (doctor, nurse, or pharmacist) may have a significant chance of exposure, but the major concern is for those patients who receive or use the drug or device. Various centers of the FDA are the primary U.S. regulators.

What is needed is a careful consideration of what the optimum product safety assessment (including safety pharmacology assessments for pharmaceu-

ticals and biologicals) strategy would be. The framework for such a strategy calls for considering each of the issues to be resolved or data points to be generated as a separate box or compartment in a flowchart. As in any flowchart, the individual components need to be arranged in a logical order so that work is not duplicated and the data from earlier "cells" (studies) are available and utilized to help better design, execute, and evaluate the results from subsequent cells. These component studies can each be considered a tool for generating required data, and the entire collection as arranged can be thought of as a data generation toolbox. Many (most) of the components that constitute each of the data generation toolboxes (screens, confirmatory tests, higher tier tests, and mechanistic evaluations) are common to all safety assessment programs in some form. But what is actually used for each of these tasks is not common to all of these programs, nor is how the decision points or notes in the chart operate (acceptance criteria and risk–benefit judgment for proceeding with the development of the candidate drug or device). The selection of these details is what constitutes the actual formulation of a strategy. Before formulating such a strategy and deciding what mix of tests should be used, it is first necessary to decide criteria for what would constitute and ideal (or acceptable) test program.

22.4.1 Considerations in Adopting New Test Systems

Conducting toxicological investigations in two or more species of laboratory animals is generally accepted as a benign, prudent, and responsible practice in developing a new chemical entity, especially one that is expected to receive widespread use and to have exposure potential over human lifetimes. Adding a second or third species to the testing regimen offers an extra measure of confidence to toxicological and other professionals who will be responsible for evaluating the associated risks, benefits, and exposure limitations or protective measures (Gad, 2000; Smith, 1992). Although it undoubtedly broadens and deepens a compound's profile of toxicity, the practice of enlarging on the number of test species is, as has been demonstrated in multiple points in the literature (Gad and Chengelis, 1999), an indiscriminate scientific generalization. Moreover, such a tactic is certain to generate the problem of species-specific toxicoses; that is, a toxic response or an inordinately low biological threshold for toxicity is evident in one species or strain, whereas all other species examined are either unresponsive or strikingly less sensitive. The investigator confronting such findings must be prepared to address the all-important question: Are humans likely to react positively or negatively to the test agent under similar circumstances?

Assuming that numerical odds prevail and that humans automatically fit into the predominant category, whether on the side of being safe or at risk, would be scientifically irresponsible. Far from being an irreconcilable nuisance, however, such a confounded situation can be an opportunity to advance more quickly into the heart of the search for predictive information. Species-specific toxicosis can frequently contribute toward better understanding of the general case if the

underlying biological mechanisms either causing or enhancing toxicity are defined, especially if it is discovered to uniquely reside in the sensitive species.

A mention of species-specific toxicosis usually implies that either different metabolic pathways for converting and excreting xenobiotics or anatomical differences are involved. The design of our current safety evaluation tests appear to serve society reasonably well (i.e., significantly more times than not) in identifying hazards that would be unacceptable in a confirmatory manner. However, the process can just as clearly be improved from the standpoints of both improving our protection of society and performing necessary screening and exploratory research in a manner that used fewer animals in a more humane manner.

22.4.2 In Vitro Models

In vitro models, at least as screening tests, have been used in toxicology for some 25 years. The years since 1980 have brought a great upsurge in interest in such models. This increased interest is due to economic and animal welfare pressures and technological improvements (Rowan and Stratmann, 1980; Borenfreund and Puerner, 1984; Tyson and Frazier, 1993; Salem and Baskin, 1993) and has led to the development and (in some cases) successful utilization of numerous new test methods. Tissues can now be engineered for specific model development, such as tubular heart tissue (Franchini et al., 2007).

In addition to potential advantages, in vitro systems also have a number of limitations that can contribute to their not being acceptable modes:

1. The chemical is not absorbed at all or is poorly absorbed in in vivo studies.
2. The chemical is well absorbed but is subject to “first-pass effect” in the liver.
3. The chemical is distributed so that less (or more) reaches the target tissue than would be predicted on the basis of its absorption.
4. The chemical is rapidly metabolized to an active or inactive metabolite that has a different profile of activity and/or different duration of action than the parent drug.
5. The chemical is rapidly eliminated (e.g., through secretory mechanisms).
6. Species of the two test systems used are different.
7. Experimental conditions of the in vitro and in vivo experiments differed and may have led to different effects than expected. These conditions include factors such as temperature or age, sex, and strain of animal.
8. Effects elicited in vitro and in vivo by the particular test substance in question differ in their characteristics.
9. Tests used to measure responses may differ greatly for in vitro and in vivo studies, and the types of data obtained may not be comparable.

10. The in vitro study may not use adequate controls (e.g., pH, vehicle used, volume of test agent given, samples taken from sham-operated animals), resulting in "artifacts" of methods rather than results.
11. In vitro data cannot predict the volume of distribution in central or peripheral compartments.
12. In vitro data cannot predict the rate constants for chemical movement between compartments.
13. In vitro data cannot predict the rate constants of chemical elimination.
14. In vitro data cannot predict whether linear or nonlinear kinetics will occur with a specific dose of a chemical in vivo.
15. Pharmacokinetic parameters (e.g., bioavailability, peak plasma concentration, half-life) cannot be predicted based solely on in vitro studies.
16. In vivo effects of a chemical are due to an alteration in the higher order integration of an intact animal system, which cannot be reflected in a less complex system.

At the same time, as pointed out in this chapter, there are substantial potential advantages in using in vitro systems. Using cell or tissue culture in toxicological testing results in (1) isolation of test cells or organ fragments from homeostatic and hormonal control, (2) accurate dosing, and (3) quantitation of results. It is important to devise a suitable model system that is related to the mode of toxicity of the compound. Tissue and cell cultures have the immediate potential to be used in two very different ways by industry: (1) to examine a particular aspect of the toxicity of a compound in relation to its toxicity in vivo (i.e., mechanistic or explanatory studies) and (2) as a form of rapid screening to compare the toxicity of a group of compounds for a particular form of response. Indeed, the pharmaceutical industry has used in vitro test systems in these two ways for years in the search for new potential drug entities.

The author has already addressed the theory and use of screens in toxicology (Gad, 1988a) and the general concepts associated with their integration into the pharmaceutical and device development process (Gad, 1995a). Mechanistic and explanatory studies are generally called for when a traditional test system gives a result that is unclear or whose relevance to the real-life human exposure is doubted. In vitro systems are particularly attractive for such cases because they can focus on defined single aspects of a problem or pathogenic response, free of the confounding influence of the multiple responses on an intact higher level organism. Note, however, that first one must know the nature (indeed the existence) of the questions to be addressed.

22.4.3 Current Case: Mixed Battery

The current situation reflects the significant advances made in toxicology since 1985. It is rare to see a pharmaceutical or device researched and developed

with the use of other than an extensively commingled in vivo and in vitro test battery. This is reflected in the use of what may be termed a mixed test battery. The principles behind the development of these batteries are as follows:

1. Pharmaceutical and device development (particularly the product safety assessment aspects of it) cannot continue to be performed as it has been traditionally (on ethical, economic, or competitive grounds).
2. While there are no generally accepted in vitro test systems immediately available to completely replace all (or, indeed, any) of the regulatorily mandated in vivo testing requirements, there are test systems that can replace distinct component in vivo tests or, just as important, preclude their having to be performed by providing information quickly that makes difficult "go–no go" decisions viable at an earlier (and cheaper) stage of development.
3. Some steps can be taken to move development and acceptance of additional in vitro systems along, including wider industry utilization of available test methodologies, increased public regulatory acceptance of in vitro data where appropriate, and continued multilab validation/evaluation studies of alternatives. The single most helpful step, however, would be the clear definition of what constitutes an acceptance criterion for new test designs by regulatory authorities.
4. Some modifications to current in vivo testing methods both can and should be adopted. A current example of this would be in medical devices where a substantial portion of the requirements under the governing regulatory body [International Organization for Standardization (ISO)] can be met with in vitro alternatives (cytogenicity, muscle cell implantation, the limulus test for pyrogens, and in vitro mutagenicity assays).

22.4.4 Continuing Incremental Advances: How to Get Them

Great progress has been made in conducting safety assessment tests in intact animals and in developing an array of promising in vitro replacements, supplements, and candidates for the in vivo test. We want to have in place (that is, accepted and used by industry and accepted without question by regulatory agencies) a battery of in vitro systems that would preclude or reduce the need for intact animal testing to necessary cases. We also want duplicate or unnecessary testing of materials to be reduced to a minimum. These goals are dictated as much by economic reasons and the need to do better science as they are by ethical and humane concerns. The efficient and effective safety assessment/toxicology laboratory of the very near future will have as its "front door" an in vitro screening shop that will draw validated specific target organ screens from a library as needed to perform the initial go–no go evaluations on new compounds (or at least provide guidance as to where further evaluation is

required). This same shop would also provide (again, from its established collection) in vitro system models to elucidate mechanistic questions later in the assessment process.

Some would say that this is the current state of the art. Much of the necessary library could be assembled from test systems that have been extensively evaluated and have already undergone extensive validation (Gad, 2000, 2001b). Three critical steps must be taken for the eventual fulfillment of these objectives: (1) acceptance of a scientific approach to the problem of safety assessment, (2) development of an operative validation and acceptance process for new test procedures, and (3) clear enunciation of an acceptance criterion for new test designs by regulatory authorities.

A scientific approach to safety assessment, such as the one presented in this chapter, does have proponents and adherents. Such an approach requires those involved in both the management and conduct of the safety assessment process to continually question (and test) both the efficacy and the validity of their evaluation systems and processes. More to the point, it requires recognition of the fact that "we have always done it this way" is not a reason for continuing to do so. This approach asks first what is the objective behind the testing and then how well our testing is meeting this objective.

Currently, the second step, a collaborative process involving industrial, academic, and regulatory agencies for the validation and "acceptance" for new test systems, is totally absent. The general model of peer recognition leading to acceptance by the scientific community is not working in this case, as should have been expected from a situation where politics, social policy, and litigation have as much influence as science itself.

22.4.5 Conclusion

The first principle in hazard assessment is to have the data correspond as closely as possible to the real-life situation; that is, the nearer the model to humnas, the better the quality of the prediction of any potential hazards. The second principle should now also be clear: To be able to translate toxicity to hazard and to be able to manage such hazards, it is essential to know how the agent is to be used and the marketplace it is to be part of. It is hoped that this section has made these relationships clear.

Finally, alternatives of both in vitro and in vivo types are in the process of development for almost all the different endpoints of concern in safety assessment. Many of these have promise and could be used as screens for many of the uses presented here or as mechanistic tools, but complete replacement is clearly not near at hand, particularly for the more complicated endpoints. How these tests can (and should) be integrated into strategies for product safety assessment is the key scientific and managerial challenge for the next decade. Not only are there strong reasons against continuing where we are, there is also the possibility of tremendous competitive advantage to those who successfully manage to integrate in vitro tools as both efficient screens and effec-

tive means of isolating and understanding the mechanistic underpinning for toxic and pathogenic processes. These advantages are not primarily a matter of each piece of testing (data generation point) being less expensive, but rather that multitudes of information can be on hand much earlier in the research (discovery) and development process (before the bulk of expenses associated with individual compounds has been incurred) to allow the elimination of noncompetitive candidates. At the same time, each practicing toxicologist should feel both a moral and ethical compulsion to reduce the number of animals used in research and testing to the fullest extent possible and to ensure that those that are used are maintained and used in as humane a manner possible.

22.5 LETHALITY

Many endpoints of interest in toxicology present a fundamental limitation to the development and use of an in vitro or nonmammalian system in place of established in vivo methods. While cytotoxicity is a component mechanism in many of these toxic responses, disruption or diminution of the integrated function of multiple cells and systems is just as important.

The evaluation of lethality [symbolized in the public mind by the median lethal dose (LD_{50}) test] would seem to offer a unique opportunity for the development and use of alternatives. Approaches to alternatives for lethality testing include no living materials at all [the structure–activity relationship (SAR) or computer model approaches], those that use no intact higher organisms (but rather cultured cells or bacteria), and those that use lower forms of animal life (e.g., invertebrates and fish). Each of these presents a different approach to the objective of predicting acute lethality in humans or, rarely, economic animals and will be examined in turn.

There are systems that do not directly use any living organisms but, rather, seek to predict the lethality (in particular, the LD_{50}) of a chemical on the basis of what is known about structurally related chemicals. Such SAR systems have improved markedly over the last 10 years (Enslein et al., 1983; Lander et al., 1984; Blagg, 2006) but are still limited. Accurate predictions are usually possible only for those classes of structures where data have previously been generated on several members of the classes. For new structural classes, the value of such predictions is minimal. Accordingly, this approach is valuable when working with analogues in a series but not for novel structures. It is also a strong argument for getting as much data as possible into the published literature.

A more extensive and once promising approach has been the use of various cultured cell systems. Kurack et al. (1986), for example, have developed and suggested a system based on cultured mammalian hepatocytes. The system does metabolize materials in a manner like mammalian target species and has shown promise in a limited battery of chemicals. Such mammalian cell culture and bacterial screening systems have significant weaknesses for assessing the

lethality of many classes of chemicals since they lack any of the integrative functions of a larger organism. Thus, they would miss all agents that act by disrupting functions such as the organophosphate pesticides, most other neurologically mediated lethal agents, and agents that act by modifying hormonal or immune systems.

Clive et al. (1979) have reported on the correlation of the median lethal concentration (LC_{50}) of a variety of chemicals in mouse lymphoma cell cultures with their oral LD_{50} in mice, as shown in Figure 22.2. No linear correlation is present, but highly cytotoxic substances (in this group) are significantly more toxic orally. Given the impression of some LD_{50} values, due to such factors as steepness of slope of the lethality curve, the lack of linear correlation should be no surprise. Most recently, Ekwall et al. (1989) have reported on the MEIC program system, which utilizes a battery of five cellular systems. For a group of 10 chemicals, the system provided good correlation with or predictive power of rat LD_{50}.

Recently Parce et al. (1989) reported on a biosensor technique in which cultured cells are confined to a flow chamber through which a sensor measures the rate of production of acidic metabolites. It is proposed to use this as a functional measure of cytotoxicity and as a screening technique for a number of uses, including in vivo lethality.

Three lower species of intact animals have been proposed for use in screening or testing of the lethal effects of chemicals. First, some researchers have shown a good correlation between the LD_{50} of the same chemicals in rats. This correlation is nonlinear but still suggests that more toxic materials could be at least initially identified and classified in some form of screening system based

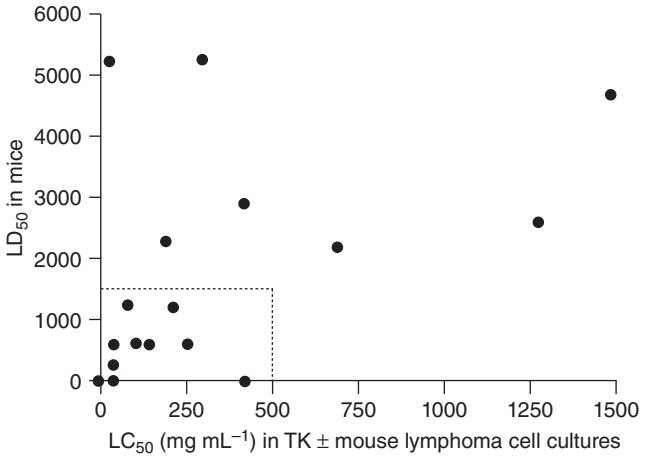

Figure 22.2 *Graph showing comparison of lethality of group of 18 drugs of diverse structure in in vivo (mouse) and in vitro (cultured mouse lymphoma cells) test systems. Correlation of these LD_{50}/LC_{50} values is very poor, though extreme high- and low-scale values seem to be more closely associated in two systems.*

on *Daphnia*. A broader range of chemical structures will need to be evaluated, however, and some additional laboratories will need to confirm the finding. It must also be kept in mind that the metabolic systems and many of the other factors involved in species differences (as presented in Gad and Chengelis, 1999; Gad, 2007) contribute to a nonlinear correlation and may also make the confidence in prediction of human effects in cases somewhat limited.

Earthworms have been one of the more common species used to test chemicals for potential hazardous impact on the environment. The 48-h contrast test has proved to be a fast and resource-effective way of assessing acute toxicity of chemicals in earthworms and is outlined in Table 22.4. The standardized method, approved by the EEC, is discussed by Neuhauser et al. (1986). This test is for environmental impact assessment where cross-laboratory comparisons are important. If, however, one wishes to adopt this technology for the purpose of screening new chemicals or releasing batches of antibiotics, then variants of this method may be acceptable, as internal consistency is more important than interlaboratory comparisons. There are two important considerations. First, because of seasonal variation in the quality of earthworms obtained from suppliers, positive controls or comparator chemicals should be included on every assay run. Second, distilled water must be used, as worms are quite sensitive to contaminants that may occur in chlorinated water. The filter paper should completely cover the sides of the vessel; otherwise the worms will simply crawl up the sides to escape the adverse stimulus the chemical contact may provide.

Using these techniques, Roberts and Dorough (1984, 1985) and Neuhauser et al. (1986) have compared acute toxicity in a variety of organic chemicals in several earthworm species. While there are some obvious differences between worm species, in general the rank order of toxicity is about the same. *Lumbricus rubellus* tends to be the most sensitive species. All earthworms are very sensitive to carbofuran under the conditions of this test. Neuhauser et al.

TABLE 22.4 Earthworm 48-h Contact Test—Acute Lethality

1. Place filter paper of known size (9 cm, or 12 × 6.7 cm) in a Petri dish or standard scintillation vial.
2. Dilute test article in acetone or some other volatile solvent.
3. Slowly and evenly deposit known amounts of test article solution onto filter paper.
4. Dry thoroughly with air or nitrogen gentle stream.
5. Add 1.0 mL of distilled water to filter paper.
6. Add worm (*L. rubellus*). Use 400–500 mg body weight range.
7. Ten replicate vials per concentration.
8. Store/incubate in the absence of light at 15–20 °C for 48 h.
9. Examine for lethality (swollen, lack of movement upon warming to room temperature, lack of response to tactile stimulation).
10. Express dose as micrograms per square centimeter and mortality as usual. Calculate LD_{50} using standard techniques.
11. Always include negative and positive (benchmark) controls.

(1985a,b) have proposed a toxicity-rating scheme based on acute lethality in the earthworms which is similar to the more familiar scheme based on acute toxicity in rodents (Table 22.5). Roberts and Dorough (1985) and Neuhauser et al. (1986) have published extensive compilations of acute lethality in worms and compared these with acute lethality in rats and mice. A selection of these is shown in Table 22.6. Applying the rating scheme of Neuhauser, most chemicals receive about the same toxicity rating based on results in *Eisenia foetida* and mice. This may suggest that replacing the LD_{50} with the LC_{50} for rating toxicity (e.g., for transportation permits) deserves serious consideration.

The main advantages of the 40-h contact test are the savings of time and money. The cost savings fall into three categories. First, earthworms are cheap. One hundred *L. rubellus* will cost about U.S. $2.00. The 100 mice they could replace in screens and quality control (QC) testing, for example, would cost $300–$400 (£150–£200) at an exchange rate of $2.00 = £1.00. Second, earthworms require no vivarium space, and their use could reduce the number of rodents used, resulting in a net decrease in vivarium use. Third, adapting the 48-h contact test would require little capital investment, other than a dedicated under-the-counter refrigerator set at 15–20 °C. Otherwise, the assay can be easily performed in a standard biochemistry laboratory. With regard to time savings, the standard lethality test with rodents requires 7–14 days of postdosing observations. The 48-h contact test is completed in 48 h. Not only is the turnaround time faster, but also the amount of time that technical personnel will have to spend observing animals and recording observations will be

TABLE 22.5 Earthworm Toxicity: Toxicity Rating

Rating	Designation	Rat LD_{50} (mg kg^{-1})	*Eisenia foetida* LC_{50} ($\mu g\,cm^{-2}$)
1	Supertoxic	<5	<1.0
2	Extremely toxic	5–50	1.0–10
3	Very toxic	50–500	10–100
4	Moderately toxic	500–5000	100–1000
5	Relatively nontoxic	>5000	>1000

Source: From Neuhauser et al. (1985a,b).

TABLE 22.6 Earthworm Acute Lethality: Comparative Values

Chemical	*Eisenia foetida* (LC_{50})	Mouse (LD_{50})
2,4-Dinitrophenol	0.6	45
Carbaryl	14	438
Benzene	75	4,700
1,1,1-Trichloroethane	83	11,240
Dimethylphthalate	550	7,200

reduced. An incidental advantage of earthworms is that they are cold-blooded vertebrates and thus exempt from current animal welfare laws.

There are two main disadvantages to the use of earthworms in acute toxicity testing. First, there are a limited number of endpoints. Other than death and a few behavioral abnormalities (Stenersen, 1979; Drewes et al., 1984), the test does not yield much qualitative information. Second, there probably is some institutional bias. Because the test is basically low technology (no tissue culture) and uses a nonmammalian model, it may be easy to dismiss the utility of the test.

Finally, the use of smaller species of fish as a surrogate for humans has gained some supporters. Currently, the zebra fish has been shown to be a significant surrogate model for mammalian species toxicity (Hill et al., 2005; Chiu et al., 2008; Jeong et al., 2008; Tanguay and Reimers, 2008) and as a screen for carcinogens (Stern and Zon, 2003; Berghmans et al., 2005). There is certainly no reason why they could not be used for screening water-soluble compounds for extreme acute toxicity.

Although the intact organisms would seem to be the most utilitarian on the face of it, they still will not totally replace mammalian systems, owing to the need to be concerned about those systems that are significantly different in the higher organisms. Still, it would appear that for those compounds for which human exposure is not intentional, testing in an intact lower organism system (or perhaps even in a cell culture system) should be sufficient to identify agents of significant concern. In these cases, lethality testing in intact mammals is probably unwarranted.

22.5.1 Irritation of Parenterally Administered Pharmaceuticals

Intramuscular (IM) and intravenous (IV) injection of parenteral formulations of pharmaceuticals can produce a range of discomfort resulting in pain, irritation, and/or damage to the muscular or vascular tissue. These are normally evaluated for prospective formulations before use in humans by evaluation in intact animal models—usually the rabbit (Gad and Chengelis, 1999).

Currently, a protocol utilizing a cultured rat skeletal muscle cell line (the L6) as a model has undergone an interlaboratory validation program among more than 10 pharmaceutical company laboratories. This methodology (Young et al., 1986) measures creatine kinase levels in media after exposure of the cells to the formulation of interest and predicts in vivo intramuscular damage based on this endpoint. It is reported to give excellent rank-correlated results across a range of antibiotics (Williams et al., 1987) and in a recent multilaboratory evaluation a broader structural range of compounds (PMA, 1994).

Another proposed in vitro assay for muscle irritancy for injectable formulations is the red blood cell hemolysis assay (Brown et al., 1989). Water-soluble formulations are gently mixed at a 1:2 ratio with freshly collected human blood for 5 s, then mixed with a 5% w/v dextrose solution and centrifuged for 5 min. The percentage of red blood cell survival is then determined by

measuring differential absorbance at 540 nm, and this is compared with values for known irritants and nonirritants. Against a very small group of compounds (four), this is reported to be an accurate predictor of muscle irritation.

There is no current candidate alternative for the venous irritation test, but the in vitro alternative for pyrogenicity testing—the *Limulus* test—is one of the success stories for the alternatives movement. It has totally replaced the classical intact rabbit test in both research and product release testing. The test is based on the jelling or color development of a pyrogenic preparation in the presence of the lysate of the amebocytes of the horseshoe crab (*Limulus polyphemus*). It is simpler, more rapid, and of greater sensitivity than the rabbit test it replaced (Cooper, 1975).

22.5.2 Phototoxicity

ICI (Imperial Chemical Industries) has conducted studies on an in vitro phototoxicity assay which involves using three cultured cell lines: the A431 human epidermal cell line (a derived epidermal carcinoma), normal human epidermal keratinocytes (a primary cell line derived from cosmetic surgery), and the 3T3 Swiss mouse fibroblast cell line. The protocol for this assay involves subculturing the particular cell type into microtiter tissue culture grade plates and incubating them over a period of 24 h. Following incubation, the cultures are exposed to the test compound at a concentration predetermined as nontoxic. After 4 h exposure to the compound, the cell cultures are exposed to either ultraviolet (UV) A (320–400 nm) or UV A/B (280–400 nm) radiation for varying lengths of time. The degree of enhanced toxicity effected by either UV A or UV A/B radiation in the presence of the test compound relative to the control is assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, which undergoes a reduction reaction specific to mitochondrial dehydrogenases in viable cells. Work on validation of this test using 30 compounds of known phototoxic potential has shown a high degree of correlation between in vitro and in vivo results (Jackson and Goldner, 1989).

22.5.3 Hepatocyte

The liver extensively metabolizes and biotransforms xenobiotics, and the subsequent hepatotoxicity due to exposure to parent compound or its metabolites is a common clinical event. Isolated hepatocytes, in either suspension or monolayer culture, have been utilized to study the hepatotoxicity of many compounds (Klaasen and Stacey, 1982; Suolinna, 1982; Holme, 1985; Guillouzo, 1986). The utilization of monolayer cultures has several advantages: (1) monolayer cultures consist of viable cells while suspensions typically contain a mixed population of viable and dead cells; (2) monolayer cells can be maintained for a longer period of time, which is an important factor in studying chronic exposure of a compound; and (3) monolayer cultures allow cell-to-cell contact, which is important for studying the processes necessary for cellular

organization. The following section discusses the methods employed in the establishment of primary hepatocyte cultures and provides insights as to how hepatocyte cultures are a valuable model for short-term studies involving the safety assessment of xenobiotics.

Methods The utility of primary hepatocyte cultures is most aptly illustrated by the number of species from which primary cultures can be derived. Isolation of hepatocytes from livers of small animals such as mice, hamsters, guinea pigs, and rats is generally performed in situ (Williams, 1976a,b; Dougherty et al., 1980; McQueen and Williams, 1981). In large animals such as rabbits, dogs, and monkeys, and even in human tissue, isolation of hepatocytes is performed by perfusion of biopsy liver specimen (Reese and Byard, 1981; Strom et al., 1982; Smolarek et al., 1990a,b).

Preparation of Hepatocytes In general, the harvesting of hepatocytes from the liver or liver specimen is performed by tissue perfusion using a peristaltic pump with two solutions: one consisting of 0.5 mM ethylene glycol bis (β-aminoethyl ether) *N,N′*-tetraacetic acid (EGTA) in Hank's balanced salt solution without Ca^{2+} or Mg^{2+} and the other consisting of 100 untis collagenase per milliliter Williams medium E (WME) buffered with 10 mM *N*-2-hydroxyethylpiperazine-*N*-1-ethanesulfonic acid (HEPES) and adjusted to pH 7.35 with 1 *N* sodium hydroxide (Maslansky and Williams, 1985; McQueen and Williams, 1985). The solutions are filter sterilized and kept at 37 °C. In situ perfusion involves cannulation of the portal or of the biopsy liver specimen; perfusion involves implantation of a large-gauge needle into the sinus cavities of the lobe followed by perfusion at a slow rate with the solution containing EGTA. The rate of perfusion and the volume of perfusate will vary depending on the size of the tissue. In the in situ method, the subhepatic inferior vena cava is severed to prevent excessive swelling of the tissue and to allow the perfusate to leave as waste. In the tissue specimen, perfusate diffuses freely out of the tissue. After uniform blanching of the liver is observed with the first solution, the solution containing collagenase is perfused through the tissue at a rate dependent of the size of the tissue. The liver is covered to keep it moist and a lamp is positioned above the liver to keep it warm. The liver is removed and placed into a sterile petri dish containing cold WME. Hepatocytes are dispersed into the medium by gentle teasing of the tissue. The cell suspension is then washed several times in WME containing 5–10% calf serum and 50 $\mu g\,mL^{-1}$ gentamycin sulfate. The viability is determined by trypan blue exclusion, and suspensions having viabilities of 9–100% are plated.

Culture Conditions As previously noted, the viability of freshly isolated hepatocytes in suspension does not usually exceed 5–6 h (Guguen-Guillouzo et al., 1988). Thus, the extended viability of hepatocytes requires culture techniques initially involving cell attachment to a support structure (Guguen-Guillouzo et al., 1988). There are a number of matrices that have been used

to maintain primary hepatocyte cultures: laminin (Ledbetter et al., 1984), collagen type IV derived from rat tail tendons (Seglen and Fossa, 1978), a preparation of collagen type IV and laminin in a solubilized basement membrane derived from a transplantable mouse tumor (Kleinman et al., 1986), commercially prepared plastic flasks that incorporate amide and amino function groups (Pittner et al., 1985), and cocultivation of hepatocytes with undifferentiated epithelial cells (Ratanasavahn et al., 1988). The success in maintaining primary hepatocyte cultures from different species on these matrices may vary considerably. In general, freshly derived hepatocytes are most easily maintained on collagen type IV–coated tissue culture flasks.

The tissue culture medium is another important factor in supporting survival and proliferation of primary hepatocyte cultures. Typically, hepatocytes are incubated at 37 °C, 5% CO_2–95% air in either WME media containing 50 μg mL^{-1} gentamycin and 1–10% fetal calf serum (McQueen and Williams, 1985) or Eagle's minimum essential medium supplemented with Earle's salts, nonessential amino acids, 50 μg mL^{-1} gentamycin, and 5% fetal calf serum (Seglen, 1976). The initial culture medium is removed 2–4 h postplating and replenished with fresh media with serum or without serum. Due to the significant losses of cytochrome P-450 content during the first day in culture, much effort has been devoted to developing culture media devoid of fetal calf serum and supplemented with a variety of substances. Supplements such as aminolevulinic acid (Hockin and Paine, 1983), ascorbic acid (Guzelian and Bissell, 1974), adenine (Guzelian and Bissell, 1974), nicotinamide (Decad et al., 1977), dimethylsufoxide (Isom et al., 1985), and selenium (Paine and Hockin, 1980) are reportedly important for the maintenance of P-450 activities in primary hepatocyte cultures. In addition, long-term exposure of the hepatocyte cultures to fetal calf serum has been found to be deleterious by inhibiting expression of liver-specific functions, being cytostatic at all plating densities and being cytotoxic at low seeding densities (Enat et al., 1984).

Specific Applications

Cytotoxicity The liver is the primary target organ for a variety of drugs and chemicals (Haseman et al., 1984; Farland et al., 1985). The prevalence of drug- and chemical-induced liver injury is of concern because some xenobiotics can produce liver damage at dose levels that are magnitudes below that which causes cell death (Plaa, 1976). Environmental and commercial chemicals can increase this effect by as much as 100-fold (Plaa and Hewitt, 1982; Plaa, 1976). Studies of early cell injury caused by exposure to a toxicant can be undertaken easily in monolayer cultures of hepatocytes, whereas early cell injury is very difficult to assess in vivo.

The methods that have been used to determine cytotoxicity in primary hepatocyte cultures include morphological changes, assessment of enzyme leakage, and membrane blebbing (Jewell et al., 1982; Story et al., 1983; McQueen et al., 1984). Together, these endpoints depend on the compound

tested and, in general, at least two endpoints should be monitored. Commonly used indicators of hepatocyte toxicity that suggest impaired cell function but not necessarily cell death include leakage of lactate dehydrogenase (Story et al., 1983), changes in cell morphology (McQueen et al., 1984), and inhibition of protein or DNA synthesis (Shaw et al., 1975; Seglen et al., 1980). Indicators of cell death include exclusion of neutral red dye, inclusion of trypan blue (Williams, 1977), and the loss of cell attachment (Simmerman et al., 1974). Gomez-Lechon et al. (1988) studied the use of primary hepatocyte cultures in predicting the hepatotoxicity of xenobiotics. Four well-documented indirect hepatotoxins (α-amanitin, D-galactosamine, thioacetamide, and acetaminophen) were studied in cultured rat hepatocytes and the results compared with the toxicity in vivo (Gomez-Lechon et al., 1988). The data indicated that when functional parameters were utilized, toxicity occurred in vitro at concentrations at which no effects were seen in vivo. These results indicate the sensitivity of the assay and the necessity of selecting the correct indicators of toxicity.

Primary hepatocyte cultures have been used as a tool to predict the hepatotoxicity of many compounds such as nonsteroidal anti-inflammatory drugs (Castell et al., 1988), psychotropic drugs (Boelsterli et al., 1987), immunosuppressant drugs (Boelsterli et al., 1988), and salicylates (Tolman et al., 1978). Rat primary hepatocyte cultures have also been shown to be a good model for examining the mechanisms of metallothionein-induced tolerance to cadmium toxicity (Liu et al., 1990). Guillouzo et al. (1988) developed a coculture system of rat or human hepatocytes with rat liver epithelial cells that maintains the hepatocytes in a differentiated state for extended periods of time, thereby allowing studies involving chronic treatment with the test substance to be conducted. Primary cultures of hepatocytes can therefore provide a useful model for short- and long-term studies involving the safety assessment of xenobiotics.

Comparative Metabolism Since the liver is the major organ involved in the biotransformation of xenobiotics, primary hepatocyte cultures provide an excellent model for in vitro metabolism studies. Primary hepatocyte cultures provide useful tools with which to study the comparative metabolism of xenobiotics by both humans and laboratory animals.

Primary hepatocyte cultures undergo a significant decrease in the activities of phase I and II enzymes that correlates with time in culture. Croci and Williams (1985) compared, during the first 24 h in culture representative phase I and phase II biotransformation pathways in hepatocyte primary cultures isolated from male and female rats to freshly isolated hepatocytes. Hepatocytes lost 50% of cytochrome P-450 activity during the first 24 h in culture but maintained high mixed-function activities; 75% of aryl hydrocarbon hydroxylase and 65% of benzphetamine demethylase activities were preserved in hepatocytes from male rates. Uridine 5′-diphosphate (UDP)–glucuronosyl transferase activities were slightly increased during 24 h of culture to levels higher than present in liver tissue before perfusion. Glutathione transferase

activity after 24 h diminished to 20% of the initial enzyme activity for one form while another form was stable. Donato et al. (1990) showed that in human hepatocytes 3-methylcholanthrene, phenobarbital, and ethanol can increase the activity of cytochrome P-450 monooxygenases, aryl hydrocarbon hydroxylase, and 7-ethoxycoumarin *O*-de-ethylase.

Hepatocyte cultures are not affected by some of the variables that influence in vivo studies such as absorption and distribution; accordingly, the exact concentration of the parent compound is known, and the total number and amount of each metabolite formed over time can be accurately determined. Species-specific differences in the metabolism of xenobiotics may, in fact, play a major role in determining relative susceptibilities to chemical toxicants. As an example, studies were recently conducted utilizing primary hepatocyte cultures derived from the perfusion of livers from rats, dogs, and monkey to determine if the species-specific differences in acetaminophen (APAP)–induced cytotoxicity were correlated with species-specific differences in the amounts of APAP metabolized and the formation of APAP conjugates (Smolarek et al., 1990a). In vivo studies on the hepatotoxicity of APAP have shown a species-specific difference in susceptibility to its hepatotoxic effects (Davis et al., 1974). The dog, hamster, and mouse are very sensitive, but the rabbit, guinea pig, and rat are resistant. Rat, rabbit, dog, and monkey hepatocyte cultures were exposed to 2 mM APAP for 24 h (Smolarek et al., 1990a). Aliquots of hepatocyte culture media containing APAP and its metabolites were analyzed by reverse-phase high-pressure liquid chromatography (HPLC). The sensitivity of the dog hepatocytes to APAP was directly related to low conjugating enzyme activity. In contrast, monkey hepatocyte cultures had a very large capacity to transform APAP to glucuronide conjugates and a very high level of glutathione *S*-transferase activity, which correlated with their resistance to cytotoxicity. These studies indicate that the competing pathways of APAP conjugation in hepatocyte cultures from different species explain the differences observed in APAP-induced cytotoxicity (Smolarek et al., 1990a). Similar comparisons have also been made relating species-specific tetrahydroaminoacridine (THA)–induced hepatotoxicity to differences in the rate and extent of THA biotransformation in cultured hepatocytes from rat, dog, and monkey (Smolarek et al., 1990b). These studies demonstrated that the hepatocytes from these three species differed in their sensitivity to concentration-dependent cytotoxicity, with the monkey cells being most sensitive and the canine cells least sensitive to THA-associated cytotoxicity. Tetrahydroaminoacridine biotransformation also differed among the three species, with the canine hepatocytes most effective and monkey hepatocytes least effective in the conversion of THA to most polar metabolites (Smolarek et al., 1990b). These in vitro studies correlate differences in the cytotoxicity of THA to its biotransformation. They also suggest that the monkey would be a good animal model for in vivo THA toxicity testing (Smolarek et al., 1990b).

Green et al. (1986) compared the metabolism of amphetamine in isolated hepatocyte suspensions from rat, dog, squirrel, monkey, and human livers. The

metabolite profile of hepatocytes from each species corresponded to the profile of urinary metabolites identified previously. These results indicate that species-specific differences in the metabolic activation of compounds seen in vivo can be reproduced in vitro by the utilization of primary hepatocyte cultures.

Primary hepatocyte cultures have been used in vitro to metabolically activate toxins for evaluation with target tissues since the early 1980s. Cocultures of rat embryos with hepatocytes have been used to study the role of metabolism in teratogenesis (Oglesby et al., 1986). Lindahl-Kiessling et al. (1989), in an attempt to bring test conditions closer to in vivo conditions, developed an assay utilizing primary rat hepatocytes and human peripheral lymphocytes to detect metabolism-medicated mutagenesis. Doehmer (2006) has published on the development and use of genetically engineered cells as more metabolically relevant predictors for human drug metabolism.

Genotoxicity The genotoxicity of drugs and chemicals can be detected by measurement of their interactions with cellular DNA. Many test chemicals are not genotoxic by themselves and thus may require metabolic activation to a DNA-reactive metabolite. Primary hepatocyte cultures function (1) metabolic activating systems for chemicals and (2) target cells for the interaction of reactive metabolites with hepatocellular DNA. Indirect assessment of human genotoxicity can be implemented when primary human hepatocytes are used to assess a chemical genotoxicity. In primary hepatocyte cultures, studies to resolve the extent of xenobiotic–DNA interaction products formed as a result of exposure to a test chemical determine the total amount of xenobiotic bound per milligram DNA (Poirier et al., 1980) and quantify the number of single-strand beaks in DNA by alkaline elution (Bradley et al., 1982). An indirect method for determining the genotoxicity of xenobiotics from a wide variety of structural classes in primary hepatocyte cultures is to measure DNA repair by autoradiography (Williams, 1976a). McQueen and Williams (1983) tested a large number of chemicals utilizing this technique and reported that all of the known noncarcinogens tested were negative in eliciting DNA repair, while approximately 90% of the known carcinogens were positive. The suggested reasons for 10% of the carcinogens not eliciting DNA repair are that many carcinogens are of the epigenetic type and therefore will not produce DNA damage and, second, that the carcinogen may inhibit DNA repair. Audioradiographic measurement of DNA repair allows cells undergoing replicative DNA synthesis to be readily distinguished from those in repair, and it allows DNA repair to be studied at concentrations of compound that are not cytotoxic.

Species-specific differences in DNA repair by primary hepatocyte cultures have been demonstrated by McQueen and Williams (1983). The relative resistance of the mouse to aflatoxin β_1–induced carcinogenesis was shown by the 10–100 times higher concentration of aflatoxin β_1 necessary to cause maximum DNA repair in the mouse than in other species tested. Administration of safrole to primary hepatocyte cultures elicited a positive response in DNA repair for the hamster and mouse hepatocyte cultures but was negative in the

rat hepatocyte cultures, suggesting the importance of multispecies genotoxicity testing of a compound (McQueen and Williams, 1987). With respect to the toxicological testing tier, genotoxicity testing contributes in the overall safety assessment of new drug candidates, and it can also be applied at the preproject stages of evaluation.

Summary It is clear from the preceding discussion that the primary hepatocyte in culture represents a versatile in vitro tool in the safety assessment process. The applications of the hepatocyte are summarized in Table 22.7. First, they provide a useful model for studying drug- and chemical-induced hepatotoxicity. Second, they afford the potential to generate and examine the toxicity of phase I and II metabolites to liver cells or other target organs in vitro. Third, they provide a mechanism to examine potential species differences in metabolism of drug candidates prior to in vivo studies. Early in the drug discovery process, primary hepatocyte cultures can therefore aid in the selection of a chemical series based on favorable metabolic and toxicological profiles. The choice of the animal species appropriate for in vivo toxicology studies could also be based on data obtained from primary hepatocyte cultures of several species. Lastly, utilization of primary hepatocyte cultures derived from livers of humans is becoming more commonplace. The toxicologist's ultimate goal of determining hepatotoxic (and genotoxic) risk to humans should be greatly enhanced through the utilization of primary human hepatocyte cultures.

Needs for the Future Primary hepatocyte is a well-established technology for studying the pharmacology and toxicology of different classes of xenobiotics. However, since there are currently no well-defined experimental conditions for establishing primary hepatocyte cultures, it is difficult to compare experimental results from different laboratories. The major variable consists of the cell culture media used for maintaining hepatocytes. Although each specialized medium has been shown to be useful for a specific application, a real need exists to establish an optimal medium that can be standardized. A common objective of most of the varieties of cell culture media is to define a system that maintains cytochrome P-450 activity and the hepatocellular differentiated state. Conventional cell culture conditions have only maintained approximately 20–40% of cytochrome P-450 activity in rodent hepatocytes cultured for two days, while human hepatocyte culture seemed to be more stable and maintained approximately 50–60% of their initial P-450 activity after five to six days in culture (Guguen-Guillouzo et al., 1988). Development

TABLE 22.7 In Vitro Testing Utilizing Hepatocyte Cultures

Cytotoxicity mechanisms → Cellular damage and necrosis
Comparative metabolism → Species specificity
Teratogenic mechanisms → Proteratogens
Genotoxic mechanisms → DNA repair

of a well-defined matrix upon which to grow primary hepatocytes also needs further consideration. The greater degree of loss of P-450 activity in primary hepatocytes in suspension relative to monolayer cultures suggests that cell-to-cell contact is an important issue. A procedure that should contribute some degree of uniformity to primary hepatocyte culture as a test system is to verify the status of the cultures prior to utilization in a particular way in a particular assay. This could be accomplished by routinely checking standard cytotoxic parameters such as leakage of cytoplasmic enzymes or cell survival as well as metabolic parameters such as protein synthesis and gluconeogenesis.

In light of the interest and value of studying the responses of human hepatocytes relative to other species, an important need for the future is to develop successful techniques to culture and freeze preparations of human hepatocytes which, upon thawing, maintain their viability and metabolic capabilities. Typically, human liver tissue is obtained in large quantities. Since only a small portion of the total tissue is needed to provide sufficient material for a given experiment, the remaining portion could be frozen for future use if the proper freezing techniques were established. As previously noted, there has been some success in coculturing human hepatocytes with liver epithelial cells for long-term cultures useful in chronic toxicity testing (Guillouzo et al., 1988) and for maintaining the viability of previously frozen human hepatocytes (Li et al., 1990). Recent advances in culturing techniques should provide a well-defined experimental system for culturing primary hepatocytes in the near future. The role of primary human hepatocyte cultures in safety assessment will become increasingly important, and therefore successful efforts in freezing hepatocytes will help to meet the increasing needs in pharmacological and toxicological studies.

22.5.4 Ocular Alternatives

In recent years, much attention and effort have been directed toward the search for non-whole-animal tests to predict ocular irritation of drugs and chemicals. A variety of in vitro assays as well as "nonexperimental" approaches have been proposed (Williams, 1985; Gad, 2000). These model systems run the gamut of responses observed in vivo using biological systems encompassing a range of organization (from cells to whole eye) and measure a multitude of responses. In addition to these experimental tests, one may also think about predilection of ocular irritation by means that require no additional animal work. The use of literature or computer databases, computer modeling, and prediction from physical or chemical parameters or other toxicity data (such as dermal or acute toxicity) are examples of such an approach. Given a plethora of possibilities, where does one begin in trying to develop a program for establishing ocular irritation potential without the use of live animals? What are the general strengths and weaknesses of the existing techniques and what developmental work needs to be done? How does one "validate" a model system and use it in the decision-making process? These issues are addressed in this section.

Methods

"Nonexperimental" Techniques Perhaps the most obvious and practical starting point for irritation potential to the eye is to ask what is already known about a compound or related structures. If enough data exist, perhaps further experimental work will be unnecessary. Such data might include (1) information from the literature or other databases, (2) data generated by computer modeling, (3) physical and chemical characteristics of the test compound, or (4) previously obtained toxicity data such as acute and/or dermal toxicity. The use of each of these approaches has its advantages and disadvantages, which are discussed below.

PUBLISHED DATA One might assume that previously collected data appearing in the literature should be quite useful and reliable in evaluating potential ocular irritancy. In practice, however, two basic issues detract from the utility of the literature databases. The first is illustrated in a paper by Weil and Scala (1971). In their study analysis a number of laboratories tested the same set of compounds for ocular irritation potential in both a standard protocol and in the routine protocol of the particular laboratory. Comparisons of the results showed considerable variability between laboratories, and to some degree within the laboratories, in the ranking of test compounds. These findings are not surprising in light of the subjective nature of the scoring systems, which makes it difficult to achieve consistent results among individuals and laboratories. How, then, does one determine which literature data to accept and which to reject?

The second issue involves the nature of published material itself. Due to journal space limitations and/or other factors, it is rare to find enough detail presented for a reader to make an independent evaluation of the value of the irritation data. Sometimes the test methodology is not defined, making direst comparisons to results obtained in other laboratory situations tenuous at best. Often raw data are lacking, and the results are reduced to either a plus–minus score or a broad categorization (mild, moderate, severe), so that a more specific appraisal cannot be made. These factors preclude the resolution and evaluation of seemingly conflicting data that one often finds in the literature.

A possible means of overcoming these problems is to use information generated within one's own company. In-house data are usually more consistent since the methodology generally does not change substantially over time, and one usually has the further advantage that the original raw data are available. The in-house database is also more likely to contain information on compounds whose chemistry is similar to the unknowns to be tested. However, irrespective of the source of background data on related substances, such information can only provide a starting point for the investigation of an unknown compound. While the data may be potentially useful in raising a warning flag, they are unlikely to be sufficient for a definitive judgment.

COMPUTER MODELING Computer modeling shares essentially the same problems as literature data. A computer simulation can only be as reliable as the data used in its construction. A model system called TOPKAT is available commercially from Health Designs. According to its own literature (*HDI Toxicology Newsletter*, 1987; TOPKAT manual), eye irritation has been quite difficult to model due to the inherent variability of classification of compounds. Health Designs has, in fact, had to design two sets of equations: one to separate nonirritants from all other compounds and the second to separate severe irritants from the rest, leaving a fair number of compounds broadly classified between these extremes. Furthermore, the model is predicted to be unsuitable for approximately 30% of chemical structures, and the number of indeterminates is higher than the developers would like. It appears that computer modeling, while promising, is somewhat limited as a definitive predictive tool. As with data from the literature, one can probably use the computer model to obtain a rough idea of irritation potential, but more definitive categorization will likely require further testing.

PHYSICAL AND CHEMICAL PARAMETERS Knowledge of the physical and chemical parameters of a compound should be a useful point of reference in predicting irritation potential. In fact, many companies have already reduced animal testing by using a rule of thumb with regard to the pH of a material. Compounds with an exceedingly high or low pH (for instance <3 or >12) are presumed to be irritants and would simply be labeled as such without further testing. This practice has some support in the literature (Guillot et al., 1982b), and further work in this area is ongoing in a study sponsored by the Soap and Detergent Association (SDA). A preliminary finding in the SDA study is that the alkalinity of a compound (i.e., the strength of the acid or base) may be more important than the simple measure of pH (Booman et al., 1989).

The correlation (or lack of correlation) of other physiochemical characteristics has not yet been established. For instance, are all surfactants irritants? Can one classify severity by the size of the molecule? Can octanol–water partition coefficients predict irritation potential; does a propensity to partition out of the ocular fluid mean that a compound presents more of an irritation hazard than one which is more water soluble? Theoretically, these data should reflect the ability of a compound to penetrate the eye and cause an irreversible lesion. However, until definitive data are available, physical and chemical parameters will probably have limited utility in an overall assessment of irritation.

DATA FROM OTHER TOXICOLOGICAL TESTING Another question under study has been whether dermal or systemic (acute) toxicity is predictive of ocular irritant potential. Little work has been reported in the literature regarding the correlation between ocular irritation and acute toxicity, so one can only speculate as to this relationship. When one considers the multiplicity of physiological mechanisms involved in acute lethal reactions [e.g., at the cardiovascular and central nervous system (CNS) levels], it is difficult to envision homology with equally

complex responses of tissue damage and inflammation observed at the ocular site. Thus, from a correlative viewpoint, it is unlikely that mechanisms of lethality operating in acute toxicity tests would also occur in ocular injury, thereby making any predictive analysis strictly fortuitous.

However, more extensive information is available comparing dermal and ocular irritation. Gillman et al. (1983), for example, examined dermal and ocular irritation data on selected petrochemicals and consumer products and reported no reliable correlation between ocular and dermal irritation scores. Guillot et al. (1982a,b) examined ocular and dermal irritation of 56 compounds, comparing different protocols. If one combines the data from both papers, one finds that of 11 dermal irritants, all showed ocular irritation, but the extent of irritation was not predictable. Of the 45 compounds that were nonirritating to slightly irritating dermally, only 18 were nonirritating or slightly irritating to the eye. The remaining 27 compounds ranged from mild or moderate to extremely irritating upon ocular exposure. Similar results were reported by Williams (1984, 1985), who examined 60 severe dermal irritants and found 39 to be severe ocular irritants, while 6 were moderate irritants and 15 mild to nonirritant in the eye. Lastly, in a study by Gad et al. (1986), it was revealed that correlations between dermal and ocular data differed dramatically depending on the scale of irritation used for the comparison. When the compounds were classified simply as positive or negative in both dermal and ocular irritation, the correlation was better than when one attempted to predict a specific classification (i.e., negligible, mild, moderate, severe). The only generalization that can be made is that if a compound is a severe dermal irritant, it is likely to be a strong ocular irritant. However, as alluded to previously, severe irritancy can be predicted, in many cases, on the basis of chemical properties alone (e.g., pH). Nonetheless, it appears that dermal irritancy data can be predictive of ocular irritation for severe dermal irritants. However, as cited above, a number of false positives will occur. Caution is therefore advised in the use of this parameter.

To summarize the utility of "nonexperimental" methods, it is obvious that the more available information there is about a compound, the more likely one will be able to substantially reduce the amount of testing involved in prediction of ocular irritation potential. However, at this point in time, none of the individual methods, alone or in combination, are sufficiently predictive to provide a definitive assessment of in vivo ocular irritation. There is definitely a place, however, for consideration of the above factors in a battery of tests as well as for prioritizing compounds to be tested further.

In Vitro Methods In view of the limitations of extrapolation of irritancy by nonexperimental methods, it is clear that in most cases new experimental data will need to be generated. This new information can be obtained using in vitro approaches in conjunction with in vivo data, when necessary. With respect to selecting an in vitro procedure, the first step would be to evaluate what processes one is attempting to model followed by the development of

a methodology to mimic that particular biochemical effect. However, while the broad processes of inflammation, opacity, and the like are known to be involved in ocular injury and irritation, a detailed understanding of the underlying mechanisms of these processes in the eye is lacking. As previously mentioned, in examining criteria for in vitro test systems, it is extremely useful to know enough about the underlying mechanism(s) to identify and measure a specific endpoint that is (preferably) causal to the in vivo effect. For instance, is the process of opacification due to osmotic imbalances, protein coagulation, necrosis of cells within the epithelial organization, or stromal swelling due to ionic interference with cell-to-cell junctional complexes or (more likely) does the mechanism differ with the compound being tested? And what is really known about inflammation at the cellular and molecular levels? Which components of the arachidonic acid cascade are involved? Do these components act synergistically or antagonistically, and what about specificity of cell response? Should corneal epithelial or endothelial cells, conjunctivae, or stroma be examined as the primary tissue affected? Some of the answers are known, but our deficiencies in knowledge and understanding highlight the complexity of modeling ocular responses to toxins.

Given the mechanistic complexity of the ocular response to xenobiotics, attempting to predict irritation with one assay may not be realistic at this time. The type of approach more likely to be immediately fruitful, as suggested by a number of people [Fielder et al., 1987; European Centre for Exotoxicology and Toxicology of Chemicals (ECETOC), 1988], is to use a tiered approach, taking advantage of as wide a diversity of methods as is practical to get a comprehensive picture of irritation potential. Indeed, various regulatory agencies have unofficially stated that they would like to see all components of the Draize score addressed before they would consider in vitro data in lieu of animal data. Thus, a logical approach would be to examine an assay(s) for each component of ocular irritation (i.e., opacity, inflammation, necrosis, or toxicity).

Because the materials tested for ocular irritancy in the pharmaceutical industry are diverse and many are novel chemical entities, it is advantageous to identify an in vitro assay that closely mirrors the target tissue biochemistry and physiology and monitors the specific endpoint of interest. The expectation of such a system would be that the methodology applied would detect compounds exerting toxic effects by a variety of mechanisms. An extensive list of proposed alternatives has been compiled and categorized by Frazier et al. (1987). While this listing can serve as a starting point as to the available technology, many of these methods do not possess any obvious correlation with the target tissue of interest and/or are not well validated. The following sections provide an overview of one or two representative methodologies for each component of the Draize system along with reported advantages and disadvantages.

OPACITY Corneal opacity is the most heavily weighted of the components of the Draize eye score (80 out of 110 possible points) (Conquet et al., 1977).

Thus, an in vitro system that provides an accurate measure of opacification should contribute substantially toward in vitro modeling of the classical Draize test. Two assays attempting to model this process are discussed.

A bovine corneal opacity (BCO) assay described by Muir (1984, 1985) seems to be quite promising as a model for studying opacification. Recently, this technology has been further developed for the prediction of drug- and chemical-induced ocular irritation (Gautheron and Sina, 1990; Gautheron et al., 1992). Basically, the BCO assay uses freshly isolated bovine eyes obtained from an abattoir. Alternatively, whole corneas may be obtained for experimental purposes from other species such as the rabbit (Elgebaly, personal communication, 1990) or pig (Igarashi et al., 1989). Corneas are removed, allowed to equilibrate in medium for a period of time, then mounted in a special chamber in which both sides of the cornea are bathed in fluid. Test compound is added to the epithelial side of the cornea for a defined period of time and opacity is measured at intervals using an opacitometer. An evaluation of commercially available compounds (Table 22.8) as well as a substantial number of pharmaceutical candidates and process intermediates has shown an excellent correlation between the BCO assay and in vivo irritation scores. A few severe ocular irritants give a false-negative reading in this test due to the fact that they cause the entire epithelial layer to slough from the cornea, thereby producing a lower opacity reading than expected. This difficulty can be overcome by simple examination of the cornea postexposure. The only other drawback encountered has been observed with some insoluble compounds. These materials are generally not a problem (although the highest dose tested is limited), since the corneas can be washed prior to obtaining an opacity reading. However, in a few cases compound can adhere too tightly for effective rinsing, thus giving an artificially high opacity reading. Overall, the BCO assay has been found to be an excellent first-line test in a battery of alternatives, and interlaboratory evaluation studies are currently being initiated in Europe.

A commercially available methodology (Eytex) has recently been put forth as an alternative corneal opacification assay. The method, based upon the presumption that opacification is due to coagulation or denaturation of protein, measures the precipitation caused in a protein matrix by the introduction of a test compound (Gordon and Kelly, 1989). The developers have tested a number of materials, a substantial number of which are surfactants or surfactant-based products, and claim a high level of correlation with ocular irritation. However, evaluation of the methodology has been undertaken by various researchers with mixed results. For instance, on the one hand, Lawrence-Beckett and James (1990) and Soto et al. (1989) found the test useful for evaluating industrial chemicals. On the other hand, Bruner and Parker (1990) found that the Eytex assay was not very predictive of household product irritancy compared with five other in vitro assays. One potential reason for this type of discrepancy may be found in the evaluation performed by Thomson et al. (1989a). These researchers tested a series of compounds within a variety of groups of formulations and found good correlations with in vivo data when

TABLE 22.8 Comparison between In Vivo Ocular Irritancy and Opacity Induced in Bovine Cornea

Compound	In Vivo Irritancy	Opacity at 100% concentration[a]
Tween 20	Mild[b]	1.7
Dimethylsulfoxide	Mild	11.7
Ethanol	Mild/moderate[b]	20.0
Ethylene glycol monomethyl ether	Mild/moderate[b]	24.0
Isopropyl alcohol	Mild/moderate[b,c]	30.1
Methanol	Mild/moderate[b]	32.1
Ethylene glycol monoethyl ether	Mild/moderate[d]	33.2
Carbitol	Mild/moderate[b]	36.5
Formamide	Mild/moderate[b]	51.8
Acetone	Mild/moderate[b]	65.5
Tetrahydrofurfuryl alcohol	Moderate[c]	71.2
Ethylene glycol monobutyl ether	Moderate[d]	72.3
Tetramethyl ethylenediamine	Severe[b]	80.7
Solketal	Moderate[c]	87.7
Acetonitrile	Severe[e]	88.5
Pyridine	Severe[b]	92.3
Allyl alcohol	Severe[b,c]	123
Trichloroacetic acid	Severe[b]	219

[a]Opacity scaling described in Gautheron et al., 1992.
[b]Grant, 1986.
[c]Clayton and Clayton, 1981.
[d]Smyth et al., 1951.
[e]Smyth et al., 1949.

the Eytex assay was used for surfactant blends and eye-area-use products. However, the correlation was not acceptable when alcohol-containing formulations were tested. This illustrates a recurring caveat in the field of in vitro toxicology: A technique must be evaluated with materials from a variety of chemical classes and preferably with compounds that are likely to be tested as unknowns before it can be considered a "validated" assay.

CYTOTOXICITY TESTING The majority of proposed alternative tests are assays for cytotoxicity in which a target cell is exposed to test compound and some endpoint of viability is measured. The advantages of such tests are that these methods are usually very easily and rapidly performed and can be readily transferred among different laboratories. Furthermore, some investigators report that the choice of target cell is relatively unimportant to the predictivity of the assay (Borenfreund and Borrero, 1984), suggesting that the choice of the target cell–ending combination is essentially unlimited. However, despite these advantages, cytotoxicity assays cannot, in general, be considered mechanisticaly based and may not be appropriate for every situation. While it is likely that a number of compounds, particularly severe to extreme irritants, damage the eye through overt cellular toxicity, it is equally clear that a number of compounds exert their effects by different mechanisms. The need for judicious

use of the cytotoxicity endpoint, preferably as part of a battery of approaches, is borne out by a number of evaluations reported in the literature (Bracher et al., 1987; Flower, 1987; Kennah et al., 1989).

Consider, for example, the neutral red assay developed by Borenfreund and Puerner (1984, 1987). This test has been evaluated with a number of surfactants and cosmetic ingredients and has reportedly given good correlations with in vivo ocular irritation data (Bracher et al., 1987; Shopsis, 1989; Bruner and Parker, 1990). However, Thomson et al. (1989b) used this assay to examine a variety of products and found high correlations with some types of materials and poor correlations with others. Again, the basis for this variation in assay performance seems to be the class of chemical being evaluated.

Recently, the cytotoxicity of various drug candidates and process intermediates, as well as commercially available compounds, was examined in the presumed target cell—rabbit corneal epithelium—and a nontarget cell, V79 fibroblasts (Sina and Gautheron, 1990; Sina et al., 1992). Two endpoints of toxicity were monitored: leucine incorporation into protein, a general measure of toxicity, and the tetrazolium dye assay (MTT), a measure of mitochondrial dysfunction. An IC_{50} (concentration necessary to reduce endpoint to 50% of control value) was determined for each cell–endpoint combination, and correlations were drawn with the in vivo classifications (i.e., mild, mild/moderate, moderate, severe). The data showed such a broad overlapping of the IC_{50} values that no definitive cutoff could be established between classifications (Figure 22.3). Subsequently, an IC_{50} threshold was established simply to distinguish severe in vivo irritants. Again, such a value was difficult to determine. If one evaluates commercially available and in-house products separately, a threshold value that allows approximately 70–75% predictivity between these two broad categories can be established. However, this value changes dramatically with the types of compounds as well as with the different target cell–endpoint combinations. It is therefore of limited value in testing unknowns.

Thus, cytotoxicity assays are unlikely to provide an adequate predictor of in vivo irritation in every case. There is no doubt that some type of compounds (such as surfactants) will give (and have given) acceptable correlations with in vivo data. However, for the diversity of compounds common in the pharmaceutical industry, cytotoxicity assays alone are inadequate predictors of ocular irritation, though they may have a place in a battery of tests. For instance, if one is interested in a very quick assessment that will be corroborated later, cytotoxicity assays may be indicated. But each laboratory needs to make its own evaluation of the utility and value of these methods.

INFLAMMATION To complete the examination of the major components of the Draize score, one also needs to examine inflammation. Although inflammation is not a large portion of the Draize score (20 of 110 total points), most people appear to put a great deal of weight on this component due to the subjectivity inherent in the scoring. Furthermore, an evaluation of in-house historical data (J. Sina, 1994, unpublished) indicates that, of those compounds that cause

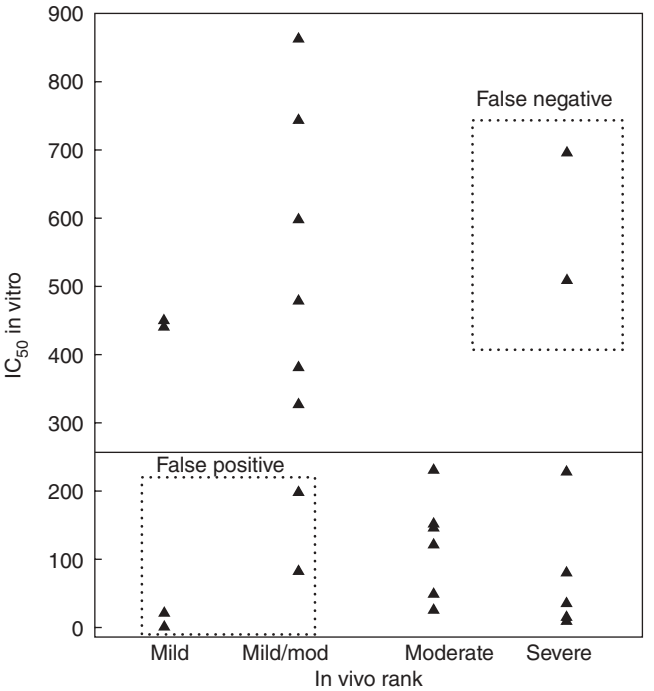

Figure 22.3 *Comparison of cytotoxicity in corneal cells (leucine incorporation) and ocular irritancy in vivo.*

opacity, the majority show moderate to severe inflammation first. This suggests that inflammation may be a more important and predictive component of ocular irritation than the Draize scoring system would suggest.

One approach to examining inflammation is the assay reported by Elgebaly et al. (1987) for the release of chemotactic factors. Earlier work has elegantly shown that neutrophil or macrophage infiltration from either the endothelial or epithelial surface can cause substantial damage to the eye (Elgebaly et al., 1985). Thus, it seems likely that release of inflammatory mediators will correlate with ocular irritation or, more important, with the potential for ocular damage. Briefly, the methodology involves the exposure of the isolated bovine cornea (or cornea from other species) to a compound, with the intact structure serving as a cup to contain the test material. After an indicated time, the exposure medium is harvested and analyzed for the release of chemotactic mediators by means of a chemotaxis assay. Conceptually, this assay is quite attractive and validation is currently underway.

Another approach that has been proposed under the category of inflammation assays [though it has been suggested (Lawrence, 1987) that the assay measures necrosis rather than the true inflammation] is the chick chorioallantoic membrane (CAM) test and its modifications (Leighton et al., 1983;

Luepke, 1985; Kong et al., 1987; Bagley et al., 1989). Basically the assay scores alterations (vascularity, necrosis, etc.) in the chorioallantoic membrane of chicken eggs upon exposure to test compounds. As with most of the other assays discussed here, the CAM assay has proven useful in some applications but inadequate in others. For instance, Bagley et al. (1989) report good predictivity of ocular irritation for surfactant-based materials with a CAM modification, the CAMVA method. By contrast, Price et al. (1986) and Lawrence et al. (1986) found the assay inadequate for their purposes. The reason(s) for these discrepancies may be due to the compound classes tested or to various methodological differences between laboratories. Another point worth mentioning with regard to this assay is that since a viable chicken embryo is part of the model, the CAM may not be truly considered an in vitro model, particularly in Europe (Lawrence, 1987).

Specific Applications How are the above-proposed tests integrated into the safety assessment process? An important issue that arises (and impinges upon the number of assays or types of data required) relative to answering this question is the scheme for decision making. Some approaches have been suggested (Fielder et al., 1987; ECETOC, 1988). For instance, the most straightforward approach would be to gather as much data as possible by nonexperimental methods such as those described above, then proceed to in vitro testing, and finally, if required, perform an animal assay. But, do all steps in the tier need to be performed? Will nonexperimental methods be sufficient? If in vitro assays are needed, how many should be performed? And if different assays are inconsistent, how does one decide which data are more accurate? Unfortunately, the answers to questions such as these are elusive. The manner in which alternative methods will be used in decision making will likely depend both on the individual company, and, within a company, on the reason for testing (i.e., how precise a measure of irritation is required and does the answer need to be definitive, subjective, or conservative?).

For example, if testing is to be done on a new entity about which little is known, one would need to perform more assays than if testing a material that is essentially a reformulation of previously tested components. Additionally, if the reason for developing in vitro assays is to completely replace animals in irritation testing, then an extensive battery of methods examining a range of endpoints and potential mechanisms of action becomes essential since no confirming animal data will be obtained. If, on the other hand, the alternative methods are used as a prescreen to reduce the number of animals used, then fewer tests would be required, since one is only attempting to approximate the irritation potential. A decision would then be made whether to simply label a compound as irritating or to proceed with animal testing for a more accurate assessment.

In addition, the type and number of assays developed may depend on why the testing is being done. If, on the one hand, the testing is to be done for

worker safety within the industrial or pharmaceutical environment, where eye protection is mandatory for all employees, it may only be necessary to establish an irritant/nonirritant label rather than a qualitative ranking (nonirritating, slight, mild, moderate, severe, extreme). In these circumstances, only a single in vitro test or reliance on a database of information on similar compounds may be required to achieve this level of information. However, because the information obtained would provide a simple "yes/no," irritant versus nonirritant label, the methods used must be highly conservative; that is to say, false positives, while undesirable, would be more acceptable than false negatives, which could have drastic consequences upon accidental exposure. If, on the other hand, the testing is to be done on an ocular product where exposure to the eye would be deliberate or on a consumer product where a large, unprotected population may be potentially exposed, a more extensive and definitive irritancy evaluation would be necessary. Thus, it seems likely that no one approach to alternative testing can be consistently applied but that the objectives of each laboratory and situation need to be considered in deciding on an appropriate test battery.

Whatever the specific need or application, the use of a tier testing scheme can significantly reduce and in some cases eliminate the use of animals. An example of a decision-making tree and its application in ocular testing is provided in Figure 22.4. First, all available data about a test material (or related compounds), including chemical characteristics, historical data, other known toxicity, and the like, are collected. Analysis of these data could provide a strong indication of irritation potential, in which case the material would be labeled a presumed positive and handled as such. Alternatively, the data may be equivocal or insufficient, in which case a battery of in vitro assays could be performed.

The in vitro battery would ideally include measures of opacity, cytotoxicity, and inflammation. The actual test method(s) will vary depending upon the experience of the laboratory, types of compounds to be tested, and so on. If the measured endpoint(s) indicates that the test material is approximately equipotent with known irritants, one would presume the unknown to be an irritant and further testing would not generally be required. One should keep in mind, however, that in many cases in vitro assays are more sensitive than whole-animal testing, so a positive response in vitro may not always indicate an in vivo irritant. If the assays give equivocal results or responses similar to those seen with nonirritants or mild irritants, some type of animal testing may be indicated as confirmation.

If animal testing is required, a full-scale Draize test may not be necessary given the background established in the beginning of the tier approach. For instance, the compound could be tested in a single sentinel animal to obtain confirmation of in vitro data. In addition, other modifications could be used, such as the administration of appropriate anesthetics to the test animals or the use of the low-volume Draize modification (Falahee et al., 1982; Freeberg et al., 1984; Griffith, 1987).

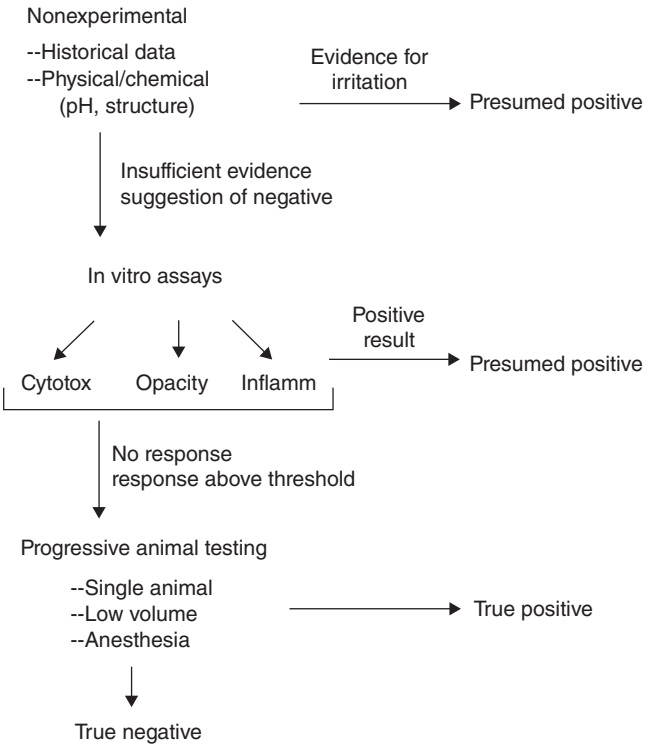

Figure 22.4 *Ocular decision tree.*

Needs for the Future One significant need in the area of ocular alternatives is for the validation of current in vitro assays. The key point is that users of the assay(s) need to have confidence in their ability to interpret and reliably use the data generated. This confidence level can only be achieved by parallel testing in one's own laboratory with compounds similar to those likely to be evaluated as unknowns. The validation process will be a long, somewhat tedious project but will be necessary before in vitro alternatives can be used responsibly.

Another area where advances need to be made is in facing certain common technical issues in the in vitro methods themselves. A number of these potential pitfalls have been outlined by Frazier and Bradlow (1989). One of the chief issues is the testing of water-insoluble materials. Many assay methods are based on material maintained in aqueous media; therefore, dosing the target cells with insoluble or immiscible test agents is difficult or impossible. Agar overlays or partitioning of test compound from a membrane have been used in some cases, but these are not universally applicable. Application of test material in various solvents may or may not allow adequate doses to be achieved. However, the toxicity of the solvents alone and in combination with test compounds must be carefully examined. Until adequate solutions to these

solubility problems are found, some materials will simply not be able to be tested with in vitro methodologies.

Finally, as previously stated, if in vitro models are to be fully effective, the underlying mechanisms of ocular irritation need to be identified. Many of the in vitro assays proposed as alternatives to in vivo testing are based on correlations rather than mechanisms of irritation. The scoring or ranking of substances utilizing the in vitro endpoint may correlate with the severity of the in vivo response, but the reason for the agreement may be unclear and strictly fortuitous for the compounds evaluated. The ideal assay would monitor several biochemical or biological events specifically evoked in the whole animal by irritants. If events that are actually causal to irritation are measured, the probability of false results would decrease. Until we fully understand the mechanisms of the irritant response seen in the whole eye, a battery of tests aimed at evaluating as many biological events as possible is likely to be the best approach.

22.5.5 Nephrotoxicity In Vitro

The kidney is a frequent site of toxic insult due to drug and chemical exposure in experimental animals and humans. Broad classes of drugs and industrial chemicals are implicated in nephrotoxic reactions. The use of various in vitro models in studying renal toxicology is well documented in the literature (Johnson and Maack, 1977; Kacew and Hirsch, 1981; Hassall et al., 1983; Hook and Hewitt, 1986; Smith et al., 1987; Tay et al., 1988; Williams, 1989). A listing of the available in vitro models is provided in Table 22.9. Each model system possesses its own advantages and disadvantages, and all have demonstrated their usefulness and application in renal toxicology.

Methods

Isolated Perfused Kidney As the name implies, the isolated perfused kidney consists of the intact organ maintained in a tissue bath apparatus. An excellent review of the methods involved in preparing this model has been published by Newton and Hook (1981). With respect to the functional integrity of the model, proximal tubule transport processes such as glucose uptake (Bowman and Maack, 1972), protein reabsorption (Maack, 1975), and amino acid uptake

TABLE 22.9 In Vitro Nephrotoxicity Models

Isolated perfused kidney
Isolated tubules
Renal cells
Cell lines
Primary cells
Kidney slices
Isolated organelles

(De Mello and Maack, 1976) have been shown to be maintained. While this model has been used widely to examine renal physiological and drug disposition issues, it has not been extensively utilized as a toxicological tool (Bekersky, 1983). Cojocel et al. (1983) have used the isolated perfused rat kidney to examine the mechanisms of proteinuria induced by various aminoglycosides, while several other investigators have studied brush-border and basolateral aspects of renal accumulation and toxic reactions to aminoglycosides in the isolated perfused kidney (Collier et al., 1979; Williams et al., 1984). In another series of investigations, Newton et al. (1982a,b) examined the metabolism of acetaminophen in relationship to the nephrotoxicity of this agent. Using the isolated perfused kidney, they were able to show that the kidney was capable of metabolizing acetaminophen to the toxic chemical species *para*-aminophenol and of generating electrophilic intermediates capable of depleting glutathione.

Kidney Slices Renal tissue slice technology has been extensively exploited for renal pharmacology and toxicology assessments. This methodology involves the removal of longitudinal sections of kidney tissue of varying thickness and weight with a razor guided either free-hand or through the use of an apparatus known as a Stadie–Riggs (1944) microtome. Kidney slices from virtually all species can be prepared; however, rat and rabbit have been the most frequently utilized models. Renal slices maintain the architecture and cellular heterogeneity of the intact kidney, with tubular segments and surrounding interstitial and vascular elements present. Functionally, renal slices also exhibit organic ion transport, gluconeogenesis, and active maintenance of cellular sodium and potassium balance through the enzymatic activity of Na^+K^+–ATPase (Cross and Taggart, 1950; Berndt et al., 1984).

Typically, the functional and morphological integrity of renal slices has a relatively short life span of approximately 2 h. However, one laboratory has succeeded in prolonging the viability of tissue slices for as long as 24 h (Ruegg et al., 1987a).

It is contended that the renal slice technique measures primarily basolateral uptake of substrates or nephrotoxins based on histological evidence of collapsed tubular lumens. This results in the inaccessibility of brush-border surfaces for reabsorptive transport (Burg and Orloff, 1969; Cohen and Kamm, 1976). This observation limits the ability of this model to accurately reflect reactions to nephrotoxins that occur as the result of brush-border accumulation of an injurious agent. Ultrastructurally, a number of alterations, particularly in the plasma membrane and mitochondrial compartments, have been shown to occur over a 2-h incubation period (Ware et al., 1975; Martel-Pelletier et al., 1977). This deterioration in morphology is very likely a consequence of the insufficient diffusion of oxygen, metabolic substrates, and waste products in the innermost regions of the kidney slice (Cohen and Barac Neito, 1973; Cohen and Kamm, 1976). Such factors also limit the use of slices in studying renal metabolism and transport functions.

Renal cortical slices have been used to study numerous nephrotoxins including gentamicin (Hsu et al., 1977; Kluwe and Hook, 1978), cisplatin (Goldstein et al., 1981; Safirstein et al., 1981; Phelps et al., 1987), cephaloridine (Goldstein et al., 1986, 1987), chloroform (Smith and Hook, 1983; Smith et al., 1983), potassium dichromate (Kacew and Hirsch, 1981; Ruegg et al., 1987b), and the mycotoxin citrinin (Berndt et al., 1984; Baggett and Berndt, 1984), to name a few. While congruence between biochemical effects of nephrotoxins on in vitro and ex vivo renal slice function has been achieved, correlations of morphological features of nephrotoxin-induced damage in kidney slices with in vivo effects are largely lacking.

Isolated Tubules Isolated renal proximal tubule segments are most commonly prepared from either rats or rabbits by collagenase digestion of the kidneys followed by dispersion of the tissue into an appropriate buffer and filtration through sieves. Although this technique yields a tubule suspension that is highly contaminated with cellular debris, glomeruli, and distal tubules, the preparation can be further purified in a number of ways (Balaban et al., 1980; Vinay et al., 1981; Taub, 1984; Hatzinger and Stevens, 1989).

Renal tubular fragments appear to retain most of their in vivo functions and morphology. Balaban et al. (1980) reported that less than 3% of the tubular cells had any structural pathology except for the removal of the basement membrane. An important feature of this preparation was that the tubular lumina were open (Balaban et al., 1980). Functionally, isolated proximal tubular fragments transport *para*-aminohippurate, tetraethylammonium, α-methylglucoside, phosphate, and ^{86}Rb in a fashion similar to that reported in vivo, demonstrating the functional integrity of membrane processes of the preparation (Vinay et al., 1981; Dantzler and Brokl, 1984; Tessitore et al., 1986; Dantzler et al., 1989). With respect to renal cortical glutathione (GSH), isolated tubules are approximately 50–70% GSH depleted when compared to control tissue. However, suspensions of isolated rabbit tubules will synthesize GSH from the constituent amino acids—glutamate, cysteine, and glycine.

Isolated proximal tubules have been utilized to study the mechanisms of nephrotoxicity induced by antibiotics (Sina et al., 1985, 1986), radiocontrast dyes (Humes et al., 1987), metals (Rylander et al., 1985), anoxia (Weinberg, 1985; Weinberg et al., 1987), cellular oxidants (Messana et al., 1988), cysteine conjugates (Rylander et al., 1985; Schnellman et al., 1987; Zhang and Stevens, 1989), and a variety of nephrotoxic bromobenzene metabolites (Schnellman and Mandel, 1986; Schnellman et al., 1987).

Renal Cells A variety of isolated cellular models exist for studying renal function and injury. These models can generally be divided into two categories: models derived from permanent renal cell lines and cellular models derived from freshly isolated renal tissue.

CELL LINES Cell lines, derived from tissue of various species, are commercially available from tissue culture banks. These cell populations are "immortalized" in that they possess the capacity to permanently proliferate in culture. Such cellular models can be studied in short-term suspension (hours) or longer term monolayer culture (days, weeks, months). Since cell lines have been extensively cultured or passaged for multiple generations, the degree or retention (or loss) of kidney-specific morphology and function is an important limitation that is not thoroughly addressed for a number of renal dell lines. One renal cell line that has been relatively well characterized is the pig kidney cell line LLC-PK_1.

The LLC-PK_1 (porcine kidney) cell line (Hull et al., 1976) exhibits a range of morphological and functional properties of proximal tubule epithelium. For example, Na^+-dependent glucose and amino acid transport has been demonstrated in LLC-PK_1 cells (Rabito and Ausiello, 1980; Rabito and Karish, 1982, 1983). LLC-PK_1 cells have also demonstrated the ability to transport organic cations such as tetraethylammonium (TEA) (Inui et al., 1985) but do not appear to possess the capacity for organic anion transport (Rabito, 1986). Morphologically, LLC-PK_1 cells have been shown to be polarized with apical microvilli, apical junctional complexes, desmosomes, and basolateral unfoldings (Cereijido et al., 1978), which is typical of transporting epithelial cells. The expression of brush-border morphology is striking in LLC-PK_1 cells, although the amount of brush border is less than that observed in the proximal tubule in vivo. High activities of proximal tubule brush-border membrane enzymes have also been observed in LLC-PK_1 cells (Gstraunthaler et al., 1985). LLC-PK_1 cells also possess significant levels of the basolateral enzyme marker Na^+K^+–adenosine triphosphatase (ATPase), which is thought to result in the formation of fluid-filled "blisters" or domes in monolayer culture via transepithelial sodium and water transport (Gstraunthaler et al., 1985).

Renal cell lines have been utilized to a limited extent for evaluation of nephrotoxins. A rabbit kidney cell line (LLC-RK_1) has been utilized for evaluating nephrotoxic antibiotics (Viano et al., 1983; Hottendorf et al., 1987; Williams et al., 1988). LLC-PK_1 cells have by far been the most widely employed cell line for studying drug-induced nephrotoxicity, specifically in the evaluation of aminoglycoside antibiotics (Hori et al., 1984; Schwertz et al., 1986; Williams et al., 1986b; Holohan et al., 1988). The morphological alterations induced by aminoglycosides in LLC-PK_1 cells correlated well with in vivo histological findings in the kidney, including the formation of secondary lysosomal inclusions referred to as myeloid bodies.

PRIMARY CELLS Cells from freshly isolated renal tissue can be obtained by explant cultures, cultures of isolated renal tubules discussed in the previous section, and digestion and isolation of individual renal cells. Cultures of isolated renal fragments such as the proximal tubule (Fine and Sakhrani, 1986) and collecting tubule (Grenier, 1986) have been the most extensively characterized models to date. Primary cultures of proximal tubules have been

isolated from rabbit (Chung et al., 1982), dog (Goligorsky et al., 1986), rat (Bellemann, 1980; Hatzinger and Stevens, 1989), and human kidneys (Detrisac et al., 1984; Wilson et al., 1985). Primary cultures prepared from rabbit proximal tubules have been shown to exhibit polarity and transport characteristics consistent with proximal tubule function. As with certain renal cell lines such as the LLC-PK_1 and MDCK, primary cultures also possess tight junctions and basolateral Na^+K^+–ATPase activity that results in the formation of fluid-filled "blisters" or domes in monolayer culture. Transport functions including phlorizin-sensitive Na^+-dependent glucose uptake (Sakhrani et al., 1984) and organic anion and cation transport (Yang et al., 1988) are present in rabbit primary cultures. Primary cultures of rabbit proximal tubules have also been shown to be responsive to parathyroid hormone (Chung et al., 1982), to maintain cellular glutathione levels (Aleo et al., 1987), and to exhibit brush-border enzyme activity (Ford et al., 1987).

Primary renal cell culture has been utilized to study a number of nephrotoxic agents, including mercuric chloride (Inamoto et al., 1976), cadmium (Cherian, 1982), lead (McLachlin et al., 1980), cisplatin (Tay et al., 1988), aminoglycoside antibiotics (Chatterjee et al., 1984; Sens et al., 1988), and cyclosporin (Trifillis et al., 1984). Studies reported by Tay et al. (1988) in rabbit proximal tubule cultures with cisplatin revealed biochemical effects upon DNA synthetic activty that correlated with in vivo histochemical effects of this antitumor agent in animals. With respect to studies involving mercuric chloride and aminoglycoside antibiotics in primary renal cultures, light and electron microscopy revealed similar patterns of cellular pathology in vitro as compared to in vivo exposure in animals (Chatterjee et al., 1984; Aleo et al., 1987).

Suspensions rather than cultures of renal cell lines and primary cells are also available for short-term evaluations. Procedures for the isolation and purification of specific renal cell types include density gradient centrifugation, flow cytometry, free flow electrophoresis, enzymatic digestion, and monoclonal antibodies (Dworzack and Grantham, 1975; Kreisberg et al., 1977; Heidrich and Dew, 1977; Eveloff et al., 1980; Endou et al., 1982; Smith and Garcia-Perez, 1985). While primary renal cell suspensions possess the advantage of being freshly isolated and derived from intact kidney tissue, a major disadvantage is their lack of cellular polarity that is exhibited with cells in culture. Relatively few reports of renal suspension cultures being used in nephrotoxic evaluations are in the literature at this time (Holohan et al., 1988).

One of the unique advantages of renal cell culture rests in the ability to study the directional aspects of drug exposure and cellular injury that operate in vivo. The technology to grow renal epithelial cells on filter inserts for this purpose became available in the late 1990s (Figure 22.5). This potential provides the opportunity to study compounds that interact or accumulate within the renal tubular epithelium in vivo via tubular reabsorption from the luminal surface or extraction from the basolateral or blood surface. Preliminary data involving the directional aspects of antibiotic toxicity to LLC-PK_1 cells have recently been obtained, indicating that LLC-PK_1 cells are significantly more

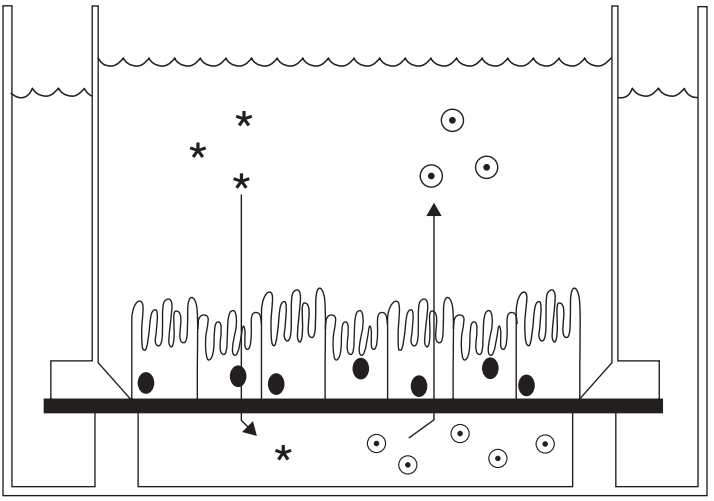

Figure 22.5 *Developing technology for bidirectional exposure/transport in polarized epithelial cells. Shown are kidney cells grown on porous membrane filters. Symbols represent chemical exposure from basolateral (⊙) and brush-border (*) surfaces.*

sensitive to the nephrotoxin cephaloridine when the antibiotic was applied to the basolateral surface of the kidney cell (Williams et al., 1993). These data are consistent with the evidence that cephaloridine is accumulated in renal cells predominantly through basolateral transport. This cellular accumulation is then proposed to precipitate cellular injury and death.

Biochemical Systems In addition to the isolated organ, tubule, and cellular models described above, biochemical systems derived from renal tissue represent valuable resources for in vitro evaluations of nephrotoxins. Particularly, the use of isolated cellular organelles such as mitochondria and plasma membranes has been applied to the study of nephrotoxins (Williams and Hottendorf, 1985, 1986; Tune et al., 1988, 1989). The examination of mechanisms of accumulation of nephrotoxins in the kidney has been accomplished utilizing purified renal brush-border and basolateral membrane vesicles for aminoglycosides (Williams and Hottendorf, 1986; Williams et al., 1987), cephalosporins (Kasher et al., 1983; Williams et al., 1985), and cisplatin (Williams and Hottendorf, 1985). Williams and co-workers (1987) reported that the affinity for renal membrane binding sites correlated well with the nephrotoxic potential of aminoglycosides. Thus, this membrane model could be utilized to assess the relative nephrotoxic potential of new aminoglycosides. Furthermore, isolated renal membranes were successfully employed in the identification of in vivo nephrotoxicity inhibitors for aminoglycosides (Williams et al., 1986a). The correlation between the ability of polyamino acids to inhibit in vitro binding and in vivo nephrotoxicity of aminoglycosides with

aminoglycosides coadministered with polyamino acids demonstrated the utility of in vitro models to contribute to the drug discovery process.

Needs for the Future Challenges for the future involve the continued development of current in vitro models to provide physiological and morphological characteristics that correlate more closely with the kidney in vivo. These challenges will include, for example, defining conditions that facilitate and optimize the retention of renal transport and metabolizing capabilities in cell culture. Perhaps most challenging is the need to establish in vitro systems that reproduce the dynamic features of the nephron in vivo. The kidney, like other organs, exists in a dynamic rather than static environment. The fluid dynamics and vasculature of the renal nephron most certainly play a role in the sensitivity of the kidney in terms of both dose and time responses to nephrotoxins.

22.6 VALIDATION

Perhaps one of the biggest issues in developing alternative test strategies is the validation of the test methods. One reason that this topic has become controversial is that validation has different meanings to different people. In the strictest scientific and linguistic sense, validation is the process of proving adherence to principles, logic, and facts free from error or superficiality. As applied to in vitro test procedures designed to mimic in vivo responses, this definition would require adherence to the principles and facts of in vivo toxicity, according to the criteria previously outlined (Table 22.2). However, test reproducibility, simplicity, and transferability are frequently viewed as the critical ingredients to test validation at the expense of any mechanistic or scientific validity. It can be argued that such components, though important in test standardization and acceptance, may have little bearing on the true scientific validation and rigor of new test procedures. Despite divergent opinions on validation, much has been written about this topic, and a number of validation or evaluation projects are currently underway within various organizations such as the Soap and Detergent Association (Booman et al., 1988), the Fund for the Replacement of Animals in Medical Experiments (FRAME), the German Federal Health Office (Kalweit et al., 1987), and the Cosmetics, Toiletries, and Fragrances Association as well as within the laboratories of individuals working with specific assays.

What constitutes acceptable validation? As alluded to above, there is no general agreement on this point; the answer is likely to be different for each company or individual performing the tests. If one generalization is to be made concerning validation, it would be that one needs to test enough compounds with different characteristics and mechanisms of action to develop confidence in the in vitro data. And this in turn will depend upon the individual user. If one simply requires a toxicity classification for purposes of prescreening, the validation process may be less involved than if a more definitive classification

is required of a test system. Stated another way, the more one expects in terms of predictability from the in vitro data, the more testing that needs to be done to develop confidence in the methods. The cornerstone to this confidence lies in the fulfillment of the scientific criteria previously outlined (Table 22.2).

Additionally, the test materials used in the validation process should be as closely related as possible to the characteristics of the unknowns to be tested. It is clear from the literature, for instance, that many cytotoxicity assays give good correlations with the in vivo ocular irritancy data for surfactants but that the correlations fail when compounds from other chemical classes are tested. Since any particular assay may be used differently by individual safety assessment programs, each user must evaluate potential methods under conditions likely to be encountered in his or her own situation.

22.7 THE FUTURE

The future of in vitro techniques in toxicological assessment takes us back to our earlier discussion of the philosophical and scientific considerations operating in the evolution of alternative methods.

Scientifically, the future will depend on the level of confidence achieved that in vitro systems provide information that is representative of the in vivo processes the toxicologist seeks to model and predict. The degree to which this level of confidence will evolve is directly proportional to the fulfillment of the scientific criteria outlined in Table 22.2 which determine the strength of the in vitro–in vivo correlations obtained. Because gaps remain in our knowledge with respect to these important criteria, the future will also depend on advancements in available knowledge and technology with respect to biochemical or in vitro toxicological events. Specifically, challenges for the future involve the continued development of current in vitro models to provide psychological and morphological characteristics that correlate more closely with target organs in vivo. These challenges will include defining media constituents that will facilitate the retention of normal morphology and metabolizing capabilities in vitro as well as the establishment of systems that reproduce the dynamic features or organs in vivo. With in vitro–in vivo correlations playing a key role in in vitro test development, the growth of technologies that focus on differentiated functions, cellular relationships, human models, and other in vivo properties, such as fluid dynamics, will be critical to the future applications of in vitro techniques in toxicology. An example of a scientific and technological advancement that promises to contribute to the field of in vitro pharmacology and toxicology is the development of a human skin equivalent model. This commercially available material possesses the qualities of differentiation, cellular relationship, and incorporation of human tissue and has already shown usefulness in the study of dermal absorption and irritancy (Bell et al., 1981, 1983, 1989).

Philosophically, where will the future lead us? Assuming that the industrial toxicologist continues to play a central role in the evaluation and prediction of product safety, one can envision continuing involvement in performing extrapolations from animal data to potential human risk. At the same time, one may also expect increasing public pressure to reduce animal testing. With these toxicological and societal goals in mind, the value and application of human tissue in predicting human metabolism and target organ effects are likely to grow. This approach not only reduces animal use but also may significantly enhance our correlative abilities with the ultimate species of concern—humans. Perhaps challenges in risk assessment in the future will involve extrapolations from human tissues to human safety. However, as alluded to above, the ultimate value of using human tissues in vitro relies on the technological and intellectual advances in performing and interpreting in vitro biochemical data. Thus, the challenges in extrapolating in vitro data from human tissues to human responses in vivo are identical to current issues in the use of in vitro models to predict toxicity to animals. As predictability and correlation of in vitro models with specific in vivo events is achieved, toxicologists can look forward to performing meaningful species comparisons in vitro, involving direct preclinical assessments in humans (Figure 22.6).

In summary, an examination of the current state of the art in the pharmaceutical industry illustrates exciting and important roles that in vitro systems can play in toxicological assessments.

While in Europe there are numerous validated [by the European Centre for the Validation of Alternative Methods (ECVAM) standards] in vitro alternatives for personal care products and industrial and agricultural chemicals (though only two—the LAL assay for pyrogenicity and the 3T3 cell assay for phototoxicity—have acceptance for use with pharmaceutical regulators) with regulatory acceptance (as summarized in Table 22.10), this is not the case in

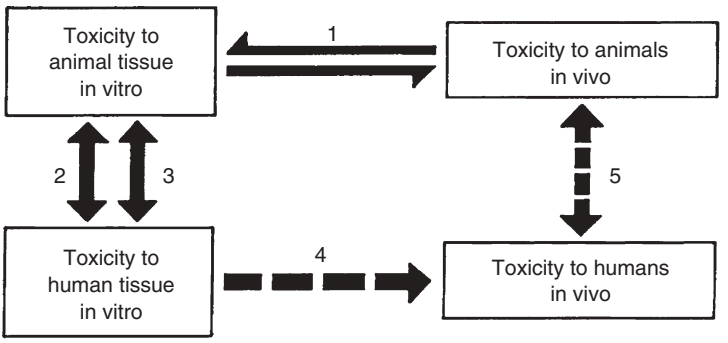

Figure 22.6 *Use of in vitro techniques in performing interspecies comparisons: 1, correlation of in vitro with in vivo observations; 2, development of parallel in vitro systems in humans; 3, comparison of in vivo sensitivity of different species; 4, potential toxicity in vivo; 5, comparison of in vivo sensitivity of different species.*

TABLE 22.10 Status of Nonanimal Methods That Have Regulatory Standing

Test Method	Test System	Endpoint	OECD/TG (Test Group) or other Regulatory Comments
In Vitro Test Methods for Which There Are OECD Health Effects Test Guidelines[a]			
Transcutaneous electrical resistance test (TER)	Monitors changes in electrical resistance as measure of loss of corneum integrity and barrier function, involves skin disks from euthanized rats	Skin corrosion (topical agents)	TG 430
Human skin models (EpiDerm, EPISKIN)	Reconstructed human epidermal equivalent (commercial system) used to assess cell viability, involving MTT reduction test	Skin corrosion (topical agents)	TG 431
3T3 NRU (neutral red uptake) phototoxicity test	BALB/c 3T3 (murine) cell line cytotoxicity based on neutral red uptake to measure cell viability; not direct replacement alternative, as there is no in vivo equivalent test	Phototoxicity	TG 432/FDA guidance
Corrositex membrane barrier test	Artificial barrier system coupled to pH-based chemical detection system	Skin corrosion (topical agents)	Draft TG 435
Bacterial reverse mutation test (Ames)	Revertant bacteria detected by their ability to grow in absence of amino acid	Genotoxicity	TG471/ICH, ISO, FDA guidance
In vitro mammalian chromosome aberration test	Microscopic detection of chromosomal damage to cells in culture	Genotoxicity	TG473/ICH, ISO, FDA guidance

TABLE 22.10 ***Continued***

Test Method	Test System	Endpoint	OECD/TG (Test Group) or other Regulatory Comments
In vitro mammalian cell gene mutation test	Functional bioassays to monitor mutations in enzyme encoding genes	Genotoxicity	TG476
Sister chromatid exchange assay	Cells in culture are examined after two rounds of division by metaphase arrest and chromosomal preparation; chromatid exchange is monitored by microscopy	Genotoxicity	TG479/ICH, ISO, FDA guidance
Gene mutation assay in yeast	*Saccharomyces cerivisiae* exposed to test substance grown under different culture conditions used to monitor mutagenic potential (cf. Ames test)	Genotoxicity	TG 480
Mitotic recombination assay in yeast	Crossover or gene conversion following exposure of yeast to test substance; relies on different growth requirements of mutated and wild-type yeast strains	Genotoxicity	TG 481
Unsheduled DNA synthesis in mammalian cells	Measures DNA repair synthesis after deletions caused by test substance based on incorporation of radioactive nucleotides into newly synthesized DNA	Genotoxicity	TG 482/ICH, ISO, and FDA guidance

TABLE 22.10 ***Continued***

Test Method	Test System	Endpoint	OECD/TG (Test Group) or other Regulatory Comments
In vitro micronucleus test	Cell-based assay; supplement to TG 474 (in vivo micronucleus test); detection of chromosome damage and formation of micronuclei in interphase	Genotoxicity	Draft TG 487/ICH guidance
Sex-linked recessive lethal test	*Drosophila* exposed to test substance; germline transmission of mutations is monitored through two successive generations	Reproductive toxicity	TG 477
	Validated Methods Yet to be Introduced Into Regulatory Use		
EpiOcular	Human keratinocyte–derived model of corneal epithelium barrier function	Eye irritation (topical application)	Retrospective (weight-of-evidence) validation (ECVAM)
In vitro micronucleus test	CHL/IU, CHO, SHE, or V79 cell lines commonly used, with or without metabolic activation, to monitor damage and formation of micronuclei in interphase	Mutagenicity	Retrospective (weight-of-evidence) validation (ECVAM)
Embryonic stem cell test	3T3 cell cytotoxicity and differentiation of embryonic stem murine cell lines used to examine teratogenic potential	Developmental toxicity	Endorsed as screening test (EU)
Postimplantation rat whole-embryo test	Morphological assessment of rat embryos	Developmental toxicity	Endorsed as screening test (EU)

TABLE 22.10 ***Continued***

Test Method	Test System	Endpoint	OECD/TG (Test Group) or other Regulatory Comments
Micromass test	Micromass cultures of rat limb are bud monitored for inhibition of cell proliferation and differentiation	Developmental toxicity	Endorsed as screening test (EU)
	Methods Undergoing Valitation		
EPISKIN	Reconstructed human skin system used with MTT assay to monitor barrier function	Skin irritation	Report stage in EU
EpiDerm	Similar to EPISKIN	Skin irritation	Report stage in EU
	Prevalidated Methods		
SkinEthic eye model	Epithelial corneal cell line used for cytotoxicity testing based on MTT reduction assay	Eye irritation	Appraisal stage in EU
	Methods Undergoing Development, Prevalidation, or Evaluation		
Tissue culture models	Neutral red release and silicon microphysiometry or fluorescein leakage bioassays with human keratinocytes and MDCK cells, respectively; red blood cell (RBC) hemolysis test	Eye irritation	Being reviewed by ICCVAM for possible retrospective (weight-of-evidence) validation
Organotypic models	Bovine corneal opacity and permeability (BCOP) assay, with postmortem corneas; hen's egg test on the chorioallantoic membrane (HET-CAM assay); isolated rabbit and chicken eye tests (IRE and ICE)	Eye irritation	Being reviewed by ICCVAM for possible retrospective (weight-of-evidence) validation

TABLE 22.10 ***Continued***

Test Method	Test System	Endpoint	OECD/TG (Test Group) or other Regulatory Comments
Modified Leydig cell line	Analysis of progesterone production as measure of test substance effects on steroid hormone production	One- or two-generation study	For use as part of test battery
Testis slices	Assessment of steroid production capacity of Leydig cells upon exposure of ex vivo rat tissue to toxicants	One- or two-generation study	For use as part of test battery
Human adrenocortical carcinoma cell line	Assay to allow entire steroid pathway effects to be mapped	One- or two-generation study	For use as part of test battery
Placental microsomal aromatase assay	Monitors ability of substances to affect steroid production; subcellular microsomal assay is used industrially	One- or two-generation study	For use as part of test battery (Stigson et al., 2007)

Note: This is a comprehensive list of methods that have been validated or that are at various stages of development for toxicity testing. More information about these methods and how they can be applied is available from http://www2.defra.gov.uk/research/project_data/more?!=CB01067amp;M=KWSamp;V=reachamp;scope=0and (OECD, 2005).

[a]Including draft guidelines under review for acceptance, can be found at http://www.oecd.org/home/.

the United States. Indeed, only four alternatives have been approved by the Interagency Coordinanating Committee for the Validation of Alternative Methods (ICVAM) to date (Gaul, 2008). Though there is considerable research continuing on the development (and putatively validation) of many more methods (Kuehn, 2008), progress in obtaining regulatory acceptance of such methods to take the place of animal-testing methods is very slow, and those methods that are accepted are limited in scope screens for eye and skin irritation pyrogenicity, phototoxicity, and genotoxicity.

This regulatory acceptance is, indeed, as it has been for 25 years, the final frontier and true obstacle to any further significant reduction in animal use.

22.8 SUMMARY

The tools are currently at hand (or soon will be) to provide the practicing toxicologist with unique opportunities for both identifying potentially toxic compounds in a much more rapid and efficient manner than before and teasing apart the mechanisms underlying such toxicities on an integrated basis (from the level of the molecule to that of the intact organism). The in vitro systems overviewed here, once understood (by investigators and regulators) in how they function and fail (just as in vivo systems have come to be understood), will allow this to happen while reducing the need to have recourse to intact mammalian test systems. However, the intact animal models—and, indeed, humans for pharmaceuticals—will still be an essential element in the safety assessment armamentarium for the foreseeable future.

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23

Evaluation of Human Tolerance and Safety in Clinical Trials: FIM Trials and Beyond

23.1 PHARMACEUTICAL DEVELOPMENT PROCESS AND SAFETY

As introduced in the beginning of this volume, the pharmaceutical development process is a long (6–16 years from drug inception to market approval) and costly ($100 million to $1 billion, depending on how one allocates costs) process, even when successful. It is shaped by medical needs, regulatory requirements, economics, our understanding of sciences and diseases, and limitations of technology. All of these interact to shape a process which serves to iteratively reduce risks (both economic and human safety), with the probability of failure being reduced in a stepwise fashion (Matoren, 1984; PhRMA, 2000). Figure 23.1 briefly summarizes this process.

For our purposes (that is, for a safety assessment perspective), the purpose of all nonclinical (animal and in vitro) testing is to reduce the risks and probability of adverse events in humans. But between initial nonclinical testing (and concurrent with additional animal testing) and a drug reaching the marketplace, the potential for having adverse effects in the general patient population it is intended for is further guarded against by a scheme of increasingly more powerful human (or "clinical") trials (Piantadosi, 1997). How safety is

Drug Safety Evaluation, Second Edition, by Shayne Cox Gad

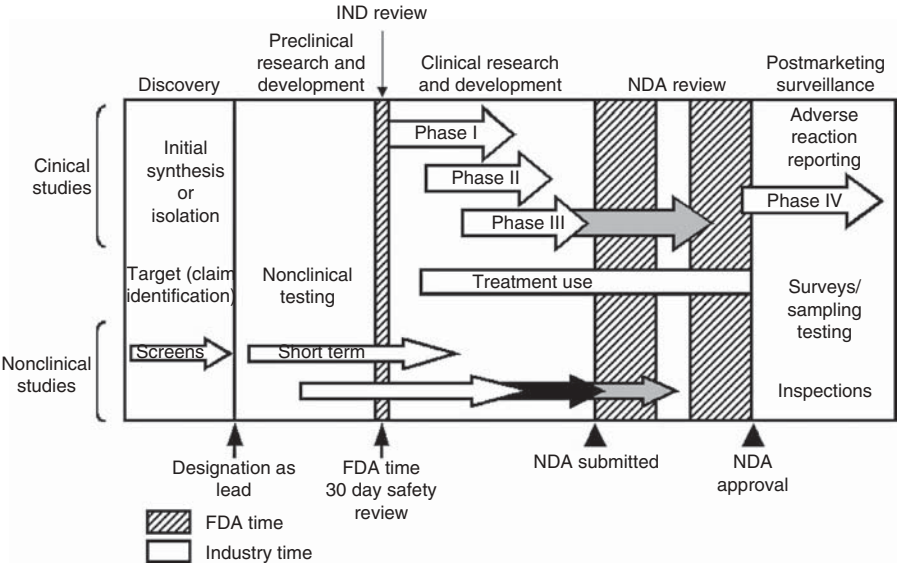


Figure 23.1 *Pharmaceutical development process viewed as four stages (discovery, preclinical development, clinical development, and NDA review) as well as important postmarket surveillance phase.*

evaluated in these is the subject of this chapter. The most common "unexpected" (from nonclinical trial results) safety findings in initial trials involve the skin (dermatitis of one form or another) and liver (Kaplowilz, 2001).

Except for those cases where there is substantial potential to save or extend lives [such as anticancer amyotrophic lateral sclerosis (ALS) and anti-AIDS drugs] or where the intended target diseases are chronic and severe [Parkinson's or multiple sclerosis (MS)] or the routes of administration are invasive (intrathecal), the initial evaluations in humans are performed in "normal," healthy volunteers with the primary objective being limited to defining the limits of tolerance (safety) of the potential drug and its pharmacokinetic characteristics. These trials may also seek to detect limited (usually surrogate—i.e., indirect) indicators of efficacy but are severely limited in doing so (Biomarkers Definitions, Working Group, 2001). Later trials look at the drug's actions on carefully defined groups of patients.

Something should be said about the special classes of studies (for cancer, HIV, ALS, and other life-threatening diseases) for which first-in-humans are not performed in normal healthy volunteers but rather in patients. Most frequently, these are patients that have failed other available forms of therapy. Even the design of such trials is different, though the monitored safety factors and pharmacokinetics are the same. As the current designs for oncology are proving to have exceptional failure rates, new "adaptive" study designs are actively being pursued (Meille et al., 2008).

With the number of drugs withdrawn from the marketplace since 1990[1] (or, perhaps, the degree of media coverage of such withdrawals), public concern with the workings of the drug safety evaluation aspects of the development process has risen sharply (Ganter, 1999; Wechsler, 2001). It is currently estimated that in the United States adverse drug reactions (ADRs) rank between the fourth to sixth leading cause of death (Eikelblom et al., 2001). While improvements in the nonclinical aspects of drug safety assessment (as covered in the first 19 chapters of this book) are possible and even likely, clearly the clinical aspects (the subject of this chapter) are likely to be where most improvement is likely. This will come from both better selection of subjects for inclusion in trials and a better understanding of individual or subpopulation differences in human responses to drugs.

While there is much press about the concern that the "increased pace of drug approval" has caused the release onto the market of less safe drugs (Willman, 2000), the causes are more mundane and of much longer standing. Additionally, the actual flow of new drugs entering the market is reduced. An important reason for the high incidence of serious and fatal ADRs is that the existing drug development paradigms do not generate adequate information on the mechanistic sources of marked variability in pharmacokinetics and pharmacodynamics of new therapeutic candidates, precluding treatments from being tailored for individual patients (Ozdemir et al., 2001).

Pharmacogenetics is the study of the hereditary basis of person-to-person variations in drug response. The focus of pharmacogenetic investigations has traditionally been unusual and extreme drug responses resulting from a single gene effect. The Human Genome Project and recent advancements in molecular genetics now present an unprecedented opportunity to study all genes in the human genome, including genes for drug metabolism, drug targets, and postreceptor second-messenger mechanisms, in relation to variability in drug safety and efficacy. In addition to sequence variations in the genome, high throughput and genomewide transcript profiling for differentially regulated messenger RNA (mRNA) species before and during drug treatment will serve as important tools to uncover novel mechanisms of drug action. Pharmacogenetic-guided drug discovery and development represent a departure for the conventional approach which markets drugs for broad patient populations, rather than smaller groups of patients in whom drugs may work more optimally.

Pharmacogenetics provides a rational framework to minimize the uncertainty in the outcome of drug therapy and clinical trials and thereby should significantly reduce the risk of drug toxicity. The reader is referred to the Internet sources in Table 23.1 for more details on pharmacogenetics and drug development. Potential improvements in patient inclusion criteria will be addressed later in this chapter.

[1] See Table 1.1.

TABLE 23.1 Educational Sources on Pharmacogenetics and Drug Development Available on WorldWide Web

Source	Focus	Web Address
Affymetrix	DNA microarray technology	www.affymetrix.com
Celera Genomics	Human genome sequencing and variation	www.celera.com
Center for Drug Development Science	Drug development	www.dml.georgetown.edu/depts/pharmacology/cdds/index.html
Center for Ecogenetics and Environmental Health	Gene–environment interactions	depts.washington.edu/ceeh
Cold Spring Harbor Laboratory	Genetics education	www.cshl.org
Food and Drug Administration	Drug development and regulation	www.fda.gov
Genaissance Pharmaceuticals	Human genetic variation	www.genaissance.com
Genset Corporation	Genomics and drug development	www.genxy.com/index/html
Human Genic Bi-allelic Sequences Database	SNPs	http://hgbase.cgr.ki.se
Human Genome Project	Human genetic variation	www.ornl.gov/TechResources/Human_Genome/home.html
Karolinska Institute	Genetics of drug metabolism	www.imm.ki.se/CYPalleles
National Institutes of Health	Glossary of genetic terms	www.nhgri.nih.gov/DIR/VIP/Glossary
Nature Genetics	Genomics	http://www.nature.com/genomics
Orchid Biocomputer	SNPs	www.snps.com
Pharsight Corporation	Drug development	www.pharsight.com
SNP Consortium	Human genetic variation	Snp.cshl.org
Stanford University	Genome resources	www-genome.stanford.edu/index.html
Whitehead Institute	Genome resources	www-genome.wi.mit.edu

Note: SNPs = single nucleotide polymorphisms.

23.1.1 Pharmacokinetics

Current costs and time pressures for developing a new therapeutic motivate companies to make the best possible decision as to whether to continue or abandon the development of a new drug based on a likelihood matrix for three factors: does it work (efficacy), is it acceptably tolerated at therapeutic doses (safety), and is it possible to deliver those therapeutic doses to the target sites/organs economically via the desired route (bioavailability). These likelihoods change most rapidly in early phase development and most compounds abandoned during clinical development are abandoned in this phase, with the most common reason begin unsuitable pharmacokinetics (is it absorbed and does it stay at therapeutic levels for an optimal or near-optimal period—Rolan,

1997). Hence the assessment of pharmacokinetics in early-phase drug development is strategically important. Some of the drug development issues which are likely to be answered at least in part by a thoughtful interpretation of pharmacokinetic data include the following:

1. Is the compound adequately absorbed to be likely to have a therapeutic effect?
2. Is the compound absorbed with a speed consistent with the desired clinical response?
3. Does the compound stay in the body long enough to be consistent with the desired duration of action?
4. Is the within- or between-subject variability acceptable given the likely therapeutic index of the compound?
5. Is there evidence of a formulation problem?
6. Is there a dose range which produces plasma (or tissue) concentrations which are likely to be associated with a desired clinical response or which gives rise to safety concerns?
7. Is there a relationship between plasma concentrations and a relevant measure of drug effect?
8. Are metabolites produced which may confound the therapeutic response or safety profile?
9. From the absorption, metabolism, and excretion profile, are there subsets of the target population which may behave differently from expected?
10. Considering the above issues, what is a suitable dosing regimen for clinical efficacy trials?

As most drugs are preferably given orally, absorption which is complete, consistent, and predictable is desirable. Although it may be possible from solubility, lipophilicity, pK_a, molecular size, and animal data to make some prediction about likely absorption, only a study in humans will give quantitative data as the mechanisms of drug absorption are complex and still incompletely understood (Washington et al., 2001). It may be helpful here to distinguish between the terms *absorption* and *bioavailability*.

Absorption refers to the fraction of the administered dose which is taken into the body. If a drug is taken up into intestinal cells but then extensively metabolized, it is still regarded as having been absorbed. However, for a drug to be bioavailable, unchanged drug must reach the systemic circulation. Hence a drug with a very high first-pass metabolism might be well absorbed but poorly bioavailable. Although in therapeutic terms poor absorption and poor bioavailability pose similar problems, it is important to distinguish between them because there are likely to be different possible solutions. Poor absorption might be approached by reformulation, change in the route of administration, or development of a prodrug; extensive presystemic metabolism might only be avoided by change in the route of administration or chemical modifica-

tion. Poor absorption is still frequently encountered in modern drug development because the rational drug discovery process often puts more emphasis on potency and selectivity (because these programs are run by biochemists and pharmacologists) than on factors likely to be associated with good absorption. This can result in lead compounds which perform very well in vitro but which may present major bioavailability and/or formulation problems (see discussion of this by Rolan et al., 1994).

Quantitative assessment of the extent of absorption (absolute bioavailability) is most rigorously obtained by comparison of the areas under the plasma concentration–time curves (after adjusting for dose) following intravenous (IV) and oral administration. However, even after oral administration alone some idea of absorption or bioavailability can be obtained in the following ways:

1. If a drug is not substantially metabolized, urinary excretion of unchanged drug may be a useful measure of absorption and bioavailability.
2. If a drug is substantially metabolized but it is reasonable to assume that metabolites are not produced in the gut lumen, urinary recovery of drug and metabolites might be a useful measure of absorption.
3. If the "apparent" plasma clearance (dose/area under the plasma concentration–time curve; equivalent to true clearance/fraction of dose absorbed) gives an implausibly high value of clearance (e.g., greater than hepatic and renal plasma flow), it is likely the bioavailability is low. However, this could be due to presystemic metabolism in addition to low absorption.
4. If there is a very large within- or between-subject variability in "apparent" clearance, this might indicate variable absorption or bioavailability, which in turn is often seen when absorption or bioavailability is low.

Whether absorption is related to the formulation or to an intrinsic property of the molecule can be determined by comparing absorption from a solid formulation and an oral solution, ideally with an IV solution as a reference.

Some idea of the rate of absorption can be obtained from examination of the plasma concentration–time profile. It should be remembered, however, that the time to maximum plasma concentration (t_{max}) is not when absorption is complete but when the rates of drug absorption and elimination are equal. Thus two drugs with the same absorption rate will differ in t_{max} if elimination rates differ. Assessment of the rate of absorption can also be confounded by complex or slow drug distribution. For example, the calcium channel blocker amlodipine has a much later t_{max} than other similar drugs. This is not due to slow absorption but to partitioning in the liver membrane with slow redistribution. A quantitative assessment of the rate of absorption can be obtained by deconvolution of plasma profiles following IV and oral administration.

Relating Time Course of Plasma Concentrations to Time Course of Effect A critical decision to be made after the first human study is whether the compound's speed of onset and duration of action are likely to be consistent with the desired clinical response. Speed of onset is clearly of interest for treatments which are taken intermittently for symptom relief, for example, acute treatments for migraine, analgesics, or antihistamines for hay fever. Duration of action is particularly important when the therapeutic effect needs to be sustained continuously, for example, anticonvulsants. The first information on the probable time course of action often comes from the plasma pharmacokinetic profile. However, it has become increasingly evident that the profile alone may be misleading, with the concentration–time and the effect–time curves being substantially different. Some reasons for this, including examples, are as follows:

1. The effect may be delayed with respect to plasma concentration because of slow uptake into the target tissue from the plasma. A well-known example is digoxin, where there is a delay of several hours between peak plasma concentration and peak effect.
2. The effect may wane faster than the plasma elimination curve due to tolerance, for example, benzodiazepines and nitrates.
3. The effect may persist despite apparent elimination from plasma. This can occur with an irreversible effect of the drug (e.g., acetylation of platelet cyclooxygenase by aspirin). Another reason is very tight binding of the drug near the receptor (e.g., salmeterol) or concentration and trapping in the target tissue (omeprazole).
4. The formation of active metabolites may also contribute to a delay in onset and/or prolongation of action.

Some of these mechanisms may become apparent during animal pharmacology studies, but the clinical pharmacologist must always be aware of the possible discrepancy between concentration and effect–time curves. Clearly, if a relevant drug effect can also be measured in early human studies, establishing a relationship between plasma concentration and effect may be possible. If the desired clinical effect can be measured directly (e.g., blood pressure for an antihypertensive drug), the pharmacokinetic profile may not contribute greatly to the assessment of the time course of action, but these circumstances are the exception rather than the rule. Because of the many causes of discrepancies between the time course of drug concentrations and effect, and often the difficulty in measuring the clinical effects directly, a potentially useful approach comes from the use of surrogate markers of drug effect (discussed elsewhere in this book) combined with pharmacokinetic–pharmacodynamic (PK/PD) modeling to explore the relationships between dose, plasma concentrations, and effects.

23.1.2 Safety of Clinical Trial Subjects

While there continues to be increased interest in and concern about the safety of marketed ("approved") drugs [Ioannides and Lau (2001), e.g., have published a study showing that adverse safety findings are frequently low compared to actual numbers], as of this writing the public's confidence in the safety of participants in trials is at a low level (Lee et al., 2006). Certainly, the fialuridine (FIAU) tragedy (Meinert, 1996) and the case of the death of a healthy volunteer in a Johns Hopkins trial brought this issue to the forefront of the public mind. Amid estimates of as many as 5000 subject deaths per year in federally funded clinical trials (out of seven million individuals enrolled in such trials) (Shamoo, 2000; Wilson, 1998; Davis, 1998; Association of American Universities, 2000; Henney, 2000), the current guidelines and procedures should be clearly understood and carefully adhered to but are likely to be changed. As a starting place, Table 23.2 presents a glossary of key terms employed in this discussion.

There are international regulations which govern (on a country-by-country basis) the conduct of clinical trials, with many national governments expecting researchers to follow specific guidelines, such as the International Conference on Harmonization (ICH, 1997) Guideline for Good Clinical Practice. Regulations and guidelines are generally based on the principles of the Nuremberg Convention. The Nuremberg Code was written in 1946 in an effort to prevent recurrence of the human experimentation atrocities of World War II. This document states that all research in humans should be done with the well-being of the subject being the primary concern (Schmidt, 2001; O'Donnell, 2005).

The 1964 Declaration of Helsinki includes significant detail about clinical trial practices and the rights of potential subjects to be informed about risks, benefits, and alternative therapies (World Medical Association, 2001). It has been amended several times, most recently in 2000, when the use of placebos in trials employing patients was pronounced to be unethical (Mackintosh, 2001). Together, several parts of the U.S. Code of Federal Regulations (21 CFR 50, 21 CFR 54, 21 CFR 56, 21 CFR 312) constitute the good clinical practice (GCP) regulations for studies conducted in the United States. The regulations detail the responsibilities of sponsors, investigators, and IRBs (institutional review boards) and also outline monitoring practices to ensure regulatory and study design compliance and subject safety. Similarly, the ICH guidelines on GCPs provide detailed instructions for investigators, institutions, sponsors, and IRBs.

As with preclinical matters, during the 1990s, the ICH has brought together regulatory agencies and industry representatives from the United States, Europe, and Japan—and observers from all over the world—to agree to a single set of technical requirements for the registration of pharmaceuticals for human use. This process is now almost complete. The ICH Guideline for Good Clinical Practice has been adopted by the three lead regions and by many other countries (ICH, 1997). As developing nations begin establishing

TABLE 23.2 Key Terms

Adverse event (or adverse experience): Any untoward medical occurrence in a patient or clinical investigation subject administered a pharmaceutical product and which does not necessarily have to have a causal relationship with this treatment.

Adverse drug reaction (ADR): In the preapproval clinical experience with a new medicinal product or its new usages, particularly as the therapeutic dose(s) may not be established "all noxious and unintended responses to a medicinal product related to any dose should be considered adverse drug reactions."

IND: Investigational new drug application filed with FDA after preclinical testing is complete asking for permission to proceed with human tests.

Efficacy pharmacology: Evaluation of a drug's characteristics, effects, and uses with regard to the target illness and its interactions with living organisms.

Healthy volunteer: A healthy person who agrees to participate in a clinical trial for reasons other than medical and receives no direct health benefit from participating.

Human subject: An individual who is or becomes a participant in research either as a recipient of the test article or as a control. A subject may be either a healthy human or a patient (21 CFR 50.3).

Nonclinical studies: Studies in living systems (animals or cells) other than humans.

Phase I: Initial safety trials on a new medicine in which investigators attempt to establish the dose range tolerance for single and multiple doses in about 20–80 healthy volunteers.

Phase II: Pilot clinical trials to evaluate efficacy, safety, and therapeutic dose ranges in selected populations of about 100–300 subjects who have the disease or condition to be treated, diagnosed, or prevented.

Phase III: Multicenter studies in populations of perhaps 100–3000 subjects (or more) for whom the medicine is eventually intended.

Phase IV: Postmarketing trials to provide additional details about the product's safety, efficacy, and additional uses.

Preclinical studies: Animal studies that support phase I safety and tolerance studies and must comply with good laboratory practice (GLP). Other preclinical studies are done in discovery research laboratories to support drug efficiency claims.

Serious adverse event: A serious adverse event (experience) or reaction is any untoward medical occurrence that at any dose:

- Results in death.
- Is life threatening.
- Requires inpatient hospitalization or prolongation of existing hospitalization.
- Results in persistent or significant disability/incapacity.
- Is a congenital anomaly/birth defect.

Subject/trial subject: An individual who participates in a clinical trial, either as recipient of the investigational product(s) or as a control. (ICH) See also healthy volunteers, human subject (ICH 1.57).

Unexpected adverse drug reaction: An adverse reaction, the nature or severity of which is not consistent with the applicable product information (e.g., investigator's brochure for an unapproved investigational medicinal product).

Source: Machin et al., 2004; Fletcher et al., 2006.

practices for the testing and registration of new molecular entities, many are using ICH guidelines as standards.

Thus, during the past 50 years, the conduct of clinical drug research has improved because of regulations, guidelines, and policies put in place to protect subjects. Individual pharmaceutical companies have used these guidelines and

regulations as the basis for their standard operating procedures (SOPs), technical operations policies, and training programs to direct work processes and staff in their research. Most companies have created quality assurance (QA) units to oversee their researchers' adherence to agency guidelines and regulations and to their own company policies and practices.

In the United States, no clinical drug research can begin without prior Food and Drug Administration (FDA) review of the investigational new drug (IND) application, which includes the human testing protocol and associated preclinical testing results. While formal FDA approval of such an application is not legally a requirement, assent before proceeding is a prudent goal. Regulators in some countries require only notification of intent to initiate first-in-human studies. Before moving on to phase II or phase III studies, pharmaceutical companies and other sponsors must submit the information gathered to date for agency reviews.

For new chemical entities (NCEs), new indications or new formulation companies must file an IND which must be approved by the FDA before a drug can be used in humans. The requirements for ADR reporting for INDs are thus known as the IND regulations. Before a new product can be marketed, companies must file a new drug application (NDA) and have it approved by the FDA. The requirements for ADR reporting after marketing are thus known as the NDA regulations. Both sets of regulations can apply to a drug at the same time; for instance the NDA regulations apply to any marketed forms, but the IND regulations apply to a new indication or formulation. At the time of writing, the current regulations are in 21 CFR as follows:

- 21 CFR 312.32: Safety reports for investigational products subject to an IND application (published 1987)
- 21 CFR 314.80: Postmarketing reporting of ADEs (NDA) (published 1985)
- 21 CFR 600.80: Postmarketing reporting of adverse experiences for licensed biological products (includes vaccines) (published 1994)

See also CFR website: http://www.access.gpo.gov/nara/cfr/index.html. The August 1997 guideline "Post-marketing adverse experience reporting for human drug and licensed biological products: Clarification of what to report" defined the minimum data relevant for a safety report as:

- An identifiable patient
- An identifiable reporter
- A suspect drug or biological product
- An adverse event or fatal outcome

If any of these items remain unknown after being actively sought, a report should not be submitted to the FDA. The guideline also clarifies that adverse

experiences derived during planned contacts and active solicitation of information from patients (e.g., company-sponsored patient support programs, disease management programs) should be handled as safety information from a postmarketing study (i.e., for expedited reporting, events must be serious and unexpected and there must be a reasonable possibility that the drug caused the event).

See also the Center for Drug Evaluation and Research (CDER) guidance page http://www.fda.gov/cder/guidance/index.htm.

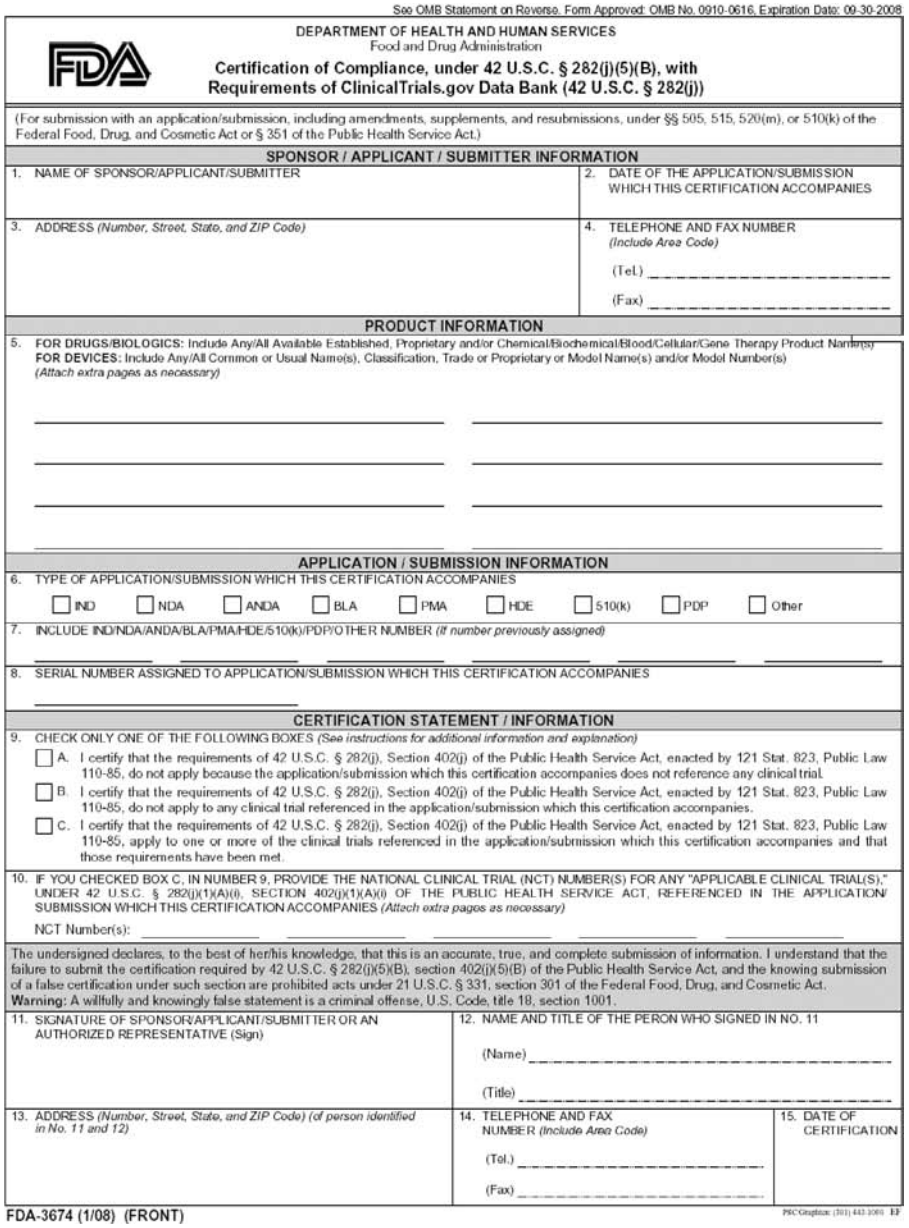
In the *Federal Register* of October 27, 1994, the FDA published a proposed rule to amend the regulations to provide consistency with certain standardized definitions, procedures, and formats developed by the ICH and the Council for International Organizations of Medical Sciences (CIOMS). The FDA received many comments on these proposals and finally published detailed amended expedited safety reporting regulations, implementing the ICH in October 1997 (*Federal Register*, October 7, 1997, Volume 62, Number 194, pages 52237–52253). A revision of the associated guideline has also been proposed by the FDA but was not available at the time of writing.

These new regulations for expedited reporting are effective 180 days later on April 6, 1998, but companies may comply with the provisions of this final rule before its effective date. Key points from the new regulations are described below. The amendments to the periodic postmarketing safety reporting regulations are delayed awaiting further consideration of the ICH E2C guideline. More recently, as of the start of 2008, all new drug clinical trials must be registered with ClinicalTrials.gov as part of the IND process. The form required to register, FDA-3674, is provided as Figure 23.2.

IND Regulations In 1994 the FDA proposed to amend requirements for clinical study design, conduct, and annual sponsor reporting in the IND regulations as a result of events with FIAU. In the light of comments received, the FDA withdrew the proposed amendments and will develop a guidance document with recommendations on study design and monitoring of investigational drugs used to treat serious and potentially fatal illnesses, with particular attention to detection of adverse events similar to those caused by underlying disease.

Increased Frequency Reports The requirement for increased frequency reports for serious expected ADRs with marketed products is revoked. This was also published in the *Federal Register*, June 25, 1997 (Volume 62, Number 122, pages 34166–34168). The rationale for this was that despite receiving many such reports, only a small number of drug safety problems were identified.

Reporting Forms FDA form 3500/3500A (refer to Figure 23.3) is the standard form for notifying expedited reports and can also be used by companies to submit IND safety reports. Foreign cases may be reported on the CIOMS I form.



See OMB Statement on Reverse. Form Approved: OMB No. 0910-0616, Expiration Date: 09-30-2008

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

Certification of Compliance, under 42 U.S.C. § 282(j)(5)(B), with Requirements of ClinicalTrials.gov Data Bank (42 U.S.C. § 282(j))

(For submission with an application/submission, including amendments, supplements, and resubmissions, under §§ 505, 515, 520(m), or 510(k) of the Federal Food, Drug, and Cosmetic Act or § 351 of the Public Health Service Act.)

SPONSOR / APPLICANT / SUBMITTER INFORMATION

1. NAME OF SPONSOR/APPLICANT/SUBMITTER
2. DATE OF THE APPLICATION/SUBMISSION WHICH THIS CERTIFICATION ACCOMPANIES
3. ADDRESS *(Number, Street, State, and ZIP Code)*
4. TELEPHONE AND FAX NUMBER *(Include Area Code)*
   (Tel.) _____
   (Fax) _____

PRODUCT INFORMATION

5. FOR DRUGS/BIOLOGICS: Include Any/All Available Established, Proprietary and/or Chemical/Biochemical/Blood/Cellular/Gene Therapy Product Name(s)
   FOR DEVICES: Include Any/All Common or Usual Name(s), Classification, Trade or Proprietary or Model Name(s) and/or Model Number(s)
   (Attach extra pages as necessary)

APPLICATION / SUBMISSION INFORMATION

6. TYPE OF APPLICATION/SUBMISSION WHICH THIS CERTIFICATION ACCOMPANIES
   ☐ IND ☐ NDA ☐ ANDA ☐ BLA ☐ PMA ☐ HDE ☐ 510(k) ☐ PDP ☐ Other
7. INCLUDE IND/NDA/ANDA/BLA/PMA/HDE/510(k)/PDP/OTHER NUMBER *(if number previously assigned)*
8. SERIAL NUMBER ASSIGNED TO APPLICATION/SUBMISSION WHICH THIS CERTIFICATION ACCOMPANIES

CERTIFICATION STATEMENT / INFORMATION

9. CHECK ONLY ONE OF THE FOLLOWING BOXES *(See instructions for additional information and explanation)*
   ☐ A. I certify that the requirements of 42 U.S.C. § 282(j), Section 402(j) of the Public Health Service Act, enacted by 121 Stat. 823, Public Law 110-85, do not apply because the application/submission which this certification accompanies does not reference any clinical trial.
   ☐ B. I certify that the requirements of 42 U.S.C. § 282(j), Section 402(j) of the Public Health Service Act, enacted by 121 Stat. 823, Public Law 110-85, do not apply to any clinical trial referenced in the application/submission which this certification accompanies.
   ☐ C. I certify that the requirements of 42 U.S.C. § 282(j), Section 402(j) of the Public Health Service Act, enacted by 121 Stat. 823, Public Law 110-85, apply to one or more of the clinical trials referenced in the application/submission which this certification accompanies and that those requirements have been met.
10. IF YOU CHECKED BOX C, IN NUMBER 9, PROVIDE THE NATIONAL CLINICAL TRIAL (NCT) NUMBER(S) FOR ANY "APPLICABLE CLINICAL TRIAL(S)," UNDER 42 U.S.C. § 282(j)(1)(A)(i), SECTION 402(j)(1)(A)(i) OF THE PUBLIC HEALTH SERVICE ACT, REFERENCED IN THE APPLICATION/SUBMISSION WHICH THIS CERTIFICATION ACCOMPANIES *(Attach extra pages as necessary)*
    NCT Number(s): _____

The undersigned declares, to the best of her/his knowledge, that this is an accurate, true, and complete submission of information. I understand that the failure to submit the certification required by 42 U.S.C. § 282(j)(5)(B), section 402(j)(5)(B) of the Public Health Service Act, and the knowing submission of a false certification under such section are prohibited acts under 21 U.S.C. § 331, section 301 of the Federal Food, Drug, and Cosmetic Act.
Warning: A willfully and knowingly false statement is a criminal offense, U.S. Code, title 18, section 1001.

11. SIGNATURE OF SPONSOR/APPLICANT/SUBMITTER OR AN AUTHORIZED REPRESENTATIVE (Sign)
12. NAME AND TITLE OF THE PERON WHO SIGNED IN NO. 11
    (Name) _____
    (Title) _____
13. ADDRESS *(Number, Street, State, and ZIP Code) (of person identified in No. 11 and 12)*
14. TELEPHONE AND FAX NUMBER *(Include Area Code)*
    (Tel.) _____
    (Fax) _____
15. DATE OF CERTIFICATION

FDA-3674 (1/08) (FRONT)

Figure 23.2 *FDA form 3674.*

Instructions for Completion of Form FDA 3674

Certification of Compliance, under 42 U.S.C. § 282(j)(5)(B), with Requirements of ClinicalTrials.gov Data Bank (42 U.S.C. § 282(j))
Form 3674 must accompany an application/submission, including amendments, supplements, and resubmissions, submitted under §§ 505, 515, 520(m), or 510(k) of the Federal Food, Drug, and Cosmetic Act or § 351 of the Public Health Service Act.

1. **Name of Sponsor/Applicant/Submitter -** This is the name of the sponsor/applicant/submitter of the drug/biologic/device application/submission which the certification accompanies. The name must be identical to that listed on the application/submission.

2. **Date -** This is the date of the application/submission which the certification accompanies.

3. **& 4. -** Provide complete address, telephone number and fax number of the sponsor/applicant/submitter.

5. **Product Information - For Drugs/Biologics:** Provide the established, proprietary name, and/or chemical/biochemical/blood product/cellular/gene therapy name(s) for the product covered by the application/submission. Include all available names by which the product is known. **For Devices:** Provide the common or usual name, classification, trade or proprietary or model name(s), and/or model number(s). Include all available names/model numbers by which the product is known.

6. **Type of Application/Submission -** Identify the type of application/submission which the certification accompanies by checking the appropriate box. If the name of the type of application/submission is not identified, check the box labeled "Other."

7. **IND/NDA/ANDA/BLA/PMA/HDE/510(k)/PDP/Other Number -** If FDA has previously assigned a number associated with the application/submission which this certification accompanies, list that number in this field. For example, if the application/submission accompanied by this certification is an IND protocol amendment and the IND number has already been issued by FDA, that number should be provided in this field.

8. **Serial Number -** In some instances a sequential serial number is assigned to the application. If there is such a serial number, provide it in this field.

9. **Certification -** This section contains three different check-off boxes.
   Box A should be checked if the sponsor/applicant/submitter has concluded that the requirements of 42 U.S.C. § 282(j), section 402(j) of the Public Health Service Act, do not apply because no clinical trials are included, relied upon, or otherwise referred to, in the application/submission which the certification accompanies.

   Box B should be checked if the sponsor/applicant/submitter has concluded that the requirements of 42 U.S.C. § 282(j), section 402(j) of the Public Health Service Act, do not apply at the time of submission to any clinical trials that are included, relied upon, or otherwise referred to, in the application/submission which the certification accompanies. This means that, at the time the application/submission is being made, the requirements of 42 U.S.C. § 282(j), section 402(j) of the Public Health Service Act, do not apply to any of the clinical trials included, relied upon, or otherwise referred to, in the application/submission which this certification accompanies.

   Box C should be checked if the sponsor/applicant/submitter has concluded that the requirements of 42 U.S.C. § 282(j), section 402(j) of the Public Health Service Act, do apply at the time of submission to some or all of the clinical trials that are included, relied upon, or otherwise referred to, in the application/submission which the certification accompanies. This means that, at the time the application/submission is being made, the requirements of 42 U.S.C. § 282(j), section 402(j) of the Public Health Service Act, apply to one or more of the clinical trials included, relied upon, or otherwise referred to, in the application/submission which this certification accompanies.

10. **National Clinical Trial (NCT) Numbers -** If you have checked Box C in number 9 (Certification), provide the NCT Number obtained from www.ClinicalTrials.gov for each clinical trial that is an "applicable clinical trial" under 42 U.S.C. § 282(j)(1)(A)(i), section 402(j)(1)(A)(i) of the Public Health Service Act, and that is included, relied upon, or otherwise referred to, in the application/submission which the certification accompanies. Type only the number, as NCT will be added automatically before number. Include any and all NCT numbers assigned to the clinical trials included, relied upon, or otherwise referred to, in the application/submission which this certification accompanies. Multiple NCT numbers may be required for a particular certification, depending on the number of "applicable clinical trials" included, relied upon, or otherwise referred to, in the application/submission which the certification accompanies.

11. **Signature of Sponsor/Applicant/Submitter or an Authorized Representative -** The person signing the certification must sign in this field.

12. **Name and Title of Person Who Signed In number 11. -** Include the name and title of the person who is signing the certification. If the person signing the certification is not the sponsor/applicant/submitter of the application/submission, he or she must be an authorized representative of the sponsor/applicant/submitter.

13. **& 14. -** Provide the full address, telephone and fax number of the person who is identified in number 11 and signs the certification in number 11.

15. Provide the date the certification is signed. This date may be different from the date provided in number 2.

Paperwork Reduction Act Statement

Public reporting burden for this collection of information is estimated to average 15 minutes and 45 minutes (depending on the type of application/submission) per response, including time for reviewing instructions. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to the applicable address below.

Food and Drug Administration
Center for Drug Evaluation and Research
Central Document Room
Form No. FDA 3674
5901-B Ammendale Road
Beltsville, MD 20705-1266

Food and Drug Administration
Center for Biologics Evaluation and Research
1401 Rockville Pike
Rockville, MD 20852-1448

Food and Drug Administration
Center for Devices and Radiological Health
Program Operations Staff (HFZ-403)
9200 Corporate Blvd.
Rockville, MD 20850

An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information, unless it displays a currently valid OMB control number.

FDA-3674 (1/08) (BACK)

Figure 23.2 *Continued*

Form Approved: OMB No. 0910-0291, Expires: 10/31/08
See OMB statement on reverse.

U.S. Department of Health and Human Services
Food and Drug Administration

For use by user-facilities, importers, distributors and manufacturers for MANDATORY reporting

Mfr Report #
UF/Importer Report #
FDA Use Only

MEDWATCH

FORM FDA 3500A (10/05)

Page ____ of ____

PLEASE TYPE OR USE BLACK INK

A. PATIENT INFORMATION

1. Patient Identifier — In confidence
2. Age at Time of Event: or Date of Birth:
3. Sex — ☐ Female ☐ Male
4. Weight — ____ lbs or ____ kgs

B. ADVERSE EVENT OR PRODUCT PROBLEM

1. ☐ Adverse Event and/or ☐ Product Problem *(e.g., defects/malfunctions)*
2. Outcomes Attributed to Adverse Event *(Check all that apply)*
   - ☐ Death: ____ *(mm/dd/yyyy)*
   - ☐ Life-threatening
   - ☐ Hospitalization - initial or prolonged
   - ☐ Required Intervention to Prevent Permanent Impairment/Damage (Devices)
   - ☐ Disability or Permanent Damage
   - ☐ Congenital Anomaly/Birth Defect
   - ☐ Other Serious (Important Medical Events)
3. Date of Event *(mm/dd/yyyy)*
4. Date of This Report *(mm/dd/yyyy)*
5. Describe Event or Problem
6. Relevant Tests/Laboratory Data, Including Dates
7. Other Relevant History, Including Preexisting Medical Conditions *(e.g., allergies, race, pregnancy, smoking and alcohol use, hepatic/renal dysfunction, etc.)*

Submission of a report does not constitute an admission that medical personnel, user facility, importer, distributor, manufacturer or product caused or contributed to the event.

C. SUSPECT PRODUCT(S)

1. Name *(Give labeled strength & mfr/labeler)* — #1 / #2
2. Dose, Frequency & Route Used — #1 / #2
3. Therapy Dates *(If unknown, give duration)* from/to *(or best estimate)* — #1 / #2
4. Diagnosis for Use *(Indication)* — #1 / #2
5. Event Abated After Use Stopped or Dose Reduced? — #1 ☐ Yes ☐ No ☐ Doesn't Apply; #2 ☐ Yes ☐ No ☐ Doesn't Apply
6. Lot # — #1 / #2
7. Exp. Date — #1 / #2
8. Event Reappeared After Reintroduction? — #1 ☐ Yes ☐ No ☐ Doesn't Apply; #2 ☐ Yes ☐ No ☐ Doesn't Apply
9. NDC# or Unique ID
10. Concomitant Medical Products and Therapy Dates *(Exclude treatment of event)*

D. SUSPECT MEDICAL DEVICE

1. Brand Name
2. Common Device Name
3. Manufacturer Name, City and State
4. Model # / Lot # / Catalog # / Expiration Date *(mm/dd/yyyy)* / Serial # / Other #
5. Operator of Device — ☐ Health Professional ☐ Lay User/Patient ☐ Other:
6. If Implanted, Give Date *(mm/dd/yyyy)*
7. If Explanted, Give Date *(mm/dd/yyyy)*
8. Is this a Single-use Device that was Reprocessed and Reused on a Patient? ☐ Yes ☐ No
9. If Yes to Item No. 8, Enter Name and Address of Reprocessor
10. Device Available for Evaluation? *(Do not send to FDA)* ☐ Yes ☐ No ☐ Returned to Manufacturer on: ____ *(mm/dd/yyyy)*
11. Concomitant Medical Products and Therapy Dates *(Exclude treatment of event)*

E. INITIAL REPORTER

1. Name and Address
Phone #
2. Health Professional? ☐ Yes ☐ No
3. Occupation
4. Initial Reporter Also Sent Report to FDA ☐ Yes ☐ No ☐ Unk.

Figure 23.3 *FDA Form 3500A.*

MEDWATCH

FORM FDA 3500A (10/05) *(continued)* Page ____ of ____

FDA USE ONLY

F. FOR USE BY USER FACILITY/IMPORTER ***(Devices Only)***

1. **Check One** ☐ User Facility ☐ Importer
2. **UF/Importer Report Number**
3. **User Facility or Importer Name/Address**
4. **Contact Person**
5. **Phone Number**
6. **Date User Facility or Importer Became Aware of Event** *(mm/dd/yyyy)*
7. **Type of Report** ☐ Initial ☐ Follow-up # ____
8. **Date of This Report** *(mm/dd/yyyy)*
9. **Approximate Age of Device**
10. **Event Problem Codes** *(Refer to coding manual)* Patient Code ____ - ____ - ____ Device Code ____ - ____ - ____
11. **Report Sent to FDA?** ☐ Yes ____ *(mm/dd/yyyy)* ☐ No
12. **Location Where Event Occurred** ☐ Hospital ☐ Home ☐ Nursing Home ☐ Outpatient Treatment Facility ☐ Other: ____ *(Specify)* ☐ Outpatient Diagnostic Facility ☐ Ambulatory Surgical Facility
13. **Report Sent to Manufacturer?** ☐ Yes ____ *(mm/dd/yyyy)* ☐ No
14. **Manufacturer Name/Address**

G. ALL MANUFACTURERS

1. **Contact Office - Name/Address** ***(and Manufacturing Site for Devices)***
2. **Phone Number**
3. **Report Source** *(Check all that apply)* ☐ Foreign ☐ Study ☐ Literature ☐ Consumer ☐ Health Professional ☐ User Facility ☐ Company Representative ☐ Distributor ☐ Other: ____
4. **Date Received by Manufacturer** *(mm/dd/yyyy)*
5. (A)NDA # ____ IND # ____ STN # ____ PMA/510(k) # ____ Combination Product ☐ Yes Pre-1938 ☐ Yes OTC Product ☐ Yes
6. **If IND, Give Protocol #**
7. **Type of Report** *(Check all that apply)* ☐ 5-day ☐ 30-day ☐ 7-day ☐ Periodic ☐ 10-day ☐ Initial ☐ 15-day ☐ Follow-up # ____
8. **Adverse Event Term(s)**
9. **Manufacturer Report Number**

H. DEVICE MANUFACTURERS ONLY

1. **Type of Reportable Event** ☐ Death ☐ Serious Injury ☐ Malfunction ☐ Other: ____
2. **If Follow-up, What Type?** ☐ Correction ☐ Additional Information ☐ Response to FDA Request ☐ Device Evaluation
3. **Device Evaluated by Manufacturer?** ☐ Not Returned to Manufacturer ☐ Yes ☐ Evaluation Summary Attached ☐ No *(Attach page to explain why not)* or provide code: ____
4. **Device Manufacture Date** *(mm/yyyy)*
5. **Labeled for Single Use?** ☐ Yes ☐ No
6. **Evaluation Codes** *(Refer to coding manual)* Method ____ - ____ - ____ - ____ Results ____ - ____ - ____ - ____ Conclusions ____ - ____ - ____ - ____
7. **If Remedial Action Initiated, Check Type** ☐ Recall ☐ Repair ☐ Replace ☐ Relabeling ☐ Notification ☐ Inspection ☐ Patient Monitoring ☐ Modification/Adjustment ☐ Other: ____
8. **Usage of Device** ☐ Initial Use of Device ☐ Reuse ☐ Unknown
9. **If action reported to FDA under 21 USC 360i(f), list correction/removal reporting number:**
10. ☐ **Additional Manufacturer Narrative** and / or 11. ☐ **Corrected Data**

The public reporting burden for this collection of information has been estimated to average 66 minutes per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to:

Department of Health and Human Services
Food and Drug Administration - MedWatch
10903 New Hampshire Avenue
Building 22, Mail Stop 4447
Silver Spring, MD 20993-0002

Please DO NOT RETURN this form to this address.

OMB Statement:
"An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number."

Figure 23.3 *Continued*

Definitions The definition of "serious" has been revised to make it consistent with ICH E2A and is the same for INDs and NDAs (see above).

The definition of "unexpected" for IND reporting is as follows:

> Any adverse drug experience, the specificity or severity of which is not consistent with the current investigator brochure; or if an investigator brochure is not required or available, the specificity or severity of which is not consistent with the risk information described in the general investigational plan or elsewhere in the current application, as amended. For example, under this definition, hepatic necrosis would be unexpected (by virtue of greater severity) if the investigator brochure only referred to elevated hepatic enzymes or hepatitis. Similarly, cerebral thromboembolism and cerebral vasculitis would be unexpected (by virtue of greater specificity) if the investigator brochure only listed cerebral vascular accidents. "Unexpected," as used in this definition, refers to an adverse drug experience that has not been previously observed (e.g., included in the investigator brochure) rather than from the perspective of such experience not being anticipated from the pharmacological properties of the pharmaceutical product.

Time Frames The time period for submitting written IND safety reports has been revised from 10 working days to 15 calendar days. For telephone reports (fatal and life-threatening unexpected reactions), it has been revised from 3 working days to 7 calendar days. Such reports can also be made by fax. Telephone reporting was previously restricted to clinical studies conducted under the IND, but under the new rule, telephone reporting within 7 calendar days applies to any unexpected fatal or life-threatening reaction from any source.

The time period for submitting NDA alert reports (serious and unexpected) has been revised from 15 working days to 15 calendar days.

Wherever human drug research is conducted, national regulations call for an independent ethical review of the study plan. In countries where a guideline on GCPs is used, ethics review bodies are made up of medical professionals from the institution, nonmedical personnel, and community members. Sponsor companies and involved investigators have no voting representation on these review boards, and they may not be present during the voting on the research approval. The investigator conducting the study may, however, present the protocol and answer questions at the IRB review meeting. The company sponsoring the trial is not allowed to participate in IRB meetings as a matter of routine, although a representative might be invited to explain or clarify the protocol to the ethics review body.

Informed consent must be obtained from study participants—in writing—before any study-related activities are performed. Regulations clearly describe the required elements of the consent document and the consent process to be followed. A good informed consent process can help ensure that potential subjects understand the nature of the studies they will enter, the type of treat-

ments they will undergo, alternative therapies currently available, and any particular hazards they might experience. They must be informed that they can withdraw from the study at any time without penalty. Subjects are to be asked for their consent to release information from their medical records and told that the medical information may be inspected by sponsor, company, and regulatory agency representatives. They are to be informed that the results of the trials may be used publicly, but anonymously.

Drug supplies must be accounted for throughout the trial and reconciled at the end of the trial. These practices are designed to prevent the misuse or inappropriate redistribution of the investigational drug and to help ensure compliance with the protocol.

Adverse events, unexpected drug reactions, and drug side effects experienced and reported by subjects or observed by clinical investigators are all to be recorded and promptly reported to sponsor companies. It is clear, however, that there are differences in reporting standards between companies and between countries (Hayachi and Walker, 1996). There is also, however, a marked difference in reporting standards and rates in U.S. clinical trials between different medical areas (Ioannides and Lau, 2001). The investigators involved with subject care and the pharmaceutical company sponsor are then to analyze each event for "causality" and "relatedness" to administration of the drug—did this reaction occur because of the drug or because of something else such as the progression of the disease symptoms, other medications being taken, or unrelated causes? Such safety information is then to be forwarded to the appropriate IRB or ethics committee and regulatory agencies. The sponsor company is to send periodic updates to investigators, alerting them to new serious or unexpected drug reactions.

Company physicians are expected to continuously analyze the adverse drug event data coming in from worldwide trials for trends and patterns that could foretell a drug safety problem. Drug companies frequently set up data and safety monitoring boards (DSMBs) composed of noncompany medical experts and statisticians who impartially evaluate safety as the study progresses and are responsible for alerting the sponsor to unanticipated problems. Regulatory agencies also watch for trends because they are often in the best position to see safety trends across classes of drugs from any different companies.

Sponsor companies use quality assurance units independent of the clinical research group to audit medical operations. Their role is to ensure that regulatory standards and company policies and procedures for clinical research are being followed in all countries where research is being conducted.

Regulatory agencies oversee sponsor organizations, clinical trial processes, and clinical trial sites to verify that sponsors are conducting trials appropriately. Existing FDA regulations are conforming to ICH guidelines. When deficiencies are noted, agency inspectorates can restrict and penalize the offending academic IRB, investigator, and/or sponsor company. Investigator sites (including, in extreme cases, entire universities) can be prohibited from

conducting clinical research. Company studies can be rejected by regulatory agencies.

Continuous Safety Monitoring Medical staff members at the clinical trial site and at the sponsor company are expected to be continuously alerted to adverse drug reactions or unexpected and serious medical problems that might be attributed to the new medication begin tested. The investigator and the site staff examine subjects and take vital sign measurements on the schedule designated by the protocol, the guide for study conduct. Each drug, as shown in its preclinical studies, has unique characteristics and the potential for adverse events or side effects that investigators need to watch for during the clinical trial. Because of this, safeguard activities are built into the protocol, such as the time intervals between subject visits, how often subjects are to be questioned and examined, the specific medical tests to be run at various time points, and the special diagnostic tests or interviews to be conducted. Staff members at the clinical site must record the medical information from these tests and from interviews and medical histories. Site staff must also transfer information from the medical source documents to the case report forms (CRFs) specific to the study. The CRFs contain key information required for the protocol. Clinical research associates and physicians are required to review the information regularly and to immediately report anything alarming to the IRB and regulatory agencies for further evaluation.

Sponsor Pharmacovigilance Dedicated departments in pharmaceutical companies—often called pharmacovigilance groups—receive, review, analyze, follow up on, and appropriately distribute safety-related information from new drug trials. These groups sometimes staff hot lines and question-and-answer services to provide up-to-date answers to drug-related questions. Safety information is to be reported to regulatory agencies at specified intervals and at milestone time points throughout all phases of drug development. The postapproval aspects of this effort are the core of the subject of Chapter 22.

Sponsor monitoring is another important oversight process to ensure quality, compliance, and subject safety. Monitors may be employees of the sponsor's medical staff or a contract research organization or may be independent contractors. In each case, they represent the sponsor and visit investigator sites regularly, perhaps every four to eight weeks. They examine subject records in detail and verify that the correct information was transferred to the clinical trial case report forms—a process called source data verification. During their site visits, monitors also examine administrative and regulatory documents, including drug supply and dosing records, adverse-event documentation and reporting, the informed consent process and forms, and case report forms.

Ensuring subject safety must be of foremost concern during the entire drug development process, especially in clinical trials. The gradual dosing of a drug

candidate in healthy human volunteer subjects is performed under tightly controlled conditions and under the direction and scrutiny of physicians trained in clinical pharmacology. A drug candidate's progress toward broader testing in a population of individuals with the disease or condition to be treated also moves prudently and in well-defined steps.

23.1.3 Limitations on/of Clinical Trials

Before looking more closely at the definition, structure, and designs of trials, one should understand their limitations. These are regulatory, economic, legal, and due to custom.

First, the most recent (October 2000) revision of the Declaration of Helsinki (World Medical Association, 2000) called for discontinuing the use of placebo-controlled trials in patients. While this is not currently binding on U.S. trials (the FDA has specifically said that they will not mirror this as a requirement) and is intended to protect the health of participating patient subjects by precluding having some denied existing efficacious treatments (which would be the effect in most, but not all, cases). It will also likely cause the numbers of subjects required in a trial to increase. This will further stretch the economic aspects of limitations on the power of trials to assess potential drug safety in what will be the intended patient population. Trials are already very expensive—each additional subject enrolled costs $15,000 or more in a phase II or III trial.

The legal (or rather, litigation) limitations are that any adverse event in a trial (or resulting from it) exposes a sponsor to potential litigation. Accordingly, trials are designed to exclude not only those individuals who are not in the precisely designed subject disease population but also those who represent potential additional risk subpopulations (the elderly, the young, those currently taking other drugs, minorities and women who are or may become pregnant) who are likely to eventually use the drug when it enters the marketplace.

Custom (continuing to do things as they have previously been done) also limits the power of trials to identify safety issues. While there are now regulatory inducements to include more women, the young, and ethnic minorities in trials, the first two groups still are not proportionately incorporated because of both the perceived risks of adverse events that they represent and because historically they have not been included. Ethnic minorities, particularly African Americans, present a different problem in that there is a historically based resistance to participation in such trials.

23.2 CLINICAL TRIALS PROCESS

While the numbers of animals involved in research is tracked closely and is well known, such is not the case for human subjects involved in clinical trials.

We simply do not know how many are involved in such trials in the United States, much less worldwide. Though the National Institutes of Health (NIH) does track closely how many dollars and individuals are involved in research it funds ($18.0 billion and eight million subjects in 1997), the same is not true for privately funded research (where the numbers are greater). And while there is now a website where one can examine the numbers and types of efficacy trials open, the same is not true for phase I tolerance and pharmacokinetic trials—where most potential drugs cease development.

Clinical drug development is often described as consisting of four distinct phases (I–IV). It is important to recognize that the phase of development provides an inadequate basis for classification of clinical trials because one type of trial may occur in several phases (see Figure 23.2). Table 23.3 presents a preferable (objective-based) classification of trial types. It is important to appreciate that the phase label is a description, not a set of requirements. It is

TABLE 23.3 Approach to Classifying Clinical Studies According to Objective

Type of Study	Objective of Study	Study Examples
Human pharmacology (phase I)	• Assess tolerance • Define/describe pharmacokinetics and pharmacodynamics • Explore drug metabolism and drug interactions • Estimate activity	• Dose–tolerance studies • Single- and multiple-dose PK and/or PD studies • Drug interaction studies
Therapeutic exploratory (phase Ib/II)	• Explore use for targeted indication • Estimate dosage for subsequent studies • Provide basis for confirmatory study design, endpoints, methodologies	• Earliest trials of relatively short duration in well-defined narrow patient populations using surrogate or pharmacological endpoints or clinical measures • Dose–response exploration studies
Therapeutic confirmatory (phase III)	• Demonstrate/confirm efficacy • Establish safety profile • Provide adequate basis for assessing benefit–risk relationship to support licensing • Establish dose–response relationship	• Adequate and well-controlled studies to establish efficacy • Randomized parallel dose–response studies • Clinical safety studies • Studies of mortality/morbidity outcomes • Large simple trials • Comparative studies
Therapeutic use (phase III/IV)	• Refine understanding of benefit–risk relationship in general or special populations and/or environment • Identify less common adverse reactions • Refine dosing recommendation	• Comparative effectiveness studies • Studies of mortality/morbidity outcomes • Studies of additional endpoints • Large simple trials • Pharmacoeconomic studies

also important to realize that these temporal phases do not imply a fixed order of studies since for some drugs in a development plan the typical sequence will not be appropriate or necessary. For example, although human pharmacology studies are typically conducted during phase I, many such studies are conducted at each of the other three stages but nonetheless sometimes labeled as phase I studies. Figure 23.4 demonstrates this close but variable correlation between the two classification systems. The distribution of the points of the graph shows that the types of study are not synonymous with the phases of development.

Drug development is ideally a logical, stepwise procedure in which information from small early studies is used to support and plan later larger, more definitive studies. To develop new drugs efficiently, it is essential to identify characteristics of the investigational drugs in the early stages of development and to plan an appropriate development based on this profile.

Initial trials provide an early evaluation of short-term safety and tolerability and can provide pharmacodynamic and pharmacokinetic information needed to choose a suitable dosage range and administration schedule for initial exploratory therapeutic trials. Later confirmatory studies are generally larger and longer and include a more diverse patient population. Dose–response information should be obtained at all stages of development, from early tolerance studies, to studies of short-term pharmacodynamic effect, to large efficacy studies. Throughout development, new data may suggest the need for additional studies that may commonly be part of an earlier phase. For example, blood-level data in a late trial may suggest a need for a drug–drug interaction

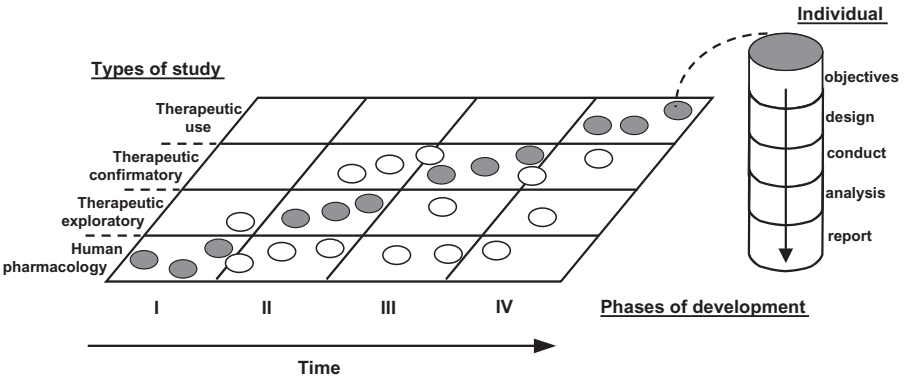


Figure 23.4 *Matrix illustrating relationship between phases of development and types of study by objective that may be conducted during each clinical development of new medicinal product. The shaded circles show the types of the study most usually conducted in a certain phase of development; the open circles show certain types of study that may be conducted in that phase of development but are less usual. Each circle represents an individual study. To illustrate the development of a single study, one circle is joined by a dotted line to an inset column that depicts the elements and sequence of an individual study.*

study or adverse effects may suggest the need for further dose finding and/or additional nonclinical studies. In addition, to support a new marketing application approval for the same drug, for example, for a new indication, pharmacokinetic or therapeutic exploratory studies are considered to be in phase I or II of development (Stone, 2006; Green et al., 2003; Gallin and Ognibene, 2007).

Phase I starts with the first-in-man administration of an IND. Although human pharmacology studies are typically identified with phase I, they may also be indicated at other points in the development sequence. Studies in this phase of development usually have nontherapeutic objectives and may be conducted in healthy volunteer subjects or certain types of patients, for example, patients with mild hypertension. Drugs with significant potential toxicity (cytotoxic drugs) or risks due to route of administration (such as intrathecal) are usually studied in patients. Studies in this phase can be open or baseline controlled or may use randomization and blinding to improve the validity of observations.

Studies conducted in phase I typically may involve one or a combination of the following:

(a) *Estimation of Initial Safety and Tolerability* The initial and subsequent administration of an IND into humans is usually intended to determine the tolerability of the dose range expected to be needed for later clinical studies and to determine the nature of adverse reactions that can be expected. These studies typically include both single- and multiple-dose administration.

(b) *Pharmacokinetics* Characterization of a drug's absorption, distribution, metabolism, and excretion continues throughout the development plan. Such preliminary characterization is an important goal of phase I. Pharmacokinetics may be assessed via separate studies or as a part of efficacy, safety, and tolerance studies. Pharmacokinetic studies are particularly important to assess the clearance of the drug and to anticipate possible accumulation of parent drug or metabolites and potential drug–drug interactions. Some PK studies are commonly conducted in later phases to answer more specialized questions. For many orally administered drugs, especially modified release products, the study of food effects on bioavailability is important. Obtaining PK information in subpopulations such as patients with impaired elimination (renal or hepatic failure), the elderly, children, women, and ethnic subgroups should be considered. Drug–drug interaction studies are important for many drugs and are generally performed in phases beyond phase I. But studies in animals and in vitro studies of metabolism and potential interactions may lead to doing such studies earlier.

(c) *Assessment of Pharmacodynamics* Depending on the drug and the endpoints studied, PD studies and studies relating drug blood levels to response (PK/PD studies) may be conducted in healthy volunteer subjects or in patients with the target disease. In patients, if there is an appropriate

measure, PD data can provide early estimates of activity and potential efficacy and may guide the dosage and dose regimen in later studies.

(d) *Early Measurement of Drug Activity* Preliminary studies of activity or potential therapeutic benefit may be conducted in phase I as a secondary objective. Such studies are generally performed in later phases but may be appropriate when drug activity is readily measurable with a short duration of drug exposure in patients at this early stage. Frequently such evaluations are done in what are called "phase Ib" studies.

Phase II is usually considered to start with the initiation of studies in which the primary objective is to explore therapeutic efficacy in patients.

Initial therapeutic exploratory studies may use a variety of study designs, including concurrent controls and comparison with baseline status. Subsequent trials are usually randomized and concurrently controlled to evaluate the efficacy of the drug and its safety for a particular therapeutic indication. Studies in phase II are typically conducted in a group of patients who are selected by relatively narrow criteria, leading to a relatively homogeneous population and are closely monitored.

An important goal for this phase is to determine dose levels and regimen for phase III trials. Early studies in this phase often utilize dose escalation designs to give an early estimate of dose response, and later studies may confirm the dose–response relationship for the indication in question by using recognized parallel dose–response designs (could also be deferred to phase III). Confirmatory dose–response studies may be conducted in phase II or deferred until phase III. Doses used in phase II are usually but not always less than the highest doses used in phase I.

Additional objectives of clinical trials conducted in phase II may include evaluation of potential study endpoints, therapeutic regimens (including concomitant medications) and target populations (e.g., mild versus severe disease) for further study in phase II or III. These objectives may be served by exploratory analyses, examining subsets of data, and including multiple endpoints in trials.

Phase III usually is considered to begin with the initiation of studies in which the primary objective is to demonstrate or confirm therapeutic benefit.

Studies in phase III are designed to confirm the preliminary evidence accumulated in phase II that a drug is safe and effective for use in the intended indication and recipient population. These studies are intended to provide an adequate basis for marketing approval. Studies in phase III may also further explore the dose–response relationship or explore the drug's use in wider populations, in different stages of disease, or in combination with another drug. For drugs intended to be administered for long periods, trials involving extended exposure to the drug are ordinarily conducted in phase III, although they may be started in phase III. ICH E1 and ICH E7 describe the overall clinical safety database considerations for chronically administered drugs and drugs used in the elderly. These studies carried out in phase III complete the

information needed to support adequate instructions for use of the drug (official product information).

Phase IV begins after drug approval. Once rare, there are now commonly required therapeutic use studies that go beyond the prior demonstration of the drug's safety, efficacy, and dose definition.

Studies in phase IV are all studies (other than routine surveillance) performed after drug approval and related to the approved indication. They are studies that were not considered necessary for approval but are often important for optimizing the drug's use. They may be of any type but should have valid scientific objectives. Commonly conducted studies include additional drug–drug interaction, dose response or safety studies, and studies designed to support use under the approved indication, for example, mortality/morbidity studies, epidemiological studies.

Development of Application Unrelated to Original Approved Use After initial approval, drug development may continue with studies of new or modified indications, new dosage regimens, and new routes of administration or additional patient populations. If a new dose, formulation, or combination is studied, additional human pharmacology studies may be indicated, necessitating a new development plan.

The need for some studies may be obviated by the availability of data from the original development plan or from therapeutic use.

23.2.1 Special Considerations

A number of special circumstances and populations require consideration on their own when they are part of the development plan.

Studies of Drug Metabolites Major active metabolite(s) should be identified and deserve detailed PK study. Timing of the metabolic assessment studies within the development plan depends on the characteristics of the individual drug.

Drug–Drug Interactions If a potential for drug–drug interaction is suggested by metabolic profile, by the results of nonclinical studies, or by information on similar drugs, studies on drug interaction during clinical development are highly recommended. For drugs that are frequently coadministered it is usually important that drug–drug interaction studies be performed in nonclinical and, if appropriate, in human studies. This is particularly true for drugs that are known to alter the absorption or metabolism of other drugs or whose metabolism or excretion can be altered by effects by other drugs.

Special Populations Some groups in the general population may require special study because they have unique risk–benefit considerations that need to be taken into account during drug development or because they can be

anticipated to need modification of use of the dose or schedule of a drug compared to general adult use. Pharmacokinetic studies in patients with renal and hepatic dysfunction are important to assess the impact of potentially altered drug metabolism or excretion. Specific ICH and FDA documents address such issues for geriatric patients and patients from different ethnic groups. The need for nonclinical safety studies to support human clinical trials in special populations is addressed in the ICH M3 document.

A key issue is thus when to perform kinetic studies in special patient groups (elderly, patients with renal or hepatic disease) and how. As the elderly are the majority users of many medicines, the subject of evaluating new drugs in the elderly is a major issue which is discussed in detail elsewhere. Unless a medication is unlikely to be used in the elderly, some data will be required by regulators for registration. However, an important issue in early phase development is whether to perform a separate elderly volunteer kinetic/tolerability study before elderly patients are included in later phase clinical trials. The major argument for doing so includes the possible reluctance of clinical investigators to enroll elderly patients without such data being available because of safety concerns; however, the utility of such studies has been questioned. The subjects in elderly volunteer studies are usually in much better health than the general population they are intended to represent. Also, the elderly may differ from the young not so much in terms of mean kinetic parameters but because the variability in the elderly may be much greater. The relatively small sample size (typically 12–18) may not allow a good estimation of the variability within the elderly population. For these reasons the FDA has recommended that information about the kinetics of a drug in the elderly should come from a larger group representative of the target population and this can be done in the efficacy clinical trials. Although these data are useful, it is not always an acceptable substitute for a specific elderly volunteer PK study because the information is only available after many patients have been exposed rather than before and clinical investigators may be reluctant to enroll patients without such data in advance. In practice, for a drug likely to be given to the elderly, an elderly volunteer study should be performed soon after a young healthy volunteer study to expand the potential population for efficacy studies as much as possible. If elderly patients are then included in the main efficacy/safety studies, the population approach can then be used to explore the pharmacokinetic variability in this subset of the population and whether this is associated with an altered clinical outcome.

A similar rationale can be used to decide whether special kinetic (and possibly dynamic) studies should be performed in patients with renal or hepatic disease. For example, if the compound is largely metabolized to inactive metabolites, renal function can reasonably be expected not to have a major effect on kinetics. However, regulators usually will want some information as some expectations do exist to the above assumption. An example is the "futile cycle" involving some nonsteroidal anti-inflammatory drugs (NSAIDs)—where prolonged residence of inactive acyl glucuronide metabolites in the

plasma in patients with renal disease allows breakdown back to the parent molecule resulting in accumulation (Sallustio et al., 1989). As outlined above, a population approach could be used to screen for an effect of disease on drug kinetics, but some investigators may need reassurance before enrolling patients in trials. A small study in patients with advanced renal disease may be able to provide this reassurance. Liver disease can be handled similarly for drugs which are primarily eliminated renally.

The safety of marketed drugs could be significantly improved if the subject groups involved in phase II and III trials better reflected the patient populations that will use drugs. By excluding "representatives" for what will clearly be subpopulations utilizing a drug (those using other drugs or with other diseases), many clear safety questions go unasked. The same is, to some degree, true about preclinical animal studies where only healthy young animals are employed rather than (perhaps) some disease model groups which might serve as better predictors of patient safety concerns.

23.3 INSTITUTIONAL REVIEW BOARDS (IRBS) IN CLINICAL TRIAL PROCESS

Clinical drug trials represent research with human subjects (Cato, 1988). All research involving human subjects that is supported by the federal government or the results of which are to be used in applications for drug or device approval must be conducted in accordance with regulations promulgated by the Department of Health and Human Services (DHHS) (45 CFR 46) and the FDA (21 CFR 56). The regulations of both the DHHS and the FDA require that an IRB "shall review and have authority to approve, required modifications in (to secure approval), or disapprove all research activities covered by [the] regulations" (45 CFR 46, 21 CFR 56).

The review of clinical drug trials by IRBs raises a number of interesting and difficult issues. These relate to the origin and sponsor of the proposed trial, the nature of the institution the IRB serves, and the manner in which the norms for determining ethical conduct in clinical trials can be applied to specific trials.

Here the ethical principles underlying research involving human subjects, the legal authority for IRBs, and the regulatory requirements affecting the operations of IRBs are reviewed. It will then discuss the role of IRBs in reviewing clinical trials by examining how IRBs can assess the scientific design of trials, the competency of the investigator, the manner of selecting subjects for the trial, the balance of risks and benefits, informed consent, and provisions for compensating for research-related injuries.

Legal Authority for IRBs The legal authority for IRBs derives from two parallel sets of federal regulations. One set of regulations was promulgated by

the DHHS and implements the 1974 amendments to the Public Health Services Act (National Research Act, 1974). These regulations are codified in 45 CFR 46. The second set of regulations was promulgated by the FDA under the federal Food, Drug and Cosmetic (FD&C) Act. These regulations are codified in 21 CFR; regulations pertaining to IRBs are in Part 50 and those pertaining to informed consent are in Part 56.

The FDA has the legal authority to regulate clinical investigations in the United States when the investigational products move across state or national boundaries. Under the FDA regulations, review and approval by an IRB is required for any experiment that involves a test article and one or more human subjects, either patients or healthy persons, and that is subject to the requirements for prior submission to the FDA (21 CFR 50.3). Such review is also required for any experiment the results of which are intended to be submitted later to, or held for inspection by, the FDA.

The regulations of the FDA are identical or similar to those of the ICH and DHHS in nearly all essential respects. Such differences as do exist reflect the different statutory authority under which the separate sets of regulations were promulgated and the difference in mission between the FDA and the NIH, the agency within DHHS charged with overseeing the implementation and enforcement of the DHHS regulations. The difference in mission between the FDA and the NIH is reflected in the FDA's approach to compliance with its regulations utilizing its traditional tools of inspections and audits.

The FDA regulations specify requirements for IRB membership, function, and operation, and the criteria according to which approval may be given for conducting research. Since these requirements are similar, a single committee should be established to undertake the activities required by both sets of regulations. Additionally, the FDA regulations allow a wide variety of ways in which private practitioners not affiliated with an institution can obtain necessary IRB review of their clinical research activities. The basic ethical tenets governing the actions of an IRB should include (Sharp, 2001):

1. Risks to subjects are minimized: (i) by using procedures which are consistent with sound research design and which do not unnecessarily expose subjects to risk; and (ii) whenever appropriate, by using procedures already being performed on the subjects for diagnostic or treatment purposes.
2. Risks to subjects are reasonable in relation to anticipated benefits, if any, to subjects, and the importance of the knowledge that may reasonably be expected to result. …
3. Selection of subjects is equitable. …
4. Informed consent will be sought from each prospective subject or the subject's legally authorized representative. …
5. Informed consent will be appropriately documented. …

Additional requirements are that adequate provisions exist for monitoring the data collected, adequate provisions exist to protect the privacy of subjects and maintain the confidentiality of the data, and appropriate safeguards be included to protect the rights and welfare of subjects who are "vulnerable to coercion or undue influence … or persons who are economically or educationally disadvantaged" (45 CFR 46.1).

Before a trial initiates, formal review by an IRB that agrees to assume this additional function or by IRBs formed by a local or state health agency, a medical school, a medical society, a state licensing board, or a nonprofit or for-profit independent group is required. All IRBs, regardless of sponsorship, that are assuming responsibilities for reviewing and approving clinical research protocols subject to FDA authority must comply with the IRB regulations set out by the FDA.

Duties of IRBs IRBs are required to review and have the authority to approve, require modifications in, or disapprove all research activities covered by the regulations [45 CFR 46.109(b)]. They must require that information given to subjects as part of informed consent is in accordance with the general requirements for informed consent that are set out in the regulations. Additionally, they may require that other information be given to subjects when they judge that such information would further protect the rights and welfare of the subjects [45 CFR 46.109(c)].

IRBs must require documentation of informed consent in all studies except those specified in the regulations in which documentation may be waived. Clinical drug trials are not among the classes of studies in which documentation of informed consent may be waived.

IRBs must provide written notification to investigators and institutions of their decisions to approve, require modifications in, or disapprove proposed research activities. Decisions to disapprove a proposed research proposal must be accompanied by a statement of reasons for the decision and provide the investigator an opportunity to respond in person or in writing.

IRBs must conduct continuing reviews of research they approve at least once each year. More frequent reviews may be required if the risk of a particular research project so warrants. IRBs have the authority to suspend or terminate approval of research that is not being conducted in accordance with their requirements or that has been associated with unexpected serious harm to subjects. Such action must be accompanied by a statement of reasons for it and be communicated to the investigator, appropriate institutional officials, and the secretary of DHHS.

The regulations require that IRBs must follow the written procedures that are set out in the assurances they have filed with DHHS, review proposed research at convened meetings at which a majority of the IRB members are present, vote approval by a majority of members present at the meeting, and be responsible for reporting to the appropriate institutional official and the

secretary of DHHS "… any serious or continuing noncompliance by investigators with the requirements and determination of the IRB" (45 CFR 46.109(b)).

Institutions that are cooperating in multiinstitutional studies, such as clinical drug trials, must each review and approve the proposed studies. Such institutions may, however, use joint review, rely on the review of another qualified IRB, or utilize similar arrangements to avoid duplication of efforts (Cato, 1988; Fletcher et al., 2002).

Informed Consent Assuring that adequate provisions exist for securing informed consent is a central duty of IRBs and that which is seemingly the most visualized when it fails (Office of Inspector General, 2000a,b). The requirements for informed consent are specified in international guidelines and the federal regulations. These require that investigators "shall seek such consent only under circumstances that provide the prospective subject … sufficient opportunity to consider whether or not to participate and that minimize the possibility of coercion or undue influence. The information that is given to the subject … shall be in language that is understandable to [him]" (45 CFR 46.116). The regulations further stipulate that "no informed consent, whether oral or written, may include any exculpatory language through which the subject … is made to waive or appear to waive any of the subject's legal rights, or releases or appears to be release the investigator, the sponsor, the institution or its agents from liability from negligence."

The federal regulations specify the information that shall be provided to each subject:

1. A statement that the study involves research, an explanation of the purposes of the research and the expected duration of the subject's participation, a description of the procedures to be followed, and identification of any procedures which are experimental
2. A description of any reasonably foreseeable risks or discomforts to the subjects
3. A description of any benefits to the subject or to others which may reasonably be expected from research
4. A disclosure of appropriate alternative procedures or courses of treatment, if any, that might be advantageous to the subject
5. A statement describing the extent, if any, to which confidentiality of records identifying the subject will be maintained
6. For research involving more than minimal risk, an explanation as to whether any compensation and an explanation as to whether medical treatments are available if injury occurs and, if so, what they consist of or where further information may be obtained
7. An explanation of whom to contact for answers to pertinent questions about the research and research subject's rights and whom to contact in the event of a research-related injury to the subject

8. A statement that participation is voluntary, refusal to participate will involve no penalty or loss of benefits to which the subject is otherwise entitled, and the subject may discontinue participation at any time without penalty or loss of benefits to which the subject is otherwise entitled

In addition to these basic elements of informed consent, IRBs also require that information be provided, where indicated, to the effect that (1) the particular treatment or procedure being tested may involve risks to the subject that are currently unforeseeable; (2) foreseeable circumstances may exist under which continued participation by the subject may be terminated by the investigator without regard to the subject's consent; (3) additional costs to the subject may result from participation in the research; (4) the consequences of a decision to withdraw; and (5) significant findings that may influence a subject's continued participation will be related to the subject.

In addition to the elements enumerated in the federal regulations, IRBs must consider whether consent forms should include the fact of randomization in the case of prospective randomized clinical trials.

Those who feel that the fact of randomization need not be disclosed to prospective subjects argue that since the alternative treatments to be tested are not known to produce significantly different results and since the physician would have to make an arbitrary selection of one treatment or the other for a particular patient, notification that selection of treatment is by computer rather than by the patient's own physician does not provide additional protection for the subjects and is unnecessary. The response to this contention is that a subject's ability to exercise full autonomy over what will be done with his or her own body is best served by notifying the subject as to how the treatment will be selected and by whom, even if the selection process is equally arbitrary whatever process is used.

The weight of the arguments favors the notion that for consent to be fully informed subjects must be notified that their treatments will be allocated in a random manner, that is, selected by a process other than the judgment of their own physician. The meaning of the concept of randomization and the fact that it will be the manner by which treatment is selected is therefore considered to be an important and integral part of informed consent for participation in randomized clinical trials.

Implicit in the elements that comprise informed consent for subjects participating in clinical trials is that subjects will be notified of the nature of their disease. Current bioethical thinking views this to be essential in order for patients/subjects to give legally effective informed consent. The current practice in the United States is that informed consent to participate in clinical trials requires that patients be notified of their diagnosis. Accordingly, a statement regarding the diagnosis is required in consent forms for participation in clinical trials that are sponsored by national cooperative groups. The Tuskegee study on syphilis is an excellent example of the results of not informing patients

(Jones, 1993). It is of interest that other Western countries do not feel that it is necessary or even appropriate to inform patients of their diagnosis as part of the consent process.

Increased incidents in clinical trials have led to recognition of the weaknesses of informed consent procedures. Actually, the FDA has reported that such deficiencies have been the poorest area of GCP compliance for more than 12 years (FDC Reports, 2000).

23.4 DRUG FORMULATIONS AND EXIPIENTS

It should never be lost sight of that one of the major reasons for the 1938 FD&C Act was a public health disaster caused by a drug formulation mistake. In the 1930s, the Massengill Company's use of diethylene glycol in an elixir of sulfanilamide led to 105 deaths. This same disaster was, by the way, repeated in Haiti in 1995 and 1996 (O'Brien et al., 1998). Such considerations are also overlooked in clinical safety evaluations, though the history of them directly and indirectly causing problems, even to the current day, is extensive (Winek, 2000).

Preclinical animal studies are usually performed with simple formulations which are appropriate for the route investigated in the (nonhuman) species involved. While similar simple formulations or approaches (such as capsules) are also employed for first-in-man studies, as development proceeds, efforts are made to develop formulations which optimize bioavailability. This may lead to effects not seen in earlier animal (or, indeed, human) studies—a factor that should be kept in mind in both study design and interpretation.

It is essential that formulations used in clinical trials should be well characterized, including information on bioavailability wherever feasible. The formulation should be appropriate for the stage of drug development. Ideally, the supply of a formulation will be adequate to allow testing in a series of studies that examine a range of doses. During drug development, different formulations of a drug may be tested. Links between formulations established by bioequivalence studies or other means are important in interpreting clinical study results across the development program.

Safety limitations on formulations usually arise from local tissue tolerance concerns at the site of administration for drugs other than oral.

23.5 PHASE I DESIGNS

Phase I clinical trials are the first studies in which a new drug is administered to human subjects. The primary purpose of phase I studies of new drugs is to establish a safe dose and schedule of administration (O'Grady and Linet, 1990). Other purposes are to determine the types of side effects and toxicity and organ systems involved, to assess evidence for efficacy, and to

investigate basic clinical pharmacology of the drug. Not all of these goals can be met completely in any phase I trial in part because the number of patients treated is small. However, well-conducted phase I studies can achieve substantial progress toward each of these goals. Phase I trials are not synonymous with dose–response studies, but they have many characteristics in common.

The initial phase of clinical testing has the following objectives:

(a) Establish a dose–response pharmacodynamic profile by using initial doses projected to be therapeutic in humans. The dose required is predicted on the basis of blood levels found in animal screens.
(b) Determine the pharmacokinetic profile for initial titration and maintenance of steady state for chronically administered drugs.
(c) Design a safe dosage regimen for efficacy testing in adults, the pediatric, or the elderly.
(d) Estimate efficacy information necessary to make sample size determinations for phase II studies and establish adequate duration of treatment.
(e) Determine the drug interaction potential when concurrent medications are administered as well as food interaction, assess the enzyme induction potential, and assess the need for therapeutic drug monitoring during efficacy testing.
(f) Establish the requirements for the final formulation.

The initial strategy for phase I is to conduct a single-dose safety study in normal volunteers. The first trial demands close 24-h supervision in a clinical setting. Ethical considerations may, however, demand that only patients be used—for example, when evaluating an anticancer agent with predictable toxicity. A repeat-dose tolerance and pharmacokinetic study in normal or patient volunteers is then conducted for chronically administered drugs. These studies will provide the necessary safety information to support efficacy testing.

Sometimes investigators say that phase I studies are not "clinical trials" because there is no treatment comparison being made (except that frequently a placebo is employed). Such treatment comparisons are not a prerequisite for experiments. Because Phase I trials rely on investigator-controlled treatment administration and subsequent structured observations, they are clinical trials.

In the development of cytotoxic drugs in oncology, dose finding usually means establishing a maximum tolerated dose (MTD). This is the dose associated with serious but reversible side effects in a sizable proportion of patients and the one that offers the best chance for a favorable therapeutic ratio. Side effects from cytotoxic drugs tend to be serious and are referred to as toxicities.

Investigators are interested not only in the organ systems involved but also in the duration, reversibility, and probability of specific toxicities. In this setting, evidence of efficacy is usually weak or nonexistent because many patients receive what turn out to be subtherapeutic doses of the drug.

For all phase I studies, learning about basic pharmacokinetics (clinical pharmacology) is important and includes measuring drug uptake, metabolism, distribution, and elimination. This information is vital to the future development and use of the drug and is helpful in determining the relationship between blood levels and side effects, if any. These goals indicate that the major areas of concern in designing phase I trials will be selection of patients, choosing a starting dose, rules for escalating doses, and methods for determining the MTD or safe dose.

If basic pharmacology were the only goal of a phase I study, the patients might be selected from any underlying disease and without regard to functioning of specific organ systems. However, phase I studies are usually targeted for patients with the specific condition under investigation. For example, in phase I cancer trials, patients are selected from those with a disease type targeted by the new drug. Because the potential risks and benefits of the drug are unknown, patients often are those with relatively advanced disease. It is usually helpful to enroll patients with a normal cardiac, hepatic, and renal function. Because bone marrow suppression is a common side effect of cytotoxic drugs, it is usually helpful to have normal hematological function as well when testing new drugs in cancer patients. In settings other than cancer, the first patients to receive a particular drug might have less extensive disease or even be healthy volunteers.

23.5.1 First Administration: Single Dose

First-time administration of single doses of new drugs is undertaken using a wide range of study design, but essentially there are several basic designs available which are modified to meet the needs of a given study. Fundamental to all designs is that in the interests of safety successive subjects are exposed to increasing doses of the drug. The fact that doses are titrated upward either in the same subject or in groups of subjects and not randomized to remove the potential for bias can be argued as a design weakness, but there is no alternative. Nevertheless, an ordered dose response can be taken as reasonable evidence of a drug-related effect. In addition, the use of placebo, which enables studies to be conducted on either a single- or a double-blind basis, will help to minimize bias. For this reason, placebo control is an integral part of a phase I study. Unwanted feelings or sensations are common occurrences in every-day life; hence it is to be expected that adverse events will be encountered during phase I studies. Adverse events may be drug related, study related, or result from something which has nothing to do with the drug or the study. They may act singly or in combination. For example, headache, which is one of the com-

monest if not the commonest symptom reported by volunteers taking part in phase I studies, can result from any one of the following: fasting, caffeine withdrawal, feeling anxious about the study, an impending attack of influenza, or a combination of all four factors. Thus without placebo control it becomes difficult to differentiate between headache which is drug related and headache which is not drug related. But a placebo is not only of value in helping to distinguish between drug- and non-drug-related subjective effects, it also plays a role in the interpretation of results from laboratory and other safety tests and pharmacological tests which may be influenced by diverse factors such as diet, physical activity, mental state, circadian or other biological rhythms, and asymptomatic illnesses, for example, subclinical viral infections.

The different designs available for a first-in-man study all have their own advantages and disadvantages (Fleiss, 1986; Spilker, 1991; Nylen, 2000; Gallin and Ognibene, 2007; Rallin and Ognibene, 2007). At the end of the day it is up to the investigator to weigh the pros and cons of each and then to choose the design which best meets the aims of the study. In an attempt to examine their strengths and weaknesses, let us consider some designs open to an investigator who wishes to undertake a single rising-dose safety and tolerability study with a new drug. A typical protocol might require:

- Placebo control
- The dose be increased from x (first dose) to $64x$ (top dose)
- Twofold increases in successive doses (within or between subjects)
- A seven-day within-subject washout period
- A minimum of four subjects to receive each dose level

Some design options are shown in Tables 23.4–23.8, while the implications for going with one or the other in terms of subject numbers, number of clinic visits, highest first dose given to a subject, biggest increment in dose, and time to complete the study are given in Table 23.9.

TABLE 23.4 Phase I Study: Type A

	Number of Subjects Who Received Each Treatment							
Group	Placebo	x	$2x$	$4x$	$8x$	$16x$	$32x$	$64x$
1	2	3						
2	2		3					
3	2			3				
4	2				3			
5	2					3		
6	2						3	
7	2							3

Note: x = first dose.

TABLE 23.5 Phase I Study: Type B

	Number of Subjects Who Received Each Treatment							
Group	Placebo	*x*	2*x*	4*x*	8*x*	16*x*	32*x*	64*x*
1	2	3	1					
2	2		2	1				
3	2			2	1			
4	2				2	1		
5	2					2	1	
6	2						2	1
7	2							2

Note: *x* = first dose.

TABLE 23.6 Phase I Study: Type C

No. Visits[a]	Volunteer No.	Treatment			
2	1–4	*x*	2*x*	4*x*	(P)
2	5–8	4*x*	8*x*	16*x*	(P)
2	9–12	16*x*	32*x*	64*x*	(P)

Note: *x* = first dose; (P) = randomized placebo.
[a]Volunteers receive three doses of drug on one visit and placebo on the other visit.

TABLE 23.7 Phase I Study: Type D

No. Visits[a]	Volunteer No.	Treatment			
4	1–4	*x*	2*x*	4*x*	(P)
4	5–8	4*x*	8*x*	16*x*	(P)
4	9–12	16*x*	32*x*	64*x*	(P)

Note: *x* = first dose; (P) = randomized placebo.
[a]Volunteers receive single dose of drug on each of three visits and placebo on one visit.

TABLE 23.8 Phase I Study: Type E

Visit	Volunteer No.		Volunteer No.		Volunteer No.	
1	1	P	5	*x*	9	2*x*
	2	*x*	6	P	10	2*x*
	3	*x*	7	2*x*	11	P
	4	*x*	8	2*x*	12	4*x*
2	1	4*x*	5	P	9	4*x*
	2	4*x*	6	4*x*	10	P
	3	4*x*	7	4*x*	11	8*x*
	4	P	8	4*x*	12	8*x*
3	1	8*x*	5	16*x*	9	P
	2	8*x*	6	16*x*	10	16*x*
	3	P	7	16*x*	11	16*x*
	4	16*x*	8	P	12	16*x*
4	1	16*x*	5	32*x*	9	64*x*
	2	P	6	32*x*	10	64*x*
	3	32*x*	7	P	11	64*x*
	4	32*x*	8	64*x*	12	P

Note: *x* = first dose; P = placebo.

TABLE 23.9 Comparisons of Study Types

	A	B	C	D	E
No. of volunteers	12	12	12	42	42
No. of clinic visits	4	4	2	1	1
Highest first dose to subject	16*x*	8*x*	16*x*	64*x*	64*x*
Largest within-subject increment in dose	×2	×16	×2	—	—
Times to do study	9 weeks	4 weeks	3 weeks	3–6 weeks[a]	3–6 weeks[a]

[a]Dependent upon whether dosing takes place once or twice weekly.

Design types A and B require more than threefold the number of volunteers needed for the other designs but also give the clearest picture of the pharmacokinetics and tolerance of a single dose. This requirement is compounded by the fact that one-third of the volunteers will receive placebo. The number of volunteers on placebo in each group is open to the investigator's choice, but a balanced (even number of each) design again is easiest to interpret. Whichever way, types A and B require large numbers of subjects, which could present problems when recruiting suitable subjects. The situation is made more difficult when numbers on the volunteer panel are limited (which often is the case) and when one is attempting to recruit the best available volunteers who also satisfy the inclusion/exclusion criteria for a first-in-man study. It can also be argued that if the drug under test proved to be toxic, then more subjects would be exposed to its harmful effects. On the other hand, if the drug turns out to be well tolerated, it can be argued equally well that exposing a larger number of subjects is a better basis on which to proceed to the next study.

Types A and B, however, have two clear advantages over the other designs. First, as only one visit to the clinic is required, this will encourage the volunteer to take part in and complete the study. Second, they are ideal designs for drugs with long (or unclear) pharmacological, clinical, or chemical half-lives when a seven-day washout period is an inadequate time for the drug effects to disappear or for it to be cleared from the body.

In the interests of safety, the lower the dose the volunteer is given on the first exposure to the drug, the better. However, as it is impractical to start everyone off with dose x, the next best thing one can do is to keep the first dose given to a volunteer in each group as low as possible within the confines of the design of the study. In this respect, type E works best and types A and B do badly.

With types D and C, twofold increments in dose are uniformly made throughout the whole dose range. This is in contrast to type E, in which the size of dosage increments over the dose range within subjects varies between 2- and 16-fold. Thus type E might be an unwise choice for a drug anticipated to have a narrow therapeutic index or a steep dose response.

Assuming the study goes according to plan (which is often not the case in first-administration studies) and depending upon the study design used, it will take between three and nine weeks to complete. However, although type C (in which the dose is increased stepwise on the same study day) offers the advantage of speed, the fact that it can only really be used for drugs given by the intravenous route and for drugs with rapid onsets and offsets of action limits its usefulness in practice.

23.5.2 First Administration in Humans: Repeat Dose

In clinical practice, drugs are often prescribed for illnesses which require regular treatment for days, weeks, months, or years. For drugs used in this way (or for which off-label use is likely to be this way), testing on a repeat-dose basis in volunteers is required to evaluate safety and tolerability before treating patients. As with first-time single-dose studies, first-time repeat-dose studies can be undertaken using different designs but with the emphasis again on safety and tolerability. The cornerstone design is a randomized, rising-dose, placebo-controlled group comparative evaluation. Whichever design is used, the investigator has to decide upon an appropriate dosing schedule. The choice of a unit dose and dosing interval depends primarily upon the results from the single-dose study. To illustrate this point, if one assumes that the top dose (i.e., 64*x*) given in the previously described single-dose study proved to be well tolerated, then one might opt for the dosing schedule given in Table 23.10. Of course, the frequency of dosing will depend upon the pharmacodynamic and/or pharmacokinetic profile of the drug. Ideally, dosing should be continued until steady-state plasma concentrations of drug have been achieved, but this may not be practical for drugs with long half-lives. More often than not volunteers are dosed for 7–10 days, but in certain circumstances if toxicological clearance is available and there is a definite need to do so, volunteers may be dosed for 4 weeks. Even if the intent is to dose more than once daily [as in Table 23.10 where twice-daily (bid) dosing is required], giving single doses on the mornings of day 1 and the last day of dosing (i.e., day 7) offers certain advantages. For example, it allows for a longer period to assess tolerability before the second dose of drug is given to a volunteer who more than likely will not have been exposed to the drug previously. It also enables comparisons

TABLE 23.10 Design and Dosing Schedule for First Repeat-Dose Phase I Study

Group	Day 1	Day 2–6	Day 7
1	8*x*	8*x* bid	8*x*
2	16*x*	16*x* bid	16*x*
3	32*x*	32*x* bid	32*x*

Note: bid = twice daily.

to be made between drug plasma concentration–time profiles over 24 h and the elimination kinetics of the drug at the start and end of dosing.

In the interests of safety, doses are increased between groups sequentially and as a rule dosing is completed in the previous group before dosing is started in the next group. However, if groups are to be dosed for more than 7–10 days or a large number of increments in dose is planned, particularly if more than one dosing frequency is under test, the investigator might choose to overlap dosing between one group and the next, thus enabling the study to be completed in a reasonable time frame. Within each group, volunteers are randomly allocated to receive drug or placebo. The size of the groups usually varies between 6 and 12 with the numbers of subjects receiving drug and placebo in a group being subject to investigator preference.

Number of Subjects In a phase I trial, a sufficient number of subjects must be included in a study if valid conclusions are to be drawn from the results. Studies in healthy volunteers and patients are inherently flawed when it comes to assessing safety and tolerability because of the small numbers of subjects involved, and only the most guarded of conclusions are possible. It is easier to draw valid conclusions with respect to drug action involving pharmacodynamic, surrogate, or clinical endpoints because one is able to specify before hand the magnitude of the difference which constitutes a useful drug effect and thus calculate the numbers of studies, except that regulatory authorities rather than the investigator specify the criteria which have to be met to enable different formulations to be judged bioequivalent.

23.5.3 Route of Administration

Just as new drugs must be tested in animals by the route to be used in humans, so must they be tested in volunteers using the intended route for patients. But there are clear benefits in testing all drugs when going into humans for the first time using intravenous infusions, even if systemic exposure in patients will be achieved by another route. These benefits relate primarily to the fact that intravenous infusion allows for precise control of drug administration:

- In the event of a serious or otherwise distressing adverse event during the infusion, drug delivery can be halted.
- As the drug is delivered directly into the bloodstream, this ensures 100% exposure and overcomes problems relating to bioavailability which may occur with other routes but in particular dosing by the oral route when the drug may be destroyed in the gastrointestinal (GI) tract or metabolized presystemically in the gut wall or in the liver.
- Delivery of the full dose into the bloodstream coupled with a uniform delivery rate results in less variability in plasma or tissue concentrations of drug than is possible using oral dosing, where not only the extent but also the rate of absorption from the GI tract can vary considerably

between subjects. Less intersubject variability in plasma concentrations of drug in turn enables the study to be done using smaller numbers of subjects and also offers advantages for drugs it is anticipated might have narrow therapeutic ratios.

- Intravenous dosing allows the true disposition kinetics of the drug to be evaluated and makes the assessment of PK/PD relationships easier to perform.
- Pharmacokinetic scaling between species, that is, animals to humans, is made simpler as fewer assumptions need to be made about extent and rate of exposure in humans. This in turn helps in further dose selection for human studies.
- Blinding of studies is made easier when intravenous dosing is used, that is, there is no need to produce matching placebos while intravenous dosing overcomes any problems relating to taste, which can make it difficult in blind studies involving oral dosing.

The primary disadvantage of using intravenous dosing for first-in-human studies is that additional resource will be needed to be spent in toxicology, establishing dosage form stability, and mutation development on a drug which might fail at the first hurdle in humans, as indeed many do. For this reason investigators often prefer to administer drugs for the first time in healthy volunteers using the route to be used in patients and dose intravenously to establish the drugs' pharmacokinetic profile only when they feel reasonably certain that it is likely to be a candidate for further development.

23.6 CLINICAL TRIAL SAFETY INDICATORS

One major purpose of preclinical (animal) toxicity studies of a potential new drug is to identify the toxic effects which most commonly occur at doses nearest to those to be used in humans. These observations serve to help ensure that care is taken to detect any such effects in humans. Additionally, a broad range of other indicators of adverse drug action may be identified to ensure that their occurrence is looked for. These are also commonly called safety parameters.

Because of the relatively small numbers of volunteers and patients involved, only the most common of drug-related adverse events are likely to be detected during early studies (O'Grady and Joubert, 1997). For example, to have a 95% chance of picking up three subjects who have experienced an adverse reaction (with no background incidence) which occurs in 1 in every 100 subjects treated with the drug, it would need to be given to 650 subjects. Matters are made worse when the adverse event in question also occurs in the general population, which is usually the case with the kind of symptoms reported by volunteers and patients taking part in drug studies. No matter how good the

study design is, nothing can compensate for this problem of inadequate numbers. In this respect, all of the study designs described earlier are more or less equally adequate or inadequate as the case may be.

Monitoring for drug-related adverse events employs the same or similar methods in both volunteers and patients. In both cases assessments of tolerability and safety are based upon symptom reports, routine laboratory safety screens, electrocardiogram (ECG) monitoring, and on occasion special tests designed to detect unwanted effects associated with a particular class of drug. The chances of obtaining reliable information on a drug's safety profile are enhanced by detailed and careful monitoring, such as special biomarkers for cardiotoxicity (Braunwald, 2008). Symptoms may be reported spontaneously or elicited in reply to standard questions. Open questions such as "how are you feeling?" are to be preferred to leading questions on the basis that they result in fewer reports of adverse events. If leading questions are used, they need to be carefully worded. A certain amount of basic information is required on all adverse events, that is, type, severity, time of onset in relation to time of dosing, duration, and causality. Attributing the cause of an unwanted effect to the drug or some other factor can be difficult, particularly when little is known about the drug, as is often the case at the state of initial studies in volunteers or patients. Rechallenge with the drug ideally using the same dose or, if need be (because the event caused a degree of discomfort), a reduced dose is probably the single best way of proving or disproving a causal relationship. But if done the rechallenge procedure must be designed using placebo as the comparator under double-blind conditions. Obviously rechallenges can be done only if the adverse event was reversible, did not cause excessive discomfort, and most importantly was not life threatening. The question of assessing attributions or causality is considered in detail later in this chapter.

23.6.1 Overall Approach to Assessing Safety

Choosing Safety Parameters Choosing the appropriate safety parameters for a clinical trial depends on a number of factors. A selected list of examinations and tests commonly used to assess the safety of medicines is given in Table 23.11. The majority of these tests will not be conducted in most drug trials. An assessment of the quantity and quality of prior experience and previous data obtained with the therapeutic is essential to enable one to decide which specific safety tests to incorporate in a medicine trials. The choice of safety parameters requires both data in areas where there are indications of potential (or actual) safety problems to monitor and additional experience and data with a new drug. Until a sufficient body of safety data has accumulated, more laboratory parameters of safety are generally included than will be needed at a later date. The nature of the clinical trials and efficacy tests used may dictate that certain safety parameters should or should not be included (e.g., in testing a new anticancer medicine, it may be necessary to perform a bone marrow biopsy and smear to confirm the lack of toxicity, and

TABLE 23.11 Selected List of Examinations and Tests Used to Evaluate Safety

A. Clinical examinations
   1. Physical
   2. Vital signs (usually considered part of physical examination)
   3. Height and weight (state of dress is usually specified, e.g., socks)
   4. Neurological or other specialized clinical examinations

B. Clinical Laboratory examinations
   1. Hematology (see Table 23.15)
   2. Clinical chemistry (see Table 23.14)
   3. Urinalysis (see Table 23.15)
   4. Virology (viral cultures or viral serology)
   5. Immunology or immunochemistry (e.g., immunoglobulins, complement)
   6. Serology
   7. Microbiology (including bacteriology and mycology)
   8. Parasitology (e.g., stool for ova and protozoa)
   9. Pulmonary function tests (e.g., arterial blood gas)
   10. Other biological tests (e.g., endocrine, toxicology screen)
   11. Stool for occult blood (specify hemoccult or guaiac method)
   12. Skin tests for immunological competence
   13. Medicine screen (usually in urine) for detection of illegal or non-protocol-approved medicines
   14. Bone marrow examination
   15. Gonadal function (e.g., sperm count, sperm motility)
   16. Genetic studies (e.g., evaluate chromosomal integrity)
   17. Stool analysis using in vivo dialysis

C. Probe for adverse reactions

D. Psychological and psychiatric tests and examinations
   1. Psychometric and performance examinations
   2. Behavioral rating scales
   3. Dependence liability

E. Examinations requiring specialized equipment (selected examples)
   1. Audiometry
   2. Electrocardiogram (ECG)
   3. Electroencephalogram (EEG)
   4. Electromyography (EMG)
   5. Stress test
   6. Endoscopy
   7. Computed tomography (CT) scans
   8. Ophthalmological examination
   9. Ultrasound
   10. X-rays
   11. Others

in assessing an agent in anesthetized patients, the appropriate tests to ensure the patient's safety while under anesthesia must be performed). If, on the basis of preclinical pharmacological or toxicological data, any toxicity is either anticipated or considered possible, then an attempt should be made to evaluate patients for those possible problems. The anticipated use(s) of a therapeutic will also influence which safety parameters are chosen for evaluation (e.g., ophthalmological tests would be included for drugs intended for ocular use).

Measuring Safety Parameters After specific safety parameters are chosen, it is necessary to determine how thorough an evaluation of each parameter should be conducted. It is also possible that different types of examinations would be suitable at different points of a clinical trial. For example, a physical examination may be specified to include more or fewer measurements or facets, and a complete examination may not be necessary or even suitable during some periods of clinical trial.

Vital signs may be measured with the patient in a supine, seated, and/or erect position. Both supine and erect positions are usually used if orthostatic changes are being evaluated. The need for such data will depend on the situation, but the position of the patients for this examination, as well as the period of time desired for stabilization, should be noted in the protocol.

Parameters That Measure Either Safety or Efficacy Certain parameters may, of course, be either safety or efficacy parameters or both. The electroencephalogram (EEG) is an example. Blood pressure is another. It is thus important to clearly establish in the protocol whether each parameter is begin incorporated in the protocol for safety or efficacy evaluation. Almost any safety parameter can be used for measuring efficacy.

Appropriateness of Each Parameter for Clinical Trial and Patient There are four categories of appropriateness of safety tests used in clinical trials:

1. Appropriate for patients but not necessary for the clinical trial. All of these tests should be included in the clinical trial. They indirectly benefit the trial because they may be monitored for progress or trends or they may simply ensure that patients are receiving appropriate care.
2. Appropriate for the clinical trial but not necessary for the patients. These tests should be included in the clinical trial if they do not place the patient at unacceptable risk or discomfort. If any tests are deemed unethical in the context of the trials and the patients enrolled, then they should be excluded.
3. Appropriate for both patients and the clinical trial. All of these tests should be included in the clinical trial.
4. Appropriate for neither patients nor the clinical trial. All of these tests should be identified and excluded from the clinical trial.

23.6.2 Precautions

Clinical laboratory parameters must be specified individually in the protocol. Abbreviations such as SMA-6 or SMA-12 are not acceptable, as different laboratories include different tests in their SMA-6 (or SMA-12) battery, and using these abbreviations without an explanation can adversely affect the clarity of the protocol and possibly lead to the collection of data on divergent

parameters at different sites. Other precautions to consider prior to initiating a clinical trial are to decide if (1) severely abnormal results should be routinely confirmed, (2) samples should be divided and sent to two separate laboratories when specified abnormalities are determined, (3) additional tests should be routinely requested if specified abnormalities are observed, (4) medical consultants should examine patients whenever severe abnormalities are observed, and (5) aliquots of known concentrations of standard drugs should be sent to laboratories for confirmatory measurements and interlaboratory evaluation.

Summary of Tests Common dermatological tests are shown in Table 23.12 and ophthalmological tests in Table 23.13. Note that any of these tests could be utilized as measures of efficacy if they addressed the clinical trial objectives. Selected pointers are given in Table 23.14. Specific tests that may be used in hematology, clinical chemistry, and urinalysis are shown in Table 23.15, adult and pediatric behavioral rating scales in Tables 23.16 and 23.17, and psychometric and performance tests in Table 23.18.

Choosing Laboratory Tests There is no standardized series of laboratory parameters that are evaluated in all clinical trials, nor is there a single standard for drugs in phases I, II, or III. There are, however, broad general guidelines for laboratory tests that are performed at each stage of clinical development.

Tests in Phase I In Phase I clinical trials, there is the greatest need to obtain a wide variety of laboratory evaluations as part of developing the safety profile on a new medicine. This entails an evaluation of the basic hematology, clinical chemistry, and urinalysis parameters (Table 23.15). There will never be 100% agreement among investigators and/or clinical scientists as to which specific tests constitute a "basic" workup.

TABLE 23.12 Deleterious Effects of Biomarkers of Inflammation in Heart Failure

Known
Left ventricular dysfunction
Pulmonary edema
Cardiomyophathy
Decreased skeletal-muscle blood flow
Endothelial dysfunction
Anorexia and cachexia
Potential[a]
Receptor uncoupling from adenylate cyclase
Activation of the fetal-gene program
Apoptosis of cardiac myocytes

[a]Effects shown in animals but not yet in humans.

Source: Adapted from Mann.

TABLE 23.13 Biomarkers in Heart Failure

Inflammation[a,b,c]
C-reactive protein
Tumor necrosis factor α
Fas (APO-1)
Interleukins 1, 6, and 18
Oxidative stress[a,b,d]
Oxidized low-density lipoproteins
Myeloperoxidase
Urinary biopyrrins
Urinary and plasma isoprostanes
Plasma malondialdehyde
Extracellular matrix remodeling
Matrix metalloproteinases
Tissue inhibitors of metalloproteinases
Collagen propeptides
Propeptide procollagen type I
Plasma procollagen type III
Neurohormones[a,b,d]
Norepinephrine
Renin
Angiotensin II
Aldosterone
Arginine vasopressin
Endothelin
Myocyte injury[a,b,d]
Cardiac-specific troponins I and T
Myosin light-chain kinase I
Heart-type fatty acid protein
Creatin kinase MB fraction
Myocyte stress[b,c,d,e]
Brain natriuretic peptide
N-terminal probrain natriuretic peptide
Midregional fragment of proadrenomedullin
ST2
New biomarkers[b]
Chromogranin
Galectin 3
Osteoprotegerin
Adiponectin
Growth differentiation factor 15

[a]Biomarkers in this category aid in elucidating the parthenogenesis of heart failure.
[b]Biomarkers in this category provide prognostic information and enhance risk stratification.
[c]Biomarkers in this category can be used to identify subjects at risk for heart failure.
[d]Biomarkers in this category are potential targets of therapy.
[e]Biomarkers in this category are useful in the diagnosis of heart failure and in monitoring therapy.

Source: Adapted from Braunwald, 2008.

TABLE 23.14 Selected Considerations Pertaining to Laboratory Data

1. Ask the laboratory to maintain assayed samples that are of particular importance; if questions arise as to the accuracy of results, it might be possible to retest the original samples.
2. If laboratory problems are anticipated, divide the initial (and subsequent) samples and send them to two different laboratories or to the same laboratory at two different times.
3. If laboratory samples for a complete blood count are going to remain unexamined for a long period of time (e.g., sample obtained on Sunday), prepare a fresh smear so that a comparison may be made with one made 24 or more hours later because abnormalities may occur when a sample lies around even when it is kept at an appropriate temperature.

TABLE 23.15 Hematology, Clinical Chemistry, and Urinalysis Parameters Usually Evaluated During Development of New Therapeutic Agent

A. Hematology
   1. Red blood cell (RBC) count
   2. Hemoglobin
   3. Hematocrit
   4. White blood cell (WBC) count and differential
   5. Platelet estimate or platelet count
   6. Red blood cell indices (MCV, MCH, MCHC)[a]
   7. Prothrombin (PT) and partial thromboplastin (PTT) times
   8. Reticulocytes
   9. Fibrinogen
   10. Any additional tests suggested by previous data

B. Clinical chemistry
   1. Albumin
   2. Albumin/globulin ratio
   3. Alkaline phosphatase (and/or its isoenzymes)
   4. Amylase
   5. Bilirubin, total and direct
   6. Bicarbonate (carbon dioxide)
   7. BUN/creatinine ratio
   8. Calcium
   9. Chloride
   10. Cholesterol (and/or a lipid panel)
   11. Creatinine
   12. Creatine phosphokinase (CPK)
   13. γ-Glutamyl transferase (GGT)
   14. Globulin
   15. Glucose, nonfasting or fasting
   16. Glucose-6-phosphate dehydrogenase (G6PD)
   17. Glutamate oxaloacetic transaminase (SGOT), now frequently referred to as aspartate aminotransferase (AST)
   18. Glutamate pyruvate transaminase (SGPT), now frequently referred to as alanine aminotransferase (ALT)
   19. Iron (and/or other related parameters such as ferritin, total iron-binding capacity)
   20. Lactic acid dehydrogenase, total (LDH, and/or its isoenzymes)
   21. Inorganic phosphorus
   22. Potassium
   23. Sodium
   24. Total iron-binding capacity
   25. Total protein
   26. Triglycerides
   27. Urea nitrogen (BUN)
   28. Uric acid

TABLE 23.15 ***Continued***

C. Hormones and/or other chemical substances in blood
D. Urinalysis[b]
    1. Appearance and color
    2. Specific gravity
    3. Acetone
    4. Protein
    5. Glucose
    6. PH
    7. Bile
    8. Irobilinogen
    9. Occult blood
    10. Microscopic evaluation of sediment
        a. Red blood cells (number per high-power field)
        b. White blood cells (number per high-power field)
        c. Casts (describe and give number per high- or low-power field)
        d. Crystals (describe and give number per high-power field)
        e. Bacteria (generally rated as few, many, or loaded)
        f. Epithelial cells (number per low-power field)
E. Other urine tests sometimes evaluated
    1. Creatinine (actual values are preferable to estimated values)
    2. Electrolytes (usually sodium, potassium, and chloride)
    3. Protein
    4. Specific hormones or chemicals
    5. 24-h collections for specific evaluations

[a]MCH, mean corpuscular hemoglobin = hemoglobin divided by RBC count; MCHC, mean corpuscular hemoglobin concentration = hemoglobin divided by hematocrit; MCV, mean corpuscular volume = hematocrit divided by RCB count.
[b]Sample codes used to quantify several parameters in the urinalysis are the following. Protein, glucose, ketones, bilirubin: 0, none or negative; 0.5, trace or positive (qualitative); 1, + or 1+; 2, ++ or 2+; 3, +++ or 3+; 4, ++++ or 4+. Epithelial cells, crystal, WBC, RBC, casts: 0, none or negative; 0.5, rare, occasional, few present, trace (1–5); 1, several, mild (6–10); 2, moderate (11–25); 3, many, much (26–50); 4, loaded, severe (>50). Bacteria: 0, none or negative; 0.5, rare, trace, occasional, few several (1–10); 1, mild (11–50); 2, moderate (51–75); 3, many, numerous (76–100); 4, loaded, severe (>100).

TABLE 23.16 Procedures and Tests Performed in Ophthalmological Examination

1. Ophthalmological history (attention is paid to patient family history plus patient's diseases and drug reactions
2. Visual acuity corrected (i.e., with glasses present)
3. External ocular examination (i.e., check for inflammation, ptosis, nystagmus, tearing, proptosis, and other abnormalities)
4. Extraocular muscle testing
5. Pupil size and evaluation (in darkened room with controlled illumination)
6. Slit-lamp biomicroscopy (with dilated pupils)
7. Tonometry (ocular pressure)
8. Ophthalmoscopy with fundus photographs
9. Visual field testing and color vision testing
10. Goniscopy[a]
11. Lacrimation[a] (Schirmer test)

[a]These tests are of minimal value in determining ocular toxicity and are not recommended for routine use in ophthalmological examination to detect drug toxicities.

TABLE 23.17 Selected Examples of Safety Measurements and Tests for Specialized Dermatological Examination

1. Biopsy
2. Erythema at site of lesion
3. Absorption of medications systemically (e.g., blood levels)
4. Signs and symptoms of absorption
5. Interactions with standard treatment (e.g., ultraviolet light)

TABLE 23.18 Adult Behavioral Rating Scales

	Scale Rated By		
Scale	Professional		Subject
1. Anxiety Status Inventory (ASI)	X		
2. Beck Depression Inventory (Beck)			X
3. Brief Psychiatric Rating Scale (BPRS)	X		
4. Carroll Depression Scale			X
5. Clinical Global Impression (CGI)	X	or	X
6. Clyde Mood Scale	X		
7. Covi Anxiety Scale	X		
8. Crichton Geriatric Rating Scale	X		
9. Depression Status Inventory	X		
10. Hamilton Anxiety Scale (HAMA)	X		
11. Hamilton Depression Scale (HAMD)	X		
12. Hopkin Symptom Checklist (HSCL)			X
13. Inpatient Multidimensional Psychiatric Scale (IMPS)	X		
14. Nurses Observation Scale for Inpatient Evaluation (NOSIE)	X		
15. Plutchik Geriatric Rating Scale (PLUT)	X		
16. Profile of Mood States (POMS)			X
17. Sandoz Clinical Assessment—Geriatric	X		
18. Self-Report Symptom Inventory (SCL-90)			X
19. Wittenborn Psychiatric Rating Scale (WITT)	X		
20. Zung Self-Rating Anxiety Scale (SAS)			X
21. Zung Self-Rating Depression Scale (SDS)			X

Note: Standard abbreviations are used (see Guy, 1976). Additional tests are described in Buros (1978).

Tests in Later Phases The total number of normal laboratory values that is sufficient to collect on a new drug to demonstrate safety is impossible to specify. Numerous factors must be considered, such as the toxicological profile on other safety parameters and the expected use of the drug in patients. It is important to determine if a therapeutic agent is to be used topically or parenterally, whether it is to be used in generally healthy patients or in seriously ill patients, whether it is a "me-too" drug or a totally novel drug chemically, and whether it will be life saving or provide a minimal therapeutic effect. The number of laboratory tests performed usually decreases as an investigational drug moves closer to the market, but one or more tests may be added to the list in Table 23.15 and studied in great detail.

TABLE 23.19 Pediatric Behavioral Rating and Diagnostic Scales

1. Children's Behavior Inventory (CBI)
2. Children's Diagnostic Classification (CDC)
3. Children's Diagnostic Scale (CDS)
4. Children's Psychiatric Rating Scale (CPRS)
5. Clinical Global Impression (CGI)
6. Conners Parent Questionnaire (PO)
7. Conners Parent–Teacher Questionnaire (PTO)
8. Conners Teacher Questionnaire (TO)
9. Devereux Child Behavior Rating Scale
10. Devereux Elementary School Behavior Rating Scale
11. Dosage Record and Treatment Emergent Symptoms (DOTES)
12. Stereotyped Behavior in Retarded

TABLE 23.20 Psychometric and Performance Tests

	For Use In	
Test	Adults	Children
1. Bender–Gestalt Test	X	X
2. Conceptual Clustering Memory Test	X	X
3. Digital Symbol Substitution Test	X	X
4. Embedded Figures Test	X	X
5. Frostig Development Test of Visual Perception		X
6. Goodenough–Harris Figure-Drawing Test (GOOD)		X
7. Peabody Picture Vocabulary Test		X
8. Porteus Mazes	X	X
9. Reaction Time	X	X
10. Vigilance Tests	X	X
11. Wechsler Adult Intelligence Scale (WAIS)	X	
12. Wechsler Intelligence Scale for Children (WISC)		X
13. Wechsler Memory Scale (WMEN)	X	X
14. Wide Range Achievement Test (WRAT)		X

Note: Additional tests are described in Buros (1978).

Tests in Medical Practice The ordering of laboratory tests in medical practice (as opposed to phase I clinical trials) is extremely inefficient and often irrational. This suggests the need in some clinical situations to develop logical protocols and algorithms for physicians to follow in ordering tests, particularly when the technology is changing (e.g., hepatitis), in therapeutic areas in which an excessive number of tests are often ordered (e.g., thyroid tests), or when hospitals have developed their own approaches to diagnosis (e.g., use of cardiac isoenzymes in diagnosing a myocardial infarction).

Less Commonly Used Methods Evaluations of virtually any biological fluid, tissue, or sense (taste, smell, hearing, sight, and touch) can be conducted to ascertain the safety of a drug (several have been reported to affect taste in some patients, and there are many other examples involving medicine-induced

effects on one of the other senses). The choice of tests will depend on experience with the medicine and suspicions about possible problems. Drugs should also be reviewed for teratogenic potential, drug dependence, liability, and carcinogenicity.

Identifying Most Important Laboratory Analytes to Monitor in Clinical Trial A choice often must be made among the numerous laboratory analytes that could be measured in a clinical trial. This choice is based on (1) past experience with the treatment(s) being evaluated, (2) therapeutic claim, (3) cost of the tests, (4) convenience of obtaining samples, (5) resources available, (6) state-of-the-art concept of the data's importance, and (7) the ability of data obtained to convince both regulators and medical practitioners. Arriving at a decision given these and other previously discussed factors may be difficult.

Uses of Specific Laboratory Tests to Discover, Confirm, and/or Exclude a Disease Some tests can confirm the diagnosis of a disease (e.g., tissue histology from a bronchoscopic biopsy to confirm lung cancer) but cannot be used to exclude the disease or discover the disease in routine screening. Other tests can be used both to confirm and to exclude the diagnosis of a disease (e.g., glucose tolerance test for diabetes mellitus) but are too inconvenient to be used to discover the disease in routine screening. The uses of each laboratory test to discover, confirm, or exclude a disease should be considered before a test is simply added to a clinical trial protocol. This ensures that the test is appropriate in the context of the planned clinical trial.

Hematology A basis hematology evaluation usually includes determination of hemoglobin, hematocrit, red blood cell (RBC) count, white blood cell (WBC) count, RBC indices [mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and mean corpuscular volume (MCV)], and platelet count. The WBC differential count is usually not required as part of a basic hematological workup unless a specific parameter of the differential count is being evaluated. Nonetheless, a WBC differential count is often obtained in phase I and generally provides useful (though often negative) information. Other hematological parameters (some of which are indicated in Table 23.15) are not usually obtained unless there is a specific reason to do so.

23.6.3 Clinical Chemistry

A measurement of renal function [creatinine and/or blood urea nitrogen (BUN)] is an "essential" test for most clinical studies, as is the inclusion of a panel of liver function tests [serum glutamic–oxaloacetic acid transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), lactic dehydrogenase (LDH), creatine phosphokinase (CPK), gamma-glutamyl transferase (GGT),

and/or alkaline phosphatase]. The specific tests chosen to be included in a study are somewhat dependent on both the investigator's and/or clinical scientist's experiences and the characteristics of the drug. Other important parameters to measure include serum electrolytes and at least some of the tests listed in Table 23.15.

Drug Levels in Plasma Drug levels may also be measured in a clinical trial. Such levels are usually part of a pharmacokinetic analysis but also provide important safety data. This information would be particularly relevant in cases of suspected or actual drug overdosage or drug interactions, to correlate medicine levels with toxic events, or in other situations. It must be clarified whether free levels of the drug and/or the protein bound will be measured by the laboratory.

Total Blood That May be Taken from Patients The total amount of blood that may be taken from a subject in most therapeutic trials should be limited to one unit (about 460 mL) per eight-week period.

23.6.4 Urinalysis

Most clinical laboratories have established a standard battery of tests that includes most or all of the basic parameters listed in Table 23.15. If a dipstick is used to test the urine for several parameters, it is useful to use one that measures occult blood, even if a microscopic examination will count the number of RBCs per high-power field. The means of obtaining the specimen should be indicated (i.e., normal voiding sample, clean catch, midstream, catheterization, suprapubic tap, or cytoscopy), especially in clinical trials in which an antidiuretic or antibiotic (or other relevant drug) is being tested.

It is usually unnecessary to obtain a microscopic examination on all urinalyses unless there are reasons to believe that important information and data may be lost. This is particularly true after it has been demonstrated that the test treatment does not affect the parameters measured in the microscopic evaluation of urine.

23.6.5 Urine Screens

A urine screen can be used to confirm generally that patients being screened or entering the baseline period of the clinical trial are not using agents (legal or otherwise) contraindicated in the protocol. It can also be used on a scheduled or random basis during the study to confirm that patients are not using such agents. The urine screen is limited in that it is unable to detect positive compliance with the protocol and only measures certain aspects of compliance failure. If a urine test will be conducted at unannounced times in the clinical trial, then this point must be mentioned in the informed consent.

The number of agents tested in the urine screen is generally determined individually for each clinical trial, since there is a wide variety of possible drugs

that may be measured. The choice of drugs to screen will be based on their relative importance for the trial plus the cost and reliability of the methodology. Results of urine screens are usually best viewed in qualitative (i.e., present or absent) rather than quantitative terms. The identification of specific drugs in a patient's urine may help in explaining unusual adverse reactions, laboratory abnormalities, or other events. Urine screens may detect the presence of the therapeutic under study. If the urine screen is able to detect the presence of the study drug, and this is reported as an unknown drug that is present or as a false-positive for another drug, then it could essentially unblind a double-blind clinical trial. To prevent this situation from occurring, data from urine screens may be reported to a nonblinded monitor rather than to the investigator. If a sample of the study drug is put in urine at a physiological concentration and sent to the laboratory, the possibility of cross-reactivity with known agents may be assessed prior to initiation of the trial.

Type of Container to be Used The specific type of container used to collect blood or urine samples is sometimes indicated in a protocol, especially if a special anticoagulant or additive is required or if other specific conditions of sample collection and handling are required. It is generally not necessary to provide this information for commonly requested laboratory tests.

23.6.6 Use of International System Units

Although the international system of laboratory analyte units is almost universally agreed upon, many people in the United States resist using it. Typically, these are physicians (and others) who desire to retain the system with which they were trained, which makes more sense to them.

23.6.7 Identifying New Diagnostic Laboratory Tests

Numerous laboratory tests are periodically performed as aids in the diagnosis of disease states. The standards that must be met before a new test is accepted are extremely high, particularly in terms of calculated rates of false-positive and false-negative results. A five-step process is proposed leading up to acceptance of a new diagnostic test.

23.6.8 Ophthalmological Examination

Various parts of the ophthalmological examination are shown in Table 23.13. The most important common ophthalmological test to evaluate patients for the occurrence of chronic drug-induced toxicity is slit-lamp examination. Specific types of drugs with known potential for ocular toxicity may require that special attention be directed to other evaluations shown in Table 23.13. Most drugs that are to be taken systemically require at least some evaluation of ocular safety prior to approval for marketing.

23.6.9 Dermatological Examinations

A few selected safety measurements and tests for specialized dermatological examination are listed in Table 23.12.

In evaluating the safety of drugs using laboratory or other tests, it is important to develop data that help establish the nature and magnitude of any issue or problem (real or potential) that arises with abnormal laboratory data. Data obtained must also measure the strength of the association between the drug and the event noted or of the serial trends that are observed. While this information is being collected, the definitive courses of action in dealing with the issue or problem can be developed and evaluated. These countermeasures may take the form of (1) periodic monitoring [i.e., prothrombin (PT) or partial thromboplastin (PTT) times for patients receiving anticoagulants], (2) cessation of medicine treatment, (3) decreasing the dose or changing the dose schedule, (4) initiating countertreatment, (5) specific antidotes that may be used to counter or reverse medicine effects, (6) increasing surveillance of the patient, or (7) various other alternatives.

23.6.10 Deaths in Clinical Trials

Certain ADRs may be sufficiently alarming so as to require very rapid notification to regulators in countries where the medicinal product or indication, formulation, or population for the medicinal product is still not approved for marketing because such reports may lead to consideration of suspension of or other limitations to a clinical investigation program. Fatal or life-threatening, unexpected ADRs occurring in clinical investigations qualify for very rapid reporting. Regulatory agencies should be notified (e.g., by telephone, by facsimile transmission, or in writing) as soon as possible, but no later than seven calendar days after first knowledge by the sponsor that a case qualifies, followed by a report that is as complete as possible within eight additional calendar days. This report must include an assessment of the importance and implication of the findings, including relevant previous experience with the same or similar medicinal products.

Determining the cause of death in clinical trials is extremely important, but this goal is often difficult or impossible to achieve. Investigators should be prepared to present reasons to family members to convince them of the importance of conducting an autopsy. Such an autopsy should include examination of the brain, whenever possible.

Any history of drug or alcohol abuse by a patient should trigger a request for appropriate blood and urine tests. Blood samples should always be taken to assess the levels of study drugs and any concomitant agents used. The drug containers should always be analyzed to confirm their contents. This usually entails sending these drugs to their manufacturer.

The circumstances surrounding the patients' death should be as well documented as possible, including a description of all possible influences of the

clinical trial procedures on the death, even influences that are clearly independent of the medicine(s) being tested. Even procedures in a clinical trial apparently unrelated to a patient's death may have contributed to the death in some way. For example, these procedures could include (1) the requirement for excessive physical exertion, (2) prolonged periods of psychologically difficult testing that lead to extreme fatigue, or (3) giving patients many (e.g., 30) large capsules to ingest per day that lead to choking or aspiration.

Evaluation of the data surrounding the death by physicians who are unassociated with the clinical trial lends additional credibility to the report and conclusions. Physician biases probably will strongly influence their decision regarding the association of a patient's death with the clinical trial, and this factor must be considered in interpreting their report. This is particularly true for developing survival curves in cancer or other often fatal diseases, when deaths unrelated to the disease or to the treatment are excluded from the analysis.

23.6.11 Behavioral Rating Scales, Performance, Personality, and Disability Tests

A number of behavioral rating scales and psychometric and performance tests, listed in Tables 23.18–23.20, are briefly summarized below, since many of these scales and tests may be used to evaluate safety as well as efficacy. The following comments on the tests provide only a few highlights; readers interested in more details are advised to obtain additional information before choosing the tests that appear most relevant to their particular protocol.

These scales may be used either as part of a clinical trial or as major endpoints in an efficacy trial. Here they are described as a means of obtaining ancillary data on psychological factors in a clinical trial. If these scales are used to demonstrate efficacy, it is mandatory to include only those scales known to be valid.

Unless otherwise noted, all of the adult and children's behavioral scales are given once pretreatment and at least once posttreatment (depending on the trial design, subject drug pharmacokinetics, and length of the trials). Investigators may schedule additional evaluations with these tests, but this is usually not done at less than weekly or biweekly intervals. Many tests provide data on both a total score and subtest (factor) scores. The times given to complete tests are subject to significant variation depending on the anxiety and characteristics of the patient and/or the experience of the professional. The times listed do not include either scoring or preliminary and/or necessary observations of the patient.

23.6.12 Adult Behavioral Rating Scales

Anxiety Status Inventory The Anxiety Status Inventory (ASI) scale is the professional-rated version of the Zung Self-Rating Anxiety Scale (SAS). Both

tests (ASI and SAS) contain 20 items, each with a four-point scale, and are designed for use in adults diagnosed as having anxiety neurosis. Both assess anxiety as a clinical disorder rather than a "feeling state." The tests rate either the present time or the average status of the patient during the week preceding the evaluation. The ASI takes up to 15–20 min to complete and gives two scores: state anxiety and trait anxiety.

Beck Depression Inventory The Beck Depression Inventory (Beck) test may be used to measure the depth of depression as a rapid screen for depressed patients. It is a self-rating scale of 21 items (13 in a shortened form), with each item rated on a four-point scale. It measures the immediate present and has been used in antidepressant medicine trials. The original 21-item scale can be completed in about 10 min and the test is able to discriminate between anxiety and depression. No subtests are present in the Beck.

Brief Psychiatric Rating Scale The Brief Psychiatric Rating Scale (BPRS) is used primarily in adult inpatients to evaluate treatment response in medicine trials and in nonmedicine clinical treatment, but it is also used in some outpatient trials. Abbreviated instructions are printed on the form. Ratings are based on observations of patients. Originally developed for psychopharmacological research, this test contains 18 symptoms, each rated on a seven-point severity scale. It requires approximately 20 min to complete and rates the period of time since the last test. If the test is being used for the first time, it rates the previous week. Five separate subscales are obtained: anxiety–depression, anergia, thought disturbance, activation, and hostility–suspiciousness.

Carroll Rating Scale for Depression The Carroll Rating Scale for depression (52-item self-rating scale) is scored with yes or no answers by patients. It was designed to match closely the information content and specific items included in the Hamilton rating scale. It has been validated by comparisons with both the Hamilton Depression Scale (HAMD) and Beck and requires approximately 20 min to complete. Seventeen components of depression are measured.

Clinical Global Impressions Although the *ECDEU Assessment Manual for Psychopharmacology* (Guy, 1976) provides a formal test for the Clinical Global Impression (CGI) Scale, numerous investigators have modified the three major questions as well as the scales used in order to fit this test to their own clinical trials. The three questions, which may be applied in almost all phase II and III clinical trials, are:

1. *Severity of Illness* "Considering your total clinical experience with this particular population, how mentally ill [the investigator may substitute a more appropriate term if this is not applicable] is the patient at this time?"

2. *Global Improvement* "Rate total improvement, whether or not in your judgment it is due entirely to medicine treatment."
3. *Efficacy Index* "Rate this item on the basis of medicine effect only." This utilizes a rating of both efficacy and adverse reactions and divides the efficacy score by the adverse reaction score to form a ratio (*efficacy index*).

Severity of illness is the only one of these three that is rated pretreatment. All three questions may be rated posttreatment, and additional ratings are possible during a clinical trial. The CGI measure, which is widely used in all types of medicine trials, is generally well accepted.

A scale of two to nine gradations is usually used for questions 1 and 2, although five or so gradations are probably most common. A typical five-point scale for question 2 would be that the patient is rated as 1 (much worse), 2 (minimally worse), 3 (unchanged), 4 (minimally improved), or 5 (markedly improved).

Clyde Mood Scale The Clyde Mood Scale test may be used as either a self-rated or observer-rated scale. It contains 48 items to measure mood and has been shown to be sensitive to medicine effects. The test takes 5–15 min to complete and measures the immediate present in a patient or normal individual. The test gives six scores: friendly, aggressive, clear thinking, sleepy, unhappy, and dizzy.

Covi Anxiety Scale The Covi Anxiety Scale is a global observer's rating scale of patient anxiety. There were three items that are each rated on a 0–5 scale. The test is simple to use and requires only a few minutes to complete.

Crichton Geriatric Rating Scale The Crichton Geriatric Rating Scale test measures the level of behavioral function in elderly psychiatric patients using a five-point scale on 11 items. It rates either the present or the period within the last week and takes 5–10 min to complete.

Depression Status Inventory The Depression Status Inventory (DSI) scale is the professional's version of the Zung Self-Rating Depression Scale (SDS). Each of the two scales (DSI and SDS) consists of the same 20 items rated on a four-point scale and is applied to adults with depressive symptomatology. The DSI is completed by the professional, and the SDS is completed by the patient. Both tests take about 5–10 min to complete. The DSI rates either the present situation or the situation for the last week prior to the test, and a total score is obtained.

Hamilton Anxiety Scale The Hamilton Anxiety (HAMA) scale was designed to be used in adult patients who already have a diagnosis of anxiety neurosis rather than for making a diagnosis of anxiety in patients who have

other problems. The test contains 14 items, each with a five-point scale, and is completed by a physician or psychologist. The test emphasizes the patient's subjective state. The two subscales determined are somatic anxiety and psychic anxiety.

Hamilton Depression Scale The HAMD is one of the most widely used tests to quantitatively evaluate the severity of depressive illness in adults. The most widely used form of this test contains 21 items covering a broad range of symptomatology, with a three- to five-point scale for most items. The minimum time required to complete this test is usually 10–20 min, and it requires a skilled interviewer. Either the present time or the period within the last week is rated. Six subscales are obtained in the HAMD: anxiety/somatization, weight, cognitive disturbance, diurnal variation, retardation, and sleep disturbance.

Hopkins Symptom Checklist The Hopkins Symptom Checklist (HSCL) is a scale that has been used to measure the presence and intensity of various symptoms in outpatient neurotic patients. It is a 58-item self-rating scale and has generally been replaced by the Self-Report Symptom Inventory (SCL-90). It measures the symptoms during the past week and requires approximately 20 min to complete. There are five subtests: somatization, obsessive–compulsive, interpersonal sensitivity, depression, and anxiety.

Inpatient Multidimensional Psychiatric Scale The Inpatient Multidimensional Psychiatric Scale (IMPS) is used to measure psychotic syndromes in hospitalized adults capable of being interviewed. The 89 items are rated on the basis of a psychiatric interview. This test has been well validated and requires 10–15 min following a 35–45-min interview. There are 10 scores: excitement, hostile belligerence, paranoid projection, grandiose expansiveness, perceptual distortions, anxious intropunitiveness, retardation and apathy, disorientation, motor disturbances, and conceptual disorganization.

Nurses Observation Scale for Inpatient Evaluation The Nurses Observation Scale for Inpatient Evaluation (NOSIE) (30-item test) is used by nursing personnel to rate a patient's behavior on the ward, with a five-point scale for each item. This test is widely used and is well accepted for adult inpatients. The test, which rates the most recent three days, is relatively easy to use and requires 3–5 min to complete.

Plutchik Geriatric Rating Scale The Plutchik Geriatric Rating Scale (PLUT) (31-item test) is designed to measure the degree of geriatric functioning in terms of both physical and social aspects. The three-point scale for each item is completed on the basis of direct observation of the patient's behavior and takes 5–10 min to complete. The subscales measure overall dysfunction,

aggressive behavior, sleep disturbance, social isolation, sensory impairment, work and activities, and motor impairment.

Profile of Mood States The Profile of Mood States (POMS) self-rating scale is used in both normal and psychiatric outpatients to evaluate feelings, affect, and mood. It has been widely used in medicine trials. The 65 adjectives included in this test may be used to rate the present and/or previous week. This test requires approximately 5–10 min to complete and provides scores for six subtests: tension–anxiety, depression–dejection, anxiety–hostility, vigor, fatigue, and confusion.

Sandoz Clinical Assessment—Geriatric The Sandoz Clinical Assessment—Geriatric (SCAG) test measures 18 individual symptoms plus a global rating using a seven-point scale similar to those used in the Brief Psychiatric Rating Scale. It measures the present period or that within the last week, requires about 10–15 min to complete, and does not contain subtests.

Self-Report Symptom Inventory Each of the 90 items in the SCL-90 uses a five-point scale of distress. It was designed as a general measure of symptomatology for use by adult psychiatric outpatients in either a research or a clinical setting. It rates either the present or previous week. It requires about 15 min for the patient to complete this form and about 5 min for a technician to verify identifying information. This test is sensitive to drug effects and may be used with inpatients. Nine subscales are measured: somatization, obsessive–compulsive, interpersonal sensitivity, depression, anxiety, anger–hostility, phobic anxiety, paranoid ideation, and psychoticism.

Wittenborn Psychiatric Rating Scale The ECDEU version [Wittenborn Psychiatric Rating Scale (WITT)] is a 17-item test shortened from the original 72-item test. All but one item use a four-point scale, and the test takes 5–10 min to complete. It is used in both inpatients and outpatients and rates either the present or previous week. This test is not intended to make diagnoses but to reflect changes within one patient and to provide a basis for comparing different patients. This test provides descriptive, as opposed to etiological or prognostic, information on patients and includes the following subscales: anxiety, somatic–hysterical, obsessive–compulsive–phobic, depressive retardation, excitement, and paranoia.

Zung Self-Rating Anxiety Scale The SAS test requires approximately 5–10 min to complete.

Zung Self-Rating Depression Scale The SDS test requires approximately 5–10 min to complete.

23.6.13 Pediatric Behavioral Rating and Diagnostic Scales

Many of the behavioral rating scales described for adults are not suitable for use in the pediatric population. Special tests have been designed, and a number of pediatric behavioral rating scales are presented in Table 23.17. General comments on these tests are presented below. A further description of rating scales used in pediatric medicine trials is given in the *ECDEU Assessment Manual for Psychopharmacology* by Guy (1976). His article is a practical guide to identifying appropriate scales for a particular situation. Conners discusses the two broad approaches of many pediatric rating scales as either "rating current behaviors, symptoms or states; or … describing basic traits, dispositions, and personality characteristics." The choice of one of these two approaches depends in part on the purpose of using a scale in a medicine trial. Three general purposes have been suggested for using a behavioral test (prediction, measurement of change, and classification). The choice of one of these three purposes usually implies that one of the two specific approaches implicit in the pediatric behavioral scales will be more appropriate:

1. To be able to predict something about a patient, choose a scale that rates basic traits.
2. To measure change in a patient, choose a scale that rates current symptoms.
3. To assess a patient's classification, choose a scale that rates either basic traits or current symptoms, depending on the purpose of the classification.

The type of patient population and the desired format of the test to be used in a clinical trial also influence the particular scale(s) chosen.

An evaluation system that can be used in a wide variety of pediatric inpatients is the Children's Behavior Inventory.

Children's Behavior Inventory The Children's Behavior Inventory (CBI) is an 139-item, two-point (yes–no) scale to record maladaptive behavior in children aged 1–15 years. Relatively little training is needed to administer this test. It is easily used by nurses, teachers, graduate students, psychologists, and others. This test usually requires at least 2 h of observation of the child, but better reliability is achieved if behavior is observed over an 8-h period. Nine subtest scores are provided: anger–hostility, conceptual dysfunctioning, fear and worry, incongruous behavior, incongruous ideation, lethargy–dejection, perceptual dysfunctioning, physical complaints, and self-deprecation.

Children's Diagnostic Classification The Children's Diagnostic Classification (CDC) test may be used instead of the Children's Psychiatric Rating Scale (CPRS) to arrive at a diagnosis. This differs from the CPRS in that it is highly directed and leads the observer to a diagnosis. It rates the current status

of the child and may be used at pretreatment and/or the termination of the clinical trial.

Children's Diagnostic Scale The Children's Diagnostic Scale (CDS) is used in children up to 15 years of age to assist in the diagnosis and classification of the child's condition. It contains 13 items, 8 of which have a seven-point scale. The others are specific diagnostic questions. It measures current status only and is mainly used at the start of a study, although it may be used at the termination of the study as well.

Children's Psychiatric Rating Scale The CPRS is a comprehensive scale to assess a wide range of psychopathologies in children up to age 15. It contains 63 items, with a seven-point scale derived from the BPRS. This test rates 28 items by direct observation of the child based on behavior expressed during the interview and rates other items based on the child's reports of events that occurred either over the preceding week or during the interview. Scores of 15 separate clusters of the rated items are provided as well as the overall score.

Clinical Global Impression See adult behavioral rating scale description of the CGI.

Conners Parent Questionnaire The Conners Parent Questionnaire (PQ) is a 94-item checklist of symptoms that evaluates common behavior disorders using a four-point scale in children up to 15 years of age and takes 15–20 min to complete. It is used once pretreatment and may be repeated but is often replaced after the first use by the 11-item Conners Parent–Teacher Questionnaire (PTQ). There are eight subscales: conduct problem, anxiety, impulsive–hyperactive, learning problem, psychosomatic, perfectionism, antisocial, and muscular tension.

Conners Parent–Teacher Questionnaire See descriptions above for Conners Parent Questionnaire and below for the Conners Teacher Questionnaire (TQ). The PTQ is used in conjunction with either the PQ to TQ and yields a total score only (i.e., no subscales are given). The PTQ takes about 5 min to complete and is not used pretreatment.

Conners Teacher Questionnaire The TQ form was designed to obtain teacher evaluations of children up to age 15 in terms of their interactions with peers and their ability to cope with the school environment and requirements. There are 41 items, and the first 39 have a four-point scale. Question 40 deals with the teacher's evaluation of the child's severity of illness, and question 41 deals with global improvement in four different areas. This test is used once at pretreatment and as needed afterward. It takes about 15 min to complete and covers either the present or any interval period up to 1 month. A shorter 11-item PTQ is often used after the initial use of the 41-item TQ. The five

subscales included are conduct, inattentive–passive, tension–anxiety, hyperactivity, and social ability.

Devereux Child Behavior Rating Scale The Devereux Child Behavior Rating Scale contains 97 items and is similar to the Devereux Teacher Scale. It is used for emotionally disturbed and mentally retarded children aged 8–12 years. Besides being easy to use, this scale is well researched and discussed in the literature. It requires 10–20 min to complete by clinicians, child care workers, parents, or others and gives 17 scores. There is a Devereux Adolescent Behavior Rating Scale for children from ages 13 to 18.

Devereux Elementary School Behavior Rating Scale The Devereux Elementary School Behavior Rating Scale is a widely used test incorporating 47 items that have high test–retest reliability. It uses a checklist format and is easy to use (requires 10 min). There are 11 factor scores and 3 item scores.

23.6.14 Psychometric and Performance Tests

The psychometric and performance tests presented in Table 23.18 may be grouped as being applicable for use in either children or adults. In children, the tests measure intellect [GOOD, Porteus Mazes, Wechsler Intelligence Scale for Children (WISC), Peabody], achievement [Wide Range Achievement Test (WRAT)], and motor performance (vigilance tests, reaction time). There are other tests that may be used to measure learning, although many of these tests utilize equipment and are not described. All of these tests (unless otherwise noted) are given once pretreatment, at least once posttreatment, and at additional times if desired by the investigator. The contribution of learning in the scores obtained at second and third testings is usually unknown. The methods used to motivate patients to perform to the best of their ability in all tests must be standardized and reported.

Bender–Gestalt Test The Bender–Gestalt is a nonverbal performance test in which the individual copies a design shown on a card. It is often used to identify a problem of visual perception and/or motor performance or minimal brain dysfunction in children.

The scoring used for children (age 4 or 5–11 years) differs from that used for adults (age 15 years to adult). This test measures perceptual maturity, possible neurological impairment, and emotional adjustment in children. It measures maturation, intelligence, psychological disturbance, and cortical impairment in adults. The test requires 10 min to complete. Scores may fluctuate from test to test and thus must be interpreted carefully.

Conceptual Clustering Memory Test For the Conceptual Clustering Memory Test, patients are given a list of 24 specific words from a number of different categories such as birds, cars, or types of drinks. The words are pre-

sented one at a time over 2 min, after which patients are asked to recall as many of the specific words as possible. The test measures the total recall as well as the degree to which words of a specific category (e.g., animals) are recalled from the cluster of words given in that category (e.g., dog, cat, cow).

Digital Symbol Substitution Test A subtest of the Wechsler Adult Intelligence Scale (WAIS), the Digital Symbol Substitution test, measures sensorimotor integration and learning relationships of symbols. It has been used in many psychopharmacological studies. Subjects are given different forms of this test at each session. The test requires the patient to match as many of 100 symbols to their respective numerals, found in a code key, as possible within 60 s.

Embedded Figures Test For the Embedded Figures Test, patients are shown a complex design and must identify as quickly as possible a simple figure that is "embedded" within the design. Twenty-four embedded figures are included, and a maximum of 3 min is allowed for each one.

Frostig Developmental Test of Visual Perception The Frostig Developmental Test of Visual Perception (FROST) measures the development of perceptual skills in children from four to eight years of age or in older children with learning difficulties. It may be administered individually (requires 30–45 min) or to groups (requires 40–60 min).

Goodenough–Harris Figure-Drawing Test The Goodenough-Harris Figure-Drawing Test is a brief (10–15-min) easy-to-use test for children 4–15 years of age to measure intellectual maturity.

Peabody Picture Vocabulary Test The Peabody Picture Vocabulary Test is a rapid 10–15-min intelligence test for children aged 2.5–18 years that is useful when there is inadequate time to give the WISC.

Porteus Mazes The Porteus Mazes is a nonverbal test that has been shown to be sensitive to medicine effects in both children (over three years) and adults. The test has three series of mazes to prevent score improvement on retesting with the same test. It requires about 25 min and provides both a qualitative and a quantitative score.

Reaction Time There are many different tests used to measure reaction time. These tests measure the period of time between the presentation of a stimulus to a patient and the onset of the resulting response. The signal is usually a visual or auditory stimulus, and the onset of a motor reaction, such as the lifting of a finger, arm, or leg or the pressing of a buzzer, is used to measure the speed of response.

In simple reaction times, a stimulus is presented that always requires the same response, even if the nature of the stimulus changes. A complex reaction time requires the patient to respond to some stimuli but not to others.

Vigilance Tests Numerous tests have been designed to measure vigilance. In these tests, patients are requested to respond in some manner to certain stimuli or occurrences but not to others. The stimuli may be controlled to present minimally perceived signals that require vigilance on the part of the patient.

Wechsler Adult Intelligence Scale The WAIS consists of 11 subtests—6 verbal tests and 5 performance tests. This provides an age-related IQ in adults from 16 to 75 years of age; that is, the test measures intelligence of the person in relation to his age group and not to the entire population. It may be used either as an initial assessment or as a tool to measure change. The test, which takes 40–60 minutes to complete, provides 13 scores in verbal and performance categories plus a total score.

Wechsler Intelligence Scale for Children The WISC was extensively revised in 1974, and it became the WISC-R, which requires 40–60 minutes to complete. This widely used scale in children from 6 to 16 years of age may be used for either screening or baseline data or as a measure of change. There is a "preschool and primary scale of intelligence" version that may be used in children from 4 to 6½ years of age (requires 50–75 min). The WISC-R has six verbal and six performance subtests.

Wechsler Memory Scale The Wechsler Memory (WMEM) Scale is a brief test that is used to measure memory deficits. There are two forms of the test, and they are generally alternated to avoid a training effect in children taking the test on two or more occasions.

Wide Range Achievement Test The WRAT is used in children from age five years to adult in college. It assesses basic skills in reading, spelling, and mathematics. It is simple and easy to administer and requires 20–30 min to complete.

23.6.15 Personality Tests

In addition to the above behavioral and performance tests, there are a number of well-known tests of personality that may provide useful information in select clinical studies. The most well known of these tests is the Minnesota Multiphasic Personality Inventory (MMPI). This test consists of 550 affirmative statements to which a true or false response is given and requires about 1 h to complete. It is given to adults over the age of 16 and is scored for 10 scales: depression, hysteria, hypochondriasis, psychopathic deviate, masculinity–femininity, paranoia, hypomania, schizophrenia, psychasthenia, and social introversion.

23.7 ASSESSMENT OF UNWANTED DRUG EFFECTS

Because of the relatively small numbers of volunteers and patients involved, only the most common of drug-related adverse events are likely to be detected during early studies. For example, to have a 95% chance of picking up three subjects who have experienced an adverse reaction (with no background incidence) which occurs in 1 in every 100 subjects treated with the drug, it would need to be given to 650 subjects (Gad, 1998). Matters are made worse when the adverse event in question also occurs in the general population, which is usually the case with the kind of symptoms reported by volunteers and patients taking part in drug studies. No matter how good the study design is, nothing can compensate for this problem of inadequate numbers. In this respect, all of the study designs described earlier are more or less equally adequate or inadequate, as the case may be.

Monitoring for drug-related adverse events employs the same or similar methods in both volunteers and patients. In both cases assessments of tolerability and safety are based upon symptom reports, routine laboratory safety screens, ECG monitoring, and on occasion special tests designed to detect unwanted effects associated with a particular class of drug. The chances of obtaining reliable information on a drug's safety profile are enhanced by detailed and careful monitoring. Symptoms may be reported spontaneously or elicited in reply to standard questions. Open questions such as "how are you feeling?" are to be preferred to leading questions on the basis that they result in fewer reports of adverse events. If leading questions are used, they need to be carefully worded. A certain amount of basic information is required on all adverse events, that is, type, severity, time of onset in relation to time of dosing, duration, and causality. Attributing the cause of an unwanted effect to the drug or some other factor can be difficult, particularly when little is known about the drug, as is often the case at the stage of initial studies in volunteers or patients. Rechallenge with the drug ideally using the same dose or if need be (because the event caused a degree of discomfort) a reduced dose is probably the single best way of proving or disproving a causal relationship; but if done, the rechallenge procedure must be designed using placebo as a comparator under double-blind conditions. Obviously rechallenges can be done only if the adverse event was reversible, did not cause excessive discomfort, and most importantly was not life threatening.

Separation of Adverse Reactions from Placebo Reactions Since adverse nondrug symptoms are common (Reidenberg and Lowenthal, 1968) and are not easily separated from drug-induced symptoms, both must be collected for analysis if a complete profile of adverse reactions is to be made. However, this technique can only be sued in controlled studies, ideally with placebo, as well as with other standard drugs. The temptation to subtract the number of the particular adverse reactions in the placebo group from the number in the active drug group as

$$\text{Drug group} - \text{placebo group} = \text{number of adverse reactions to drugs}$$

should be resisted because:

- The difference may not be statistically significant and may have arisen by chance.
- Although the total number of events may be statistically different in the two treatment groups, it is also necessary to establish whether the number of patients afflicted with the adverse event is different and vice versa.
- Having established that there is a significant difference between the two treatment groups for the number of events and the number of patients afflicted, the severity of the ADRs in the two groups should be compared.

A further problem is that due to classification; some terms may include more than one type of abnormality (e.g., the incidence of "blurred vision" may be equal in both groups, but there may be several cases of tunnel vision with the trial drug but because there is no code for tunnel vision it is coded under more general terms). Another problem is that the symptoms forming a syndrome are often coded separately and individually and there may be no difference between two drugs, but when the cases are examined there may be a combination of symptoms with one drug that warrant being called a syndrome. It is therefore essential to read the individual original description of the adverse events (AEs) before making a judgment. This area has been explored more fully by Bernstein and he has added bias to the equation:

$$\text{Attributable AEs} = \text{drug group AEs} - \text{placebo group AEs} \pm \text{bias}$$

where bias is equal to the baseline (B) frequency and severity of the AE multiplied by the pharmacological clinical activity of the drug (AD) minus the pharmacological clinical activity of the placebo (AP):

$$\text{Bias} = B(AD - AP)$$

The argument is that the disease or a symptom or sign of the disease and the drug ADR may interact as follows:

- Compliance—Early improvement may cause the patient to stop the drug and the improvement of the ADR may be inappropriately assigned to tachyphylaxis of the ADR; failure of the disease to improve may persuade the patient to add a rescue drug or increase the dose of the study drug or even stop the drug; impaired mental or cognitive function due to the disease may affect compliance.
- The disease may alter the absorption, distribution, metabolism, or elimination of the drug (e.g., alteration of the blood–brain barrier by the disease may allow the drug to affect the brain).

- Observational bias of convalescence (e.g., severe pain causing insomnia may require morphine, causing compensatory hypersomnia in excess of that caused by morphine alone).
- Observational bias by halo effects. Perception of an ADR may be swamped by the symptoms of the disease; thus as the disease symptoms resolve, the ADR becomes apparent.
- Unblinding—If the patient or physician is unblinded due to rapid improvement of the disease or an ADR, he or she may be led to expect ADRs with the active treatment.
- Pharmacological clinical activity bias—An AE that is already present due to the disease may be increased if it is also an ADR of the drug or vice versa. For example, the diarrhea of gastroenteritis may be alleviated by codeine-containing preparations given to relieve pain while the inertia of a severely depressed patient may be sufficiently resolved by an antidepressant to enable the patient to commit suicide.

Adverse drug reactions that are similar to common nondrug AEs are rarely described or investigated sufficiently for a causal relationship for each individual event to be established. If they cannot be distinguished qualitatively, the correct quantitative procedure is to compare them using nonparametric statistics, giving the confidence limits for the incidences of ADRs. Small studies ($n < 30$) have little chance of separating ADRs from placebo or nondrug events unless they are very common and specific to the drug. The situation is worsened by the fact that members of a placebo group have a tendency to "catch" AEs from the active drug group, therefore changing a relatively specific ADR to a nonspecific event.

23.7.1 Base-Case Causality of Single-Event Adverse Drug Reactions

The analysis and evaluation of ADRs are major problems in both the development of new drugs and the postmarketing surveillance period. Just as there are standards and requirements established as guidelines in the chemical, pharmacological, and toxicological phases preceding the marketing of a new drug, there are also guidelines for causality assessment of individual human cases in both pre- and postapproval for new drugs.

The most common problem of assessment is the single-event ADR case. Presented here is an approach to such single-event ADR cases. This methodology, relating to the use of therapeutic, diagnostic, and prophylactic-type drugs in a clinical setting, should permit the diagnostician to make one of three responses after an assessment of an ADR case: an assured *yes*, a firm *no*, or a reasoned admission of *uncertainty*. The clinicopathological picture presented by the ADR case is often not readily distinguishable from non-drug-induced diseases. The clinical and morphological findings of ADRs have the same

limited number of final common paths that characterize these other (nondrug) human illnesses.

There are three major requirements for establishing the occurrence of an ADR:

1. The possibility and likelihood of a causal relationship between the drug and the ADR must be confirmed by establishing its eligibility.
2. Linkage of the drug with the clinicopathological findings.
3. The degree of certainty of this drug linkage should be determined.

As an initial background for developing this algorithm or methodology, Figure 23.4 is offered for consideration and orientation. This figure has the basic elements of a "time flow chart," which has considerable utility in evaluating ADR cases.

In this graphical representation of an ADR, the ordinate (Q) represents any of the findings of an ADR. *Specifically, Q* may be a symptom (pain, nausea, etc.), a sign, a clinical laboratory result, a radiological finding, a morphological finding, or any combination of these. Synonyms for Q include marker, disease marker, signal, indicator, parameter, detector, response, and effect.

The abscissa is the time element (T) related to both the time of drug administration and the dating of disease marker data. Both are usually plotted on the same time flow chart in a particular case.

This graphical representation of an ADR case will be sued frequently in the assessment of eligibility and linkage determinations of ADRs. The four eligibility criteria are also listed in Figure 23.5.

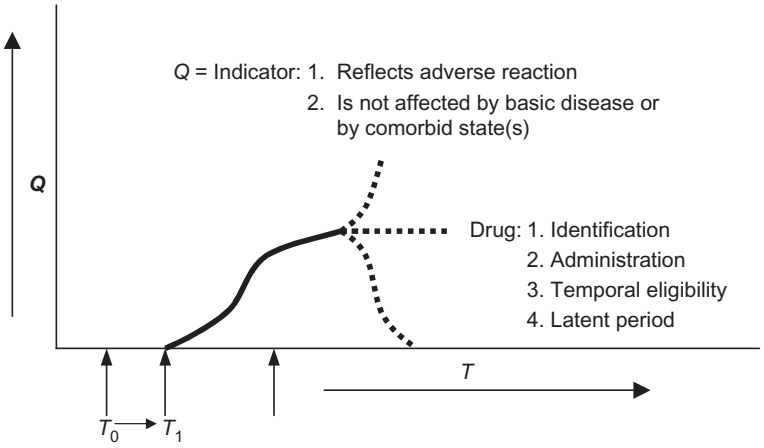


Figure 23.5 *Adverse drug reaction (curve* Q*) plotted against time (abscissa* T*). Dashed lines show three courses an ADR can take: increasing severity to death; leveling off to chronicity; or return to abscissa, indicating recovery. Four criteria that must be met before drug is eligible to be empiric correlate of* Q *(adverse drug reaction) are listed.*

Administration of the Drug As it is with accurate identification of a drug, so it is that its "administration" must at times be held in question. Subject compliance with the study protocol is not a rare problem in clinical trials. Complete noncompliance sometimes occurs.

Temporal Eligibility The time factor in assessment of ADRs is a very important one and in some cases is of critical diagnostic importance. This is true not only in establishing "eligibility" of the drug but also in linking the drug to the reaction. On the other hand, the time element may be equally important in denying eligibility and also makes linkage of the drug with the clinicopathological picture a most unlikely possibility.

It is quite apparent that a drug cannot be responsible for an ADR if the latter is already in progress before the drug is first administered. This dyssynchronicity is sometimes seen both in trials and later in the marketplace.

Latent Period Latent period refers to the time interval between the initial administration of the drug and the onset of the ADR (in Figure 23.5, it is the interval T_0 to T_1). The latent period is not rigidly fixed or exactly predictable, but it tends to fall within certain limits.

Characteristically, strychnine deaths occur in seconds to minutes. Most anaphylactic deaths occur within 20–30 min after contact with the lethal antigen, while jaundice associated with most drugs has its onset within three days to three weeks after the beginning of therapy. The fatal pancytopenia following chloramphenicol appears in one to three months, while hepatic angiosarcoma related to aflotoxin has a latent period of one to several decades. The ultimate in length of latency is one to several generations from a drug-induced mutational germ cell change to its manifestation in a conceptus.

Consideration of the latent period in an ADR is of use in an ADR assessment in one of two ways: The latent period may be too long or too short.

In summary, identification, administration, temporal eligibility, and latent period are the four criteria for establishing the eligibility of a drug to have caused an ADR. Emphasis should be placed on obtaining sufficiently detailed time-related data on drug administration and the appearance of ADR markers. These data are a *sine qua non* in the assessment of drug eligibility.

Linking Drug with Clinicopathological Findings The second major task in analyzing an ADR case is to establish a connection or linkage between the drug and the clinicopathological findings (making empiric correlates of the drug and these findings).

Figure 23.6 is a time flow chart representing an ADR that itemizes six ways of making this linkage.

Exclusion Exclusion consists of selecting one drug from a group of drug candidates by the use of the time flow chart.

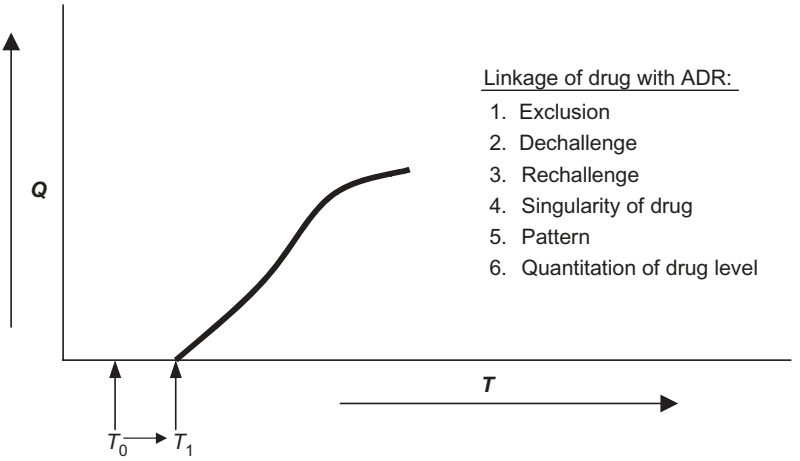


Figure 23.6 *Six methods of linking drug with adverse drug reaction.*

The exclusion method also includes instances in which drug candidates are themselves excluded from causation status because a nondrug etiology is clearly demonstrable (environmental or occupational factors, radiation injury, the underlying disease of the patient, or a comorbid state) and can reasonably account for the clinicopathology findings.

Dechallenge The principle involved in the dechallenge method of linkage is that if there is a reversible effect present, then removing the cause will eliminate the effect.

Rechallenge The principle involved in the rechallenge method of linkage of a therapeutic to an ADR is implied in the phrase *post hoc ergo propter hoc* ("after this therefore because of this").

As applied, if a drug has been incriminated with a reaction and the ADR disappeared when the drug was discontinued, a rechallenge with this drug followed by a return of the ADR would increase the probability that the drug and the ADR were empiric correlates. While intentional challenge is not often done, such a rechallenge may occur inadvertently.

Singularity of Drug The principle involved in the singularity method of linking a drug with an ADR is based on two assumptions: Only one drug was administered and there was no basic disease or comorbid state that could be related to the ADR marker being used in the assessment.

Pattern The pattern method of linking a drug with an ADR shifts the focus of attention to the clinicopathological findings in an ADR and away from the identification of the causative drug. This shift of emphasis is necessary when

detailed time-related drug and disease marker data are unavailable to the evaluator of the case. The site-process profile may then be used as a guideline for searching past experience and the literature for cases that have matching features. Matching features found in the literature may include associations with certain drugs or chemicals, which serves as a guideline for a focused examination of the patient's history for the causative agent.

This "pattern" method may also be used in excluding drugs. If the drugs or chemicals suggested by the morphological findings are not identified or disclosed by historical or toxicological efforts, then the morphological changes appear to remain non–drug or non–chemical related.

Quantitation of Drug Level Assessing an ADR case by quantitation of drug level brings our focus back to the search for and identification of the causative agent by quantitative and objective data based on laboratory analysis of body fluids and/or viscera (Ozdemir et al., 2001). This method is applicable and strongest in the case of higher dose level. The feasibility of this approach is based on the availability of dependable information on lethal levels from past experience or preclinical work. Without this comparison information, there is no judgmental significance to toxicological levels in the case at hand.

Quantitated levels of drugs have limitations in diagnostic value. In adverse reactions in the hypersensitivity, idiosyncratic, and pharmacogenetic categories, drugs have been administered in therapeutic (not toxic) amounts, and blood and other body fluids and tissue levels have been found to lie within therapeutic ranges. Such analyses will confirm any prior administration of the drugs, but the problem of the etiological differential diagnosis will still remain.

In addition to a quantitative approach, qualitative identification can be of value in appropriate instances. In some cases of ADRs, more than one of the six methods of drug linkage that are listed in Figure 23.5 may be used in causation analysis. In fact, multiple methods in the same case strengthen the confirmation of the rejection of an ADR and its etiology.

Difficulties in Assessing ADRs Requirements for establishing eligibility and methods of linking a drug with an illness have been presented in the preceding discussion. This algorithm should constitute a blueprint for solving many, if not most, of the ADR problems in this area of medical diagnostics.

However, in the hands-on practice of the assessment of ADR cases, there are at least four major difficulties that stand in the way of such high diagnostic expectations:

1. *Incomplete Information* Incomplete information is not unique to ADR evaluation but is common to all areas of medical practice. The lack of sufficiently detailed, time-related data on drug administration and disease markers may make it impossible to render a reasoned judgment on many ADR cases, leaving them in their original and unsatisfactory anecdotal status.

Denial to the evaluation of access to these required facts makes it impossible to make judgments on latent period and temporal eligibility; time flow charts cannot be utilized in exclusion, dechallenge, and rechallenge techniques. The diagnostic database should also include information on any other drugs being administered or taken, concurrent comorbid states, and the existence of any preexisting occupational and environmental hazards.

2. *Polypharmacy* In recent times, polypharmacy is the rule rather than the exception. Patients with complicated and prolonged illnesses may have 20–30 medications in their medical background. Cases of this sort may be of such complexity that even with ideally complete drug and disease marker information, diagnostic success may be elusive.
3. *Lack of Objective Means of Linking Drug to ADR* Tests and procedures that specifically and causally connect a drug to an illness are lacking. Our high-technology laboratory instrumentation is capable of identifying and quantifying extremely low levels of drugs and chemicals, but this type of information falls short of establishing causation.
4. *Limited Number of Toxicological Responses in Human Disease* There are a limited number of generic morphological reaction patterns that diseases fit into (inflammatory, congenital, neoplastic, degenerative, infiltrative, vascular, and functional). In parallel, there are also a rather limited number of clinical symptoms and signs (pain, nausea, fever, lumps, etc.) that come to the attention of the practicing physician. There are a multitude of causes and a multitude of clinical conditions that funnel into these clinicopathological "final common paths." The algorithm previously described is an attempt to move from the generic to the specific in analyzing ADR causation.

Of the above four difficulties, only the first (incomplete information) is subject to at least some degree of improvement.

Degree of Certainty The third major task in analyzing and assessing ADRs is determining the degree of certainty one has as to the causal relationship between the drug and the clinicopathological findings. Interposed between the definitive causative and negative categories are three shades of certainty (probable, possible, and coincidental) that titrate between these two extremes. These degrees of certainty are defined as follows:

1. *Causative* Cases in this class are those in which there is no doubt that a drug has caused the reaction. This category is essentially limited to drug overdose cases or those cases in which the causative agent can be objectively identified (asbestos bodies, granuloma-encapsulated silica, etc.). Parenthetically, the overdose cases with drug levels in lethal ranges should have important negative findings: no anatomical cause of death at autopsy.

2. *Probable* This term is equivalent to the phrase "consistent with," and cases in this category of certainty fall short of the "causative" designation because they lack an objective and quantitative laboratory finding that is the sine qua non of the causative category. Cases placed in this category have the following characteristics:
   (a) The criteria of temporal eligibility and appropriateness of latent period have been met.
   (b) The clinicopathological features are consonant with previous experience and literature precedent for the drug in question.
   (c) Other causes (the basic disease, comorbid states, and other modalities of therapy) have been eliminated from consideration.
   (d) One or several means of linkage of the drug to the ADR have been utilized: exclusion, dechallenge, rechallenge, singularity of the drug, and pattern.
3. *Possible* Cases are put in this category when the relationship between the drug and the clinicopathological findings can be neither confirmed nor denied. There are three subdivisions in this category:
   (a) Cases with potential causes other than the drug in question. The clinicopathological picture could have been produced by the basic disease, a comorbid state, or some other modality of therapy.
   (b) Cases in which some of the criteria for eligibility and linkage have been met but some have not because of lack of adequate information. Such a case could be put in this category temporarily while awaiting more information or placed here permanently if it were evident that further data would not be forthcoming.
   (c) Cases that have met all the criteria of eligibility and linkage but for which there is no known precedent literature. Such a case might be a new and emergent ADR. It could be placed in the "possible" group, awaiting the appearance of similar cases for cluster studies at a later time.
4. *Coincidental* Cases in this category include those that were indeed exposed to the drug in question but in which assessment of the case clearly reveals only an anecdotal association.
5. *Negative* This category applies to those cases in which the alleged drug was not or could not have been in the patient's system at the time of the ADR. This circumstance could be related to noncompliance, mislabeling of the drug, or historical misinformation.

23.8 CONCLUSION

The roles of the toxicologist in clinical trials are as follows:

1. Prospectively and retrospectively evaluate, explain, and extrapolate from the relationships between nonclinical trial findings and adverse clinical trial events.

2. Provide mechanistic insight into the causes, treatment, and avoidance (of further) undesired effects in a clinical trial.
3. Guide selection and refinement of subject profiles for clinical trials.

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Postmarketing Safety Evaluation: Monitoring, Assessing, and Reporting of Adverse Drug Responses

Once a new drug is approved, it proceeds to market either with or without postmarketing evaluation requirements. While this represents the end of a long road, it is also the start of yet another. While careful work during development (both in animals and humans) serves to provide the tools to greatly reduce the potential safety issues around a new drug, it cannot totally eliminate them. One needs only to look at Table 24.1 to appreciate the history of market withdrawals due to safety issues in the modern era (1961–2001) or turn to Table 24.2 to verify that the problem is still present in the first decade of the twenty-first century and comparable to the past.

Tracking and continuing to evaluate the safety of a therapeutic agent once it is on the market represent a complex task. Manufacturers are legally required to collect, analyze, and report such data both nationally [by the U.S. Food and Drug Administration (FDA) and by equivalent organizations in other countries, but here the emphasis will be on the U.S. situation] and internationally

Drug Safety Evaluation, Second Edition, by Shayne Cox Gad

TABLE 24.1 Therapeutic Products Withdrawn from Marketplace Due to Safety Reasons in United Kingdom and/or United States, 1961–1995

Drug (INN)	Trade Name	Therapeutic Class	Reason(s) for Withdrawal/Suspension of Product License	Launch Date	Country	License Suspension or Market Withdrawal	Years on Market
Acetylsalicylic acid (pediatric)	Aspirin	Analgesic	Reye's syndrome	1899	U.K.	1986	87
Alclofenac	Prinalgin	Nonsteroidal anti-inflammatory drug (NSAID)	Skin and renal reactions, mutagenic metabolite	1972	U.K.	1979	8
Alphoxalone	Althesin	Anesthetic	Allergic-type reaction	1972	U.K.	1984	12
Aminoglutethimide	Elipten	Anticonvulsant	Endocrinological,	1960	U.S.	1966	6
			Reintroduced—Cushing's syndrome	1978	U.S.		
Aminopyrine	—	Analgesic	Hematological	1900	U.S.	1970	70
					U.K.	1975	75
Astemizole	Hismanal	Antihistamine	Drug–drug interaction	1988	U.S.	1999	11
Azaribine	Triazure	Antipsoriatic	Neuropsychiatric, coagulation disorders	1975	U.S.	1976	1
Benoxaprofen	Opren	NSAID	Cholestatic jaundice	1980	U.K.	1982	2
			Photosensitivity	1982	U.S.	19882	<1
Benziodarone	Amplivix	Uricosuric, coronary dilator	Hepatic	1962	U.K.	1964	2
Bithionol	Actamer	Anthelminthic	Dermatological	?	U.S.	1967	?
Bronfemic sodium	Duract	NSAID	Liver damage	1996	U.S.	1998	3
Cerivastatin sodium	Baycol	Cholesterol lowerer	Muscle weakening	1999	U.S.	2001	2
Chlormadinone	Normenon	Hormone	Animal carcinogenicity	1965	U.S.	1970	5
				1966	U.K.	1970	4
Cisapride	Propulsid	Heartburn	Cardiovascular irregularities	1993	U.S.	2000	
Clioquinol	Enterovioform	Antidiarrheal	Neuropsychiatric	1930	U.K.	1981	51
				1930	U.S.	1973	43
Centoxin	HA-1A	Hu-anti-lipid A IgM monoclonal	Gram-negative septicemia	1990	U.K.	1992	2

Danthron	Dorbanex	Laxative	Animal carcinogenicity	1959	U.S.	1987	28
			Reintroduced with restrictions	1964	U.K.	1987	23
Desensitizing vaccines	Various	Vaccine	Allergic-type reactions	?	U.K.	1989	
Diamthazole	Asterol	Antifungal	Neuropsychiatric	?	U.S.	1970	17
Dihydrostreptomycin	—	Antibiotic	Neuropsychiatric	?	U.S.	1970	?
Dinoprostone	Propess	Hormone	Uterine hypertonus and fetal distress	1989	U.K.	1990	<1
Dipyrone	—	Analgesic	Hematological	1930	U.S.	1977	47
				1930	U.K.	1977	47
Dithiazanine	—	Anthelminthic	Metabolic, cardiovascular	?	U.S.	1964	
Domperidone (injection)	Motilium	Antiemetic	Cardiovascular, risk of overdose	1984	U.K.	1986	2
Doxylamine	Bendectin	Antihistamine	Fear of	1956	U.S.	1983	27
Dicyclomine	Debendox		teratogenicity—dysmorphogenicity?	1957	U.K.	1983	26
Encainide	Enkaid	Antiarrhythmic	Cardiovascular, excess mortality risk	1987	U.S.	1991	4
Factor VIII	Factorate	Coagulation factor	Manufacture problem, risk of AIDS transmission	1972	U.K.	1986	14
Fenclofenac	Flenac	NSAID	Multiple—especially skin reactions	1978	U.K.	1984	6
Feprazone	Methrazone	NSAID	Multiple	1976	U.K.	1984	8
						1984	
Flosequinan	Manoplax	Heart failure	Increased mortality, lack of long-term efficacy	1992	U.K.	1993	9 months
						1993	
Grepafloxacin	Raxar	Quinolone antibiotic	QT-interval prolongation	1997	U.S.	1999	23 months
Growth hormone (natural)	Crescormon	Hormone	Manufacture problem	1970	U.K.	1985	15
			Creutzfeldt–Jakob disease transmission	1970	U.S.	1985	
Guanethidine	Ganda (high dose)	Anti-glaucoma eye drops	Ophthalmological	1977	U.K.	1986	9
Ibufenac	Dytransin	NSAID	Hepatic	1966	U.K.	1968	2

TABLE 24.1 ***Continued***

Drug (INN)	Trade Name	Therapeutic Class	Reason(s) for Withdrawal/Suspension of Product License	Launch Date	Country	License Suspension or Market Withdrawal	Years on Market
Indomethacin-R	Osmosin form	NSAID	Multiple gastrointestinal—36 fatal small intestine perforations	1982	U.K.	1983 1983	9 months
Indoprofen	Flosint	NSAID	Gastrointestinal carcinogenicity	1982	U.K.	1983 1984	1 ?2
Iodinated casein strophantin	Coratose	Anorexiant	Metabolic	?	?	1964	?
—	Lotanex	Irritable bowel syndrome	Ischemic colitis	2000	U.S.	2000	9 months
Mebanazine	Actomol	Antidepressant	Hepatic drug interactions	1963	U.K.	1975	12
Megestrol acetate	Volidan 21	Hormone	Carcinogenicity	1963	U.K.	1970 1969 1975	7
Methandrostenolone	Dianabol	Hormone	Endocrinological	1960	U.S.	1982	12
Methapyrilene	—	H1 antihistamine	Carcinogenicity	1947 1950	U.S. U.K.	1979 1979	31 29
Metipranolol	Glauline	Antiglaucoma eye drops	Ophthalmological—uveitis (high dose) Low-dose preparation	? —	U.K. —	1990 1991	?
Metofoline	Versidyne	Analgesic	Experimental toxicity	?		1965	?
Midefradil	Posiccor	Calcium channel blocker	Lethal drug interaction (inhibited liver enzymes)`	1996	U.S.	1998	3
Mumps vaccine	Pariorix	Vaccine	Neuropsychiatric	1988	U.K.	1992	4
Urabe AM9 strain	—	—	Meningitis	1988	U.S.	1992	4
Neomycin (injection)	—	Antibiotic	Misuse, irrigation of open wounds	?	U.S.	1989	?
Nialamide	Niamid	Antidepressant (MAOI)	Hepatic Drug interactions	1959 1959	U.K. U.S.	1978 1974	19 15
Normifensin	Merital	Antidepressant	Hemolytic anemia, hepatotoxicity-fatal hepatitis	1977	U.K.	1986	9

Oxphenbutazone	Tanderil	NSAID	Hematological	1960	U.S.	1985	25
			Multiple	1962	U.K.	1984	22
Oxyphenisatin	Veripaque	Laxative	Hepatic	1955	U.K.	1978	23
				1957	U.S.	1972	15
Perhexiline maleate	Pexid	—	Hepatic damage, peripheral neuropathy	1975	U.K.	1985	10
Phenacetin	—	Analgesic	Renal carcinogenicity	<1900	U.K.	19080	80
				1900	U.S.	1983	83
Phen-fen	Fenfuramine/ dexafluramine	Diet aid	Heart valve abnormality	1973	U.S.	1997	24
Phenformin	Insoral, Dibotin	Antidiabetic	Metabolic	1959	U.K.	1982	23
				1959	U.S.	1977	18
Phenoxypropazine	Drazine	Antidepressant (MAOI)	Hepatic Drug Interactions	1961	U.K.	1966	5
Phenylpropanolamine	PPA	CTC ingredient	Hemorrhagic stroke	1936	U.S.	2001	65
Pituitary chorionic hormone	—	Hormone	Hypersensitivity	?	U.K.	1972	?
Polidexide	Secholex	Antihyperlipidemic	Experimental toxicity Toxic impurities	1974	U.K.	1975	1
Practolol	Eraldin	Beta blocker	Oculomucocutaneous syndrome Deafness Sclerosing peritonitis	1970	U.K.	1989	16
Prenylamine	Segontin, Synadrin	Antianginal	Cardiovascular	1973	U.K.	1989	16
Pronethalol	Alderlin	Beta blocker	Animal Carcinogenicity	1963	U.K.	1965	2
Remoxipride	Roxiam	Antipsychotic	Hematological—aplastic anemia	1991	U.K.	1994	3
Rotashield	—	Rotavirus vaccine	Bowel obstruction	1998	U.S.	1999	1
Somatropin	Crescormone	Natural growth hormone	Creutzfeldt-Jakob disease	1973	U.K.	1985	12
Sulfamethoxy-pyridazine	Lederkyn	Anti-infective	Hematological Dermatological	?	U.K.	1986	?
Supofren	Suprol	NSAID	Renal	1986	U.S.	1987 1987	1

TABLE 24.1 ***Continued***

Drug (INN)	Trade Name	Therapeutic Class	Reason(s) for Withdrawal/Suspension of Product License	Launch Date	Country	License Suspension or Market Withdrawal	Years on Market
Temafloxacin	Omniflox, Teflox	Anti-infective	Hepatic dysfunction, hemolytic anemia, nephrological, anaphylaxis, metabolic	1991 1992	U.K. U.S.	1992 1992	1 4 months
Terolidine	Micturin	Urinary incontinence	Cardiac arrhythmia	1986	U.K.	1991	5
Tetracycline (pediatric form)	Achromycin V	Antibiotic	Teeth discoloration	1952	U.S.	1979	27
Thalidomide	Conterganl	Sedative	Teratogenicity Phocomelia	1956	U.K.	1961	5
Thenalidine	Distaval	H_1 antihistamine	Hematological			1961	?
Ticrynafen	Selacryn, Diflurex	Diuretic	Hepatic	1979	U.S.	1980	1
Triazolam	Halcion	Hypnotic	Neuropsychiatric—memory loss, depression	1979 1983	U.K. U.S.	1991 NW	12
Triparanol	MER-29	Antihyperlipidemic	Ophthalmological	1959	U.S.	1962	3
Troglitazone	Renzulin	Type II diabetes	Liver damage	1996	U.S.	2000	5
Trovafloxacin	Trovan	Antibiotic	Liver/kidney damage	1992	U.S.	1999 (use severely restricted)	7
Tryptophan	Pacitron, Optimax	Low-protein diet	Eosinophilic myalgia syndrome	1974	U.S.	1989 1990	15
Vitamin E	E-Ferol	Vitamin	Hematological, hepatic, renal	1983	U.S.	1984	1
Zomepirac	Zomax	NSAID	Allergic-type reactions—fatal anaphylaxis	1980 1981	U.S. U.K.	1983 1983	3 2
Zimeldine	Zelmid	Antidepressant	Hepatotoxicity, neurological—peripheral neuropathy, Guillain–Barré syndrome	1982	U.K.	1983	1

TABLE 24.2 Drugs Withdrawn since 1990

Year	Drug	Indication/Class	Causative Side Effect
1991	Enkaid (4 years on market)	Antiarrhythmic	Cardiovascular (sudden cardiac death)
1992	Temafloxacin	Antibiotic	Blood and kidney
1997	Fenfluramine/ dexafluramine (combo used since 1984) (24 years on market)	Diet pill	Heart valve abnormalities
	Seldane (terfenadine)	Antihistamine	Ventricular arrhythmias
1998	Posicor (midefradil) (1 year on market)	Ca^{2+} channel blocker	Lethal drug interactions (inhibited liver enzymes)
	Duract (bronfemic sodium) (early approval warnings of elevated liver enzymes)	Pain relief	Liver damage
1999	Trovan (use severely restricted)	Antibiotic	Liver/kidney damage
2000	Hismanal	Antihistamine	Drug–drug interactions
	Rotashield	Rotavirus vaccine	Bowel obstruction
	Renzulin (approved Dec. 1996)	Type II diabetes	Liver damage
	Propulsid	Heartburn	Cardiovascular irregularities/death
	Lotronex[a]	Irritable bowel syndrome	Ischemic colitis/death
2001	Phenylpropanolamine (PPA)	OTC ingredient	Hemorrhagic stroke
	Baychlor (Baycol)	Cholesterol reducing (statin)	Muscular weakness/death
2004	Serazone	Antidepressant	Liver failure and injury
	Vioxx[b]	Arthritis (COX-2 inhibitor)	Heart attack/cardiovascular (thrombosis)
2005	Tysabri	MS (multiple sclerosis)	PML (progressive multifocal leukoencephalopathy)
	Bextra	Arthritis (CoOX-2 inhibitor)	Skin reaction (sometimes fatal)
2006	Dolophine (methadone hydrochloride)	Treatment of moderate to severe pain	Respiratory depression and cardiac arrhythmias
2007	Zelnorm	Constipation	Cardiovascular safety
	Permax	Parkinson's disease	Heart valve damage

Note: None were withdrawn in 2002/2003.

[a]Reintroduced to market in 2003.

[b]Voluntary withdrawal.

[by the World Health Organization (WHO)]. There are regulatory reporting systems (i.e., where the reports go directly to government agencies) and organizational reporting systems [organized around method of distribution, such as hospital pharmacies—American Society of Hospital Pharmacy (ASHP), 1995; Hunziker et al., 1977—or by product type, such as radiopharmaceuticals]. Poison control centers also monitor adverse drug reaction (ADR) cases and rates (Chyka and McEommon, 2000; Chyka, 1999). The regulatory systems for such pharmacosurveillance in the United States are MedWatch (for human drugs), Vaccine Adverse Event Reporting System (VAERS, for human vaccines) (Niu et al., 1998, 1999; Varrincchio, 1998), and the FDA-CVM system (for veterinary drugs) (Bukowski and Wartenberg, 1996; Keller et al., 1998). One key difference of the U.S. systems from those in other countries is that they are voluntary (largely a reflection of the primarily private—i.e., not national government—health care system in America).

There is no compulsion for physicians, hospitals, or individuals to report adverse events to either the manufacturer or the government (though the marketing companies for a therapeutic are required to periodically summarize all adverse events that they know of and report them to the federal government). It is widely held that it is this voluntary (or "spontaneous") aspect which limits the effectiveness of the U.S. systems (Piazza-Hepp and Kennedy, 1995; Sharpe, 1998; White and Love, 1998; Goldman, 1998; Kennedy and Goldman, 1997; Brewer and Colditz, 1999). Studies have identified factors which influence (and limit) physician use of such systems (LaCalamita, 1995; Figueras et al., 1999), and newly marketed drugs are subject to a higher rate of underreporting of ADRs than are established drugs (Martin et al., 1998). It should be kept in mind that what we are considering here are adverse effects caused by the use of a drug as intended, and not by a medication error. Medication errors are at least as serious a problem (and complex an issue) as ADRs (Antonow et al., 2000) but are beyond the scope of this volume. Both companies and the regulatory agencies must collect reports of adverse events, evaluate them, and then decide on a correct course of action (ranging from doing nothing through improving labeling, then on to restricting access and/or requiring ongoing or increased medical surveillance of patients, to withdrawing the drug from the market).

While new medications can save or mostly improve lives, once on the market side effects—harmful ones—are virtually certain to occur and be recognized.

24.1 CAUSES OF SAFETY WITHDRAWALS

It would be comforting to be able to state that the causes of postmarketing withdrawals from drugs were substantially different from those of failure of drugs in clinical trials. While the last few years (refer back to Table 24.2) are seemingly somewhat different from those in the past, (carcinogenicity is no

TABLE 24.3 Characteristics of Drug Safety Withdrawals (1960–August 2001)

	Drugs	% of Total
Most common classes		
NSAIDs	16	13
Nonnarcotic analgesics	10	8
Antidepressants	9	7
Vasodilators	7	6
Anorexiants	5	4
CNS stimulants	5	4
Barbiturates	5	4
Anesthetics	4	3
Antihistamines	4	3
Antibiotics	3	2
Most common causes of withdrawal		
Hepatic toxicity		26
Hematological toxicity (bone marrow suppression)		10
Cardiovascular toxicity		6
Carcinogenicity		6
Renal toxicity		5
Drug interactions		4
Neurotoxicity		4
Behavioral effects		4
Abuse potential		4

longer necessarily a major marketed drug problem) the historic causes for the modern era (the last 40 years) are by hepatic toxicity—also the primary cause for safety failures in early clinical trials (see Table 24.3).

Fung et al. (2001) have done an extensive assembly and analysis of safety withdrawal data through 1999, and Ajani et al. (2000) have also analyzed factors that increase the likelihood of safety problems. Table 24.2 presents this author's extension of their work through the time of this writing (late in the third quarter of 2001), which changes the results but a little. It should be noted that the rank order of these two lists is different than the rank orders based on numbers of adverse events (see Holland and DeGruz, 1997). Adverse events can have a wide range of causes which may be due not to unanticipated effects of a drug but to medication error or something as mundane as discrepancies between doses recommended in *The Physician's Desk Reference* and those recommended or reported in the medical literature (Cohen, 2001).

In 2005, Schuster et al . published an anaysis stating that cardiovascular toxicity accounted for 40% of current (2000–2005) safety withdrawals and hepatotoxicity for 27% (leaving all others to share 33% of the total). Failure to identify these largely predictable causes of failures in new therapeutic entities largely reflects both a continuing lack of recognition of the actual patient populations utilizing drugs with their existing pathophysiological

TABLE 24.4 Factors That Increase Patient Risk for Adverse Drug Interactions

Factor	Group/Disorder
Age	Neonates, elderly
Gender	Women
Genetic phenotype	Slow metabolizers
Chronic disease	Moderate/severe renal or hepatic impairment, CHF, cirrhosis
Acute illness	Pneumonia, influenza
Metabolic disturbances	Hypothyroidism, hypoxia
Multiple drug use	Elderly, HIV patients
Multiple prescribing physicians	Elderly
Use of drugs with a low therapeutic index	
Use of drugs that are enzyme inhibitors or inducers	

TABLE 24.5 Limitations of FDA's Current Clinical Trials

1. Too few	Prior to approval, most drugs are administered to 2000–3000 patients. (To obtain an 80% probability of detecting an adverse drug event that occurs in one out of every 10,000 recipients, 16,000 patients must receive the drug.)
2. Too simple	Premarketing trials often exclude patients with complicated medical histories or medication regimens. It is easier to demonstrate efficacy without including these complex patients.
3. Too median	Most premarketing trials exclude patient populations such as pediatric, geriatric, lactating, and pregnant patients.
4. Too narrow	Premarketing trials are generally intended to investigate a drug for a single indication. After release to the market, the drug may be used to treat other conditions in different populations with varying medical histories.
5. Too brief	Adverse drug events that occur only with chronic use will not be detected in the relatively short clinical trial.

Source: From Rogers, 1987.

characteristics (Table 24.4) and limitations of the currently employed clinical trial scheme (Table 24.5). While such efforts as mandatory assessment of safety pharmacology features will serve to improve the situation, for the foreseeable future it remains vital to ensure that our pharmacovigilance systems identify problems as soon as possible.

24.2 REGULATORY REQUIREMENTS

Regulations and guidelines concerning pharmacovigilance have been in a continuous state of change and development in recent years. A discussion of

thse should start with the understanding of the individual incident, or "case."

A case is a basic unit of drug safety surveillance. It is used to ensure, to the greatest extent possible, the safety of approved drug products that are still in use. The basic unit of all postmarketing safety submissions is the adverse drug experience (AED) case, which is an individual adverse drug experience.

The FDA has explicit requirements for reporting adverse event cases for drugs. A postmarketing adverse drug experience source can be categorized into several sample categories: clinical trial, nonclinical trial/regulatory authority, nonclinical trial/literature, and nonclinical trial/all other. This chapter only deals with spontaneous experiences—nonclinical trial adverse drug experiences reported to the industry any time after a marketed drug product achieves marketing approval from the FDA (Adams et al., 1997).

A typical case folder will contain certain types of information:

- The outside of a folder is identified by a numeric or alphanumeric code.
- There is an "initial" report.
- There is either at least one letter requesting additional information regarding the initial report or documentation reflecting the failed attempts to obtain additional information.
- There is at least one "follow-up" report.
- The spontaneous report event is categorized as serious or nonserious, expected, or unexpected.
- The source is literature, regulatory authority, or spontaneous.
- There is at least one MedWatch form or Council for International Organization of Medical Sciences (CIOMS) I form for each report.

Everything in a given case folder is present because of an FDA regulation requirement or a related company-written standard operating procedure.

The designation on the outside of the case is required to be numeric or alphanumeric rather than the name of the patient. Patient names are not permitted to be publicly disclosed in the context of a MedWatch report per 21 Code of Federal Regulations (CFR) 21.63(f). The initial report is the first reported information received by the company about an individual's adverse drug experience. There must be a "prompt" attempt to obtain follow-up information about each initial report. The attempt(s) are made per the company's written procedures. If the written safety procedures are not followed, the safety reports are not appropriately submitted, or the safety records are not appropriately kept, the FDA has the authority under Section 80 of Part 315 to withdraw the market new drug application (NDA). The follow-up report is the format for submitting additional information about an experience. Each case regards only one individual unless the experience is both temporally and clinically unrelated to a second event experienced by the same person taking the same drug product.

TABLE 24.6 How Spontaneous Drug Case Is First Submitted to FDA

Case Source/Case Type	Report Submitted
Foreign literature/not both serious and unexpected	Not 15-day, not periodic
Foreign literature/serious and unexpected	15-day
U.S. consumer/not both serious and unexpected	Periodic
U.S. consumer/serious, unexpected	15-day
Foreign consumer/not both serious and unexpected	Not 15-day, not periodic
Foreign consumer/serious and unexpected	15-day
FDA, initial/serious and unexpected	15-day
FDA, initial/serious and unexpected	Periodic, not 15-day
International regulatory authority/serious and unexpected	15-day
International regulatory authority/not serious and unexpected	Not 15-day, not periodic

Table 24.6 summarizes FDA reporting requirements of spontaneous reports in terms of how the case event is first submitted to the agency. The definitions of *serious*, *unexpected*, and so on, are in 21 CFR 314.80(a) (CFR, 1994).

The 15-Day Report versus U.S. Periodic Report Postmarketing adverse drug experiences are reported to a drug company by the public via regulatory authorities, literature, attorneys, consumers, and health professionals. Sometimes an adverse drug experience is reported directly to the FDA and the FDA then submits the report to the company. When the FDA sends the applicant an initial MedWatch report, the information does not have to be resubmitted to the FDA in an initial 15-day report if the information is serious and unexpected. This is because the FDA already has knowledge of the report. However, MedWatch and its information are incorporated into the next periodic report of the product. Follow-up to a report obtained from a non-FDA source would be submitted as an expedited 15-day report (and should reference the source of the initial report). When the FDA sends the applicant an initial MedWatch that is not both serious and unexpected, the applicant incorporates the information into the next periodic report, under the normal procedure for submitting follow-up information.

If an initial 15-day report was submitted and the first follow-up information reflects that the event is no longer classified as suitable for a 15-day report (never was serious and unexpected), the first follow-up report describes the change in the report classification but is a (first) follow-up 15-day report. Subsequent additional information is not submitted in the form of a 15-day report.

A periodic report contains certain information, such as the event terms submitted during the period, the dates that events of the period were submitted, an event term count by body system, and labeling changes made due to the period's adverse experiences. In addition (and prior) to being incorporated into a periodic report, 15-day reports are submitted within 15 calendar

days of the date the applicant received the data. All 15-day reports contain serious, unexpected events. Non-15-day reports are submitted periodically in FDA periodic reports.

If on a given day a serious, unexpected domestic report is received, it is first submitted on FDA form 3500A within 15 calendar days of receipt via the 15-day report and subsequently is incorporated (not in the form of FDA form 3500A) into a periodic report. If a report is received that is domestic but not both serious and unexpected, it is not submitted in a 15-day report but rather in the U.S. periodic report. A U.S. periodic report is submitted quarterly for the first three years after the date the product was approved by the FDA for marketing (21 CFR 314.80). However, the March 2001 FDA guidance allows an applicant to request a 21 CFR 314.90 waiver of the U.S. periodic reporting period and base the report not on the date of FDA marketing approval but instead on the international birth date (the first date the product was approved in the international community). The request for such a waiver should be submitted to Director, Office of Postmarketing Drug Risk Assessment, Center for Drug Evaluation and Research (CDER), FDA, 5600 Fishers Lane, HFD-400, Rockville, Maryland 20857. The request should include the product's name, the date of FDA marketing approval, and the product's approved application number. In addition, an applicant may request a 21 CFR 314.90 waiver of the 21 CFR 314.80(2)(ii) format of the periodic report submitted. If the waiver is granted, the International Conference on Harmonisation (ICH) E2C Periodic Safety Update Report format may be used, provided that the content of the Section 80(2)(ii) information that is not in the body of the ICH E2C periodic report is found in appendices, that is, certain reports from consumers that are not in the body of the ICH E2C periodic report submission. Among other things, 21 CFR 314.90 states that the applicant may request that the FDA waive any of the postmarketing requirements under 21 CFR 314.80.

ICH E2C and the FDA March 2001 draft guidance "Post-Marketing Safety Reporting for Human Drug and Biological Products Including Vaccines" are available at www.fda.gov/cder/guidance/index.htm; case requirements are accessible in 21 CFR 314.80.

The major change over the past few years has been a significant attempt to harmonize regulations under the aegis of the ICH, and this is covered in some depth below. The latest response to the ICH in its three participating regions (Europe, United States, and Japan) is also described together with an update on the current U.K. regulations. The ICH potentially offers real advantages to the pharmaceutical industry, but the process takes time and countries have adopted and implemented the guidance in slightly different ways and at different times. National regulations and guidelines are therefore bound to change in the near future as each country embraces the ICH.

The ICH of Technical Requirements for Registration of Pharmaceuticals for Human Use has brought together as equal partners the regulatory authorities of Europe, Japan, and the United States and experts from the pharmaceutical industry in these regions to discuss scientific and technical aspects of

product registration. The WHO, European Free Trade Area (EFTA), and Canada are observers, and the International Federation of Pharmaceutical Manufacturers Association (IFPMA) ensures contact with the research-based industry outside the ICH regions.

The aim of the ICH is to achieve greater harmonization in the interpretation and application of technical guidelines and requirements for product registration and reduce or eliminate duplicate testing. This should result in better use of resources and eliminate unnecessary delay in the global development and availability of new medicines while maintaining safety guards on quality, safety, and efficacy.

There are four broad areas within the ICH:

S—Safety (animal toxicology and pharmacology)
Q—Quality (pharmaceutical and analytical)
E—Efficacy (clinical)
M—Multidisciplinary topics

Timely, complete reporting of ADRs and medical device problems is essential to an effective national system of postmarketing surveillance. Pharmaceutical manufacturers are required by federal regulations to report all ADRs of which they are aware to the FDA. However, many health care professionals do not report adverse events to either the manufacturers or the FDA. To encourage and facilitate the reporting of serious adverse events, the FDA launched the MedWatch reporting program in June 1993. The MedWatch reporting form is used by health care professionals to voluntarily report ADRs and other problems with all FDA-regulated products used in medical therapy (drugs, biologicals, medical devices, and special nutritional agents). The ADRs associated with vaccine products is the only exception, since reporting of these ADRs is mandatory. The form used for vaccines is the joint FDA/Centers for Disease Control and Prevention (CDC) VAERS form. For drugs and therapeutic biologicals, the MedWatch (3500A) form replaces the 1639 reporting form.

The FDA does not require reports on every adverse event observed, as this would not be practical for reporters or the FDA because of the sheer number of adverse-event reports already being sent to the agency each year (about 130,000 in 1994). While 80–85% of these reports are submitted by the manufacturer, 10–15% are received by MedWatch directly from physicians, pharmacists, other health care professionals, and consumers. MedWatch encourages reporters to be selective by limiting their reports to events for which the outcome was serious (see the definition on page 876 in the previous chapter). This enables the FDA to focus on those events with potentially the largest public health impact. Reporters are encouraged to fill out the reporting form as completely and accurately as possible.

From 1978 through 1990, the CDC and the FDA divided the responsibility for postmarketing surveillance of vaccines in the United States. The FDA

received reports of adverse events after vaccines were administered in the private sector; events occurring after the administration of vaccines purchased with public funds were reported to the Monitoring System for Adverse Events Following Immunization.

The monitoring system was a stimulated passive surveillance system. In other words, when vaccines purchased with federal funds were administered in the public sector, "Important Information" forms were given to recipients or their parents or guardians instructing them to report any illnesses requiring medical attention that occurred within four weeks of vaccination. System coordinators at each immunization project/grantee site and the state health department completed standardized forms that were reviewed for consistency and completeness and then forwarded to the CDC for data entry and analysis.

In response to the National Childhood Vaccine Injury Act of 1988, which required health workers to report vaccine adverse events, the CDC and the FDA collaborated in 1990 to implement the VAERS to monitor the safety of vaccines in both sectors. Health care professionals and parents/caretakers are *encouraged* to report all clinically significant vaccine adverse events. Narrative diagnostic reports are reviewed and assigned standard codes using Coding Symbols for a Thesaurus of Adverse Reaction Terms. The source of the vaccines (public versus private provider) is recorded on the form.

The WHO system, created in response to the thalidomide disaster, seeks to capture worldwide adverse events and identify problems (WHO, 1975; Olsson, 1998). It is proposed that first all such gathered reports be analyzed for mortality effects and trends (Rose and Elnis, 2000) and then the identified most critical trends be evaluated.

24.3 MANAGEMENT OF ADR AND ADE DATA

In monitoring the safety of products, pharmaceutical companies need to comply with worldwide regulations as well as the primary requirement of helping doctors to prescribe safely. The intent in this chapter is not to provide a comprehensive review but to provide an insight into the methods of managing ADR data.

Sources of Data There can be an enormous variation in the nature and quality of data depending upon the source, and this must be considered when the data are processed, computerized, and analyzed. Safety data may come from any of the sources described below.

Clinical Trials In phase I studies, good documentation and additional investigations should be standard practice. Serious reactions are pretty unusual in these studies, which will detect only very common ADRs, in particular those

that are pharmacologically mediated (e.g., bradycardia with beta-adrenergic receptor antagonists).

Good documentation and follow-up should be possible in phase II studies, but rare reactions will not be identified due to the small numbers of patients involved. The larger numbers in phase III trials can pose problems, but these can be minimized by careful choice of investigators, good case report form design, and procedures for follow-up. Phase IV studies are designed to test the efficacy and safety of the drug in clinical practice and often share the same constraints in patient numbers as premarketing trials.

Postmarketing Surveillance Studies Any surveillance of the safety of a drug after marketing is postmarketing surveillance (PMS) (now often referred to as a postauthorization safety study). In practice the distinction between phase IV studies and PMS is blurred (e.g., German drug experience studies).

Spontaneous Reports Spontaneous reports are the most effective means of identifying rare, serious adverse reactions (usually idiosyncratic or type B) after marketing despite the underreporting that exists. Spontaneous reports are an unsolicited communication to a company, regulatory authority, or other organization that describes an adverse event in a patient given one or more medical products. These reports do not originate from a study or from any organized data collection scheme. Unless indicated otherwise by the reporter, all spontaneous adverse events are assumed to be possible ADRs. The quality and completeness of spontaneous reports is often inadequate. Pharmaceutical companies or regulatory authorities can only achieve good case documentation through effective data collection, detailed follow-up, and use of field workers for complex cases. The quality of spontaneous reports also varies from country to country. Some countries do not have a regulatory reporting form for ADRs. There are differences among countries in publicity of drug safety issues and drug regulations differ regarding the format, content, and submission time frames for ADR reporting.

Reports received by companies via regulatory authorities are often edited and poorly documented, but they cannot be ignored and should be handled alongside reports received directly. The FDA implemented the Medical Products Reporting Program (MedWatch) in 1993, which encourages health care providers to regard reporting as a fundamental professional and public health responsibility and submit serious adverse-event reports directly to the FDA on the FDA 3500A form. The FDA forwards these reports to the manufacturer, who is obliged to follow up with those reporting such events and submit any relevant information obtained to the FDA and other regulatory agencies worldwide as required. It is currently proposed that regulatory agencies (FDA) should take a more direct hand in these activities (Snidermann, 2000).

Literature The publication of case reports in medical and scientific journals is an important primary source of information on ADRs. Many ADRs are

noted in medical and scientific journals before they become well known. For example, the association of thalidomide with birth defects was first noted in a letter to *Lancet* in 1961. The quality of ADR reports in the published literature can be variable and has been the subject of much criticism and correspondence, though guidelines have been promulgated for these (Jones, 1982).

Despite the anecdotal nature and sometimes poor documentation, publication of case reports in journals remains one of the most useful primary sources of information on ADRs. ADR reports in the literature can be identified in several different ways. Prepublication manuscripts describing a spontaneous case report or an event from a clinical trial are sometimes provided by authors to the manufacturer of the drug and the regulatory authority in that country. Pharmaceutical companies are required to be aware of the literature as to the safety of their approved therapeutic products and are assumed (by law) to be cognizant of such.

Searching for ADRs in Literature With the increasing number of scientific and biomedical journals there are more sources of ADR data on many drugs. Conversely, for some drugs, particularly those recently marketed, there is a scarcity of clinical publications and frequently there is an inadequate account of the adverse reaction profile. Searching for ADRs in the literature may be assisted by online databases such as MEDLINE (Index Medicus) and EMBASE (Excerpta Medica) and secondary sources such as SEDBASE (Meyler's side effects of drugs) and ADIS online services such as REACTIONS. Many journals contain relevant information, but some specific ADR-related journals may assist in the search for information. Increasingly, the use of high-capacity storage systems such as compact disks (CD-ROM) has led to stand-alone systems for storage and search of the literature other than online systems. Integrated dictionaries have allowed the development of user-friendly information; however, due to the anecdotal nature of these reports, pharmaceutical companies should have a clear policy on how to handle them.

Information Required for Reports In order to draw a conclusion about the possible relationship between a drug and an adverse event, certain minimal information elements are required. Points considered essential for literature reports have been proposed (Jones, 1982) and some journals issue guidelines or checklists for potential authors. These can be adapted as a potential checklist for information that should be included in any ADR report as follows:

- Patient demography—age, sex, body weight, height, race, pregnancy.
- Medical history—previous medical history and concurrent conditions, known allergies (including ADRs with similar drugs), previous experience with drug.
- Timing—duration of treatment with the suspect drug before the adverse event.

- Concurrent medications—details of other drugs, including formulation, dose, and duration.
- Dechallenge—action taken with the suspect drug (stopped, continued, dose reduction).
- Outcome—outcomes of the adverse events.
- Alternative causes—what other factors could have accounted for the adverse event (diet, occupations exposure) and which were excluded?
- Rechallenge—was the patient rechallenged, and if so, what was the result?
- Relevant additional data—blood levels, laboratory data, biopsy data, and, where relevant, postmortem findings.

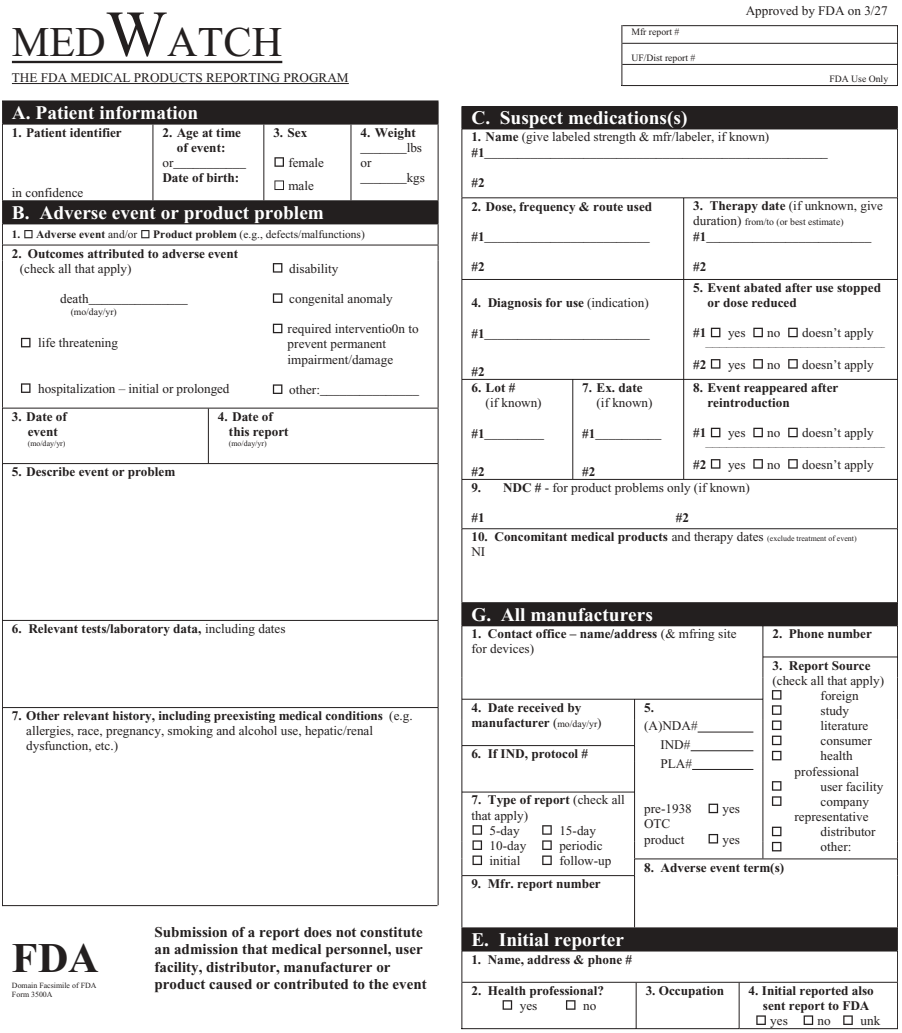
ADR Forms and Form Design. Many forms are used by different organizations to collect ADR information. Most regulatory authorities have their own form (see Figure 24.1). Although the content of these forms is similar, little attempt has been made to standardize the design other than by the CIOMS.

In order to design the best form for their needs, users must first define what data they wish to collect and which factors are of the greatest importance. In addition, all the usual factors in form design need to be considered (e.g., size, layout, color, print type, spacing, flow of questions, boxes, language, and instructions). A pilot to test the form should be carried out before formal introduction and use.

Consideration should be given to what happens to the form once it is returned. Form design will be affected depending upon whether it is intended to serve as a direct-entry document (i.e., the data elements closely match the data entry screens) or whether a transcription document will be used.

The key factor in ADR form design is the compatibility with other forms required for output, most importantly regulatory authority forms. The FDA, for example, required ADR reports to be submitted on form FDA 3500A (Figure 24.1). If the pharmaceutical company does not wish to collect data on an FDA 3500A but must submit reports to the FDA, it will need to design a form that collects the same information. Adverse-event report forms generally collect the basic data elements outlined below:

- Patient demography
- Relevant medical history and allergies
- Suspect and concurrent drugs, route, indication
- Adverse events
- Treatment and management of adverse event
- Dechallenge, rechallenge, outcome
- Relevant laboratory data
- Reporter's opinion of causality
- Report source of information



MEDWATCH

THE FDA MEDICAL PRODUCTS REPORTING PROGRAM

Approved by FDA on 3/27

Mfr report #

UF/Dist report #

FDA Use Only

A. Patient information

1. Patient identifier — in confidence
2. Age at time of event: or Date of birth:
3. Sex: ☐ female ☐ male
4. Weight: _____lbs or _____kgs

B. Adverse event or product problem

1. ☐ Adverse event and/or ☐ Product problem (e.g., defects/malfunctions)
2. Outcomes attributed to adverse event (check all that apply)
   - death_____ (mo/day/yr)
   - ☐ life threatening
   - ☐ hospitalization – initial or prolonged
   - ☐ disability
   - ☐ congenital anomaly
   - ☐ required interventio0n to prevent permanent impairment/damage
   - ☐ other:_____
3. Date of event (mo/day/yr)
4. Date of this report (mo/day/yr)
5. Describe event or problem
6. Relevant tests/laboratory data, including dates
7. Other relevant history, including preexisting medical conditions (e.g. allergies, race, pregnancy, smoking and alcohol use, hepatic/renal dysfunction, etc.)

C. Suspect medications(s)

1. Name (give labeled strength & mfr/labeler, if known) #1_____ #2
2. Dose, frequency & route used #1_____ #2
3. Therapy date (if unknown, give duration) from/to (or best estimate) #1_____ #2
4. Diagnosis for use (indication) #1_____ #2
5. Event abated after use stopped or dose reduced #1 ☐ yes ☐ no ☐ doesn't apply #2 ☐ yes ☐ no ☐ doesn't apply
6. Lot # (if known) #1_____ #2
7. Ex. date (if known) #1_____ #2
8. Event reappeared after reintroduction #1 ☐ yes ☐ no ☐ doesn't apply #2 ☐ yes ☐ no ☐ doesn't apply
9. NDC # - for product problems only (if known) #1 #2
10. Concomitant medical products and therapy dates (exclude treatment of event) NI

G. All manufacturers

1. Contact office – name/address (& mfring site for devices)
2. Phone number
3. Report Source (check all that apply)
   - ☐ foreign
   - ☐ study
   - ☐ literature
   - ☐ consumer
   - ☐ health professional
   - ☐ user facility
   - ☐ company representative
   - ☐ distributor
   - ☐ other:
4. Date received by manufacturer (mo/day/yr)
5. (A)NDA#_____ IND#_____ PLA#_____ pre-1938 OTC product ☐ yes ☐ yes
6. If IND, protocol #
7. Type of report (check all that apply) ☐ 5-day ☐ 15-day ☐ 10-day ☐ periodic ☐ initial ☐ follow-up
8. Adverse event term(s)
9. Mfr. report number

E. Initial reporter

1. Name, address & phone #
2. Health professional? ☐ yes ☐ no
3. Occupation
4. Initial reported also sent report to FDA ☐ yes ☐ no ☐ unk

FDA

Domain Facsimile of FDA Form 3500A

Submission of a report does not constitute an admission that medical personnel, user facility, distributor, manufacturer or product caused or contributed to the event

Figure 24.1 *FDA form 3500A.*

The form can be printed as a postage prepaid folding envelope for domestic use to encourage a reply. The pharmaceutical company must be able to demonstrate due diligence in seeking relevant follow-up information on each adverse-event report.

Within the next two years, several key regulatory authorities including the MCA (Medicines Control Authority) and FDA will require electronic data submission by companies for both expedited and nonexpedited case reports. The compatibility between the company's and the regulatory authority's data-

bases with regard to content and format of the key data elements for transmission is a critical factor to success of these initiatives. The adoption of internationally sanctioned standards such as a dictionary of medical terms, various code lists (e.g., countries, routes, units), file formats, and periodic safety update reports is essential to enable efficient and accurate transmission. The ICH guideline E2B defines data elements for transmission of individual case safety reports. The guideline aims to standardize the data elements for all individual case safety reports regardless of source and destination and covers reports for both preapproval and postapproval periods. It also defines the minimum information for a report and the requirements for proper processing of the report. The medium for electronic submissions will be Electronic Data Interchange (EDI)–encrypted transmissions over the Internet.

Computerization of Drug Safety Data: Data Collection and Input "Rubbish in, rubbish out" applies to safety data as to any other computerized data. The enforced control of terms at entry can be linked to checking of data, which should form part of the quality control procedures. Such controls should be driven by the business so that clinical trial data, free from all errors and needed for statistical analysis, will probably involve double data entry whereas single data entry is generally considered adequate for adverse-event databases used for signal generation and regulatory reporting.

Data are still generally typed into a database rather than electronically loaded from other systems. The first step of any data entry process should involve a check for duplicate cases. The need for decision making at the data entry stage will depend upon the type of database design. In all cases, there should be clear rules on how data should be entered into each field to ensure consistency and aid subsequent searching and outputting. This is particularly important when there are multiple users distributed over a number of international sites. Use of electronically available field-specific lists of value and well-defined coding conventions will help with this.

In the future, data will increasingly be captured electronically. Image processing and developments in optical character recognition are already proving useful. Electronic data capture (using fax or pen-based methods) is used to collect data in some clinical trial.

With the increase in licensing agreements between pharmaceutical companies, safety data frequently need to be exchanged between one or more parties. If the case volume is sufficient, it is worth considering electronic data exchange between the databases involved. In addition to preventing rekeying of data, this minimizes discrepancies between the data sets. With the adoption of proposed ICH standards in the future, this will become a much simpler process.

Medical and Drug Terminology Medical and drug terminology is at the heart of the ADR systems. Accurate and consistent input of terms is critical for retrieval and analysis of ADR information. An integrated dictionary allows the capture of original text, which is autoencoded against the dictionary

to retrieve the correct code for that piece of text. Coded information allows easy retrieval and analysis. The dictionary structure should allow different ways of grouping and analyzing data encompassing body systems at the highest level. Specific reporter's wording should meet the following needs:

- Acceptable to all users of the system.
- New terms can be easily added.
- Specificity of the reported term preserved.
- Hierarchical structure to group terms at various levels of specificity.
- Logical groupings so similar terms are not scattered.
- A default grouping for each term.
- Unambiguous to enable autoencoding on input.

Dictionaries This section compares commonly used dictionaries in monitoring drug safety. As electronic exchange of ADR data between industry and regulatory authorities in different countries increases, so does the need for standardization of terminology (Benichou et al., 1991). MedDRA (Medical Dictionary for Drug Regulatory Affairs) has completed development with version 4.0 just being available and is discussed later in this chapter. Table 24.4 presents a summary of its structure (Brown et al., 1999; Gruchalla, 1995).

Medical Term Coding Dictionaries It is logical to deal with adverse events, indications, diseases, surgeries, and procedures using one system for the following reasons:

- ADRs frequently mimic spontaneously occurring diseases; hence the same diagnosis or symptom could appear as an adverse event or disease.
- In the identification of new ADRs, it is important not to separate a possible side effect from a disease.
- Separate classifications can lead to confusion and add a layer of complexity when developing ADR systems.

Meaningful codes may or may not be needed for modern dictionaries. For example, the new Adverse Drug Reactions On-Line Information Tracking (ADROIT) dictionaries do not use meaningful codes but rely on linkage of related terms and effective text processing. Where codes are considered necessary, they should be as short as possible (Westland, 1991). Whenever a system is used for adverse events from the literature, spontaneous reports, clinical trials, or a combination of these, the needs of the users of the system will influence the selection of the dictionary.

MedDRA (Medical Dictionary for Regulatory Activities) MedDRA is a medical dictionary encompassing terms relevant to pre- and postmarketing

phases of the regulatory process. It was developed by the MCA to support its information systems and has subsequently been further developed by the MCA to support its information systems and Medical Terminology Working Group. The objective is to harmonize standards for electronic submissions among regulatory authorities and between authorities and industry within and across regions. The aims of the dictionary are:

- To address pre- and postmarketing adverse-event reporting
- To cover multiple medical product areas
- To be available in multiple languages
- To be available in multiple formats and platforms
- To be well maintained

The guiding principles are:

- To build from existing terminologies to maximize compatibility
- To focus on the international community need rather than on individual countries
- To ensure worldwide use through collaboration and participation in development
- To ensure mechanisms and structures are in place for translation into many languages
- To ensure long-term maintenance

The scope of MedDRA is as follows:

- Disease
- Diagnoses

There is a dual classification for some terms (e.g., 573.1, "Hepatitis in viral diseases classified elsewhere"), but this is not extensive. The dictionaries are very comprehensive with the exception of symptoms, which tend to be scattered. They have been widely used in coding patient histories and hospital charts.

ICD-9 CM is a clinical modification of ICD-9 and offers some advantages, particularly the inclusion of synonyms, but is constrained by systems that have used the older versions of ICD-9. ICD-10 is more comprehensive than any ICD revision to date (see www.meddramsso.com/mssoweb). It extends well beyond the traditional causes of death and causes of hospitalization. The content has been expanded to include symptoms, signs, abnormal findings, factors related to lifestyle, and other factors causing contact with health services:

TABLE 24.7 MedDRA Structure

Level of Hierarchy	Approximate Number of Terms	Definition	Example
System organ class (SOC)	26	Broadest collection of concepts for retrieval; grouped by anatomy or physiology	Cardiac disorders
High-level group term	333	Broad concepts for linking clinically related terms; can be linked to one or more SOCs	Cardiac rhythm disorders
High-level term	1,685	Groups of preferred terms related by anatomy, pathology, physiology, etiology, or function; can be linked to one or more high-level group terms or SOCs	Tachyarrhythmia
Preferred term	14,287	International level of information exchange; single, unambiguous clinical concept	Ventricular tachycardia
Lowest level term	51,083	Synonyms and quasi-synonyms; help define scope of preferred terms	Paroxysmal ventricular tachycardia

- Signs and symptoms
- Therapeutic indications
- Investigation names and qualitative results
- Medical and surgical procedures
- Medical, social, and family history
- Terms from COSTART (Coding Symbols for a Thesaurus of Adverse Reaction Times), WHO-ART (World Health Organization Adverse Reaction Terminology), ICD-9, HARTS, J-ART

The current structure of MedDRA is defined in Table 24.7. There will be a central maintenance organization responsible for development, user support, implementation, and communication as well as an international user group. A management board will oversee the activities of the central maintenance organization with direction provided by the ICH steering committee. A standard medical dictionary will facilitate electronic data exchange between industry and regulatory authorities worldwide, as recommended by the ICH.

Table 24.8 presents current annual MedDRA fees.

FDA Under the March 2001 draft guidance "Postmarketing Safety Reporting for Human Drug and Biological Products Including Vaccines" (FDA, 2001), the FDA will accept SAEs coded with MedDRA, COSTART, or WHO ART MedDRA has been implemented for SAE coding in FDA's Adverse Event Reporting System (AERS) program. While the FDA encourages

TABLE 24.8 MedDRA Annual Fees

Basic service[a]	$3,000/year
System developer (no change requests)	$5,000/year
Core service[b]	
Under $10 million in annual revenue	$7,000/year
$10–500 million in annual revenue	$12,000/year
$500 million to $1 billion in annual revenue	$23,000/year
$1–5 billion in annual revenue	$62,000/year
More than $5 billion in annual revenue	$82,000/year

[a]Reserved for nonprofit medical libraries, educational institutions, and direct patient care providers. No change requests are available for this service.
[b]Have the ability to send in proposed changes to MSSO. An individual core subscriber can send in up to 100 proposed changes a month. Any changes beyond that will be charged $325 per change. Collectively, no more than 9000 change requests from all core subscribers can be received per year. Anything beyond that will be charged $325 per request and distributed among all core subscribers. A change must be medically valid and internally acceptable.

companies to use MedDRA, the deadline for full MedDRA implementation is still pending.

European Union (EU) The European Agency for the Evaluation of Medical Products (EMEA) established January 2002 as the deadline for all electronically filed single case reports to be coded in MedDRA. All ADR reporting must be coded in MedDRA by January 2003.

Japan MedDRA/J, the Japanese version of MedDRA, was officially issued on December 28, 1999, and the Ministry of Health highly recommended its use for ADR reporting beginning at the end of March 2000. However, J-ART terms are still applicable and upon submission to the ministry are begin converted to MedDRA/J terms. No firm deadline for full implementation has been issued.

MedDRA is available in English and Japanese only. The Maintenance and Support Services Organization (MSSO) is working on translations in French, Portuguese, German, Greek, and Spanish. With their annual dues, subscribers can get MedDRA in English and one other European language (when available). Japanese or additional European languages will need to be purchased separately. The German and Portuguese translations were recently submitted to MSSO for review.

The most current version of MedDRA, 11.0, was released in March 2008. The cost of the dictionary depends on the type of organization and annual revenue. An annual subscription provides a company with all versions of MedDRA released during the year. All regulators are provided MedDRA free of charge. Otherwise, costs are as follows:

Periodic Reports Many regulatory authorities require detailed summary reports on groups of cases on a regular basis. The FDA requires annual progress reports for investigational compounds and periodic reports for marketed

drugs either quarterly or annually depending upon the length of time the product has been on the U.S. market. CIOMS II guidelines recommend submission of line listings of serious, unlabeled spontaneous cases in conjunction with a summary of the drug safety profile on a six-month basis. These reports are well defined in format, content, and submission time frame. Most major pharmaceutical companies produce them electronically.

The regulatory requirements, particularly regarding frequency of submission and content, differ in the tree regions (Europe, Japan, and the United States). In order to avoid duplication of effort and to ensure that important data are submitted with consistency to regulatory authorities worldwide, the ICH3 Topic E2C Guideline on the Format and Content for Comprehensive Periodic Safety Update Reports (PSUR, 1996) of marketed medicine products has been developed. The general principles of this guideline include:

- One report is submitted for one active substance. All dosage forms as well as indications for a given active substance should be covered in one PSUR.
- The focus is on ADRs, which include all spontaneous reports and all drug-related clinical trial and literature reports.
- An international birthdate and frequency of review and reporting are defined. The international birthdate is the date of the first marketing authorization for the product granted to any company in any country in the world. Preparation of PSURs should be based on data sets of six months or multiples thereof. The PSUR should be submitted within 60 days of the data lock point.
- The reference safety information is the company core data sheet to determine whether an ADR is listed or unlisted.
- ADR data are presented in line listings and/or summary tabulations.

24.4 CAUSALITY ASSESSMENT

Decisions have to be made by pharmaceutical companies and regulatory authorities about whether a drug can cause a particular adverse event so that an appropriate action can be taken. What does “can cause” mean? Does it imply certainty? In many cases waiting for “certainty” before taking action would entail many patients suffering unnecessarily. The degree of certainty, or “probability,” required will vary according to the situation.

There are nearly always many factors other than the administration of a drug that can cause an adverse event and will determine whether the adverse event will occur in a particular patient. The drug may be “the last straw that broke the camel’s back.” If an adverse event would not have occurred if not for the drug, then the drug caused the adverse event (Hutchinson, 1992). So with an adverse drug interaction both drugs caused the adverse event. Using this definition the drug may only be a minor factor.

Certainty is rarely obtainable; perhaps an adverse event with a positive rechallenge where there is objective evidence and an absence of confounders in an individual case would be considered as certainty due to the drug. In the majority of cases action is needed before there is absolute certainty that a drug can cause an adverse event. This lack of certainty in individual cases has been described using rather vague terms such as "almost certain," "probably," "possible," and "unlikely." These terms have also been defined, but each author has a slightly different definition (Venulet et al., 1982; Stephens et al., 1998).

Again, in epidemiological studies or clinical trials there is nearly always a degree of uncertainty due to bias, chance, and confounders. In these studies uncertainty is measured in terms of *p* values, odd ratios, and relative risks.

The differential diagnosis of adverse events associated with a drug or drug(s) is an everyday part of a practicing clinician's life (Rogers, 1987). However, the term *causality assessment* is reserved for a similar process performed at one or more stages removed from the patient and with some important differences. Clinicians do not necessarily need to find out whether a drug caused an adverse event in order to satisfy themselves and their patients. They will be more interested in resolving the event as quickly as possible. If there is a possibility that the event might be an ADR, it may be resolved either by reducing the dose or stopping the drug or by treating the ADR while waiting for tolerance to develop or it may resolve if any of the underlying factors are altered. The resolution of the adverse event might be because the event has been caused by the drug or it may have been a transient natural occurrence; either way the patient and doctor will welcome its disappearance. If, however, the doctor is interested in knowing whether it was an ADR, further investigations can be undertaken, as long as the patient is willing, until it is established or refuted.

When causality assessment is undertaken by a regulatory authority or a scientist/physician in industry, it is unlikely that the full details known to the clinic treating the patient will be reported, even after further inquiry is made. The only way to obtain all available data is usually by visiting the physician and, with permission, reading the notes and discussing the case with him or her.

Aims of Causality Assessment Of the many similar events on an adverse-event database, only a few have sufficient and relevant data to enable the assessor to decide what caused the adverse event. A preliminary assessment (sometimes referred to as a "triage") can be made by placing the event into a category (e.g., probably, possibly, or unlikely or using the EEC classification of A, B, or O) (Mezboom and Rozer, 1992). This will enable the company to extract the probable cases at regular intervals in order to consider whether there is a "signal." The possible and unlikely cases will probably not contribute much to this signal.

This preliminary assessment will need to be updated as and when further information becomes available. It should favor sensitivity over specificity so

that a borderline possible/probable case is classified as probable rather than possible to make certain that the case is not lost when at a later stage the probable cases are picked out as a signal. A full assessment when all the information is available can then rectify any misclassifications.

24.5 COURSES OF CORRECTIVE ACTION

Identification of a safety issue with a marketed drug does not necessarily (or even usually) lead to the withdrawal of that therapeutic agent from the market. As noted at the beginning of this chapter, there are a range of possible actions:

- Change in dosage or dose form (reformation)
- Change in labeling (warnings)
- Restriction of situation of use (from open prescription to either clinician administration or hospital use only)
- Monitoring of patients during use
- Restriction on use (i.e., of patients allowed to use)
- Withdrawal from the market (usually a permanent step, but not always)

Which action(s) are taken depends on severity and incidence rates of the adverse response, technical details, the existence of alternative therapies, and the benefit of the use of the drug.

24.6 LEGAL CONSEQUENCES OF SAFETY WITHDRAWAL

Although in the context of personal injury claims an HMO and other parties (e.g., doctors, hospital) may all be the target of proceedings, it is usually the pharmaceutical company, perceived as having "deep pockets," that is the prime target for claimants. Claims for negligence based upon a failure to act with reasonable care (e.g., to obtain or act upon pharmacovigilance data) and/or the supply of a product that is "defective" in legal terms (e.g., because its labeling was not amended, pursuant to the receipt and review of pharmacovigilance data so as to give adequate warnings and precautions) is always possible.

Tables 24.9 and 24.10 set out in very simple terms the necessary "ingredients" for establishing product liability, either in negligence or under statute: so-called strict liability.

All of the elements of each of these legal wrongs must be present in a given situation for liability to be established. In negligence, therefore, where the claim is made against the person alleged to owe the duty of care (in the context of this chapter, this will be the company putting the product on the market), proof of causation, without a lack of reasonable care having occurred, will not

TABLE 24.9 Criteria for Negligence—D + L + F + C = N

D: Duty of care	Owed to claimant; easy to establish in case of supplier/manufacturer vis á vis patient who uses product
L: Lack of reasonable care	Evidenced by failure to conduct operations according to accepted standards applicable at time—that is, breach of regulatory requirements or possibly failure to take account of or apply (industry) guidelines
F: Foreseeable injury	Of type likely to occur following failure (e.g., side effect of drug)
C: Causation	Lack of reasonable care must have caused/contributed to injury; if label would not have been read by patient in any event, omission from it might not have caused injury

TABLE 24.10 Criteria for Strict Liability—D + D + C = SL

D: Defect	Widely defined—product design defect, manufacturing error (so that product is less safe than persons generally would be entitled to expect), deficiency in "presentation"
D: Damage	To person or property flowing from defect
C: Causation	See Table 24.7

afford the claimant a remedy. However, the chief distinction between negligent liability and so-called strict liability is that in the case of the latter fault is not required to be shown. To establish strict liability, the claimant must establish against the "producer" (manufacturer/importer) that the product was "defective" (for the purpose of the law, this could refer to shortcomings in its presentation, design, or manufacture) and that it caused the injury suffered.

It would not be at all unusual for claimants in personal injury actions to look for a regulatory compliance failure on the part of a company defendant. The demonstration of a regulatory breach will significantly assist the plaintiff in establishing lack of reasonable care (i.e., conduct falling below acceptable standards). In fact, whether the failure is alleged to be directly relevant to the injury or not, it can be used to demonstrate a general lack of care in the operation of corporate systems with prejudicial effect. Failure to warn is a common element of many pharmaceutical product liability cases, where the pleadings (of negligence and strict liability) might be expected to assert that had the labeling accurately dealt with contraindications, precautions, and/or warnings, the patient would have avoided the injury allegedly suffered, because either the product would not have been used/administered at all or the patient would have been monitored, advised (by the treating doctor), or managed differently so as to avert injury.

In a case where pharmacovigilance omissions are identified that can be said to lead to no/or an insufficient response being adopted by the manufacturer (especially where the regulatory authorities have taken some form of action

or simply criticized a company), the plaintiff is a significant way toward establishing a case for lack of reasonable care in negligence, or that the product was defective in strict liability terms, because it was not presented accurately and was therefore less safe than persons were entitled to expect given the content of the labeling.

24.6.1 FDA Tools for Risk Management

The Food and Drug Administration Amendments Act of 2007 (PDUFA IV) gives the FDA an array of new regulatory tools to exert tighter controls over prescription drugs in the postmarketing setting (McCaughen, 2008).

The centerpiece of the new authorities is included in the section of the law allowing the FDA to impose risk management plans on new drugs under the new acronym REMS (Risk Evaluation and Mitigation Strategies). But the spirit of the REMS lives throughout the new law, providing the FDA with new authority on direct onset of consumer ads, a proposed active surveillance system, mandatory postmarketing study provisions, and new safety labeling procedures. Fundamentally, these arise from the belief that product labeling alone is not sufficient to ensure the safe, appropriate use of prescription drugs.

This belief that top FDA officials expressed publicly for a decade grew out of the agency's frustration over a series of earlier product withdrawals like the type 2 diabetes drug troglitazone (Rezulin) and the cholesterol agent cerivastatin (Baycol).

In both those cases (and several less prominent examples), FDA officials believed that the product could be safely marketed if only prescribers followed appropriate use instructions already included in the labeling. But such labeling actions did not occur and the drugs ultimately were taken off the market for safety reason.

Such concerns gave rise to the voluntary risk management plans that serve as the models for the REMS. The amendment makes the REMS the new de facto baseline for drug approval, setting a formal mechanism for the FDA to impose the programs.

The standard for the FDA to impose the REMS is quite low: It can do so if a program is necessary to ensure that the benefits of a drug outweigh the risks. That, of course, is the standard the FDA already applies for any new drug approval decisions.

That does not mean the FDA will impose the REMS for every new drug—but rather it can. So sponsors need to anticipate that every new drug review will involve a discussion of whether the REMS is necessary, in essence forcing sponsors to work with the FDA to define a program up front or prove that one is not necessary for a new drug.

Not all REMS plans are created equal. Instead, the new law allows the FDA to impose different degrees of control depending on the profile of the product under consideration.

24.6.2 Regulatory Pyramid

The FDA's new regulatory Risk Management Evaluation and Mitigation Strategies (REMS) under FDAA (Food and Drug Administration Amendments Act, Pub. L. No. 110-185, 121 Stat 823) can be envisioned as forming a pyramid (Figure 24.2), with the base representing a relatively small increase in the regulatory burdens already imposed on sponsors in the postmarketing setting and the tip of the pyramid representing the strictest distribution controls that essentially require patients to be identified and screened individually before the medicine is administered.

Though it is not explicitly defined in law, each tier of the pyramid layers new regulatory controls on top of those used in the lower tiers. The result: Products regulated on the lower tiers can reach a larger share of the potential patient population for the medicine, while products regulated on higher tiers are limited to narrower and narrower slices.

Following is one approach to arranging the FDA's new regulatory authorities that may be useful in drug development planning, along with some examples of currently marketed products that already have these kinds of restrictions in place.

Tier 1: Mandatory Studies The lowest tier in the new regulatory system will be products for which the FDA does not impose the REMS at all but does mandate postmarketing studies. The FDA's authority to mandate studies is independent of the new REMS provisions in FDAAA, and it is broadly crafted. The FDA can mandate studies to (1) assess a known safety risk identified prior to marketing, (2) investigate a safety signal about a product,

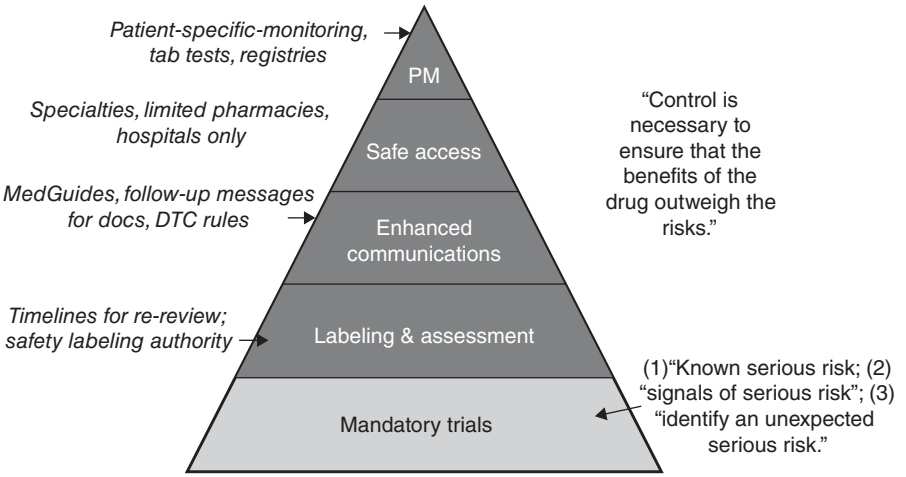


Figure 24.2 *FDA PDUFA IV risk management pyramid.*

or (3) identify "an unexpected serious risk when available data indicates the potential for a serious risk" (FDAAA).

These criteria suggest that the FDA will have the ability to argue for mandatory studies for essentially any drug.

The mandatory study authority also interacts with the separate provisions of the law directing the FDA to create an active surveillance system via a public/private partnership.

The law stipulates that the FDA is not to impose a clinical trial requirement if the active surveillance system can answer a safety question. On the other hand, the active surveillance network will be certain to generate plenty of safety signals that could serve as justification for the FDA to impose phase IV trials.

Still, products in the lowest tier of the FDA's new regulatory authority will have no special limits on their distribution or the potential access to patients. Indeed, there is nothing in the law that prevents sponsors from using studies of new indications—trials that could expand use of their medicines—to answer safety questions about existing uses.

Any product marketed with a voluntary phase IV commitment can serve as an example for this tier—in particular, products approved under the FDA's accelerated approval mechanism which face the threat of a streamlined withdrawal process if the sponsor fails to complete a postmarketing trial or if the trial fails to prove clinical benefit.

But even accelerated approval medicines are not perfect examples of the new regulatory reality, since the FDA has not so far used its enhanced power to pull a product from the market for failure to complete trials. In a pending case the FDA may be considering using that authority: The agency has written to generic drug manufacturer who market versions of Shire PLC's orthostatic hypertension therapy midrodine (ProAmatine), pointing out that the required postmarketing studies are not yet complete and inviting comments on proposals from some of the generic manufacturers to do the trials themselves.

One of the first high-profile examples of a sponsor preparing for the new reality of mandatory postmarketing commitments for already marketed products is Amgen, which will be further refining its postmarketing research program for darbepoetin (Aranesp).

Tier 2: Labeling and Assessment The next tier up the pyramid is the first layer of the new REMS authority. The law says that the minimal requirement of the REMS is a periodic assessment of a product's postmarketing safety profile. In other words, everything about the drug would be the same as a non-REMS product, but there would be predefined timelines to assess how effectively the labeling is working to ensure safe use.

The FDA's separate authority to dictate labeling changes will also come into play in this tier. After 18 months on the market (the first predefined assessment point), the FDA and the sponsor would review adverse-event

reports and other new data (eventually including the active surveillance system). The new information would be added to labeling, and warning statements might be revised. But in the event of a disagreement about the interpretation of the data, the FDA now has the upper hand—the agency can force a change (subject to due process provisions in the law).

In principle, there is nothing new about the idea that the FDA will be analyzing postmarketing safety reports for new drugs. But the deadline for a formal rereview makes a profound difference in the impact of those analyses.

The FDA is already pilot testing this authority via a series of planned drug safety "report cards" for new molecular entities. The agency published its first review in September 2008, looking at spontaneous adverse-event reports for the oral chelating agent deferasirox (Exjade). The review prompted a new round of media coverage about acute renal failure and cytopenia associated with the drug; Novartis alerted prescribers to the issue in May 2008.

Going forward, sponsors will have to be cognizant of the deadlines for reassessment and wary of the FDA's ability to dictate new labeling.

At a minimum, sponsors will need to enhance their own postmarketing monitoring of products regulated under this tier to prevent any surprises in their interactions with the FDA. In some cases sponsors may decide to take voluntary steps to further restrict use if they believe the alternative will be facing an unworkable warning in labeling.

Tier 3: Enhanced Communication The REMS authority allows the FDA to require consumer medication guides, enhanced communications for doctors, or other tools to communicate appropriate use information more effectively. Current examples of risk management tools that would fall under this authority might include special stickers to be affixed to prescriptions, agreements to limit sampling, enhanced patient compliance programs, or voluntary agreements not to advertise to consumers.

The FDA's new direct-to-consumer ad review user fee program and its separate authority to require disclaimers in broadcast ads also should be considered as part of this regulatory tier. Though not part of the REMS, the DTC (Direct to Consumer) provisions will work in the same way by giving the FDA a stronger hand in shaping the final version of safety messages included in broadcast ads.

The enhanced communication provisions obviously place a higher regulatory burden on the sponsor. Sponsors operating under an enhanced communication plan may find themselves in jeopardy if there is significant, measurable use of the product in a setting that the FDA considers inappropriate.

The agency will consider imposing a more restrictive REMS in that situation; it could also seek to hold the company accountable for the failure of the program. The new law makes REMS mandatory with potentially significant penalties for violations. A sponsor that willfully promotes a product in a manner that runs counter to the enhanced communication plan is obviously in jeopardy. But the FDA may also view the failure of the plan as suspicious

in itself, triggering a deeper investigation of a company's marketing activities.

There are a number of examples of products marketed with enhanced communication plans. Amylin Pharmaceuticals type 2 diabetes therapy pramlintide (Symlin) is noteworthy as an early example of a manufacturer voluntarily agreeing not to advertise directly to consumers.

Another instructive example is AstraZeneca PLC's rosuvastatin (Crestor). Developed as a "superstatin," Crestor had the misfortune to be pending at the FDA at the time of the Baycol withdrawal. In response to concerns about potential toxicity from high-potency statins, AstraZeneca agreed not to sample the high dose of Crestor. The product also underwent an unplanned safety reassessment after approval due in part to a petition filed by Public Citizen seeking Crestor's withdrawal. The result was a relabeling with stronger warnings followed by the resumption of direct-to-consumer advertising and the emergence of Crestor as a brand approaching $3 billion in annual sales.

Two recent Pfizer approvals also used enhanced communication plans. In the case of the inhaled insulin brand Exubera, the program was part of an effective regulatory strategy to resolve safety issues that long delayed its launch. (See "Pfizer's Exubera: Breathing New Life into Inhaled Insulin," In Vivo, October 2005). But getting the product to market turned out to be the easy part: After dismal uptake, Pfizer is giving up on the brands and writing off almost $3 billion as a result.

A happier example is the smoking cessation agent varenicline (Chantix). Pfizer markets the drug in conjunction with a voluntary patient support program and also delayed the launch of DTC advertising as part of its commitment to principles established by the Pharmaceutical Research & Manufacturers of America. Now the brand is one of the rising stars of the company's commercial portfolio, with sales jumping almost 10-fold to $241 million in the third quarter of 2007.

Tier 4: Safe-Use Restriction, Defined by Provider The next level up the pyramid is when the REMS really start to pinch market sizes. The law gives the FDA the authority to set distribution restrictions on a drug if "necessary to assure safe use of the drug, because of its inherent toxicity or potential harmfulness" (FDAAA). The FDA is supposed to apply the restrictions only when a drug could not be marketed (or would be withdrawn) without them or if less restrictive REMS fail to assure appropriate use.

The safe-use authority is one section of the law, but it really represents two tiers of regulation.

The lower tier is a restriction based on the providers. That restriction could be to allow use only by providers who complete a special training program—a burden to the sponsor, to be sure, but one with a clear upside in promoting a new medicine.

Or the restriction could be defined by physician specialty or practice setting. The FDA could allow use of a medicine only in hospitals, for example, as

was the case for Praecis Pharmaceuticals' abarelix (Plenaxis). The restriction essentially killed the product; Praecis withdrew it and was itself acquired by GlaxoSmithKline.

In other cases, such as with Genentech omalizumab (Xolair), the distribution restriction can serve more as an opportunity. Xolair's labeling stipulates that the asthma drug must be administered in a physician's office to monitor for acute adverse reactions. The product did not initially meet Genentech's expectations but is now emerging as a prime example of the "minibuster" model that will be critical to industry in the future.

Still, restricted access provisions place a significant burden on sponsors, typically requiring specialized distribution systems and close coordination with providers. In other words, the primary care blockbuster model that built the modern big pharma business does not apply.

Tier 5: Safe-Use Restiction, Defined by Patient The highest tier on the pyramid involves the other elements of a restricted distribution programs, ones that define the limits by patient-specific criteria.

The law allows the FDA to require distribution only to patients with a documented lab test or to require special monitoring of each patient given the drug or to mandate patient registries for recipients. This tier represents the tip of the regulatory pyramid, where the FDA's authority dictates the conditions for administering the drug to individual patients.

But even at this layer of regulation, commercial success is possible. Biogen Idee Tysabri is marketed under the most restrictive distribution plan approved by the FDA. The product is not at the level the sponsor hoped for when it was first launched for MS—but the company believes it could still be a billion dollar brand.

Or consider Celgene's thalidomide (Thalomid), the drug that ushered in the modern era of risk management plans when it was approved in 1998. The program has been so successful that it has essentially defined Celgene's business model—an approach that many other companies are likely to want to imitate post-FDAAA.

There is an even earlier example: Novartis's atypical antipsychotic clozapine (Clozaril), which was sold with a requirement for blood monitoring, initially tied to a single lab test provider (Caremark). Clozaril has been surpassed by many newer atypicals, but it is worth remembering that one of the biggest blockbuster classes of the past two decades began as a prototype for the era of restrictive distribution plans.

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25

Statistics in Pharmaceutical Safety Assessment

25.1 INTRODUCTION

This chapter is intended for both practicing and student toxicologists as a practical guide to the common statistical problems encountered in drug safety assessment and the methodologies that are available to solve them. The chapter has been enriched by the inclusion of discussions of why a particular procedure or interpretation is recommended, by the clear enumeration of the assumptions that are necessary for a procedure to be valid, and by discussion of problems drawn from the actual practice of toxicology and toxicological pathology.

Studies continue to be designed and executed to generate increased amounts of data. The resulting problems of data analysis have thus become more complex and toxicology has drawn more deeply from the well of available statistical techniques. Statistics has also been very active and growing during the last 35 years—to some extent, at least, because of the parallel growth of toxicology. These simultaneous changes have led to an increasing complexity of data and, unfortunately, to the introduction of numerous confounding factors which severely limit the utility of the resulting data in all too many cases.

A major difficulty is that there is a very real necessity to understand the biological realities and implications of a problem as well as the peculiarities

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of toxicological data before procedures are selected and employed for analysis. These characteristics include the following:

1. The need to work with a relatively small sample set of data collected from the members of a population (laboratory animals) which is not actually our population of interest (i.e., humans or a target animal population).
2. Dealing frequently with data resulting from a sample which was censored on a basis other than by the investigator's own design. By censoring, of course, we mean that not all data points were collected, as might be desired. This censoring could be the result of either a biological factor (a test animal that is dead or too debilitated to manipulate) or a logistic factor (inoperative equipment or tissue that is missed in necropsy).
3. The conditions under which our experiments are conducted are extremely varied. In pharmacology (the closest cousin to at least classical toxicology), the possible conditions of interaction of a chemical or physical agent with a person are limited to a small range of doses via a single route over a short course of treatment to a defined patient population. In toxicology, however, all these variables (dose, route, time span, and subject population) are determined by the investigator.
4. The time frames available to solve our problems are limited by practical and economic factors. This frequently means that there is not time to repeat a critical study if the first attempt fails. So a true iterative approach is not possible.

The training of most pathologists in statistics remains limited to a single introductory course which concentrates on some theoretical basics. As a result, the armementarium of statistical techniques of most toxicologists is limited and the tools that are usually present [*t* tests, chi square, analysis of variance (ANOVA), and linear regression] are neither fully developed nor well understood. It is hoped that this chapter will help change this situation.

As a point of departure toward this objective, it is essential that any analysis of study results be interpreted by a professional who firmly understands three concepts: the difference between biological significance and statistical significance, the nature and value of different types of data, and causality.

For the first concept, we should consider the four possible combinations of these two different types of significance, for which we find the relationship shown below:

		Statistical Significance	
		No	Yes
Biological significance	No	Case I	Case II
	Yes	Case III	Case IV

Cases I and IV give us no problems, for the answers are the same statistically and biologically. But cases II and III present problems. In case II (the "false positive"), we have a circumstance where there is a statistical significance in the measured difference between treated and control groups, but there is no true biological significance to the finding. This is not an uncommon happening, for example, in the case of clinical chemistry parameters. This is called type I error by statisticians (for an example, see Portier and Hoel, 1984), and the probability of this happening is called the α level. In case III (the "false negative"), we have no statistical significance, but the differences between groups are biologically/toxicologically significant. This is called type II error by statisticians, and the probability of such an error happening by random chance is called the β level. An example of this second situation is when we see a few of a very rare tumor type in treated animals. In both of these latter cases, numerical analysis, no matter how well done, is no substitute for professional judgment. Along with this, however, one must have a feeling for the different types of data and for the value or relative merit of each. Note that the two error types interact, and in determining sample size we need to specify both α and β levels. Table 25.1 demonstrates this interaction in the case of tumor or specific lesion incidence.

The reasons that biological and statistical significance are not identical are multiple, but a central one is certainly causality. Through our consideration of statistics, we should keep in mind that just because a treatment and a change in an observed organism are seemingly or actually associated with each other does not "prove" that the former caused the latter. Though this fact is now widely appreciated for correlation (e.g., the fact that the number of storks' nests found each year in England is correlated with the number of human births that year does not mean that storks bring babies), it is just as true in the general case of significance. Timely establishment and proof that treatment

TABLE 25.1 Sample Size Required to Obtain Specified Sensitivity at $p < 0.05$

		Treatment Group Incidence									
Background Tumor Incidence	*P*	0.95	0.90	0.80	0.70	0.60	0.50	0.40	0.30	0.20	0.10
0.30	0.90	10	12	18	31	46	102	389			
	0.50	6	6	9	12	22	32	123			
0.20	0.90	8	10	12	18	30	42	88	320		
	0.50	5	5	6	9	12	19	28	101		
0.10	0.90	6	8	10	12	17	25	33	65	214	
	0.50	3	3	5	6	9	11	17	31	68	
0.05	0.90	5	6	8	10	13	18	25	35	76	464
	0.50	3	3	5	6	7	9	12	19	24	147
0.01	0.90	5	5	7	8	10	13	19	27	46	114
	0.50	3	3	5	5	6	8	10	13	25	56

Note: *P* = power for each comparison of treatment group with background tumor incidence.

causes an effect require an understanding of the underlying mechanism and proof of its validity. At the same time, it is important that we realize that not finding a good correlation or suitable significance associated with a treatment and an effect likewise does not prove that the two are not associated—that a treatment does not cause an effect. At best, it gives us a certain level of confidence that under the conditions of the current test these items are not associated.

These points (p. 973) are discussed in greater detail in the "assumptions" section for each method along with other common pitfalls and shortcomings associated with the method. To help in better understanding the discussion, terms frequently used throughout this book should first be considered. These are presented in Table 25.2.

TABLE 25.2 Some Frequently Used Terms and Their General Meanings

Term	Meaning
95% confidence interval	Range of values (above, below, or above and below) the sample (mean, median, mode, etc.) has a 95% chance of containing the true value of the population (mean, median, mode). Also called the fiducial limit equivalent to the $P < 0.05$.
Bias	Systemic error as opposed to a sampling error. For example, selection bias may occur when each member of the population does not have an equal chance of being selected for the sample.
Degrees of freedom	Number of independent deviations, usually abbreviated df.
Independent Variables	Also known as predictors or explanatory variables.
P value	Another name for significance level; usually 0.005.
Power	Effect of experimental conditions on dependent variable relative to sampling fluctuation. When the effect is maximized, the experiment is more powerful. Power can also be defined as the probability that there will not be a type II error ($1 - \beta$). Conventionally, power should be at least 0.07.
Random	Each individual member of the population has the same chance of being selected for the sample.
Robust	Having inferences or conclusions little effected by departure from assumptions.
Sensitivity	Number of subjects experiencing each experimental condition divided by variance of scores in sample.
Significance level	Probability that a difference has been erroneously declared to be significant, typically 0.005 and 0.001 corresponding to 5% and 1% chance of error.
Type I error (false positives)	Concluding that there is an effect when there really is not an effect. Its probability is the α level.
Type II error (false negatives)	Concluding there is a particular effect when there really is another effect. Its probability is the β level.

Source: Marriott, 1991.

Each measurement we make—each individual piece of experimental information we gather—is called a datum. However, we gather and analyze multiple pieces at one time, the resulting collection being called data.

Data are collected on the basis of their association with a treatment (intended or otherwise) as an effect (a property) that is measured in the experimental subjects of a study, such as body weights. These identifiers (i.e., treatment and effect) are termed variables. Our treatment variables (those that the researcher or nature controls and which can be directly controlled) are termed independent, while our effect variables (such as weight, life span, and number of neoplasms) are termed dependent variables—their outcome is believed to depend on the "treatment" being studied.

All the possible measures of a given set of variables in all the possible subjects that exist is termed the population for those variables. Such a population of variables cannot be truly measured—for example, one would have to obtain, treat, and measure the weights of all the Fischer- 344 rats that were, are, or ever will be studied. Instead, we deal with a representative group—a sample. If our sample of data is appropriately collected and of sufficient size, it serves to provide good estimates of the characteristics of the parent population from which it was drawn.

25.1.1 Bias and Chance

Any toxicological study aims to determine whether a treatment elicits a response. An observed difference in response between a treated and a control group need not necessarily be a result of treatment. There are, in principle, two other possible explanations—*bias*, or systematic differences other than treatment between the groups, and *chance*, or random differences. A major objective of both experimental design and analysis is to try to avoid bias. Wherever possible, treated and control groups to be compared should be alike with respect to all other factors. Where differences remain, these should be corrected for in the statistical analysis. Chance cannot be wholly excluded, since identically treated animals will not respond identically. While even the most extreme difference might in theory be due to chance, a proper statistical analysis will allow the experimenter to assess this possibility. The smaller the probability of a "false positive", the more confident the experimenter can be that the effect is real. Good experimental design improves the chance of picking up a true effect with confidence by maximizing the ratio between "signal" and "noise."

25.1.2 Hypothesis Testing and Probability (*p*) Values

A relationship of treatment to some toxicological endpoint is often stated to be *statistically significant* ($p < 0.05$). What does this really mean? A number of points have to be made. *First*, statistical significance need not necessarily imply biological importance if the endpoint under study is not relevant to the ani-

mal's well-being. *Second*, the statement will usually be based only on the data from the study in question and will not take into account prior knowledge. In some situations, for example, when one or two of a very rare tumor type are seen in treated animals, statistical significance may not be achieved, but the finding may be biologically extremely important, especially if a similar treatment was previously found to elicit a similar response. *Third*, the p value does not describe the probability that a true effect of treatment exists. Rather, it describes the probability of the observed response or one more extreme occurring on the assumption that treatment actually had no effect whatsoever. A p value that is not significant is consistent with a treatment having a small effect not detected with sufficient certainty in this study. *Fourth*, there are two types of p values. A *one-tailed* (or *one-sided*) *p value* is the probability of getting by chance a treatment effect in a specified direction as great as or greater than that observed. A *two-tailed p value* is the probability of getting, by chance alone, a treatment difference in either direction which is as great as or greater than that observed. By convention p values are assumed to be two tailed unless the contrary is stated. Where, which is unusual, one can rule out in advance the possibility of a treatment effect except in one direction, a one-tailed p value should be used. Often, however, two-tailed tests are to be preferred, and it is certainly not recommended to use one-tailed tests and *not* report large differences in the other direction. In any event, it is important to make it absolutely clear whether one- or two-tailed tests have been used.

It is a great mistake, when presenting results of statistical analyses, to mark, as do some laboratories, results simply as significant or not significant at one defined probability level (usually $p < 0.05$). This poor practice does not allow the reader any real chance to judge whether or not the effect is a true one. Some statisticians present the actual p value for every comparison made. While this gives precise information, it can make it difficult to assimilate results from many variables. One practice the author recommends is to mark p values routinely using plus signs to indicate positive differences (and minus signs to indicate negative differences) as follows: +++, $p = 0.001$; ++, $0.001 \leq p < 0.01$; +, $0.01\ p < 0.05$; ±, $0.05 \leq p < 0.1$. This highlights significant results more clearly and also allows the reader to judge the whole range from "virtually certain treatment effect" to "some suspicion." Note that using two-tailed tests, bracketed plus signs indicate findings that would be significant at the conventional $p < 0.05$ level using one-tailed tests but are not significant at this level using two-tailed tests. This "fiducal limit" ($p < 0.05$) implies a false-positive incidence of 1 in 20 and, though now imbedded in regulation, practice, and convention, was a somewhat arbitrary choice to begin with. In interpreting p values it is important to realize they are only an aid to judgment to be used in conjunction with other available information. One might validly consider a $p < 0.01$ increase as chance when it was unexpected, occurred only at a low dose level with no such effect seen at higher doses, and was evident in only one subset of the data. In contrast, a $p < 0.05$ increase might be convincing if it occurred in the

top dose and was for an endpoint one might have expected to be increased from known properties of the chemical or closely related chemicals.

25.1.3 Multiple Comparisons

When a p value is stated to be <0.05, this implies that, for that particular test, the difference could have occurred by chance less than 1 time in 20. Toxicological studies frequently involve making treatment–control comparisons for large numbers of variables and, in some situations, also for various subsets of animals. Some statisticians worry that the larger the number of tests, the greater is the chance of picking up statistically significant findings that do not represent true treatment effects. For this reason, an alternative "multiple-comparison" procedure has been proposed in which, if the treatment was totally without effect, then 19 times out of 20 *all* the tests should show nonsignificance when testing at the 95% confidence level. Automatic use of this approach cannot be recommended. Not only does it make it much more difficult to pick up any real effects, but also there is something inherently unsatisfactory about a situation where the relationship between a treatment and a particular response depends arbitrarily on which other responses happened to be investigated at the same time. It is accepted that in any study involving multiple endpoints that there will inevitably be a gray area between those showing highly significant effects and those showing no significant effects, where there is a problem distinguishing chance and true effects. However, changing the methodology so that the gray areas all come up as nonsignificant can hardly be the answer.

25.1.4 Estimating Size of Effect

It should be clearly understood that a p value does not give direct information about the size of any effect that has occurred. A compound may elicit an increase in response by a given amount, but whether a study finds this increase to be statistically significant will depend on the size of the study and the variability of the data. In a small study, a large and important effect may be missed, especially if the endpoint is imprecisely measured. In a large study, on the other hand, a small and unimportant effect may emerge as statistically significant.

Hypothesis testing tells us whether an observed increase can or cannot be reasonably attributed to chance, but not how large it is. Although much statistical theory relates to hypothesis testing, current trends in medical statistics are toward confidence interval estimation with differences between test and control groups expressed in the form of a best estimate coupled with the 95% confidence interval (CI). Thus, if one states that treatment increases response by an estimated 10 units (95% CI 3–17 units), this would imply that there is a 95% chance that the indicated interval includes the true difference. If the lower 95% confidence limit exceeds zero, this implies the increase is statistically significant at $p < 0.05$ using a two-tailed test. One can also calculate, for

example, 99 or 99.9% confidence limits, corresponding to testing for significance at $p < 0.01$ or $p < 0.001$.

In screening studies of standard design, the tendency has been to concentrate mainly on hypothesis testing. However, presentation of the results in the form of estimates with confidence intervals can be a useful adjunct for some analyses and is very important in studies aimed specifically at quantifying the size of an effect.

Two terms refer to the quality and reproducibility of our measurements of variables. The first, accuracy, is an expression of the closeness of a measured or computed value to its actual or "true" value in nature. The second, precision, reflects the closeness or reproducibility of a series of repeated measurements of the same quantity.

If we arrange all of our measurements of a particular variable in order as a point on an axis marked as the values of that variable and if our sample were large enough, the pattern of distribution of the data in the sample would begin to become apparent. This pattern is a representation of the frequency distribution of a given population of data—that is, of the incidence of different measurements, their central tendency, and dispersion.

The most common frequency distribution—and one that has been talked about throughout this book—is the normal (or Gaussian) distribution. The normal distribution is such that two-thirds of all values are within one standard deviation of the mean (or average value for the entire population) and 95% are within 1.96 standard deviations of the mean. Symbols used are μ for the mean and σ for the standard deviation. Other common frequency distributions include the binomial, Poisson, and chi square.

In all areas of biological research, optimal design and appropriate interpretation of experiments require that the researcher understand both the biological and technological underpinnings of the system being studied and of the data being generated. From the point of view of the statistician, it is vitally important that the experimenter both know and be able to communicate the nature of the data and understand its limitations. One classification of data types is presented in Table 25.3.

The nature of the data collected is determined by three considerations: the biological source of the data (the system being studied), the instrumentational and techniques being used to make measurements, and the design of the experiment. The researcher has some degree of control over each of these—least over the biological system (he or she normally has a choice of only one of several models to study) and most over the design of the experiment or study. In fact, such choices dictate the type of data generated by a study.

Statistical methods are based on specific assumptions. Parametric statistics—those that are most familiar to the majority of scientists—have more stringent underlying assumptions than do nonparametric statistics. Among the underlying assumptions for many parametric statistical methods (such as the ANOVA) is that the data are continuous. The nature of the data

TABLE 25.3 Types of Variables (Data) and Examples of Each Type

Classified By	Type	Example
Scale Continuous	Scalar	Body weight
	Ranked	Severity of lesion
Discontinuous	Scalar	Weeks until first observation of tumor in carcinogenicity study
	Ranked	Clinical observations in animals
	Attribute	Eye colors in fruit flies
	Quantal	Dead/alive or present/absent
Frequency Distribution	Normal	Body weights
	Bimodal	Some clinical chemistry parameters
	Others	Measures of time to incapacitation

associated with a variable (as described above) imparts a "value" to that data, the value being the power of the statistical tests which can be employed.

Continuous variables are those which can at least theoretically assume any of an infinite number of values between any two fixed points (such as measurements of body weight between 2.0 and 3.0 kg). Discontinuous variables, meanwhile, are those which can have only certain fixed values with no possible intermediate values (such as counts of five and six dead animals, respectively).

Limitations on our ability to measure constrain the extent to which the real-world situation approaches the theoretical, but many of the variables studied in toxicology are in fact continuous. Examples of these are lengths, weights, concentrations, temperatures, periods of time, and percentages. For these continuous variables, we may describe the character of a sample with measures of central tendency and dispersion that we are most familiar with: the mean, denoted by the symbol $\bar{x}$ and also called the arithmetic average, and the standard deviation, SD, which is denoted by the symbol σ and is calculated as

$$\sigma = \sqrt{\frac{\sum X^2 - \left(\sum X\right)^2 / N}{N-1}}$$

where X is the individual datum and N is the total number of data in the group.

Contrasted with these continuous data, however, we have discontinuous (or discrete) data, which can only assume certain fixed numerical values. In these cases our choice of statistical tools or tests is, as we will find later, more limited.

25.1.5 Functions of Statistics

Statistical methods may serve to do any combination of three possible tasks. The one we are most familiar with is hypothesis testing—that is, determining if two (or more) groups of data differ from each other at a predetermined

level of confidence. A second function is the construction and use of models which may be used to predict future outcomes of chemical–biological interactions. This is most commonly seen in linear regression or in the derivation of some form of correlation coefficient. Model fitting allows us to relate one variable (typically a treatment or "independent" variable) to another. The third function, reduction of dimensionality, continues to be less commonly utilized than the first two. This final category includes methods for reducing the number of variables in a system while only minimally reducing the amount of information, therefore making a problem easier to visualize and to understand. Examples of such techniques are factor analysis and cluster analysis. A subset of this last function, discussed later under descriptive statistics, is the reduction of raw data to single expressions of central tendency and variability (such as the mean and standard deviation).

There is also a special subset of statistical techniques which is part of both the second and third functions of statistics. This is data transformation, which includes such things as the conversion of numbers to log or probit values.

25.1.6 Descriptive Statistics

Descriptive statistics are used to summarize the general nature of a data set. As such, the parameters describing any single group of data have two components. One of these describes the location of the data, while the other gives a measure of the dispersion of the data in and about this location. Often overlooked is the fact that the choice of which parameters are used to give these pieces of information implies a particular type of distribution for the data.

Most commonly, location is described by giving the (arithmetic) mean and dispersion by giving the SD or the standard error of the mean (SEM). The calculation of the first two of these has already been described. If we again denote the total number of data in a group as N, then the SEM would be calculated as

$$\text{SEM} = \frac{\text{SD}}{\sqrt{N}}$$

The use of the mean with either the SD or SEM implies, however, that we have reason to believe that the sample of data being summarized is from a population which is at least approximately normally distributed. If this is not the case, then we should rather use a set of statistical descriptions which do not require a normal distribution. These are the median for location and the semiquartile distance for a measure of dispersion. These somewhat less familiar parameters are characterized as follows.

Median When all the numbers in a group are arranged in a ranked order (i.e., from smallest to largest), the median is the middle value. If there is an odd number of values in a group, then the middle value is obvious (in the case

of 13 values, for example, the seventh largest is the median). When the number of values in the sample is even, the median is calculated as the midpoint between the $(N/2)$th and the $[(N/2) + 1]$th number. For example, in the series 7, 12, 13, 19 the median value would be the midpoint between 12 and 13, which is 12.5.

The SD and SEM are related to each other but yet are quite different.

The SEM is quite a bit smaller than the SD, making it very attractive to use in reporting data. This size difference is because the SEM actually is an estimate of the error (or variability) involved in measuring the means of samples, not an estimate of the error (or variability) involved in measuring the data from which means are calculated. This is implied by the *central limit theorem*, which tells us three major things:

- The distribution of sample means will be approximately normal regardless of the distribution of values in the original population from which the samples were drawn.
- The mean value of the collection will reflect that of a normal distribution.
- The standard deviation of the collection of all possible means of samples of a given size, called the standard error of the mean, depends on both the standard deviation of the original population and the size of the sample.

The SEM should be used only when the uncertainty of the estimate of the mean is of concern, which is almost never the case in toxicology. Rather, we are concerned with an estimate of the variability of the population, for which the standard deviation is appropriate.

Semiquartile Distance When all the data in a group are ranked, a quartile of the data contains one ordered quarter of the values. Typically, we are most interested in the borders of the middle two quartiles Q_1 and Q_3, which together represent the semiquartile distance and which contain the median as their center. Given that there are N values in an ordered group of data, the upper limit of the jth quartile (Q_j) may be computed as being equal to the $[jN/(1)/4\text{th}]$ value. Once we have used this formula to calculate the upper limits of Q_1 and Q_3, we can then compute the semiquartile distance (which is also called the quartile deviation and abbreviated as QD) with the formula $\text{QD} = (Q_3 - Q_1)/2$.

For example, for the 15-value data set 1, 2, 3, 4, 4, 5, 5, 5, 6, 6, 6, 7, 7, 8, 9, we can calculate the upper limits of Q_1 and Q_3 as

$$Q_1 = \frac{1(15+1)}{4} = \frac{16}{4} = 4 \qquad Q_3 = \frac{3(15=1)}{4} = \frac{48}{4} = 12$$

The 4th and 12th values in this data set are 4 and 7, respectively. The semiquartile distance can then be calculated as

$$\text{QD} = \frac{1}{2}(7 - 4) = 1.5$$

There are times when it is desired to describe the relative variability of one or more sets of data. The most common way of doing this is to compute the coefficient of variation (CV), which is calculated simply as the ratio of the standard deviation to the mean:

$$\text{CV} = \frac{\text{SD}}{\bar{X}}$$

A CV of 0.2 or 20% thus means that the standard deviation is 20% of the mean. In toxicology the CV is frequently between 20 and 50% and may at times exceed 100%.

25.2 EXPERIMENTAL DESIGN

Toxicological experiments generally have a twofold purpose. The first question is whether or not an agent results in an effect on a biological system. The second question, never far behind, is how much of an effect is present. It has become increasingly desirable that the results and conclusions of studies aimed at assessing the effects of environmental agents be as clear and unequivocal as possible. It is essential that every experiment and study yield as much information as possible and that the results of each study have the greatest possible chance of answering the questions it was conducted to address. The statistical aspects of such efforts, so far as they are aimed at structuring experiments to maximize the possibilities of success, are called experimental design.

The four basic statistical principles of experimental design are replication, randomization, concurrent ("local") control, and balance. In abbreviated form, these may be summarized as follows.

Replication Any treatment must be applied to more than one experimental unit (animal, plate of cells, litter of offspring, etc.). This provides more accuracy in the measurement of a response than can be obtained from a single observation since underlying experimental errors tend to cancel each other out. It also supplies an estimate of the experimental error derived from the variability among each of the measurements taken (or "replicates"). In practice, this means that an experiment should have enough experimental units in each treatment group (i.e., a large enough N) so that reasonably sensitive statistical analysis of data can be performed. The estimation of sample size is addressed in detail later in this chapter.

Randomization This is practiced to ensure that every treatment shall have its fair share of extreme high and extreme low values. It also serves to allow the

toxicologist to proceed as if the assumption of "independence" is valid. That is, there is not avoidable (known) systematic bias in how one obtains data.

Concurrent Control Comparisons between treatments should be made to the maximum extent possible between experimental units from the same closely defined population. Therefore, animals used as a "control" group should come from the same source, lot, age, and so on, as test group animals. Except for the treatment being evaluated, test and control animals should be maintained and handled in exactly the same manner.

Balance If the effect of several different factors is being evaluated simultaneously, the experiment should be laid out in such a way that the contributions of the different factors can be separately distinguished and estimated. There are several ways of accomplishing this using one of several different forms of design, as will be discussed below.

There are 10 facets of any study which may affect its ability to detect an effect of a treatment. The first 6 concern minimizing the role of chance and the last four relate to avoidance of bias.

25.2.1 Choice of Species and Strain

Ideally, the responses of interest should be rare in untreated control animals but should be reasonably readily evoked by appropriate treatments. Some species or specific strains, perhaps because of inappropriate diets (Roe, 1989), have high background tumor incidences which make increases both difficult to detect and difficult to interpret when detected.

25.2.2 Sampling

Sampling—the selection of which individual data points will be collected, whether in the form of selecting which animals to collect blood from or to remove a portion of a diet mix from for analysis—is an essential step upon which all other efforts toward a good experiment or study are based.

There are three assumptions about sampling which are common to most of the statistical analysis techniques used in toxicology: that the sample is collected without bias, that each member of a sample is collected independently of the others, and that members of a sample are collected with replacements. Precluding bias, both intentional and unintentional, means that at the time of selection of a sample to measure each portion of the population from which that selection is to be made has an equal chance of being selected. Ways of precluding bias are discussed in detail in the chapter on experimental design.

Independence means that the selection of any portion of the sample is not affected by and does not affect the selection or measurement of any other portion.

Finally, sampling with replacement means that, in theory, after each portion is selected and measured, it is returned to the total sample pool and thus has the opportunity to be selected again. This is a corollary of the assumption of independence. Violation of this assumption (which is almost always the case in toxicology and all the life sciences) does not have serious consequences if the total pool from which samples are drawn is sufficiently large (say 20 or greater) that the chance of reselecting that portion is small anyway.

There are four major types of sampling methods—random, stratified, systematic, and cluster. Random is by far the most commonly employed method in toxicology. It stresses the fulfillment of the assumption of avoiding bias. When the entire poor of possibilities is mixed or randomized (procedures for randomization are presented in a later chapter), the members of the group are selected in the order drawn from the pool.

Stratified sampling is performed by first dividing the entire pool into subsets or strata, then doing randomized sampling from each strata. This method is employed when the total pool contains subsets which are distinctly different but in which each subset contains similar members. An example is a large batch of a powdered pesticide in which it is desired to determine the nature of the particle size distribution. Larger pieces or particles are on the top while progressively smaller particles have settled lower in the container, and at the very bottom the material has been packed and compressed into aggregates. To determine a timely representative answer, proportionally sized subsets from each layer or strata should be selected, mixed, and randomly sampled. This method is used more commonly in diet studies.

In systematic sampling, a sample is taken at set intervals (such as every fifth container of reagent or taking a sample of water from a fixed sample point in a flowing stream every hour). This is most commonly employed in quality assurance or (in the clinical chemistry lab) in quality control.

In cluster sampling, the pool is already divided into numerous separate groups (such as bottles of tablets), and we select small sets of groups (such as several bottles of tablets) and then select a few members from each set. What one gets then is a cluster of measures. Again, this is a method most commonly used in quality control or in environmental studies when the effort and expense of physically collecting a small group of units is significant.

In classical toxicology studies sampling arises in a practical sense in a limited number of situations. The most common of these are as follows:

1. Selecting a subset of animals or test systems from a study to make some measurement (which either destroys or stresses the measured system or is expensive) at an interval during a study. This may include such cases as doing interim necropsies in a chronic study or collecting and analyzing blood samples from some animals during a subchronic study.
2. Analyzing inhalation chamber atmospheres to characterize aerosol distributions with a new generation system.

3. Analyzing diet in which test material has been incorporated.
4. Performing quality control on an analytical chemistry operation by having duplicate analyses performed on some materials.
5. Selecting data to audit for quality assurance purposes.

25.2.3 Dose Levels

This is a very important and controversial area. In screening studies aimed at hazard identification it is normal, in order to avoid requiring huge numbers of animals, to test at dose levels higher than those to which humans will be exposed but not so high that marked toxicity occurs. A range of doses is usually tested to guard against the possibility of a misjudgment of an appropriate high dose and that the metabolic pathways at the high doses differ markedly from those at lower doses and, perhaps, to ensure no large effects occur at dose levels in the range to be used by humans. In studies aimed more at risk estimation, more and lower doses may be tested to obtain fuller information on the shape of the dose–response curve.

25.2.4 Number of Animals

This is obviously an important determinant of the precision of the findings. The calculation of the appropriate number depends on (1) the critical difference, that is, the size of the effect it is desired to detect; (2) the false-positive rate, that is, the probability of an effect being detected when none exists (equivalent to the "α level" or "type I error"); (3) the false-negative rate, that is, the probability of no effect being detected when one of exactly the critical size exists (equivalent to the "β level" or "type II error"); and (4) some measure of the variability in the material.

Tables relating numbers of animals required to obtain values of critical size α and β are given in Kraemer and Thiemann (1987) and Gad (1998) and software is also available for this purpose. As a rule of thumb, to reduce the critical difference by a factor n for a given α and β, the number of animals required will have to increased by a factor n^2.

25.2.5 Duration of Study

It is obviously important not to terminate the study too early for fatal conditions, which are normally strongly age related. Less obviously, going on for too long in a study can be a mistake, partly because the last few weeks or months may produce relatively few extra data at a disproportionate cost and partly because diseases of extreme old age may obscure the detection of tumors and other conditions of more interest. For nonfatal conditions, the ideal is to sacrifice the animals when the average prevalence is around 50%.

25.2.6 Stratification

To detect a treatment difference with accuracy, it is important that the groups being compared are as homogeneous as possible with respect to other known causes of the response. In particular, suppose that there is another known important cause of the response for which the animals vary, so that the animals are a mixture of hyper- and hyporesponders from this cause. If the treated group has a higher proportion of hyperresponders, it will tend to have a higher response even if treatment has no effect. Even if the proportion of hyperresponders is the same as in the controls, it will be more difficult to detect an effect of treatment because of the increased between-animal variability.

Given that this other factor is known, it will be sensible to take it into account in both the design and analysis of the study. In the design, it can be used as a "blocking factor" so that animals at each level are allocated equally (or in the correct proportion) to control and treated groups. In the analysis, the factor should be treated as a stratifying variable, with separate treatment–control comparisons made at each level, and the comparisons combined for an overall test of difference. This is discussed later, where we refer to the factorial design as one example of the more complex designs that can be used to investigate the separate effect of multiple treatments.

25.2.7 Randomization

Random allocation of animals to treatment groups is a prerequisite of good experimental design. If not carried out, one can never be sure whether treatment–control differences are due to treatment or to "confounding" by other relevant factors. The ability to randomize easily is a major advantage animal experiments have over epidemiology.

While randomization eliminates bias (as least in expectation), simple randomization of all animals may not be the optimal technique for producing a sensitive test. If there is another major source of variation (e.g., sex or batch of animals), it will be better to carry out stratified randomization (i.e., carry out separate randomizations within each level of the stratifying variable).

The need for randomization applies not only to the allocation of the animals to the treatment but also to anything that can materially affect the recorded response. The same random number that is used to apply animals to the treatment group can be used to determine cage position, order of weighing, order of bleeding for clinical chemistry, order of sacrifice at terminations, and so on.

25.2.8 Adequacy of Control Group

While historical control data can, on occasion, be useful, a properly designed study demands that a relevant concurrent control group be included with which results for the test group can be compared. The principle that like should be compared with like, apart from treatment, demands that control animals

should be randomized from the same source as treatment animals. Careful consideration should also be given to the appropriateness of the control group. Thus, in an experiment involving treatment of a compound in a solvent, it would often be inappropriate to include only an untreated control group, as any differences observed could only be attributed to the treatment–solvent combination. To determine the specific effects of the compound, a comparison group given the solvent only, by the same route of administration, would be required.

It is not always generally realized that the position of the animal in the room in which it is kept may affect an animal's response. An example is the strong relationship between incidence of retinal atrophy in albino rats and closeness to the lighting source. Systematic differences in cage position should be avoided, preferably via randomization.

We have now become accustomed to developing exhaustively detailed protocols for an experiment or study prior to its conduct. A priori selection of statistical methodology (as opposed to the post hoc approach) is as significant a portion of the process of protocol development and experimental design as any other and can measurably enhance the value of the experiment or study. Prior selection of statistical methodologies is essential for proper design of other portions of a protocol, such as the number of animals per group or the sampling intervals for body weight. Implied in such a selection is the notion that the toxicologist has both an in-depth knowledge of the area of investigation and an understanding of the general principles of experimental design, for the analysis of any set of data is dictated to a large extent by the manner in which the data are obtained.

A second concept and its understanding are essential to the design of experiments in toxicology, that of censoring. Censoring is the exclusion of measurements from certain experimental units, or indeed of the experimental units themselves, from consideration in data analysis or inclusion in the experiment at all. Censoring may occur prior to initiation of an experiment (where, in modern toxicology, this is almost always a planned procedure), during the course of an experiment (when they are almost universally unplanned, resulting from, e.g., the death of animals in a test), or after the conclusion of an experiment (when usually data are excluded because of being identified as some form of outlier).

In practice, a priori censoring in toxicology studies occurs in the assignment of experimental units (such as animals) to test groups. The most familiar example is in the common practice of assignment of test animals to acute, subacture, subchronic, and chronic studies, where the results of otherwise random assignments are evaluated for body weights of the assigned members. If the mean weights are found not to be comparable by some preestablished criterion (such as a 90% probability of difference by ANOVA), then members are reassigned (censored) to achieve comparability in terms of starting body weights. Such a procedure of animal assignment to groups is known as a *censored randomization*.

The first precise or calculable aspect of experimental design encountered is determining sufficient test and control group sizes to allow one to have an adequate level of confidence in the results of a study (i.e., in the ability of the study design with the statistical tests used to detect a true difference—or effect—when it is present). The statistical test contributes a level of power to such detection. Remember that the power of a statistical test is the probability that a test results in rejection of a hypothesis, H_0 say, when some other hypothesis, H say, is valid. This is termed the power of the test "with respect to the (alternative) hypothesis H."

If there is a set of possible alternative hypotheses, the power, regarded as a function of H, is termed the *power function* of the test. When the alternatives are indexed by a single parameter θ, simple graphical presentation is possible. If the parameter is a vector θ, one can visualize a *power surface.*

If the power function is denoted by $\beta(\theta)$ and H_0 specifies $\theta = \theta_0$, then the value of $\beta(\Pi)$—the probability of rejecting H_0 when it is in fact valid—is the significance level. A test's power is greatest when the probability of a type II error is the least. Specified powers can be calculated for tests in any specific or general situation.

Some general rules to keep in mind are:

- The more stringent the significance level, the greater the necessary sample size. More subjects are needed for a 1% level test than for a 5% level test.
- Two-tailed tests require larger sample sizes than one-tailed tests. Assessing two directions at the same time requires a greater investment.
- The smaller the critical effect size, the larger the necessary sample size. Subtle effects require greater efforts.
- Any difference can be significant if the sample size is large enough.
- The larger the power required, the larger the necessary sample seize. Greater protection from failure requires greater effort. The smaller the sample size, the smaller the power, that is, the greater the chance of failure.
- The requirements and means of calculating necessary sample size depend on the desired (or practical) comparative sizes of test and control groups.

This number (N) can be calculated, for example, for equal-sized test and control groups using the formula

$$N = \frac{(t_1 + t_2)^2}{d^2} S$$

where t_1 is the one-tailed t value with $N - 1$ degrees of freedom corresponding to the desired level of confidence, t_2 is the one-tailed t value with $N - 1$ degrees of freedom corresponding to the probability that the sample size will be

adequate to achieve the desired precision, and S is the sample standard deviation, derived typically from historical data and calculated as

$$S = \sqrt{\frac{1}{N-1}\sum (V_1 - V_2)^2}$$

A number of aspects of experimental design are specific to the practice of toxicology. Before we look at a suggestion for step-by-step development of experimental designs, these aspects should first be considered as follows:

1. Frequently, the data gathered from specific measurements of animal characteristics are such that there is wide variability in the data. Often, such wide variability is not present in a control or low-dose group, but in an intermediate dosage group variance inflation may occur. That is, there may be a large standard deviation associated with the measurements from this intermediate group. In the face of such a set of data, the conclusion that there is no biological effect based on a finding of no statistically significant effect might well be erroneous.

2. In designing experiments, one should keep in mind the potential effect of involuntary censoring on sample size. In other words, though a study might start with five dogs per group, this provides no margin should any die before the study is ended and blood samples are collected and analyzed. Just enough experimental units per group frequently leave too few at the end to allow meaningful statistical analysis, and allowances should be made accordingly in establishing group sizes.

3. It is certainly possible to pool the data from several identical toxicological studies. One approach to this is meta-analysis, considered in detail later in this chapter. For example, after first having performed an acute inhalation study where only three treatment group animals survived to the point at which a critical measure (such as analysis of blood samples) was performed, we would not have enough data to perform a meaningful statistical analysis. We could then repeat the protocol with new control and treatment group animals from the same source. At the end, after assuring ourselves that the two sets of data are comparable, we could combine (or pool) the data from survivors of the second study with those from the first. The costs of this approach, however, would then be both a greater degree of effort expended (than if we had performed a single study with larger groups) and increased variability in the pooled samples (decreasing the power of our statistical methods).

4. Another frequently overlooked design option in toxicology is the use of an unbalanced design—that is, of different group sizes for different levels of treatment.

There is no requirement that each group in a study (control, low dose, intermediate dose, and high dose) have an equal number of experimental units assigned to it. Indeed, there are frequently good reasons to assign more experimental units to one group than to others, and, as we shall see later in this book,

all the major statistical methodologies have provisions to adjust for such inequalities within certain limits. The two most common uses of the unbalanced design have larger groups assigned to either the highest dose, to compensate for losses due to possible deaths during the study, or the lowest dose, to give more sensitivity in detecting effects at levels close to an effect threshold, or more confidence to the assertion that no effect exists.

5. We are frequently confronted with the situation where an undesired variable is influencing our experimental results in a nonrandom fashion. Such a variable is called a confounding variable; its presence, as discussed earlier, makes the clear attribution and analysis of effects at best difficult and at worst impossible. Sometimes such confounding variables are the result of conscious design or management decisions, such as the use of different instruments, personnel, facilities, or procedures for different test groups within the same study. Occasionally, however, such confounding variables are the result of unintentional and unrecognized factors or actions, in which as it is called a lurking variable. Examples of such variables are almost always the result of standard operating procedures being violated—water not being connected to a rack of animals over a weekend, a set of racks not being cleaned as frequently as others, or a contaminated batch of feed being used.

6. Finally, some thought must be given to the clear definition of what is meant by experimental unit and concurrent control.

The experimental unit in toxicology encompasses a wide variety of possibilities. It may be cells, plates of microorganisms, individual animals, litters of animals, and so on. The importance of clearly defining the experimental unit is that the number of such units per group is the *N*, which is used in statistical calculations or analyses and critically affects such calculations. The experimental unit is the unit which receives treatments and yields a response which is measured and becomes a datum.

A true concurrent control is one that is identical in every manner with the treatment groups except for the treatment being evaluated. This means that all manipulations, including gavaging with equivalent volumes of vehicle or exposing to equivalent rates of air exchanges in an inhalation chamber, should be duplicated in control groups just as they occur in treatment groups.

The goal of the four principles of experimental design is statistical efficiency and the economizing of resources. The single most important initial step in achieving such an outcome is to clearly define the objective of the study—get a clear statement of what questions are being asked.

For the reader who wants to further explore experimental design, there are a number of more detailed texts available which include more extensive treatment of the statistical aspects of experimental design (Cochran and Cox, 1975; Diamond, 1981; Federer, 1955; Hicks, 1982; Kraemer and Thiemann, 1987; Myers, 1972).

There are four basic experimental design types used in toxicology: randomized block, latin square, factorial design, and nested design. Other designs that

are used are really combinations of these basic designs and are very rarely employed in toxicology. Before examining these four basic types, however, we must first examine the basic concept of blocking.

Blocking is, simply put, the arrangement or sorting of the members of a population (such as all of an available group of test animals) into groups based on certain characteristics which may (but are not sure to) alter an experimental outcome. Such characteristics which may cause a treatment to give a differential effect include genetic background, age, sex, and overall activity levels. The process of blocking then acts (or attempts to act) so that each experimental group (or block) is assigned its fair share of the members of each of these subgroups.

We should now recall that randomization is aimed at spreading out the effect of undetectable or unsuspected characteristics in a population of animals or some portion of this population. The merging of the two concepts of randomization and blocking leads to the first basic experimental design, the randomized block. This type of design requires that each treatment group have at least one member of each recognized group (such as age), the exact members of each block being assigned in an unbiased (or random) fashion.

The second type of experimental design assumes that we can characterize treatments (whether intended or otherwise) as belonging clearly to separate sets. In the simplest case, these categories are arranged into two sets which may be thought of as rows (for, say, source litter of test animal, with the first litter as row 1, the next as row 2, etc.) and the secondary set of categories as columns (for, say, ages of test animals, with 6–8 weeks as column 1, 8–10 weeks as column 2, etc.). Experimental units are then assigned so that each major treatment (control, low dose, intermediate dose, etc.) appears once and only once in each row and each column. If we denote our test groups as A (control), B (low), C (intermediate), and D (high), such as assignment would appear as follows:

	Age			
Source litter	6–8 weeks	8–10 weeks	10–12 weeks	12–14 weeks
1	A	B	C	D
2	B	C	D	A
3	C	D	A	B
4	D	A	B	C

The third type of experimental design is the factorial design, in which there are two or more clearly understood treatments, such as exposure level to test chemical, animal age, or temperature. The classical approach to this situation (and to that described under the latin square) is to hold all but one of the treatments constant and at any one time to vary just that one factor. Instead, in the factorial design all levels of a given factor are combined with all levels of every other factor in the experiment. When a change in one factor produces a different change in the response variable at one level of a factor than at

other levels of this factor, there is an interaction between these two factors which can then be analyzed as an interaction effect.

The last of the major varieties of experimental design are the nested designs, where the levels of one factor are nested within (or are subsamples of) another factor. That is, each subfactor is evaluated only within the limits of its single larger factor.

25.3 DATA RECORDING

Two distinct sources of systematic bias may occur in data recording. One is that awareness of treatment may, consciously or subconsciously, affect the values recorded by the measurer. This can be avoided by organizing data recording so that observations are made blind. The second is that there is a systematic shift in the standard of measurement with time coupled with a tendency for different groups to be measured at different time points. This is particularly important when a pathologist grades a lesion for severity and when the control and high-dose animals are read before the intermediate-dose animals. In some situations it may be necessary to reread all the slides blind and in random order to be sure that diagnostic drift is avoided (Gad and Taulbee, 1996).

Valid analysis cannot be conducted unless one can distinguish animals which were examined and did not have the relevant response and animals which were not examined. It can also be important to clearly identify why data are missing. Table 25.4 identifies some basic rules for effective design of data collection forms.

25.4 GENERALIZED METHODOLOGY SELECTION

One approach for the selection of appropriate techniques to employ in a particular situation is to use a decision tree method. Figure 25.1 is a decision tree that leads to the choice of one of three other trees to assist in technique selection, with each of the subsequent trees addressing one of the three functions of statistics that was defined earlier in this chapter. Figure 25.2 is for the selection of hypothesis-testing procedures, Figure 25.3 for modeling procedures, and Figure 25.4 for reduction of dimensionality procedures. For the vast majority of situations, these trees will guide the user into the choice of the proper technique. The tests and terms in these trees will be explained subsequently.

25.5 STATISTICAL ANALYSIS: GENERAL CONSIDERATIONS

25.5.1 Variables to be Analyzed

Although some pathologist still regard their discipline as providing qualitative rather than quantitative data, it is abundantly clear that pathology, when

TABLE 25.4 Rules for Form Design and Preparation

1. Forms should be used when some form of repetitive data must be collected. They may be either paper or electronic.
2. If only a few (two or three) pieces of data are to be collected, they should be entered into a notebook and not onto a form. This assumes that the few pieces are not a daily event, with the aggregate total of weeks/months/years ending up as lots of data to be pooled for analysis.
3. Forms should be self-contained but should not try to repeat the content of the SOPs or method descriptions.
4. Column headings on forms should always specify the units of measurement and other details of entries to be made. The form should be arranged so that sequential entries proceed down a page, not across. Each column should be clearly labeled with a heading that identifies what is to be entered in the column. Any fixed part of entries (such at °C) should be in the column header.
5. Columns should be arranged from left to right so that there is a logical sequential order to the contents of an entry as it is made. An example would be date/time/animal number/body weight/name of recorder. The last item for each entry should be the name or unique initials of the individual who made the data entry.
6. Standard conditions that apply to all the data elements to be recorded on a form or the columns of the form should be listed as footnotes at the bottom of the form.
7. Entries of data on the form should not use more digits than are appropriate for the precision of the data being recorded.
8. Each form should be clearly titled to indicate its purpose and use. If multiple types of forms are being used, each should have a unique title or number.
9. Before designing the form, carefully consider the purpose for which it is intended. What data will be collected, how often, with what instrument, and by whom? Each of these considerations should be reflected in some manner on the form.
10. Those things which are common/standard for all entries on the form should be stated as such once. These could include such things as instrument used, scale of measurement (°C, °F, or K), or location where the recording is made.

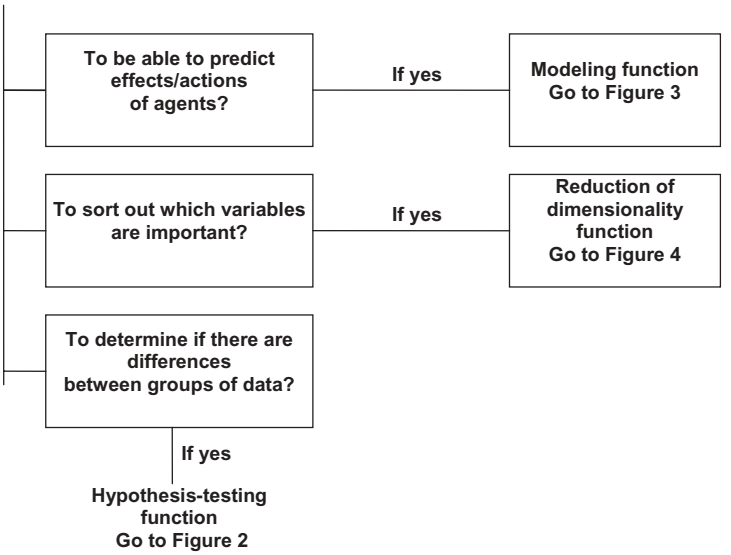
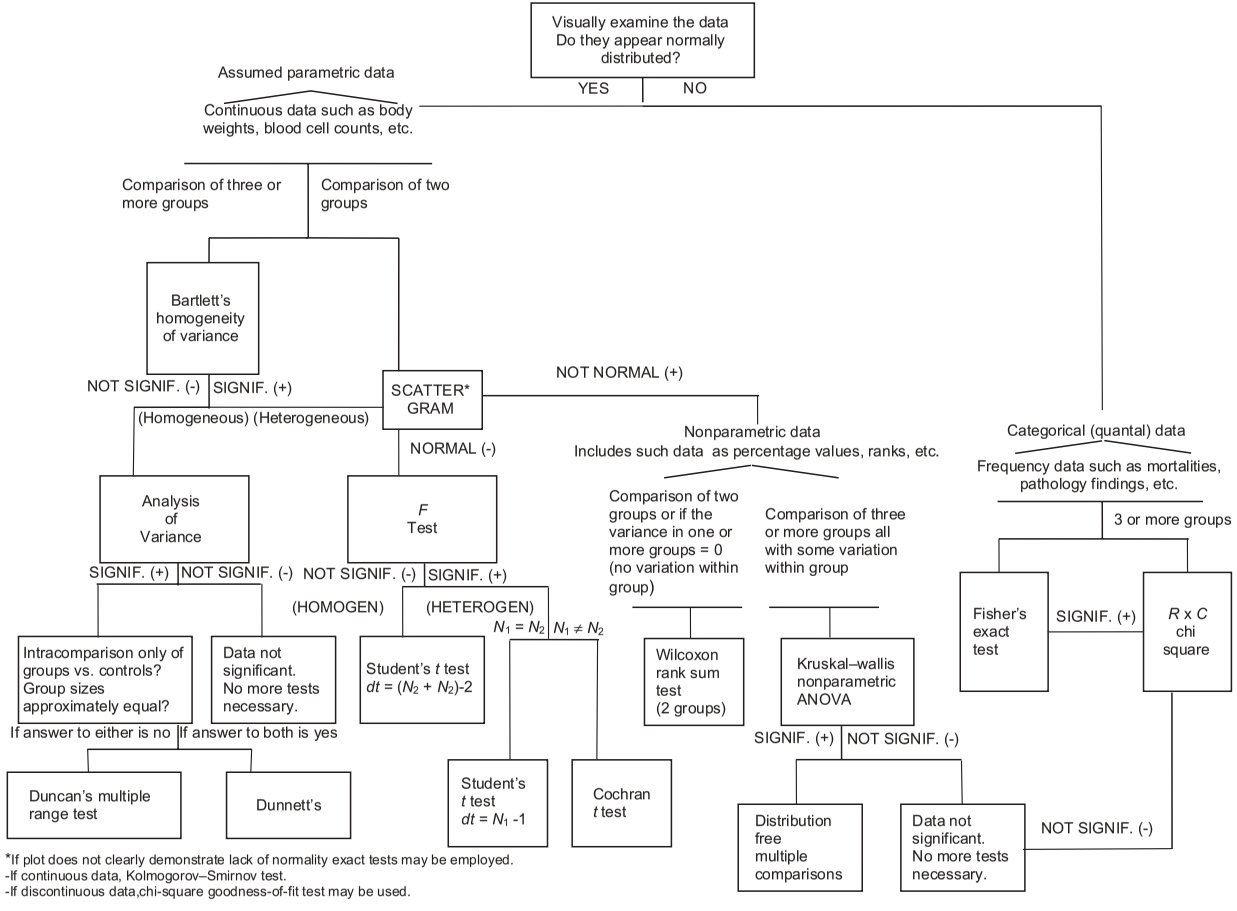


Figure 25.1 *Overall Decision Tree for Selecting Statistical Procedures.*



*If plot does not clearly demonstrate lack of normality exact tests may be employed.
-If continuous data, Kolmogorov–Smirnov test.
-If discontinuous data,chi-square goodness-of-fit test may be used.

Figure 25.2 *Decision Tree for Selecting Hypothesis-testing Procedures.*

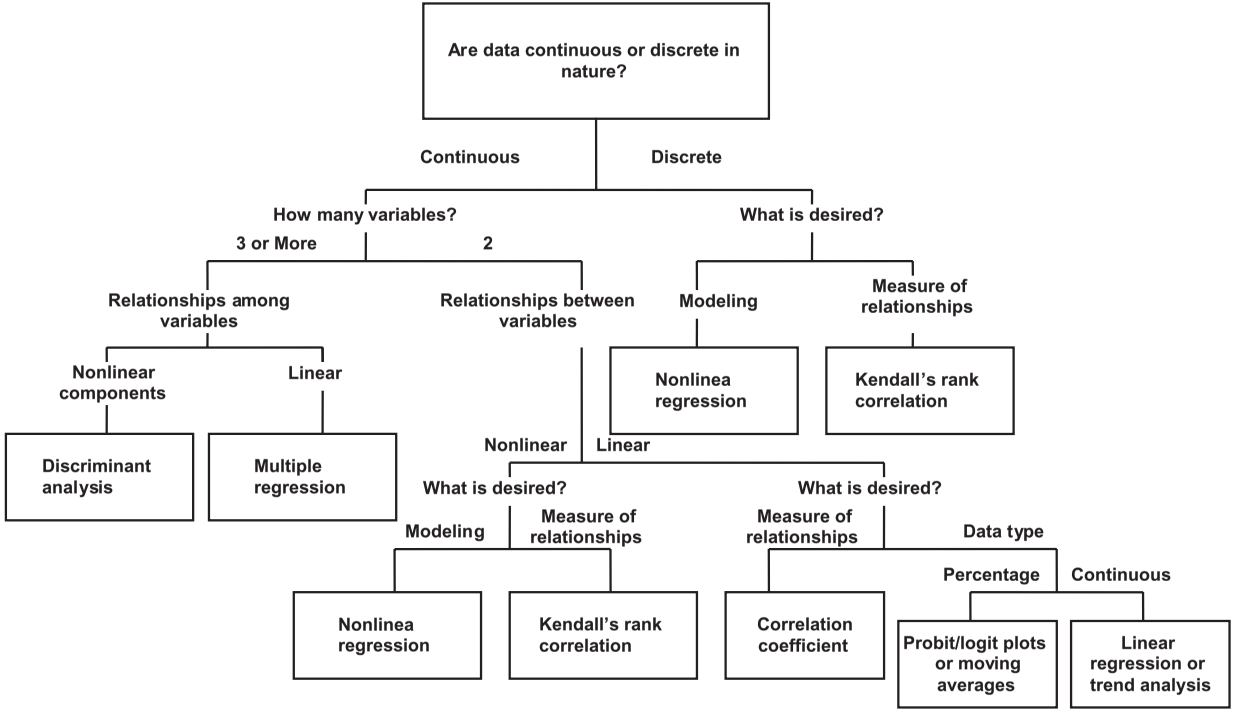


Figure 25.3 *Decision Tree for Selecting Modeling Procedures.*

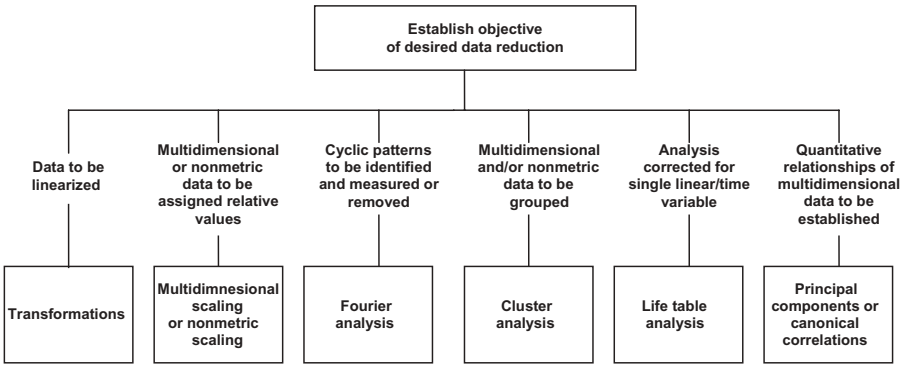


Figure 25.4 *Decision Tree for Selection of Reduction of Dimensionality Procedures.*

applied to routine screening of animal toxicity and carcinogenicity studies, has to be quantitative to at least some degree so that statistical inferences and statements can be made about possible treatment effects. Inevitably, there will be some descriptive text which will not be appropriate for statistical analysis. However, the main objective of the pathologist should be to provide information on the presence or absence (with severity grade or size where appropriate) of a list of conditions, consistently recorded from animal to animal and classified by well-defined criteria, which can be validly used in a statistical assessment.

Given that statistical analysis is worth doing and data are available that would be analyzed, should one then analyze all the endpoints recorded? Some arguments have been put forward against analyzing all the endpoints studied, none of which really holds water.

One argument is that some endpoints are not of interest. Perhaps the study is essentially a carcinogenicity study, so that nonneoplastic endpoints are not considered to be "background pathology" and almost per se unrelated to treatment. However, if the pathologist has gone to the trouble of recording the data, then, in general, they ought to be analyzed. The costs of the statistical analysis are much less than those of doing the study and the pathology. While one might justify failure to analyze nonneoplastic data where tumor analysis has already shown that the compound is clearly carcinogenic and no longer of market potential, the general rule ought to be to analyze everything that has been specifically investigated.

Another argument put forward against doing multiple analyses is that it may yield many chance significant p values that have to be considered and evaluated for biological significance in the context of the entire set of available data. The whole context of dose response, as summarized in Table 25.5, must be kept in mind. A detailed look at the data can only aid interpretation provided one is not bound by the false argument that statistical significance necessarily equates with biological importance and definitely indicates a true effect of treatment.

TABLE 25.5 Three Dimensions of Dose Response

As dose increases:
• Incidence of responders in an exposed population increases.
• Severity of response in effected individuals increases.
• Time to occurrence of response or of progressive stage of response decreases.

Finally, some endpoints occur only very rarely. One must then be clear what "very rarely" is. For a typical study with a control and three dose groups of equal size, one would get a significant trend statistics if all three cases occurred at the top dose level or in the control group (two-tailed $p \approx 0.03$), so a total of three cases will normally be enough for statistical analysis. Endpoints occurring once or twice only are not worth analyzing formally, although, if only seen in the top dose group, they may be worth noting in the report. This is especially true if they are lesions that are rarely reported.

25.5.2 Combination of Pathological Conditions

There are four main situations where one might consider combining pathological conditions in a statistical analysis.

The first is when essentially the same pathological condition has been recorded under two or more different names or even under the same name in different places. Here failure to combine these conditions in the analysis may severely limit the chances of detecting a true treatment effect. It should be noted, however, that grouping together conditions which are actually different may also result in the masking of a true treatment effect, particularly if the treatment has a very specific effect.

The second is when separately recorded pathological conditions form successive steps on the pathway of the same process. The most important example of this is for the incidence of related types of malignant tumor, benign tumor, and focal hyperplasia. It will normally be appropriate to carry out analyses of (1) incidence of malignant tumor, (2) incidence of benign or malignant tumor, and, where appropriate, (3) incidence of focal hyperplasia and benign or malignant tumor. It will not normally be appropriate to carry out analyses of benign tumor incidence only or of the incidence of hyperplasia only.

The third situation for combining is when the same pathological condition appears in different organs as a result of the same underlying process. Examples of this are the multicentric tumors (such as myeloid leukemia, reticulum cell sarcoma, and lymphosarcoma) or certain nonneoplastic conditions (such as arteritis/periarteritis and amyloid degeneration). Here analysis will normally be carried out only of incidence at any site, although in some situations site-specific analyses might be worth carrying out.

The final situation where an analysis of combined pathological conditions is normal is for analyses of overall incidence of malignant tumor at any site, of benign or malignant tumor at any site, or of multiple tumor incidence. While

analyses of tumor incidence at specific sites are normally more meaningful, since treatments often affect only a few specific sites, these additional analyses are usually required to guard against the possibility that treatment had some weak but general tumor-enhancing effect not otherwise evident.

In some situations, one might also envisage analyses of other combinations of specific tumors, such as tumors at related sites (e.g., endocrine organs if the compound had a hormonal effect) or of similar histological type.

25.5.3 Taking Severity into Account

The same line or argument that suggests that if the pathologist records data they should be analyzed also suggests that if the pathologist chooses to grade a condition for severity the grade should be taken into account in the analysis. There are two ways to carry out analysis when the grade has to be taken into account. In one, analyses are carried out not only of whether or not the animal has a condition but also of whether or not the condition is at least gade 2, at least grade 3, and so on. In the other approach, nonparametric (rank) methods are used. The latter approach is more powerful, as it uses all the information in one analysis, although the output may not be so easily understood by those without some statistical training.

Note that the analyses based on grade can be carried out only if grading has been consistently applied throughout. If a condition has been scored only as present/absent for some animals but has been graded for others, it is not possible to carry out graded analyses unless the pathologist is willing to go back and grade the specific animals showing the condition.

25.5.4 Using Simple Methods Which Avoid Complex Assumptions

Different methods for statistical analysis can vary considerably in their complexity and in the number of assumptions they make. Although the use of statistical models has its place, more so for effect estimation than for hypothesis testing and more so in studies of complex design than in those of simple design, there are advantages in using, wherever possible, statistical methods that are simple and robust and make as few assumptions as possible. There are three reasons for this. First, such methods are more generally understandable to the toxicologist. Second, there are hardly ever extensive enough data in practice to validate any given formal model fully. Third, even if a particular model were known to be appropriate, the loss of efficiency in using appropriate simpler methods is often only very small.

The methods we advocate for routine use for the analysis of tumor incidence tend, therefore, not to be based on the use of formal parametric statistical models. For example, when studying the relationship of treatment to incidence of a pathological condition and wishing to adjust for other factors (in particular, age at death) that might otherwise bias the comparison, methods involving “stratification” are recommended rather than a multiple-regression

approach or time-to-tumor models. ANOVA methods can be useful in the case of continuously distributed data for estimating treatment effects. However, they involve underlying assumptions (normally distributed variables, variability equal in each group). If these assumptions are violated, nonparametric methods based on the rank of observations, rather than their actual value, may be preferable for hypothesis testing.

25.5.5 Using All Data

Often information is available about the relationship between treatment and a condition of interest for groups of animals differing systematically with respect to some other factor. Obvious examples are males and females, differing times of sacrifice, and differing secondary treatments. While it will be necessary, in general, to look at the relationship within levels of this other factor, it will also be advisable to try to come to some assessment of the relationship over all levels of the other factor and where a combined inference is not sensible, but in far more situations this is not the case, and using all the data in one analysis allows a more powerful test of the relationship under study. Some scientists consider that conclusions for males and females should always be drawn separately, but there are strong statistical arguments for a joint analysis.

25.5.6 Combining, Pooling, and Stratification

Suppose, in a hypothetical study of a toxic agent which induces tumors that do not shorten the lives of tumor-bearing animals, the data regarding the number of animals with tumor out of number examined are as follows:

	Control	Exposed	Combined
Early deaths	1/20 (5%)	18/90 (20%)	19/110 (17%)
Late deaths	24/80 (30%)	7/10 (70%)	31/90 (34%)
Total	25/100 (25%)	25/100 (25%)	50/200 (25%)

It can be seen that if the time of death is ignored and the *pooled* data are studied, the incidence of tumors is the same in each group, resulting in the *false* conclusion that treatment had no effect. Looking within each time of death, however, an increased incidence in the exposed group can be seen. An appropriate statistical method would *combine* a measure of difference between the groups based on the early deaths and a measure of difference based on the late deaths and conclude *correctly* that incidence, after adjustment for time of death, is greater in the exposed groups.

In this example, time of death is the stratifying variable, with two strata—early deaths and late deaths. The essence of the methodology is to make comparisons only within strata (so that one is always comparing like with like except with respect to treatment) and then to combine the differences over

strata. Stratification can be used to adjust for any variable or indeed combinations of variables.

Some studies are of factorial design, in which combinations of treatments are tested. The simplest such design is one in which four equal-sized groups of animals receive (1) no treatment, (2) treatment A only, (3) treatment B only, and (4) treatments A and B. If one is prepared to assume that any effects of the two treatments are independent, one can use stratification to enable more powerful tests to be conducted of the possible individual treatment effects. Thus, to test for effects of treatment A, for example, one conducts comparisons in two strata, the first consisting of groups 1 and 2 not given treatment B and the second consisting of groups 3 and 4 given treatment B. Combination of results from the two strata is based on twice as many animals and is therefore markedly more likely to detect possible effects of treatment A than is a simple comparison of groups 1 and 2. There is also the possibility of identifying interactions, such as synergism and antagonism, between the two treatments.

In some routine long-term screening studies, the study design involved five groups of (usually) 50 animals of each sex, three of which are treated with successive doses of a compound and two of which are untreated controls. Assuming that there is no systematic difference between the control groups (e.g., the second control group in a different room or from a different batch of animals), it will be normal to carry out the main analyses with the control groups treated as a single group of 100 animals. It will usually be a sensible preliminary precaution to carry out additional analyses comparing incidences in the two control groups.

25.5.7 Trend Analysis, Low-Dose Extrapolation, and No-Observable-Effect level (NOEL) Estimation

While comparisons of individual treated groups with the control group are important, a more powerful test of a possible effect of treatment will be to carry out a test for a dose-related trend. This is because most true effects of treatment tend to result in a response which increases (or decreases) with increasing dose and because trend tests take into account all the data in a single analysis. In interpreting the results of trend tests, it should be noted that a significant trend does not necessarily imply an increased risk at lower doses. Nor, conversely, does a lack of increase at lower doses necessarily indicate evidence of a threshold (i.e., a dose below which no increase occurs).

Note that the testing for trend is seen as a more sensitive way of picking up a possible treatment effect than simple pairwise comparisons of treated and control groups. Attempting to estimate the magnitude of effects at low doses, typically below the lowest positive dose tested in the study, is a much more complex procedure and is heavils dependent on the assumed functional form of the dose–response relationship.

Deterministic trend models are based on the assumption that the trend of a time series can be approximated closely by simple mathematical functions of

time over the entire span of the series. The most common representation of a deterministic trend is by means of polynomials or of transcendental functions. The time series from which the trend is to be identified is assumed to be generated by a nonstationary process where the nonstationarity results from a deterministic trend. A classical model is the regression or error model (Anderson, 1971) where the observed series is treated as the sum of a systematic part or trend and a random or irregular part. This model can be written as

$$Z_t = Y_t + U_t'$$

where U_t is a purely random process, that is, $U_t \sim$ i.i.d. $(0, 2/u)$ (independent and identically distributed with expected value zero and variance $2/u$.)

Trend tests are generally described as k-sample tests of the null hypothesis of identical distribution against an alternative of linear order; that is, if sample I has distribution function F_i, $i = 1$, then the null hypothesis

$$\mathrm{H}_0 : \mathrm{F}_1 = F_2 - \cdots = F_k$$

is tested against the alternative

$$\mathrm{H}_1 : F_1 \geq F_2 \geq \cdots = F_k$$

(or its reverse), where at least one of the inequalities is strict. These tests can be thought of as special cases of tests of regression or correlation in which association is sought between the observations and its ordered sample index. They are also related to ANOVA except that the tests are tailored to be powerful against the subset of alternatives H_1, instead of the more general set $\{F_1 \neq F_j$, some $i \neq j\}$.

Different tests arise from requiring power against specific elements or subsets of this rather extensive set of alternatives.

The most popular trend test in toxicology is currently that presented by Tarone in 1975 because it is that used by the National Cancer Institute (NCI) in the analysis of carcinogenicity data. A simple, but efficient alternative is the Cox and Stuart (1955) test, which is a modification of the sign test. For each point at which we have a measure (such as the indidence of animals observed with tumors) we form a pair of observations—one from each of the groups we wish to compare. In a traditional NCI bioassay this would mean pairing control with low dose and low dose with high dose (to explore a dose-related trend) or each time period observation in a dose group (except the first) with its predecessor (to evaluate time-related trend). When the second observation in a pair exceeds the earlier observation, we record a plus sign for that pair. When the first observation is greater than the second, we record a minus sign for that pair. A preponderance of plus signs suggests a downward trend while an excess

of minus signs suggests an upward trend. A formal test at a preselected confidence level can then be performed.

More formally put, after having defined what trend we want to test for, we first match pairs as $(X_1 - X_{1+c})$, (X_2, X_{2+c}), … , $(X_{n'-c}, X_{n'})$, where $c = n'/2$ when n' is even and $c = (n' + 1)/2$ when n' is odd (where n' is the number of observations in a set). The hypothesis is then tested by comparing the resulting number of excess positive or negative signs against a sign test table such as are found in Beyer (1976a,b).

We can, of course, combine a number of observations to allow ourselves to actively test for a set of trends, such as the existence of a trend of increasing difference between two groups of animals over a period of time. This is demonstrated in Example 25.1.

ASSUMPTION AND LIMITATION

1. Trend tests seek to evaluate whether there is a monotonic tendency in response to a change in treatment. That is, the dose–response direction is absolute—as dose goes up, the incidence of tumors increases. Thus the test loses power rapidly in response to the occurrences of "reversals"—for example, a low-dose group with a decreased tumor incidence. There are methods which "smooth the bumps" of reversals in long data series. In toxicology, however, most data series are short (i.e., there are only a few dose levels).

Tarone's trend test is most powerful at detecting dose-related trends when tumor onset hazard functions are proportional to each other. For more power against other dose-related group differences, weighted versions of the statistic are also available (Breslow, 1984; Crowley and Breslow, 1984).

In 1985, the U.S. *Federal Register* recommended that the analysis of tumor incidence data is carried out with a Cochran–Armitage (Armitage, 1955; Cochran, 1954) trend test. The test statistic of the Cochran–Armitage test is defined as

$$T_{\mathrm{CA}} = \sqrt{\frac{N}{(N-r)r}}, \frac{\sum_{i=0}^{k}[R_1 - (n_1/N)r]d_1}{\sum_{i=0}^{k}(n_i/N)d_i^2 - \left(\sum_{i=0}^{k}(n_i/N)d_1\right)^2}$$

with dose scores d_i. Armitage's test statistic is the square of this term (T_{CA}^2). As one-sided tests are carried out for an increase of tumor rates, the square is not considered. Instead, the above-mentioned test statistic, which is presented by Portier and Hoel (1984), is used. This test statistic is asymptotically standard normally distributed. The Cochran–Armitage test is asymptotically efficient for all monotone alternatives (Tarone, 1975), but this result only holds asymptotically. And tumors are rare events, so the binominal

Example 25.1 In a chronic feeding study in rats, we tested the hypothesis that, in the second year of the study, there was a dose-responsive increase in tumor incidence associated with the test compound. Below, we utilize, a Cox–Stuart test for trend to address this question. All groups start the second year with an equal number of animals.

	Control		Low Doses			High Doses		
Month of Study	Total X, Animals with Tumors	Change $[X_{A-B}]$	Total Y, Animals with Tumors	Change $[Y_{A-B}]$	Compared to Control $(Y - X)$	Total Z, Animals with Tumors	Change $[Z_{a-b}]$	Compared to Control $(Z - X)$
12 (*A*)	1	NA	0	NA	NA	5	NA	NA
13 (*B*)	1	0	0	0	0	7	2	(+)2
14 (*C*)	3	2	1	1	(–)1	11	4	(+)2
15 (*D*)	3	0	1	0	0	11	0	0
16 (*E*)	4	1	1	0	(–)1	13	2	(+)1
17 (*F*)	5	1	3	2	(+)1	14	1	0
18 (*G*)	5	0	3	0	0	15	1	(+)1
19 (*H*)	5	0	5	2	(+)2	18	3	(+)3
20 (*I*)	6	1	6	1	0	19	1	0
21 (*J*)	8	2	7	1	(–)1	22	3	(+)1
22 (*K*)	12	4	9	2	(–)2	26	4	0
23 (*L*)	14	2	12	3	(+)1	28		
6 +								
1 –								
				$Y - X = 0$ (no trend)			$Z - X = 5$	

Reference to a sign table is not necessary for the low-dose comparison (where there is no trend) but clearly shows the high dose to be significant at the $p \leq 0.5$ level.

proportions are small. In this situation approximations may become unreliable.

Therefore, exact tests which can be performed using two different approaches—conditional and unconditional—are considered. In the first case, the total number of tumors r is regarded as fixed. As a result the null distribution of the test statistic is independent of the common probability p. The exact conditional null distribution is a multivariate hypergeometric distribution.

The unconditional model treats the sum of all tumors as a random variable. Then the exact unconditional null distribution is a multivariate binomial distribution. The distribution depends on the unknown probability.

Such low-dose extrapolation is typically only conducted for tumors believed to be caused by a genotoxic effect which some, but by no means all, scientists believe have no threshold. For other types of tumors and for many nonneoplastic endpoints a threshold cannot be estimated directly from data at a limited number of dose levels, and a NOEL can be estimated by finding the highest dose level at which there is no significant increase in effects.

A useful technique for determining if there is an effect of treatment on any toxicological parameter is the NOSTASOT method (Tukey et al., 1985; Antonello et al., 1993). This test is based on the principle that a possible toxicological effect of interest occurs with a normal dose response; that is, there is an increasing effect with increasing dosage. The data to be analyzed should be examined first to confirm that this principle is not violated. In this method, regression analysis is used to determine if there is an increased or decreased response in a parameter with increasing dosage. This method can be visualized as a plot of response versus dosage in which the analysis determines if the slope of the plotted line deviates significantly from zero.

This method can be used for essentially all parameters. Three analyses are performed each with different spacing between dosage levels. The spacing in the first analysis is based on the arithmetic values of the dosage levels. The spacing in the second, referred to as the ordinal scaling, has equal spacing between dosage levels; that is, the control through high dosage levels are assigned values of 0, 1, 2, and 3. In the third analysis, the log of the dosage level is used. Since the log of zero is impractical, the control group is assigned a value based on the spacing between the low and middle dosage levels according to a formula that assigns a log scale value to the control such that the ratio of the difference between the control and low-dose groups and the difference between the low- and middle-dose groups is equal both in absolute values and in log scale values. This places the control group at a reasonable distance from the low-dose group. The lowest p value among the three analyses—arithmetic, ordinal, and logarithmic—is taken as the p value of the overall analysis based on the assumption that, if there is a dosage-related effect, the method of analysis yielding the lowest value is the best model for that dosage response. A correction for the multiplicity of analyses can be applied. If none of the three analyses are significant at the 0.05 level, the analysis is complete and the

high dosage level is referred to as the "no statistical significance of trend dose," or the NOSTASOT dose. If there is a significant trend through the high dosage level, the data from the high dosage level is deleted and the trend test repeated. This process is repeated until a NOSTASOT dose is determined. Effects at dosage levels above the NOSTASOT dose are then considered to be statistically significant.

There are two major benefits of the NOSTASOT method. One is that spurious statistically significant results only at the low and/or middle dosage levels are eliminated, resulting in a reduction in false positives. A second benefit is that in some cases there may be real effects as multiple dosage levels that at any single dosage level are not statistically significant but will nevertheless result in a significant trend, thus providing increased sensitivity and reducing false negatives.

25.5.8 Need for Age Adjustment

Where there are marked differences in survival between treated groups, it is widely recognized that there is a need for an age adjustment (i.e., an adjustment for age at death or onset). This is illustrated in the example above, where, because of the greater number of deaths occurring early in the treated group, the true effect of treatment disappears if no adjustment is made. Thus, a major purpose of age adjustment is to avoid bias.

It is not so generally recognized, however, that, even where there are no survival differences, age adjustment can increase the power to detect between-group differences. This is illustrated in the example below:

	Control	Exposed
Early deaths	0/20	0/20
Middle deaths	1/10	9/10
Late deaths	20/20	20/20
Total	21/50	29/50

Here treatment results in a somewhat earlier onset of a condition which occurs eventually in all animals. Failure to age adjust will result in a comparison of 29/50 with 21/50, which is not statistically significant. Age adjustment will essentially ignore the early and late deaths, which contribute no comparative statistical information, and be based on the comparison of 9/10 with 1/10, which is statistically significant. Here age adjustment sharpens the contrast, rather than avoiding bias, by avoiding diluting data capable of detecting treatment effects with data that are of little or no value for this purpose.

25.5.9 Need to Take Context of Observation into Account

It is now widely recognized that age adjustment cannot properly be carried out unless the context of observation is taken into account. There are three

relevant contexts, the first two relating to the situation where the condition is only observed at death (e.g., an internal tumor) and the third where it can be observed in life (e.g., a skin tumor):

In the first context the condition is assumed to have caused the death of the animal, that is, to be *fatal*. Here the incidence rate for a time interval and a group is calculated by the number of animals dying in the interval because of the lesion divided by the number of animals alive at the start of the interval.

In the second context, the animal is assumed to have died of another cause, that is, the condition is *incidental*. Here the rate is calculated by the number of animals dying in the interval with the lesion divided by the total number of animals dying in the interval.

In the third context, where the condition is *visible*, the rate is calculated by the number of animals getting the condition in the interval divided by the number of animals without the condition at the start of the interval.

A problem with the method of Peto et al. (1980), which takes the context of observation into account, is that some pathologists are unwilling or feel unable to decide whether, in any given case, a condition is fatal or incidental. A number of points should be made here.

First, where there are marked survival differences, it may not be possible to conclude reliably whether a treatment is beneficial or harmful unless such a decision is made. This is well illustrated by the example in Peto et al. (1980), where assuming all pituitary tumors were fatal results in the (false) conclusion that *N*-nitrosodimethylamine (NDMA) was carcinogenic, while assuming they were all incidental resulted in the (false) conclusion that NDMA was protective. Using, correctly, the pathologist's best opinion as to which were and which were not likely to be fatal resulted in an analysis which (correctly) concluded NDMA had no effect. If the pathologist, in this case, had been unwilling to make a judgment as to fatality, believing it to be unreliable, no conclusion could have been reached. This state of affairs would, however, be a fact of life, and *not* a position reached because an inappropriate statistical method was being used.

Although it will normally be a good routine for the pathologist to ascribe "factors contributory to death" for each animal that was not part of a scheduled sacrifice, it is in fact not strictly necessary to determine the context of observation for all conditions at the outset. An alternative strategy is to analyze under differing assumptions: (1) no cases fatal, (2) all cases occurring in descendants fatal, and (3) all cases of the same defined severity occurring in descedants fatal, with, under each assumption, other cases incidental.

If the conclusion turns out the same under each assumption or if the pathologist can say, on general grounds, that one assumption is likely to be a close approximation to the truth, it may not be necessary to know the context

of observation for the condition in question for each individual animal. Using the alternative strategy might result in a saving of the pathologist's time by only having to make a judgment for a limited number of conditions where the conclusion seems to hand on correct knowledge of the context of observation.

Finally, it should be noted that, although many nonneoplastic conditions observed at death are never causes of death, it is, in principle, as necessary to know the context of observation for nonneoplastic conditions as it is for tumors.

25.5.10 Experimental and Observational Units

In many situations, the animal is both the "experimental unit" and the "observational unit", but this is not always so. For determining treatment effects by the methods of the next section, it is important that each experimental unit provides only one item of data for analysis, as the methods all assume that individual data items are statistically independent. In many feeding studies, where the cage is assigned to a treatment, it is the cage, rather than the animal, that is the experimental unit. In histopathology, observations for a tissue are often based on multiple sections per animal, so that the section is the observational unit. Multiple observations per experimental unit should be combined in some suitable way into an overall average for that unit before analysis.

25.5.11 Missing Data

In many types of analysis, animals with missing data are simply removed from the analysis. There are, however, some situations where this can be an inappropriate thing to do. One situation is when carrying out an analysis of a condition that is assumed to have caused the death of the animal. Although an animal dying at week 83 for which the section was unavailable for microscopic examination cannot contribute to the group comparison at week 83, one knows that it did not die because of any condition in previous weeks, so it should contribute to the denominator of the calculations in all previous weeks.

Another situation is when histopathological examination of a tissue is not carried out unless an abnormality is seen postmortem. In such an experiment one might have the following data for that tissue:

- Control group: 50 animals, 2 abnormal postmortem, 2 examined microscopically, 2 with tumor of specific type.
- Treated group: 50 animals, 15 abnormal postmortem, 15 examined microscopically, 14 with tumor of specific type.

Ignoring animals with no microscopic sections, one would compare 2/2 = 100% with 14/15 = 93% and conclude treatment nonsignificantly decreased incidence. This is likely to be a false conclusion, and it would be better here to compare the percentages of animals which had a postmortem

abnormality which turned out to be a tumor, that is, 2/50 = 4% with 14/50 = 28%. Unless some aspect of treatment made tumors much easier to detect postmortem, one could then conclude that treatment did have an effect on tumor incidence.

Particular care has to be taken in studies where the procedures for histopathological examination vary by group. In a number of studies conducted in recent years, the protocol demands full microscopic examination of a given tissue list in descedants in all groups and in terminally killed controls in high-dose animals. In other animals, that is, terminally killed low- and middose animals, microscopic examination of a tissue is only conducted if the tissue is found to be abnormal postmortem. Such a protocol is designed to save money but leads to difficulty in comparing the treatment groups validly. Suppose, for example, responses in terminally killed animals are 8/20 in the controls, 3/3 (with 17 unexamined) in the low-dose, and 5/6 (with 14 unexamined) in the middose animals. Is one supposed to conclude that treatment at the low- and middoses increased response based on a comparison of the proportions examined microscopically (40, 100, and 83%) or that it decreased response based on the proportion of animals in the group (40, 15, and 25%)? It could well be that treatment had no effect but some small tumors were missed postmortem. In this situation, a valid comparison can only be achieved by ignoring the low- and middose groups when carrying out the comparison for the age stratum "terminal kill." This, of course, seems wasteful of data, but these are data that cannot be usefully used owing to the inappropriate protocol.

25.5.12 Use of Historical Control Data

In some situations, particularly where incidences are low, the results from a single study may suggest an effect of treatment on tumor incidence but be unable to demonstrate it conclusively. The possibility of comparing results in the treated groups with those of control groups from other studies is then often raised. Thus, a nonsignificant incidence of 2 cases out of 50 in a treated group may seem much more significant if no cases have been seen in, say, 1000 animals representing controls from 20 similar studies. Conversely, a significant incidence of 5 cases out of 50 in a treated group as compared with 0 out of 50 in the study controls may seem far less convincing if many other control groups had incidences around 5 out of 50.

While not understating the importance of looking at historical control data, it must be emphasized that there are a number of reasons why variation between studies may be greater than variation within study. Differences in diet, in duration of the study, in intercurrent mortality, and in who the study pathologist is may all contribute. Statistical techniques that ignore this and carry out simple statistical tests of treatment incidence against a pooled control incidence may well give results that are seriously in error and are likely to overstate statistical significance considerably.

25.5.13 Methods for Data Examination and Preparation

The data from toxicology studies should always be examined before any formal analysis. Such examinations should be directed to determining if the data are suitable for analysis and, if so, what form the analysis should take (see Figure 25.2). If the data as collected are not suitable for analysis or if they are only suitable for low-powered analytical techniques, one may wish to use one of many forms of data transformation to change the data characteristics so that they are more amenable to analysis.

The above two objectives, data examination and preparation, are the primary focus of this chapter. For data examination, two major techniques are presented—the scattergram and Bartlett's test. Likewise, for data preparation (with the issues of rounding and outliers having been addressed in a previous chapter) two techniques are presented—randomization (including a test for randomness in a sample of data) and transformation. Exploratory data analysis (EDA) is presented and briefly reviewed later. This is a broad collection of techniques and approaches to "probe" data—that is, to both examine and perform some initial, flexible analysis of the data.

25.5.14 Scattergram

Two of the major points to be made throughout this volume are (a) the use of the appropriate statistical tests and (b) the effects of small sample sizes (as is often the case in toxicology) on our selection of statistical techniques. Frequently, simple examination of the nature and distribution of data collected from a study can also suggest patterns and results which were unanticipated and for which the use of additional or alternative statistical methodology is warranted. It was these three points which caused the author to consider a section on scattergrams and their use essential for toxicologists.

Bartlett's test may be used to determine if the values in groups of data are homogeneous. If they are, this (along with the knowledge that they are from a continuous distribution) demonstrates that parametric methods are applicable.

But, if the values in the (continuous data) groups fail Bartlett's test (i.e., are heterogeneous), we cannot be secure in our belief that parametric methods are appropriate until we gain some confidence that the values are normally distributed. With large groups of data, we can compute parameters of the population (kurtosis and skewness, in particular) and from these parameters determine if the population is normal (with a certain level of confidence). If our concern is especially marked, we can use a chi-square goodness of fit test for normality. But when each group of data consists of 25 or fewer values, these measures or tests (kurtosis, skewness, and chi-square goodness of fit) are not accurate indicators of normality. Instead, in these cases we should prepare a scattergram of the data, then evaluate the scattergram to estimate if the data

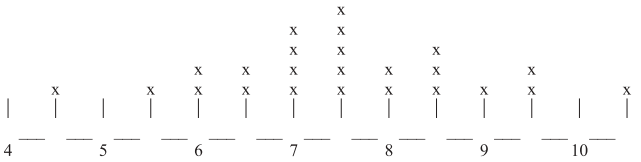
Example 25.2 Suppose we have the two data sets below:

Group 1: 4.5, 5.4, 5.9, 6.0, 6.4, 6.5, 6.9, 7.0, 7.1, 7.0, 7.4, 7.5, 7.5, 7.5, 7.6, 8.0, 8.1, 8.4, 8.5, 8.6, 9.0, 9.4, 9.5 and 10.4.

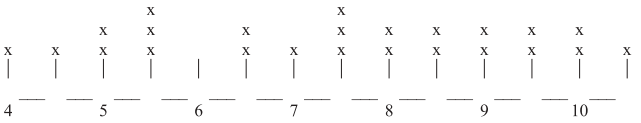
Group 2: 4.0, 4.5, 5.0, 5.1, 5.4, 5.5, 5.6, 6.5, 6.5, 7.0, 7.4, 7.5, 7.5 8.0, 8.1, 8.5, 8.5, 9.0, 9.1, 9.5, 9.5, 10.1, 10.0 and 10.4.

Both of these groups contain 24 values and cover the same range. From them we can prepare the following scattergrams:

Group 1:



Group 2:



are normally distributed. This procedure consists of developing a histogram of the data, then examining the histogram to get a visual appreciation of the location and distribution of the data.

The abscissa (or horizontal scale) should be in the same scale as the values and should be divided so that the entire range of observed values is covered by the scale of the abscissa. Across such a scale we then simply enter symbols for each of our values. Figure 25.2 shows such a plot.

Example 25.2 presents a traditional and rather limited form of scatterplot but such plots can reveal significant information about the amount and types of association between the two variables, the existence and nature of outliers, the clustering of data, and a number of other two-dimensional factors (Anscombe, 1973; Chambers et al., 1983).

Current technology allows us to add significantly more graphical information to scatterplots by means of graphic symbols (letters, faces, different shapes such as squares, colors, etc.) for the plotted data points. One relatively simple example of this approach is shown in Figure 25.5, where the simple case of dose (in a dermal study), dermal irritation, and white blood cell count are presented. This graph quite clearly suggests that as dose (variable x) is increased, dermal irritation (variable y) also increases, and as irritation becomes

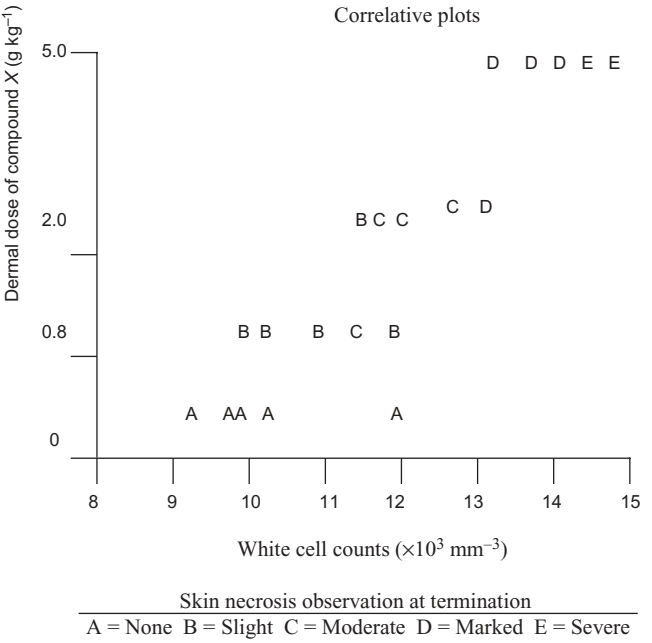


Figure 25.5 *Exploratory Data Analysis.*

more severe, white blood cell count (variable z), an indicator of immune system involvement, increases, suggesting infection or persistent inflammation, also increases. There is no direct association of variables x and z, however (Cleveland and McGill, 1984; Cleveland, 1985; Tufte, 1990).

Group 1 can be seen to approximate a normal distribution (bell-shaped curve); we can proceed to perform the appropriate parametric tests with such data. But group 2 clearly does not appear to be normally distributed. In this case, the appropriate nonparametric technique must be used.

25.5.15 Bartlett's Test for Homogeneity of Variance

Bartlett's test (Sokal and Rohlf, 1994) is used to compare the variances (values reflecting the degree of variability in data sets) among three or more groups of data, where the data in the groups are continuous sets (such as body weights, organ weights, red blood cell counts, or diet consumption measurements). It is expected that such data will be suitable for parametric methods (normality of data is assumed) and Bartlett's is frequently used as a test for the assumption of equivalent variances.

Bartlett's is based on the calculation of the corrected χ^2 (chi-square) value by the formula

$$\chi^2_{corr} = 2.3026 \frac{\sum df\left(\log_{10}\left\{\sum[df(S^2)]/\sum df\right\}\right) - \sum[df(\log_{10} S^2)]}{1+\{1/[3(K-1)]\}\left[\sum(1/df) - 1/\sum df\right]}$$

where S^2 = variance, = $\{[n\Sigma X^2 - (\Sigma X)^2]/n\}/(n-1)$
X = individual datum within each group
n = number of data within each group
K = number of groups being compared
df = degrees of freedom for each group, = $(N-1)$

The corrected χ^2 value yielded by the above calculations is compared to the values listed in the chi-square table according to the numbers of degrees of freedom (Snedecor and Cochran, 1980).

If the calculated value is smaller than the table value at the selected p level (traditionally 0.05), the groups are accepted to be homogeneous and the use of ANOVA is assumed proper. If the calculated χ^2 is greater than the table value, the groups are heterogeneous and other tests (as indicated in Figure 25.2, the decision tree) are necessary.

ASSUMPTIONS AND LIMITATIONS

1. Bartlett's test does not test for normality, but rather it tests for homogeneity of variance (also called equality of variances or homoscedasticity).
2. Homoscedasticity is an important assumption for Student's t test, ANOVA, and analysis of covariance.
3. The F test (covered in Section 25.7.3) is actually a test for the two-sample (i.e., control and one test group) case of homoscedasticity. Bartlett's is designed for three or more samples.
4. Bartlett's is very sensitive to departures from normality. As a result, a finding of a significant chi-square value in Bartlett's may indicate non-normality rather than heteroscedasticity. Such a finding can be brought about by outliers, and the sensitivity to such erroneous findings is extreme with small sample sizes.

25.5.16 Statistical Goodness-of-Fit Tests

A goodness-of-fit test is a statistical procedure for comparing individual measurements to a specified type of statistical distribution. For example, a normal distribution is completely specified by its arithmetic mean and variance (the square of the standard deviation). The null hypothesis, that the data represent a sample from a single normal distribution, can be tested by a statistical goodness-of-fit test. Various goodness-of-fit tests have been devised to

determine if the data deviate significantly from a specified distribution. If a significant departure occurs, it indicates only that the specified distribution can be rejected with some assurance. This does not necessarily mean that the true distribution contains two or more subpopulations. The true distribution may be a single distribution, based upon a different mathematical relationship (e.g., lognormal). In the latter case, logarithms of the measurement would not be expected to exhibit by a goodness-of-fit test a statistically significant departure from a lognormal distribution.

Everitt and Hand (1981) recommended use of a sample of 200 or more to conduct a valid analysis of mixtures of populations. Even the maximum-likelihood method, the best available method, should be used with extreme caution or not at all when separation between the means of the subpopulations is less than 3 SD and sample sizes are less than 300. None of the available methods conclusively establish bimodality, which may, however, occur when separation between the two means (modes) exceeds 2 SD. Conversely, inflections in probits or separations in histograms *less than* 2 SD apart may arise from genetic differences in test subjects.

Mendell et al. (1993) compared eight tests of normality to detect a mixture consisting of two normally distributed components with different means but equal variances. Fisher's skewness statistic was preferable when one component comprised less than 15% of the total distribution. When the two components comprised more nearly equal proportions (35–65%) of the total distribution, the Engelman–Hartigan (1969) test was preferable. For other mixing proportions, the maximum-likelihood ratio test was best. Thus, the maximum-likelihood ratio test appears to perform very well, with only small loss from optimality, even when it is not the best procedure.

The method of *maximum likelihood* provides estimators which are usually quite satisfactory. They have the desirable properties of being consistent, asymptotically normal, and asymptotically efficient for large samples under quite general conditions. They are often biased, but the bias is frequently removable by a simple adjustment. Other methods of obtaining estimators are also available, but the maximum-likelihood method is the most frequently used.

Maximum-likelihood estimators also have another desirable property: *invariance*. Let us denote the maximum-likelihood estimator of the parameter θ by $\hat{\sigma}$. Then, if $f(\theta)$ is a single-valued function of θ, the maximum-likelihood estimator of $f(\theta)$ is $f(\hat{\sigma})$. Thus, for example,

$$\hat{\sigma} = (\hat{\sigma}^2)1/2 .$$

The principle of maximum likelihood tells us that we should use as our estimate that value which maximizes the likelihood of the observed event.

These maximum-likelihood methods can be used to obtain *point estimates* of a parameter, but we must remember that a point estimator is a random variable distributed in some way around the true value of the parameter. The true parameter value may be higher or lower than our estimate. It is often

useful therefore to obtain an interval within which we are reasonably confident the true value will lie, and the generally accepted method is to construct what are known as *confidence limits*.

The following procedure will yield upper and lower 95% confidence limits with the property that when we say that these limits include the true value of the parameter, 95% of all such statements will be true and 5% will be incorrect.

1. Choose a (test) statistic involving the unknown parameter and no other unknown parameter.
2. Place the appropriate sample values in the statistic.
3. Obtain an equation for the unknown parameter by equating the test statistic to the upper 2½% point of the relevant distribution.
4. The solution of the equation gives one limit.
5. Repeat the process with the lower 2½% point to obtain the other limit.

One can also construct 95% confidence intervals using unequal tails (e.g., using the upper 2% point and the lower 3% point). We usually want our confidence interval to be as short as possible, however, and with a symmetric distribution such as the normal or *t*, this is achieved using equal tails. The same procedure very nearly minimizes the confidence interval with other nonsymmetric distributions (e.g., chi square) and has the advantage of avoiding rather tedious computation.

When the appropriate statistic involves the square of the unknown parameter, both limits are obtained by equating the statistic to the upper 5% point of the relevant distribution. The use of two tails in this situation would result in a pair of nonintersecting intervals. When two or more parameters are involved, it is possible to construct a region within which we are reasonably confident the true parameter values will lie. Such regions are referred to as confidence regions. The implied interval for p_1 does not form a 95% confidence interval, however. Nor is it true that an 85.7375% confidence region for p_1, p_2, and p_3 can be obtained by considering the intersection of the three separate 95% confidence intervals because the statistics used to obtain the individual confidence intervals are not independent. This problem is obvious with a multiparameter distribution such as the multinomial, but it even occurs with the normal distribution because the statistic which we use to obtain a confidence interval for the mean and the statistic which we use to obtain a confidence interval for the variance are not independent. The problem is not likely to be of great concern unless a large number of parameters is involved.

25.5.17 Randomization

Randomization is the act of assigning a number of items (e.g., plates of bacteria or test animals) to groups in such a manner that there is an equal chance for any one item to end up in any one group. This is a control against any possible

bias in assignment of subjects to test groups. A variation on this is censored randomization, which ensures that the groups are equivalent in some aspect after the assignment process is complete. The most common example of a censored randomization is one in which it is ensured that the body weights of test animals in each group are not significantly different from those in the other groups. This is done by analyzing group weights for both homogeneity of variance and ANOVA after animal assignment, then rerandomizing if there is a significant difference at some nominal level, such as $p \leq 0.10$. The process is repeated until there is no significant difference.

There are several methods for actually performing the randomization process. The three most commonly used are card assignment, use of a random number table, and use of a computerized algorithm.

For the card-based method, individual identification numbers for items (e.g., plates or animals) are placed on separate index cards. These cards are then shuffled and placed one at a time in succession into piles corresponding to the required test groups. The results are a random group assignment.

The random-number table method requires only that one have unique numbers assigned to test subjects and access to a random-number table. One simply sets up a table with a column for each group to which subjects are to be assigned. We start from the head of any one column of numbers in the random table (each time the table is used, a new starting point should be utilized). If our test subjects number less than 100, we utilize only the last two digits in each random number in the table. If they number more than 99 but less than 1000, we use only the last three digits. To generate group assignments, we read down a column one number at a time. As we come across digits which correspond to a subject number, we assign that subject to a group (enter its identifying number in a column) proceeding to assign subjects to groups from left to right filling one row at a time. After a number is assigned to an animal, any duplication of its unique number is ignored. We use as many successive columns of random numbers as we may need to complete the process.

The third (and now most common) method is to use a random-number generator that is built into a calculator or computer program. Procedures for generating these are generally documented in user manuals.

25.5.18 Transformations

If our initial inspection of a data set reveals it to have an unusual or undesired set of characteristics (or to lack a desired set of characteristics), we have a choice of three courses of action. We may proceed to select a method or test appropriate to this new set of conditions, or abandon the entire exercise, or transform the variable(s) under consideration in such a manner that the resulting transformed variates (X' and Y', e.g., as opposed to the original variates X and Y) meet the assumptions or have the characteristics that are desired.

The key to all this is that the scale of measurement of most (if not all) variables is arbitrary. Although we are most familiar with a linear scale of

measurement, there is nothing which makes this the "correct" scale on its own, as opposed to a logarithmic scale [familiar logarithmic measurements are that of pH values or earthquake intensity (Richter scale)]. Transforming a set of data (converting X to X') is really as simple as changing a scale of measurement.

There are at least four good reasons to transform data:

1. To normalize the data, making them suitable for analysis by our most common parametric techniques such as ANOVA. A simple test of whether a selected transformation will yield a distribution of data which satisfies the underlying assumptions for ANOVA is to plot the cumulative distribution of samples on probability paper (i.e., a commercially available paper which has the probability function scale as one axis). One can then alter the scale of the second axis (i.e., the axis other than the one which is on a probability scale) from linear to any other (logarithmic, reciprocal, square root, etc.) and see if a previously curved line indicating a skewed distribution becomes linear to indicate normality. The slope of the transformed line gives us an estimate of the standard deviation. And if the slopes of the lines of several samples or groups of data are similar, we accordingly know that the variance of the different groups are homogenous.
2. To linearize the relationship between a paired set of data, such as dose and response. This is the most common use in toxicology for transformations and is demonstrated in the section under probit/logit plots (Section 25.7.13).
3. To adjust data for the influence of another variable. This is an alternative in some situations to the more complicated process of analysis of covariance. A ready example of this usage is the calculation of organ weight–body weight ratios in in vivo toxicity studies, with the resulting ratios serving as the raw data for an ANOVA performed to identify possible target organs. This use is discussed in detail later in this chapter.
4. Finally, to make the relationships between variables clearer by removing or adjusting for interactions with third, fourth, and so on. Uncontrolled variables influence the pair of variables of interest. This case is discussed in detail under time series analysis (Section 25.8.5).

Common transformations are presented in Table 25.6.

25.5.19 Exploratory Data Analysis

Over the past 20 years, an entirely new approach has been developed to get the most information out of the increasingly larger and more complex data sets that scientists are faced with. This approach involves the use of a very diverse set of fairly simple techniques which comprise exploratory data analysis (EDA). As expounded by Tukey (1977), there are four major ingredients to EDA:

TABLE 25.6 Common Data Transformations[b]

Transformation	How Calculated[a]	Example of Use
Arithmetic	$x' = x/y$ or $x' = x + c$	Organ weight/body weight
Reciprocals	$x' = 1/x$	Linearizing data, particularly rate phenomena
Arcsine (also called angular)	$x' = \arcsin\sqrt{x}$	Normalizing dominant lethal and mutation rate data
Logarithmic	$x' = \log x$	pH values
Probability (probit)	x' = probability X	Percentage responding
Square roots	$x' = \sqrt{x}$	Surface area of animal from body weights
Box–Cox	$x' = \begin{cases} (x^v - 1)v & \text{for } v \neq 0 \\ \ln x & \text{for } v = 0 \end{cases}$	Family of transformsFor use when one has no prior knowledge of appropriate transformation to use
Rank transformations	Depends on nature of samples	As bridge between parametric and nonparametric statistics (Conover and Inman, 1981)

[a] x and y are original variables, x' and y' transformed values; c is a constant.
[b] Plotting a double reciprocal (i.e., $1/x$ vs. $1/y$) will linearize almost any data set. So will plotting the log transforms of a set of variables.

Displays These visually reveal the behavior of the data and suggest a framework for analysis. The scatterplot (presented earlier) is an example of this approach.

Residuals These are what remain of a set of data after a fitted model (such as a linear regression) or some similar level of analysis has been removed.

Reexpressions These involve questions of what scale would best simplify and improve the analysis of the data. Simple transformations, such as those presented earlier in this chapter, are used to simplify data behavior (e.g., linearizing or normalizing) and clarify analysis.

Resistance This is a matter of decreasing the sensitivity of analysis and summary of data to misbehavior, so that the occurrence of a few outliers, for example, will not complicate or invalidate the methods used to analyze the data. For example, in summarizing the location of a set of data, the median (but not the arithmetic mean) is high resistant.

These four ingredients are utilized in a process falling into two broad phases: an exploratory phase and a confirmatory phase. The exploratory phase isolates patterns in and features of the data and reveals them, allowing an inspection of the data before there is any firm choice of actual hypothesis testing or modeling methods.

Confirmatory analysis allows evaluation of the reproducibility of the patterns or effects. Its role is close to that of classical hypothesis testing but also often includes steps such as (a) incorporating information from an analysis of another, closely related set of data and (b) validating a result by assembling and analyzing additional data. These techniques are in general beyond the

scope of this text. However, Velleman and Hoaglin (1981) and Hoaglin et al. (1983) present a clear overview of the more important methods along with codes for their execution on a microcomputer (they have also now been incorporated into Minitab). A short examination of a single case of the use of these methods, however, is in order.

Toxicology has long recognized that no population—animal or human—is completely uniform in its response to any particular toxicant. Rather, a population is composed of a (presumably normal) distribution of individuals—some resistant to intoxication (hyporesponders), the bulk that respond close to a central value [such as the median lethal dose (LD_{50})], and some that are very sensitive to intoxication (hyperresponders). This population distribution can, in fact, result in additional statistical techniques. The sensitivity of techniques such as ANOVA is reduced markedly by the occurrence of outliers (extreme high or low values, including hyper- and hyporesponders) which, in fact, serve to markedly inflate the variance (standard deviation) associated with a sample. Such variance inflation is particularly common in small groups that are exposed or dosed at just over or under a threshold level, causing a small number of individuals in the sample (who are more sensitive than the other members) to respond markedly. Such a situation is displayed in Figure 25.6, which plots the mean and standard deviations of methemoglobin levels in a series of groups of animals exposed to successively higher levels of a hemolytic agent.

Though the mean level of methemoglobin in group C is more than double that of the control group (A), no hypothesis test will show this difference to be significant because it has such a large standard deviation associated with it. Yet this "inflated" variance exists because a single individual has such a marked response. The occurrence of the inflation is certainly an indicator that the data need to be examined closely. Indeed, all tabular data in toxicology should be visually inspected for both trend and variance inflation.

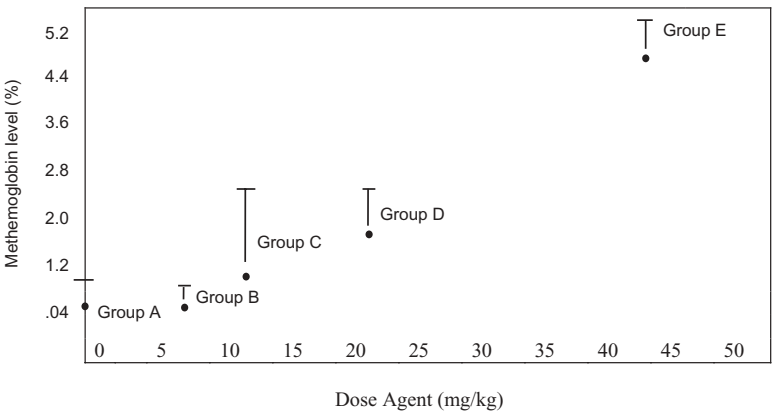


Figure 25.6 *Variance Inflation: points are means minus error bars plus one standard deviations.*

A concept related (but not identical) to resistance and EDA is that of robustness. Robustness generally implies insensitivity to departures from assumptions surrounding an underlying model, such as normality.

In summarizing the location of data the median, though highly resistant, is not extremely robust. But the mean is both nonresistant and nonrobust.

25.6 HYPOTHESIS TESTING OF CATEGORICAL AND RANKED DATA

Categorical (or contingency table) presentations of data can contain any single type of data, but generally the contents are collected and arranged so that they can be classified as belonging to treatment and control groups, with the members of each of these groups then classified as belonging to one of two or more response categories (such as tumor/no tumor or normal/hyperplastic/neoplastic). For these cases, two forms of analysis are presented: Fisher's exact test (for the 2×2 contingency table) and the $R \times C$ chi-square test (for large tables). It should be noted, however, that there are versions of both of these tests which permit the analysis of any size of contingency table.

The analysis of rank data—what is generally called nonparametric statistical analysis—is an exact parallel of the more traditional (and familiar) parametric methods. There are methods for the single-comparison case (just as Student's *t* test is used) and for the multiple-comparison case (just as ANOVA is used) with appropriate post hoc tests for exact identification of the significance with a set of groups. Four tests are presented for evaluating statistical significance in rank data: the Wilcoxon rank-sum test, distribution-free multiple comparisons, Mann-Witney *U* test, and Kruskall–Wallis nonparametric ANOVA. For each of these tests tables of distribution values for the evaluations of results can be found in any of a number of reference volumes (Gad, 1998).

It should be clearly understood that for data which do not fulfill the necessary assumptions for parametric analysis these nonparametric methods are either as powerful or in fact more powerful than the equivalent parametric test.

25.6.1 Fisher's Exact Test

Fisher's exact test should be used to compare two sets of discontinuous, quantal (all-or-none) data. Small sets of such data can be checked by contingency data tables, such as those of Finney et al. (1963). Larger sets, however, require computation. These include frequency data such as incidences of mortality or certain histopathological findings. Thus, the data can be expressed as ratios. These data do not fit on a continuous scale of measurement but usually involve numbers of responses classified as either negative or positive—that is, contingency table situation (Sokal and Rohlf, 1994).

The analysis is started by setting up a 2×2 contingency table to summarize the numbers of "positive" and "negative" responses as well as the totals of these as follows:

	Positive	Negative	Total
Group I	A	B	A + B
Group II	C	D	C + D
Totals	A + C	B + D	$A + B + C + D = N_{\text{total}}$

Using the above set of symbols, the formula for P appears as follows[1]:

$$P = \frac{(A+B)!(C+D)!(A+C)!(B+D)!}{N!A!B!C!D!}$$

The exact test produces a probability (P) which is the sum of the above calculation repeated for each possible arrangement of the numbers in the above cells (i.e., *A, B, C*, and *D*) showing an association equal to or stronger than that between the two variables.

The P resulting from these computations will be the exact one- or two-tailed probability depending on which of these two approaches is being employed. This value tells us if the groups differ significantly (with a probability less than 0.05, say) and the degree of significance.

ASSUMPTIONS AND LIMITATIONS

1. Tables are available which provide individual exact probabilities for small-sample-size contingency tables. See Zar, 1974, pp. 518–542.
2. Fisher's exact test must be used in preference to the chi-square test when there are small cell sizes.
3. The probability resulting from a two-tailed test is exactly double that of a one-tailed test from the same data.
4. Ghent (1972) has developed and proposed a good (though, if performed by hand, laborious) method extending the calculation of exact probabilities to 2×3, 3×3, and $R \times C$ contingency tables.
5. Fisher's probabilities are not necessarily symmetric. Although some analysts will double the one-tailed p value to obtain the two-tailed result, this method is usually overly conservative.

25.6.2 2 × 2 Chi Square

Though Fisher's exact test is preferable for analysis of most 2×2 contingency tables in toxicology, the chi-square test is still widely used and is preferable in a few unusual situations (particularly if cell sizes are large yet only limited computational support is available).

[1] $A!$ is A factorial. For example, for 4! this would be (4) (3) (2) (1) = 24.

The formula is simply

$$\chi^2 = \frac{(O_1 - E_1)^2}{E_1} + \frac{(O_2 - E_2)^2}{E_2}$$
$$= \sum \frac{(O_i - E_1)^2}{E_i}$$

where O are observed numbers (or counts) and E are expected numbers. The common practice in toxicology is for the observed figures to be test or treatment group counts. The expected figure is calculated as

$$E = \frac{(\text{column total})(\text{row total})}{\text{grand total}}$$

for each box or cell in a contingency table.

Our degrees of freedom are $(R - 1)(C - 1) = (2 - 1)(2 - 1) = 1$. Looking at a chi-square table (such as in Table C in Gad 1998) for one degree of freedom we see that this is greater than the test statistic at 0.05 (3.84) but less than that at 0.01 (6.64) so that $0.05 > p > 0.01$.

ASSUMPTIONS AND LIMITATIONS

Assumptions

1. Data are univariate and categorical.
2. Data are from a multinomial population.
3. Data are collected by random, independent sampling.
4. Groups being compared are of approximately the same size, particularly the for small group sizes.

When to Use

1. When the data are of a categorical (or frequency) nature
2. When the data fit the assumptions above
3. To test goodness to fit to a known form of distribution
4. When cell sizes are large

When Not to Use

1. When the data are continuous rather than categorical
2. When sample sizes are small and very unequal
3. When sample sizes are too small (e.g., when total N is less than 50 of if any expected value is less than 5)
4. For any 2×2 comparison (use Fisher's exact test instead).

25.6.3 $R \times C$ Chi Square

The $R \times C$ chi-square test can be used to analyze discontinuous (frequency) data as in the Fisher's exact or 2×2 chi-square tests. However, in the $R \times C$ test (R = row, C = column) we wish to compare three or more sets of data. An example would be comparison of the incidence of tumors among mice on three or more oral dosage levels. We can consider the data as positive (tumors) or negative (no tumors). The expected frequency for any box is equal to row total times column total divided by N_{total}.

As in the Fisher's exact test, the initial step is setting up a table (this time a $R \times C$ contingency table). This table would appear as follows:

	Positive	Negative	Total
Group I	A_1	B_1	$A_1 + B_1 = N_1$
Group II	A_2	B_2	$A_2 + B_2 = N_2$
	$\downarrow$	$\downarrow$	
Group R	A_{R}	B_{R}	$A_{\text{R}} + B_{\text{R}} = N_{\text{R}}$
Total	N_{A}	N_{B}	N_{total}

Using these symbols, the formula for chi square is

$$\chi^2 = \frac{N_{\text{tot}}^2}{N_A N_B N_k}\left(\frac{A_1^2}{N_1} + \frac{A_2^2}{N_2} + \cdots \frac{A_K^2}{N_k} - \frac{N_A^2}{N_{\text{tot}}}\right)$$

The resulting χ^2 value is compared to table values (as in Snedecor and Cochran, 1994, pp. 470–471) according to the number of degrees of freedom, which is equal to $(R - 1)(C - 1)$. If χ^2 is smaller than the table value at the 0.05 probability level, the groups are not significantly different. If the calculated χ^2 is larger, there is some difference among the groups and $2 \times R$ chi-square or Fisher's exact tests will have to be compared to determine which group(s) differ from which other group(s).

ASSUMPTIONS AND LIMITATIONS

1. Based on data being organized in a table (such as below) so that there are *cells* (below, *A, B, C*, and *D* are cells).

		Columns (*C*)		
		Control	**Treated**	**Total**
	No effect	*A*	*B*	*A + B*
Rows (*R*)				
	Effect	*C*	*D*	*C + D*
Total		*A + C*	*B + D*	*A + B + C + D*

2. None of the "expected" frequency values should be less than 5.0.
3. The chi-square test is always one tailed.
4. Without the use of some form of correction, the test becomes less accurate as the differences between group sizes increases.
5. The results from each additional column (group) is approximately additive. Due to this characteristic, chi square can be readily used for evaluating any $R \times C$ combination.
6. The results of the chi-square calculation must be a positive number.
7. The test is weak with either small sample sizes or when the expected frequency in any cell is less than 5 (this latter limitation can be overcome by "pooling"—combining cells.).
8. Test results are independent of order of cells, unlike Kolmogorov–Smirnov.
9. It can be used to test the probability of validity of any distribution.

25.6.4 Wilcoxon Rank-Sum Test

The Wilcoxon rank-sum test is commonly used for the comparison of two groups of nonparametric (inteval or not normally distributed) data, such as those which are not measured exactly but rather as falling within certain limits (e.g., how many animals died during each hour of an acute study.) The test is also used when there is no variability (variance is zero) within one or more of the groups we wish to compare (Sokal and Rohlf, 1994).

The data in both groups being compared are initially arranged and listed in order of increasing value. Then each number in the two groups must receive a rank value. Beginning with the smallest number in either group (which is given a rank of 1.0), each number is assigned a rank. If there are duplicate numbers (called "ties"), then each value of equal size will receive the median rank for the entire identically sized group. Thus if the lowest number appears twice, both figures receive a rank of 1.5. This in turn means that the ranks of 1.0 and 2.0 have been used and that the next highest number has a rank of 3.0. If the lowest number appears three times, then each is ranked as 2.0 and the next number has a rank of 4.0. Thus, each tied number gets a "median" rank. This process continues until all of the numbers are ranked. Each of the two columns of ranks (one for each group) is totaled, giving the "sum of ranks" for each group being compared. As a check, we can calculate the value $\frac{1}{2}(N)(N+1)$, where N is the total number of data in both groups. The result should be equal to the sum of ranks for both groups.

The sum-of-rank values are compared to table values (Diem, 1975; Beyer, 1976a,b, pp. 409–413) to determine the degree of significant differences, if any. These tables include two limits (an upper and a lower) that are dependent upon the probability level. If the number of data is the same in both groups ($N_1 \neq N_2$), then the lesser sum of ranks (smaller N) is compared to the table

limits to find the degree of significance. Normally the comparison of the two groups ends here and the degree of significant difference can be reported.

25.6.5 Distribution-Free Multiple Comparison

The distribution-free multiple-comparison test should be used to compare three or more groups of nonparametric data. These groups are then analyzed two at a time for any significant differences (Hollander and Wolfe, 1973, pp. 124–129). The test can be used for data similar to those compared by the rank-sum test. We often employ this test for reproduction and mutagenicity studies (such as comparing survival rates of offspring of rats fed various amounts of test materials in the diet).

Two values must be calculated for each pair of groups: the difference in mean ranks and the probability-level value against which the difference will be compared. To determine the difference in mean ranks we must first arrange the data within each of the groups in order of increasing values. Then we must assign rank values, beginning with the smallest overall figure. Note that this ranking is similar to that in the Wilcoxon test except that it applies to more than two groups.

The ranks are then added for each of the groups. As a check, the sum of these should equal $\frac{1}{2}N_{\text{tot}}(N_{\text{tot}}+1),$, where N_{tot} is the total number of figures from all groups. Next we can find the mean rank (R) for each group by dividing the sum of ranks by the numbers in the data (N) in the group. These mean ranks are then taken in those pairs which we want to compare (usually each test group vs. the control) and the differences are found ($|R_1 - R_2|$. This value is expressed as an absolute figure; that is, it is always a positive number.

The second value for each pair of groups (the probability value) is calculated lated from the expression $z[(a/K)(K-1)]\sqrt{\frac{1}{12}N_{\text{tot}}(N_{\text{tot}}+1)}\sqrt{(1/N_1)(1/N_2)}$, where a is the level of significance for the comparison (usually 0.05, 0.01; 0.001, etc.), K is the total number of groups, and Z is a figure obtained from a normal probability table and determining the corresponding "Z score."

The result of the probability value calculation for each pair of groups is compared to the corresponding mean difference $|R_1 - R_2|$. If $|R_1 - R_2|$ is smaller, there is no significant difference between the groups. If it is larger, the groups are different and $|R_1 - R_2|$ must be compared to the calculated probability values for $a = 0.01$ and $a = 0.001$ to find the degree of significance.

ASSUMPTIONS AND LIMITATIONS

1. As with the Wilcoxon rank sum, too many tied ranks inflate the false positive.
2. Generally, this test should be used as a post hoc comparison after Kruskall–Wallis.

25.6.6 Mann–Whitney *U* Test

This is a nonparametric test in which the data in each group are first ordered from lowest to highest values, then the entire set (both control and treated values) is ranked, with the average rank being assigned to tied values. The ranks are then summed for each group and U is determined according to

$$U_t = n_c n_t + \frac{1}{2}[n_t(n_t+1)] - R_t \qquad U_c = n_c n_t + \frac{1}{2}[n_c(n_c+1)] - R_c$$

where n_c, n_t are the sample sizes for the control and treated groups and R_c, R_t are the sums of ranks for the control and treated groups.

For the level of significance for a comparison of the two groups, the larger value of U_c or U_t is used. This is compared to critical values as found in tables (Siegel, 1956).

The Mann–Whitney U test is employed for the count data, but which test should be employed for the percentage variables should be decided on the same grounds as described later under reproduction studies.

ASSUMPTIONS AND LIMITATIONS

1. It does not matter whether the observations are ranked from smallest to largest or vice versa.
2. This test should not be used for paired observations.
3. The test statistics from a Mann–Whitney are linearly related to those of the Wilcoxon. The two tests will always yield the same result. The Mann–Whitney is presented here for historical completeness, as it has been much favored in reproductive and developmental toxicology studies. However, it should be noted that the author does not include it in the decision tree for method selection (Figure 25.2).

25.6.7 Kruskal–Wallis Nonparametric ANOVA

The Kruskal–Wallis nonparametric one-way ANOVA should be the initial analysis performed when we have three or more groups of data which are by nature nonparametric (either not a normally distributed population or of a discontinuous nature or all the groups being analyzed are not from the same population) but not of a categorical (or quantal) nature. Commonly these will be either rank-type evaluation data (such as behavioral toxicity observation scores) or reproduction study data. The analysis is initiated (Pollard, 1977, pp. 170–173] by ranking all the observations from the combined groups to be analyzed. Ties are given the average rank of the tied values (i.e., if two values which would tie for 12th rank—and therefore would be ranked 12th and 13th—both would be assigned the average rank of 12.5).

The sum of ranks of each group ($r_1, r_2, \ldots, r_k$) is computed by adding all the rank values for each group. The test value H is then computed as

$$H = \frac{12}{n(n+1)} \sum \left(\frac{r_1^2}{n_1} + \frac{r_2^2}{n_2} + \cdots + \frac{r_k^2}{n_k} \right) - 3(n+1)$$

where $n_1, n_2, \ldots, n_k$ are the number of observations in each group. The test statistic is then compared with a table of H values. If the calculated value of H is greater than the table value for the appropriate number of observations in each group, there is a significant difference between the groups, but further testing (using the distribution-free multiple-comparison method) is necessary to determine where the difference lies.

ASSUMPTIONS AND LIMITATIONS

1. The test statistic H is used for both small and large samples.
2. When we find a significant difference, we do not know which groups are different. It is not correct to then perform a Mann–Whitney U test on all possible combinations—rather, a multiple-comparison method must be used, such as distribution-free multiple comparisons.
3. Data must be independent for the test to be valid.
4. Too many tied ranks will decrease the power of this test and also lead to increased false-positive levels.
5. When $k = 2$, the Kruskal–Wallis chi-square value has one degree of freedom (df). This test is identical to the normal approximation used for the Wilcoxon rank-sum Test. As noted in previous sections, a chi-square with one df can be represented by the square of a standardized normal random variable. In the case of $k = 2$, the H statistic is the square of the Wilcoxon rank-sum Z test (without the continuity correction).
6. The effect of adjusting for tied ranks is to slightly increase the value of the test statistic H. Therefore, omission of this adjustment results in a more conservative test.

25.6.8 Log Rank Test

The log rank test is a statistical methodology for comparing the distribution of time until the occurrence of the event in independent groups. In toxicology, the most common event of interest is death or occurrence of a tumor, but it could just as well be liver failure, neurotoxicity, or any other event which occurs only once in an individual. The elapsed time from initial treatment or observation until the *event* is the *event time*, often referred to as "survival time," even when the event is not "death."

The log rank Test provides a method for comparing "risk-adjusted" event rates, useful when test subjects in a study are subject to varying degrees of opportunity to experience the event. Such situations arise frequently in toxicology studies due to the finite duration of the study, early termination of the animal, or interruption of treatment before the event occurs.

Examples where use of the log rank test might be appropriate include comparing survival times in carcinogenity bioassay animals which are given a new treatment with those in the control group or comparing times to liver failure for several dose levels of a new nonsteroidal anti-inflammatory drug (NSAID) where the animals are treated for 10 weeks or until cured, whichever comes first.

If every animal were followed until the event occurrence, the event times could be compared between two groups using the Wilcoxon rank-sum test. However, some animals may die or complete the study before the event occurs. In such cases, the actual time of the event is unknown since the event does not occur while under study observation. The event times for these animals are based on the last known time of study observation and are called "censored" observations since they represent the lower bound of the true, unknown event times. The Wilcoxon rank-sum test can be highly biased in the presence of the censored data.

The null hypothesis tested by the log rank test is that of equal event time distributions among groups. Equality of the distributions of event times implies similar event rates among groups not only for the clinical trial as a whole but also for any arbitrary time point during the trial. Rejection of the null hypothesis indicates that the event rates differ among groups at one or more time points during the study.

The idea behind the log rank test for comparison of two life tables is simple: If there were no difference between the groups, the total deaths occurring at any time should split between the two groups at that time. So if the numbers at risk in the first and second groups in (say) the sixth month were 70 and 30, respectively, and 10 deaths occurred in that month we would expect

$$10 \times \frac{70}{70+30} = 7$$

of these deaths to have occurred in the first group and

$$10 \times \frac{30}{70+30} = 3$$

of the deaths to have occurred in the second group.

A similar calculation can be made at each time of death (in either group). By adding together for the first group the results of all such calculations, we obtain a single number, called the extent of exposure (E_1), which represents the "expected" number of deaths in that group if the two groups had the distribution of survival time. An extent of exposure (E_2) can be obtained for the

second group in the same way. Let O_1 and O_2 denote the actual total numbers of deaths in the two groups. A useful arithmetic check is that the total number of deaths $O_1 + O_2$ must equal the sum $E_1 + E_2$ of the extents of exposure.

The discrepancy between the O's and E's can be measured by the quantity

$$x^2 = \frac{\left(|O_1 - E_1| - \frac{1}{2}\right)^2}{E_1} + \frac{\left(|O_2 - E_2| - \frac{1}{2}\right)^2}{E_2}$$

For rather obscure reasons, x^2 is known as the log rank statistic. An approximate significance test of the null hypothesis of identical distributions of survival time in the two groups is obtained by referring x^2 to a chi-square distribution on one df.

The log rank test as presented by Peto et al. (1977) uses the product limit life table calculations rather than the actuarial estimators shown above. The distinction is unlikely to be of practical importance unless the grouping intervals are very coarse.

Peto and Pike (1973) suggest that the approximation in treating the null distribution of χ^2 as a chi square is conservative, so that it will tend to understate the degree of statistical significance. In the formula for χ^2 we have used the continuity correction of subtracting ½ from $|O_1 - E_1|$ and $|O_2 - E_2|$ before squaring. This is recommended by Peto et al. (1977) when, as in nonrandomized studies, the permutational argument does not apply. Peto et al. (1977) gives further details of the log rank test and its extension to comparisons of more than two treatment groups and to tests that control for categorical confounding factors.

ASSUMPTIONS AND LIMITATIONS

1. The endpoint of concern is or is defined so that it is "right censored"—once it happens, it does not reoccur. Examples are death or a minimum or maximum value of an enzyme or physiological function (such as respiration rate).
2. The method makes no assumptions on distribution.
3. Many variations of the log rank test for comparing survival distributions exist. The most common variant has the form

$$\chi^2 = \frac{(O_1 - E_1)^2}{E_1} + \frac{(O_2 - E_2)^2}{E_2}$$

where O_i and E_i are computed for each group, as in the formulas given previously. This statistic also has an approximate chi-square distribution with one df under H_0.

A continuity correction can also be used to reduce the numerators by ½ before squaring. Use of such a correction leads to even further conservatism and may be omitted when sample sizes are moderate or large.

4. The Wilcoxon rank-sum test could be used to analyze the event times in the absence of censoring. A "generalized Wilcoxon" test, sometimes called the Gehan test, based on an approximate chi-square distribution has been developed for use in the presence of censored observations.

   Both the log rank and the generalized Wilcoxon tests are nonparametric tests and require no assumptions regarding the distribution of event times. When the event rate is greater early in the trial than toward the end, the generalized Wilcoxon test is the more appropriate test since it gives greater weight to the earlier differences.

5. Survival and failure times often follow the exponential distribution. If such a model can be assumed, a more powerful alternative to the log rank test is the likelihood ratio test.

   This parametric test assumes that event probabilities are constant over time. That is, the chance that a patient becomes event positive at time t given that he or she is event negative up to time t does not depend on t. A plot of the negative log of the event times distribution showing a linear trend through the origin is consistent with exponential event times.

6. Life tables can be constructed to provide estimates of the event time distributions. Estimates commonly used are known as the Kaplan–Meier estimates.

25.7 HYPOTHESIS TESTING: UNIVARIATE PARAMETRIC TESTS

Univariate case[2] data from normally distributed populations generally have a higher information value associated with them, but the traditional hypothesis-testing techniques (which include all the methods described in this chapter) are generally neither resistant nor robust. All the data analyzed by these methods are also effectively continuous—that is, at least for practical purposes, the data may be represented by any number and each such data number has a measurable relationship to other data numbers.

25.7.1 Student's *t* Test (Unpaired *t* Test)

Pairs of groups of continuous, randomly distributed data are compared via this test. We can use this test to compare three or more groups of data, but they must be compared by examination of two groups taken at a time and are preferentially compared by ANOVA. Usually this means comparison of a

[2]That is, where each datum is defined by one treatment and one effect variable.

test group versus a control group, although two test groups may be compared as well. To determine which of the three types of *t* tests described in this chapter should be employed, the *F* test is usually performed first. This will tell us if the variances of the data are approximately equal, which is a requirement for the use of the parametric methods. If the *F* test indicates homogeneous variances and the numbers of data within the groups (*N*) are equal, then the Student's *t* test is the appropriate procedure (Sokal and Rohlf, 1994). If the *F* is significant (the data are heterogeneous) and the two groups have equal numbers of data, the modified Student's *t* test is applicable (Cochran and Cox, 1975).

The value of *t* for the Student *t* test is calculated using the formula

$$t = \frac{\overline{X_1 - X_2}}{\sum D_1^2 + \sum D_2^2} \sqrt{\frac{N_1 N_2}{N_1 + N_2}(N_1 + N_2 - 2)}$$

where $\Sigma D^2 = [N\Sigma X^2 - \Sigma X)^2]/N$.

The value of *t* obtained from the above calculation is compared to the values in a *t*-distribution table according to the appropriate number of degrees of freedom (df). If the *F* value is not significant (i.e., variances are homogeneous), $df = N_1 + N_2 - 2$. If the *F* was significant and $N_1 = N_2$, then $df = N - 1$. Although this case indicates a nonrandom distribution, the modified *t* test is still valid. If the calculated value is larger than the table value at $p = 0.05$, it may then be compared to the appropriate other table values in order of decreasing probability to determine the degree of significance between the two groups.

ASSUMPTIONS AND LIMITATIONS

1. The test assumes that the data are univariate, continuous, and normally distributed.
2. Data are collected by randomly sampling.
3. The test should be used when the assumptions in 1 and 2 are met and there are only two groups to be compared.
4. Do not use when the data are ranked, when the data are not approximately normally distributed, or when there are more than two groups to be compared. Do not use for paired observations.
5. This is the most commonly misused test method, except in those few cases where one is truly only comparing two groups of data and the group sizes are roughly equivalent or not valid for multiple comparisons (because of resulting additive errors) or where group sizes are very unequal.
6. The test is robust for moderate departures from normality and, when N_1 and N_2 are approximately equal, robust for moderate departures from homogeneity of variances.
7. The main difference between the *Z* test and the *t* test is that the *Z* statistic is based on a known standard deviation σ while the *t* statistic uses

the sample standard deviation s as an estimate of σ. With the assumption of normally distributed data, the variance σ^2 is more closely estimated by the sample variance s^2 as n gets large. It can be shown that the t test is equivalent to the Z test for infinite degrees of freedom. In practice, a "large" sample is usually considered, $n \geq 30$.

25.7.2 Cochran *t* Test

The Cochran test should be used to compare two groups of continuous data when the variances (as indicated by the F test) are heterogeneous and the numbers of data within the groups are not equal ($N_1 \neq N_2$). This is the situation, for example, when the data, though expected to be randomly distributed, were found not to be (Cochran and Cox, 1975, pp. 100–102).

Two t values are calculated for this test, the "observed" t (t_{obs}) and the "expected" t (t'). The observed t is obtained by

$$t_{obs} = \frac{\overline{X}_1 - \overline{X}_2}{\sqrt{W_1 + W_2}}$$

$$W = \text{SEM}^2 \qquad \text{(standard error of the mean squared)}$$

$$= \frac{S^2}{N}$$

where S (the variance) can be calculated from

$$S = \frac{\left[N\sum X^2 - \left(\sum X\right)^2\right] \Big/ N}{N-1}$$

The value for t' is obtained from

$$t' = \frac{t_1' W_1 + t_2' W_2}{W_1 + W_2}$$

where t_1' and t_2' are values for the two groups taken from the t-distribution table corresponding to $N - 1$ degrees of freedom (for each group) at the 0.05 probability level (or such level as one may select).

The calculated t_{obs} is compared to the calculated t' value (or t' values prepared for more than one probability level). If t_{obs} is smaller than t', the groups are not considered to be significantly different at that probability level.

ASSUMPTIONS AND LIMITATIONS

1. The test assumes that the data are univariate, continuous, and normally distributed and group sizes are unequal.
2. The test is robust for moderate departures from normality and very robust for departures from equality of variances.

25.7.3 *F* Test

This is a test of the homogeneity of variances between two groups of data (Sokal and Rohlf, 1994). It is used in two separate cases. The first is when Bartlett's indicates heterogeneity of variances among three or more groups (i.e., it is used to determine which pairs of groups are heterogeneous). Second, the *F* test is the initial step in comparing two groups of continuous data which we would expect to be parametric (two groups not usually being compared using ANOVA), the results indicating whether the data are from the same population and whether subsequent parametric comparisons would be valid.

The *F* is calculated by dividing the larger variance (S_1^2) by the smaller one (S_2^2), where S^2 is calculated as

$$S^2 = \frac{\left[N\sum X^2 - \left(\sum X\right)^2\right]/N}{N-1}$$

where N is the number of data in the group and X represents the individual values within the group. Frequently, S^2 values may be obtained from ANOVA calculations.

The calculated *F* value is compared to the appropriate number in an *F*-value table for the appropriate degrees of freedom ($N - 1$) in the numerator (along the top of the table) and in the denominator (along the side of the table). If the calculated value is smaller, it is not significant and the variances are considered homogeneous (and the Student's *t* test would be appropriate for further comparison). If the calculated *F* value is greater, *F* is significant and the variances are heterogeneous (and the next test would be a modified Student's *t* test if $N_1 = N_2$ or the Cochran *t* test if $N_1 \neq N_2$; see Figure 25.2 to review the decision tree).

ASSUMPTIONS AND LIMITATIONS

1. This test could be considered as a two-group equivalent of the Bartlett's test.
2. If the test statistic is close to 1.0, the results are (of course) not significant.
3. The test assumes normality and independence of data.

25.7.4 Analysis of Variance

The ANOVA is used for comparison of three or more groups of continuous data when the variances are homogeneous and the data are independent and normally distributed.

A series of calculations are required for ANOVA, starting with the values within each group being added (ΣX) and then these sums being added ($\Sigma\Sigma X$).

Each figure within the groups is squared, and these squares are then summed (ΣX^2) and these sums added ($\Sigma\Sigma X^2$). Next the "correction factor" (CF) can be calculated from the formula

$$\mathrm{CF} = \frac{\left(\sum_1^K \sum_1^N X\right)^2}{N_1 + N_2 + \cdots + N_k}$$

where N is the number of values in each group and K is the number of groups. The total sum of squares (SS) is then determined as

$$\mathrm{SS}_{\mathrm{total}} = \sum_1^K \sum_1^N X^2 - \mathrm{CF}$$

In turn, the sum of squares between groups (bg) is found from

$$\mathrm{SS}_{\mathrm{bg}} = \frac{\left(\sum X_1\right)^2}{N_1} + \frac{\left(\sum X_2\right)^2}{N_2} + \cdots + \frac{\left(\sum X_k\right)^2}{N_k} - \mathrm{CF}$$

The within-group (wg) sum of squares is then the difference between the last two figures:

$$\mathrm{SS}_{\mathrm{wg}} = \mathrm{SS}_{\mathrm{total}} - \mathrm{SS}_{\mathrm{bg}}$$

Now, there are three types of degrees of freedom to determine. The first, total df, is the total number of data within all groups under analysis minus 1 ($N_1 + N_2 + \ \ldots \ + N_k - 1$). The second figure (the df between groups) is the number of groups minus 1($K - 1$). The last figure (the df within groups or "error df") is the difference between the first two figures ($\mathrm{df}_{\mathrm{total}} - \mathrm{df}_{\mathrm{bg}}$).

The next set of calculations requires determination of the two mean squares ($\mathrm{MS}_{\mathrm{bg}}$ and $\mathrm{MS}_{\mathrm{wg}}$). These are the respective sum-of-square values divided by the corresponding df figures (MS = SS/df). The final calculation is that of the F ratio. For this, the MS between groups is divided by the MS within groups ($F = \mathrm{MS}_{\mathrm{bg}}/\mathrm{MS}_{\mathrm{wg}}$).

A table of the results of these calculations would appear as follows:

	df	SS	MS	*F*
bg	3	0.04075	0.01358	4.94
wg	12	0.03305	0.00275	
Total	15	0.07380		

For interpretation, the F-ratio value obtained in the ANOVA is compared to a table of F values. If $F \leq 1.0$, the results are not significant and comparison

with the table values is not necessary. The df for the greater mean square (MS_{bg}) are indicated along the top of the table. Then one must read down the side of the table to the line corresponding to the df for the lesser mean square (MS_{wg}). The figure shown at the desired significance level (traditionally 0.05) is compared to the calculated F value. If the calculated number is smaller, there is no significant differences among the groups being compared. If the calculated value is larger, there is some difference but further (post hoc) testing will be required before we know which groups differ significantly.

ASSUMPTIONS AND LIMITATIONS

1. What is presented here is the workhorse of toxicology—the one-way ANOVA. Many other forms exist for more complicated experimental designs.
2. The test is robust for moderate departures from normality if the sample sizes are large enough. Unfortunately, this is rarely the case in toxicology.
3. ANOVA is robust for moderate departures from equality of variances (as determined by Bartlett's test) if the sample sizes are approximately equal.
4. It is not appropriate to use a t test (or a two-groups-at-a-time version of ANOVA) to identify where significant differences are within the design group. A multiple-comparison post hoc method must be used.

25.7.5 Post Hoc Tests

There is a wide variety of post hoc tests available to analyze data after finding significant result in an ANOVA. Each of these tests has advantages and disadvantages and proponents and critics. Four of the tests are commonly used in toxicology and will be presented or previewed here. These are Dunnett's t test and Williams' t test. Two other tests which are available in many statistical packages are Tukey's method and the Student–Newman–Keuls method (Zar, 1974, pp. 151–161).

If ANOVA reveals no significance, it is not appropriate to proceed to perform a post hoc test in the hope of finding differences. To do so would only be another form of multiple comparisons, increasing the type I error rate beyond the desired level.

25.7.6 Duncan's Multiple-Range Test

Duncan's (1955) is used to compare groups of continuous and randomly distributed data (body weights, organ weights, etc.). The test normally involves

three or more groups taken one pair at a time. It should only follow observation of a significant *F* value in the ANOVA and can serve to determine which group (or groups) differs significantly from which other group (or groups).

There are two alternative methods of calculation. The selection of the proper one is based on whether the number of data (*N*) are equal or unequal in the groups.

Groups with Equal Number of Data ($N_1 = N_2$) Two sets of calculations must be carried out: first, the determination of the difference between the means of pairs of groups and, second, the preparation of a probability rate against which each difference in means is compared (as shown in the first of the two examples in this section).

The means (averages) are determined (or taken from the ANOVA calculation) and ranked in either decreasing or increasing order. If two means are the same, they take up two equal positions (thus, for four means we could have ranks of 1, 2, 2, and 4 rather than 1, 2, 3, and 4). The groups are then taken in pairs and the differences between the means ($\bar{x}_1 - \bar{x}_2$), expressed as positive numbers, are calculated. Usually, each pair consists of a test group and the control group though multiple test groups may be compared if so desired. The relative rank of the two groups being compared must be considered. If a test group is ranked 2 and the control group is ranked 1, then we say that there are two places between them, while if the test group were ranked 3, then there would be three places between it and the control.

To establish the probability table, the SEM must be calculated as presented earlier or as

$$\sqrt{\frac{\text{error mean square}}{N}} = \sqrt{\frac{\text{mean square within group}}{N}}$$

where *N* is the number of animals or replications per dose level. The mean square within groups (MS_{wg}) can be calculated from the information given in the ANOVA procedure (refer to the earlier section on ANOVA). The SEM is then multiplied by a series of table values (Harter, 1960; Beyer, 1976a,b) to set up a probability table. The table values used for the calculations are chosen according to the probability levels (note that the tables have sections for 0.05, 0.01, and 0.001 levels) and the number of means apart for the groups being compared and the number of "error" df. The error df is the number of df within the groups. This last figure is determined from the ANOVA calculation and can be taken from ANOVA output. For some values of df, the table values are not given and should thus be interpolated.

Groups with Unequal Numbers of Data ($N_1 \neq N_2$) This procedure is very similar to that discussed above. As before, the means are ranked and the differences between the means are determined ($\bar{x}_1 - \bar{x}_2$). Next, weighing values

(a_{ij} values) are calculated for the pairs of groups being compared in accordance with

$$a_u = \sqrt{\frac{2N_iN_j}{(N_i + N_j)}} = \sqrt{\frac{2N_1N_2}{(N_1 + N_2)}}$$

This weighting value for each pair of groups is multiplied by $\bar{x}_1 - \bar{x}_2$ for each value to arrive at a t value. It is the t that will later be compared to a probability table.

The probability table is set up as before except that, instead of multiplying the appropriate table values by SEM, SEM^2 is used. This is equal to $\sqrt{\text{MS}_{\text{wg}}}$.

For the desired comparison of two groups at a time, either the ($\bar{x}_1 - \bar{x}_2$) value (if $N_1 = N_2$) is compared to the appropriate probability table. or if $N_1 \neq N_2$, the $\bar{x}_1 - 1 - \bar{x}_2$ value is used. Each comparison must be made according to the number of places between the means. If the table value is larger at the 0.05 level, the two groups are not considered to be statistically different. If the table value is smaller, the groups are different and the comparison is repeated at lower levels of significance. Thus, the degree of significance may be determined. We might have significant differences at 0.05 but not at 0.01, in which case the probability would be represented at $0.05 > p > 0.01$.

ASSUMPTIONS AND LIMITATIONS

1. Duncan's assures a set α level or type I error rate for all tests when means are separated by no more than ordered step increases. Preserving this α level means that the test is less sensitive than some others, such as the Student–Newman–Keuls. The test is inherently conservative and not resistant or robust.

25.7.7 Scheffé's Multiple Comparisons

Scheffé's is another post hoc comparison method for groups of continuous and randomly distributed data. It also normally involves three or more groups (Scheffé, 1959; Harris, 1975). It is widely considered a more powerful significance test than Duncan's.

Each post hoc comparison is tested by comparing an obtained test value (F_{contr}) with the appropriate critical F value at the selected level of significance (the table F value multiplied by $K - 1$ for an F with $K - 1$ and $N - K$ degrees of freedom[2]), where F_{contr} is computed as follows:

(a) Compute the mean for each sample (group).
(b) Denote the residual mean square by MS_{wg}.
(c) Compute the test statistic as

$$F_{\text{contr}} = \frac{\left(C_1 \overline{X}_1 + C_2 \overline{X}_2 + \cdots + C_k \overline{X}_k^{12}\right)}{(K-1)\text{MS}_{\text{wg}}(C_1^2/n_1 + \cdots + C_k^2/n_k)}$$

where C_k is the comparison number such that the sum of $C_1, C_2, \ldots, C_k = 0$.

ASSUMPTIONS AND LIMITATIONS

1. The Scheffé procedure is robust to moderate violations of the normality and homogeneity of variance assumptions.
2. It is not formulated on the basis of groups with equal numbers (as one of Duncan's procedures is), and if $N_1 \neq N_2$ there is no separate weighting procedure.
3. It tests all linear contrasts among the population means (the other three methods confine themselves to pairwise comparison, except they use a Bonferroni-type correlation procedure).
4. The Scheffé procedure is powerful because of its robustness, yet it is very conservative. Type I error (the false-positive rate) is held constant at the selected test level for each comparison.

25.7.8 Dunnett's *t* Test

Dunnett's *t* test (Dunnett, 1955, 1964) has as its starting point the assumption that what is desired is a comparison of each of several means with one other mean and only one other mean; in other words, one wishes to compare each and every treatment group with the control group but not compare treatment groups with each other. The problem here is that, in toxicology, one is frequently interested in comparing treatment groups with other treatment groups. However, if one wants to only compare treatment groups with a control group, Dunnett's is a useful approach. In a study with K groups (one of them being the control) we will wish to make $K - 1$ comparisons. In such a situation, we want to have a P level for the entire set of $K - 1$ decisions (not for each individual decision). The Dunnett's distribution is predicated on this assumption. The parameters for utilizing a Dunnett's table, such as found in his original article, are K (as above) and the number of degrees of freedom for the within-group mean square (MS_{wg}). The test value is calculated as

$$t = \frac{|T_j - T_i|}{\sqrt{2\text{MS}_{\text{wg}/n}}}$$

where n is the number of observations in each of the groups. The within-group mean square (MS_{wg}) is as we have defined it previously; T_j is the control group mean and T_i is the mean of, in order, each successive test group observation. Note that one uses the absolute value of the positive number resulting from subtracting T_i from T_j. This is to ensure a positive number for our final t.

ASSUMPTIONS AND LIMITATIONS

1. Dunnett's seeks to ensure that the type 1 error rate will be fixed at the desired level by incorporating correction factors into the design of the test value table.
2. Treated group sizes must be approximately equal.

25.7.9 Williams *t* Test

The Williams *t* test (Williams, 1971, 1972) is popular, although its use is quite limited in toxicology. It is designed to detect the highest level (in a set of dose/exposure levels) at which there is no significant effect. It assumes that the response of interest (such as change in body weights) occurs at higher levels, but not at lower levels, and that the responses are monotonically ordered so that $X_0 \leq X_1 \leq \ldots \leq X_k$. This is, however, frequently not the case. The Williams technique handles the occurrence of such discontinuities in a response series by replacing the offending value and the value immediately preceding it with weighted average values. The test also is adversely affected by any mortality at high dose levels. Such mortalities "impose a severe penalty, reducing the power of detecting an effect not only at level K but also at all lower doses" (Williams, 1972, p. 529). Accordingly, it is not generally applicable in toxicology studies.

25.7.10 Analysis of Covariance

Analysis of covariance (ANCOVA) is a method for comparing sets of data which consist of two variables (treatment and effect, with our effect variable being called the "variate") when a third variable (called the "covariate") exists which can be measured but not controlled and which has a definite effect on the variable of interest. In other words, it provides an indirect type of statistical control, allowing us to increase the precision of a study and to remove a potential source of bias. One common example of this is in the analysis of organ weights in toxicity studies. Our true interest here is the effect of our dose or exposure level on the specific organ weights, but most organ weights also increase (in the young, growing animals most commonly used in such studies) in proportion to increases in animal body weight. As we are not here interested in the effect of this covariate (body weight), we measure it to allow for adjustment. We must be careful before using ANCOVA, however, to ensure that the underlying nature of the correspondence between the variate and covariate is such that we can rely on it as a tool for adjustments (Anderson et al., 1980; Kotz and Johnson, 1982).

Calculation is performed in two steps. The first is a type of linear regression between the variate Y and the covariate X.

This regression, performed as described under the linear regression section, gives us the model

$$Y = a_1 + BX + e$$

which in turn allows us to define adjusted means ($\bar{Y}$ and X) such that $\bar{Y}_{1a} = \bar{Y}_1 - (\bar{x}_1 - X^*)$.

If we consider the case where K treatments are being compared such that $K = 1, 2, \ldots, K$ and we let X_{ik} and Y_{ik} represent the predictor and predicted values for each individual i in group k, we can let X_k and Y_k be the means. Then, we define the between-group (for treatment) sum of squares and cross products as

$$T_{xx} = \sum_{k-1}^{K} n_k \left(\overline{X}_K - \overline{X}\right)^2$$

$$T_{yy} = \sum_{k-1}^{K} n_k \left(\overline{Y}_K - \overline{Y}\right)^2$$

$$T_{xy} = \sum_{k-1}^{K} n_k \left(\overline{X}_k - \overline{X}\right)\left(\overline{Y}_k - \overline{Y}_k - \overline{Y}\right)$$

In a like manner, within-group sums of squares and cross products are calculated as

$$\sum xx = \sum_{k=1}^{k} \sum_{i} (X_{ik} - X_k)^2$$

$$\sum yy = \sum_{K=1}^{k} \sum_{i} (Y_{ik} - Y_k)^2$$

$$\sum xy = \sum_{k=1}^{k} \sum_{i}^{n} (X_{ik} - X_k)(Y_{ik} - Y_k)$$

where i indicates the sum from all the individuals within each group and n is the total number of subjects minus number of groups:

$$S_{xx} = T_{xx} + \Sigma_{xx} \qquad S_{yy} = T_{yy} + \Sigma_{xx} \qquad S_{xy} = T_{xy} + \Sigma_{xy}$$

With these in hand, we can then calculate the residual mean squares of treatments (St^2) and error (Se^2):

$$\mathrm{St}^2 = \frac{T_{yy} - \dfrac{S_{xy}^2}{S_{xx}} + \dfrac{\Sigma_{xy}^2}{\Sigma_{xx}}}{lc - 1} \qquad \mathrm{Se}^2 = \frac{\Sigma_{yy} - \Sigma_y^2 / \Sigma_{xx}}{f - 1}$$

*The slight difference in the results for the two approaches is due to rounding errors. It is not important biologically.

These can be used to calculate an F statistic to test the null hypothesis that all treatment effects are equal:

$$F = \frac{\mathrm{St}^2}{\mathrm{Se}^2}$$

The estimated regression coefficient of Y or X is

$$B = \frac{\Sigma_{xy}}{\Sigma_{xx}}$$

The estimated standard error for the adjusted difference between two groups is given by

$$\mathrm{Sd} = \mathrm{Se}\frac{1}{n_j} + \frac{1}{n_j} + \frac{(X_i - X_j)^2}{\Sigma_{xx}}$$

where n_0 and n_1 are the sample sizes of the two groups. A test of the null hypothesis that the adjusted differences between the groups is zero is provided by

$$t = \frac{Y_1 - Y_0 - B(X_1 - X_0)}{\mathrm{Sd}}$$

The test value for t is then looked up in the t table with $f - 1$ degrees of freedom. Computation is markedly simplified if all the groups are of equal size.

ASSUMPTIONS AND LIMITATIONS

1. The underlying assumptions for ANCOVA are fairly rigid and restrictive. The assumptions include:
   a. That the slopes of the regression lines of Y and X are equal from group to group. This can be examined visually or formally (i.e., by a test). If this condition is not met, ANCOVA cannot be used.
   b. That the relationship between X and y is linear.
   c. That the covariate X is measured without error. The power of the test declines as error increases.
   d. That there are no unmeasured confounding variables.
   e. That the errors inherent in each variable are independent of each other. Lack of independence effectively (but to an immeasurable degree) reduces sample size.
   f. That the variances of the errors in groups are equivalent between groups.
   g. That the measured data which form the groups are normally distributed. ANCOVA is generally robust to departures from normality.
2. Of the seven assumptions above, the most serious are the first four.

25.7.11 Modeling

The mathematical modeling of biological systems, restricted even to the field of toxicology, is an extremely large and vigorously growing area. Broadly speaking, modeling is the principal conceptual tool by which toxicology seeks to develop as a mechanistic science. In an iterative process, models are developed or proposed, tested by experiment, reformulated, and so on, in a continuous cycle. Such a cycle could also be described as two related types of modeling: explanatory (where the concept is formed) and correlative (where data are organized and relationships derived). An excellent introduction to the broader field of modeling of biological systems can be found in Gold (1977).

In toxicology, modeling is of prime interest in seeking to relate a treatment variable with an effect variable and, from the resulting model, predict effects at exact points where no experiment has been done (but in the range where we have performed experiments, such as "determining" LD_{50}), to estimate how good our prediction is, and, occasionally, to simply determine if a pattern of effects is related to a pattern of treatment.

For use in prediction, the techniques of linear regression, probit/logit analysis (a special case of linear regression), moving averages (an efficient approximation method), and nonlinear regression (for doses where data cannot be made to fit a linear pattern) are presented. For evaluating the predictive value of these models, both the correlation coefficient (for parametric data) and Kendall's rank correlation (for nonparametric data) are given. And finally, the concept of trend analysis is introduced and a method presented.

When we are trying to establish a pattern between several data points (whether this pattern is in the form of a line or a curve), what we are doing is interpolating. It is possible for any given set of points to produce an infinite set of lines or curves which pass near (for lines) or through (for curves) the data points. In most cases, we cannot actually know the "real" pattern. Se we apply a basic principle of science—Occam's razor. We use the simplest explanation (or, in this case, model) which fits the facts (or data). A line is, of course, the simplest pattern to deal with and describe, so fitting the best line (linear regression) is the most common form of model in toxicology.

25.7.12 Linear Regression

Foremost among the methods for interpolating within a known data relationship is regression—the fitting of a line or curve to a set of known data points on a graph and the interpolation ("estimation") of this line or curve in areas where we have no data points. The simplest of these regression models is that of linear regression (valid when increasing the value of one variable changes the value of the related variable in a linear fashion, either positively

or negatively). This is the case we will explore here using the method of least squares.

Given that we have two sets of variables, x (say milligrams per kilogram of test material administered) and y (say percentage of animals so dosed that die), what is required is solving for a and b in the equation $Y_i = a + bx_i$ [where the uppercase Y_i is the fitted value of y_i at x_i and we wish to minimize $(y_i - Y_i)^2$]. So we solve the equations

$$b = \frac{\sum x_1 y_1 - n\bar{x}\bar{y}}{\sum x_1^2 - n\bar{x}^2} \qquad a = \bar{y} - b\bar{x}$$

where a is the y intercept, b is the slope of the time, and n is the number of data points.

Note that in actuality dose–response relationships are often not linear and instead we must use either a transform (to linearlize the data) or a nonlinear regression method (Gallant, 1975).

Note also that we can use the correlation test statistic (to be described in the correlation coefficient section, 25.7.15) to determine if the regression is significant (and therefore valid at a defined level of certainty. A more specific test for significance would be the linear regression ANOVA (Pollard, 1977). To do so we start by developing the appropriate ANOVA table.

Finally, we might wish to determine the confidence intervals for our regression line; that is, given a regression line with calculated values for Y_i given x_i, within what limits may we be certain (with say a 95% probability) what the real value of Y_i is?

If we denote the residual mean square in the ANOVA by s^2, the 95% confidence limits for a (denoted by A, the notation for the true—as opposed to the estimated—value for this parameter) are calculated as

$$t_{n-2} = \frac{a - A}{\sqrt{s^2\left(\sum x^2\right)/\left(n\sum x_1^2 - n^2\bar{x}^2\right)}}$$

$$\frac{9.2 - A}{\sqrt{8.8(51)/[4(51) - (16)(10.562)]}} = \frac{9.2 - A}{\sqrt{448/35.008}}$$

$$= \frac{9.2 - A}{3.58} = -4.303$$

$$9.2 - A = -15.405$$

$$A = 9.2 - 15.405$$

ASSUMPTIONS AND LIMITATIONS

1. All the regression methods are for interpolation, not extrapolation. That is, they are valid only in the range that we have data—the experimental region. Not beyond.
2. The method assumes that the data are independent and normally distributed, and it is sensitive to outliers. The x-axis (or horizontal) component plays an extremely important part in developing the least-squares fit. All points have equal weight in determining the height of a regression line, but extreme x-axis values unduly influence the slope of the line.
3. A good fit between a line and a set of data (i.e., a strong correlation between treatment and response variables) does not imply any casual relationship.
4. It is assumed that the treatment variable can be measured without error, that each data point is independent, that variances are equivalent, and that a linear relationship does not exist between the variables.
5. There are many excellent texts on regression, which is a powerful technique. See, for example, Draper and Smith (1981) and Montgomery and Smith (1983), which are not overly rigorous mathematically.

25.7.13 Probit/Log Transforms and Regression

As we noted in the preceding section, dose–response problems (among the most common interpolation problems encountered in toxicology) rarely are straightforward enough to make a valid linear regression directly from the raw data. The most common valid interpolation methods are based upon probability ("probit") and logarithmic ("log") value scales, with percentage responses (death, tumor incidence, etc.) being expressed on the probit scale while doses (Y_i) are expressed on the log scale. There are two strategies for such an approach. The first is based on transforming the data to these scales, then doing a weighted linear regression on the transformed data (if one does not have access to a computer or a high-powered programmable calculator, the only practical strategy is not to assign weights). The second requires the use of algorithms (approximate calculation techniques) for the probit value and regression process and is extremely burdensome to perform manually.

Our approach to the first strategy requires that we construct a table with the pairs of values of x_i and y_i listed in order of increasing values of Y_i (percentage response). Beside each of these columns a set of blank columns should be left so that the transformed values may be listed. We then simply add the columns described in the linear regression procedure. Log and probit values may be taken from any of a number of sets of tables (such as provided in Appendix I) and the rest of the table is then developed from these transformed x_i' and y_i' values (denoted as x_i' and y_i'). A standard linear regression is then performed.

The second strategy we discussed has been broached by a number of authors (Bliss, 1935; Finney, 1977; Litchfield and Wilcoxon, 1949; Prentice, 1976). All of these methods, however, are computationally cumbersome. It is possible to approximate the necessary iterative process using the algorithms developed by Abramowitz and Stegun (1964) but even this merely reduces the complexity to a point where the procedure may be readily programmed on a small computer or programmable calculator.

ASSUMPTIONS AND LIMITATIONS

1. The probit distribution is derived form a common error function, with the midpoint (50% point) moved to a score of 5.00.
2. The underlying frequency distribution becomes asymptotic as it approaches the extremes of the range. That is, in the range of 16–84%, the corresponding probit values change gradually—the curve is relatively linear. But beyond this range, they change ever more rapidly as they approach either 0 or 100%. In fact, there are no values for either of these numbers.
3. A normally distributed population is assumed, and the results are sensitive to outliers.

25.7.14 Nonlinear Regression

More often than not in toxicology we find that our data demonstrate a relationship between two variables (such as age and body weight) which is not linear. That is, a change in one variable (say age) does not produce a directly proportional change in the other (e.g., body weight). But some form of relationship between the variables is apparent. If understanding such a relationship and being able to predict unknown points is of value, we have a pair of options available to us. The first, which was discussed and reviewed earlier, is to use one or more transformations to linearize our data and then to make use of linear regression. This approach, though most commonly used, has a number of drawbacks. Not all data can be suitably transformed; sometimes the transformations necessary to linearize the data require a cumbersome series of calculations, and the resulting linear regression is not always sufficient to account for the differences among sample values—there are significant deviations around the linear regression line (i.e., a line may still not give us a good fit to the data or do an adequate job of representing the relationship between the data). In such cases, we have available a second option—the fitting of data to some nonlinear function such as some form of the curve. This is, in general form, nonlinear regression and may involve fitting data to an infinite number of possible functions. But most often we are interested in fitting curves to a polynomial function of the general form

$$Y = a + bx + cx^2 + dx^2 + \cdots$$

where x is the independent variable. As the number of powers of x increases, the curve becomes increasingly complex and will be able to fit a given set of data increasingly well.

Generally in toxicology, however, if we plot the log of a response (such as body weight) versus a linear scale of our dose or stimulus, we get one of four types of nonlinear curves (Snedecor and Cochran, 1980):

1. Exponential growth, where $\log Y = A(Bx)$, such as the growth curve for the log phase of a bacterial culture.
2. Exponential decay, where $\log Y = A(B^{-x})$, such as a radioactive decay curve.
3. Asymptotic regression, where $\log Y = A - B(p^x)$, such as a first-order reaction curve.
4. Logistic growth curve, where $\log Y = A/(1 + Bp^x)$, such as a population growth curve.

In all these cases, A and B are constant while p is a log transform. These curves are illustrated in Figure 25.7.

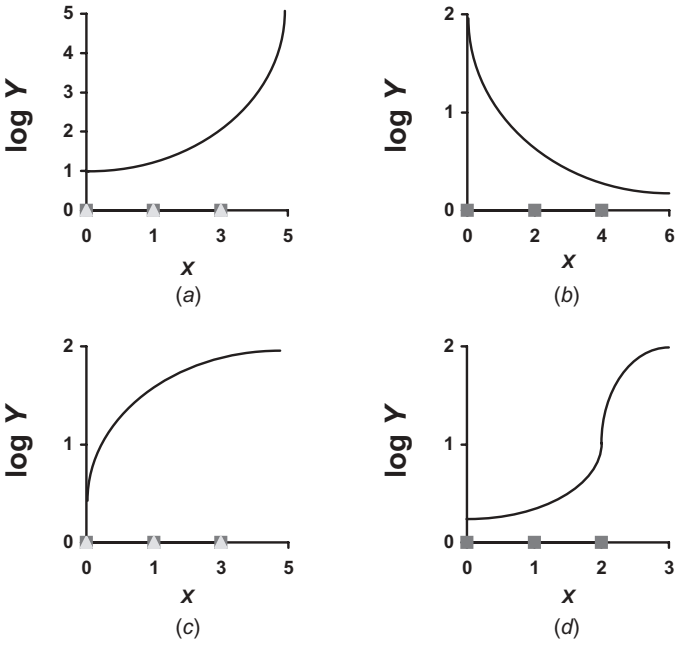


Figure 25.7 *Common Curvilinear Curves: (a) exponential growth law, log Y = A(B^x); (b) exponential decay law, log Y = A(B^-x); (c) asymptotic regression, log Y = A – B(ψ^x); (d) logistic growth law, log Y = A/(1 + Bψ^x).*

All four types of curves are fit by iterative processes—that is, best guess numbers are initially chosen for each of the constants and, after a fit is attempted, the constants are modified to improve the fit. This process is repeated until an acceptable fit has been generated. Analysis of variance or covariance can be used to objectively evaluate the acceptability of it. Needless to say, the use of a computer generally accelerates such a curve-fitting process.

ASSUMPTIONS AND LIMITATIONS

1. The principle of using least squares may still be applicable in fitting the best curve if the assumptions of normality, independence, and reasonably error-free measurement of response are valid.
2. Growth curves are best modeled using a nonlinear method.

25.7.15 Correlation Coefficient

The correlation procedure is used to determine the degree of linear correlation (direct relationship) between two groups of continuous (and normally distributed) variables; it will indicate whether there is any statistical relationship between the variables in the two groups. For example, we may wish to determine if the liver weights of dogs on a feeding study are correlated with their body weights. Thus, we will record the body and liver weights at the time of sacrifice and then calculate the correlation coefficient between these pairs of values to determine if there is some relationship.

A formula for calculating the linear correlation coefficient (r_{xy}) is as follows:

$$r_{xy} = \frac{N\sum XY - (\Sigma X)(\Sigma Y)}{\sqrt{N\Sigma X^2 - (\Sigma X)^2}\sqrt{N\Sigma Y^2 - (\Sigma Y)^2}}$$

where X is each value for one variable (such as the dog body weights in the above example), Y is the matching value for the second variable (the liver weights), and N is the number of pairs of X and Y. Once we have obtained r_{xy}, it is possible to calculate t_r, which can be used for more precise examination of the degree of significant linear relationship between the two groups. This value is calculated as follows:

$$t_r = \frac{r_{zy}\sqrt{N-2}}{\sqrt{1-r_{zy}^2}}$$

This calculation is also equivalent to r = sample covariance/(S_xS_y), as was seen earlier under ANCOVA.

The value obtained for r_{xy} can be compared to table values (Snedecor and Cochran, 1980) for the number of pairs of data involved minus 2. If the r_{xy} is

smaller (at the selected test probability level, such as 0.05), the correlation is not significantly different from zero (no correlation). If r_{xy} is larger than the table value, there is a positive statistical relationship between the groups. Comparisons are then made at lower levels of probability to determine the degree of relationship (note that if r_{xy} is either 1.0 or –1.0, there is complete correlation between the groups). If r_{xy} is a negative number and the absolute is greater than the table value, there is an inverse relationship between the groups; that is, a change in one group is associated with a change in the opposite direction in the second group of variables.

Since the comparison of r_{xy} with the table values may be considered a somewhat weak test, it is perhaps more meaningful to compare the t_r value with values in a t-distribution table for $N - 2$ degrees of freedom (df), as is done for the Student's t test. This will give a more exact determination of the degree of statistical correlation between the two groups.

Note that this method examines only possible linear relationships between sets of continuous, normally distributed data.

ASSUMPTIONS AND LIMITATIONS

1. A strong correlation does not imply that a treatment causes an effect.
2. The distances of data points from the regression line are the portions of the data not "explained" by the model. These are called residuals. Poor correlation coefficients imply high residuals, which may be due to many small contributions (variations of data from the regression line) or a few large ones. Extreme values (outliers) greatly reduce correlation.
3. X and Y are assumed to be independent.
4. Feinstein (1979) has provided a fine discussion of the difference between correlation (or association of variables) and causation.

25.7.16 Kendall's Coefficient of Rank Correlation

Kendall's rank correlation, represented by τ (tau), should be used to evaluate the degree of association between two sets of data when the nature of the data is such that the relationship may not be linear. Most commonly, this is when the data are not continuous and/or normally distributed. An example of such a case is when we are trying to determine if there is a relationship between the length of hydra and their survival time in a test medium in hours. Both of our variables here are discontinuous, yet we suspect a relationship exists. Another common use is in comparing the subjective scoring done by two different observers.

Tau is calculated as $\tau = N/n(n-1)$ where n is the sample size and N is the count of ranks, calculated as $N = 4({}^{n}C_i) - n(n-1)$, with the computing of ${}^{n}C_i$ being demonstrated in the example.

If a second variable Y_2 is exactly correlated with the first variable Y_1, then the variates Y_2 should be in the same order as the Y_1 variates. However, if the correlation is less than exact, the order of the variates Y_2 will not correspond entirely to that of Y. The quantity N measures how well the second variable corresponds to the order of the first. It has maximum value of $n(n-1)$ and a minimum value of $-n(n-1)$.

A table of data is set up with each of the two variables being ranked separately. Tied ranks are assigned as demonstrated earlier under the Kruskall–Wallis test. From this point, disregard the original variates and deal only with the ranks. Place the ranks of one of the two variables in rank order (from lowest to highest) paired with the rank values assigned for the other variable. If one (but not the other) variable has tied ranks, order the pairs by the variables without ties (Sokal and Rohlf, 1994).

The resulting value of tau will range from –1 to +1, as does the familiar parametric correlation coefficient r.

ASSUMPTION AND LIMITATION

1. A very robust estimator which does not assume normality, linearity, or minimal error of measurement.

25.7.17 Trend Analysis

Trend analysis is a collection of techniques that have been "discovered" by toxicology since the mid-1970s (Tarone, 1975). The actual methodology dates back to the mid-1950s (Cox and Stuart, 1955).

Trend analysis methods are a variation on the theme of regression testing. In the broadest sense, the methods are used to determine whether a sequence of observations taken over an ordered range of values of a variable (most commonly time) exhibit some form of pattern of change (e.g., an upward trend) associated with another variable of interest (in toxicology, some form or measure of dosage).

Trend corresponds to sustained and systematic variations over a long period of time. It is associated with the structural causes of the phenomenon in question, for example, population growth, technological progress, new ways of organization, or capital accumulation.

The identification of trend has always posed a serious statistical problem. The problem is not one of mathematical or analytical complexity but of conceptual complexity. This problem exists because the trend as well as the remaining components of a time series are latent (nonobservable) variables and, therefore, assumptions must be made on their behavioral pattern. The trend is generally thought of as a smooth and slow movement over a long term. The concept of "long" in this connection is relative and what is identified as trend for a given series span might well be part of a long cycle once the series is considerably augmented. Often, a long cycle is treated as a trend because

the length of the observed time series is shorter than one complete face of this type of cycle.

The ways in which data are collected in toxicology studies frequently serve to complicate trend analysis, as the length of time for the phenomena underlying a trend to express themselves is frequently artificially censored.

To avoid the complexity of the problem posed by a statistically vague definition, statisticians have resorted to two simple solutions: One consists of estimating trend and cyclical fluctuations together, calling this combined movement a *trend cycle*; the other consists of defining the trend in terms of the series length, denoting it as the longest nonperiodic movement.

Within the large class of models identified for trend, we can distinguish two main categories: deterministic trends and stochastic trends.

Deterministic trend models are based on the assumption that the trend of a time series can be approximated closely by simple mathematical functions of time over the entire span of the series. The most common representation of a deterministic trend is by means of polynomials or of transcendental functions. The time series from which the trend is to be identified is assumed to be generated by a nonstationary process where the nonstationarity results from a deterministic trend. A classical model is the regression or error model (Anderson, 1971) where the observed series is treated as the sum of a systematic part or trend and a random or irregular part. This model can be written as

$$Z_t = Y_t + U_t'$$

where U_t is a purely random process, that is, U_t ~i.i.d. (0, 2/u) (independent and identically distributed with expected value zero and variance 2/u).

Trend tests are generally described as k-sample tests of the null hypothesis of identical distribution against an alternative of linear order; that is, if sample I has distribution function F_i, $i = 1$, then the null hypothesis

$$H_0 : F_1 = F_2 - \cdots = F_k$$

is tested against the alternative

$$H_1 : F_1 \geq F_2 \geq \cdots = F_k$$

(or its reverse), where at least one of the inequalities is strict. These tests can be thought of as special cases of tests of regression or correlation in which association is sought between the observations and its ordered sample index. They are also related to ANOVA except that the tests are tailored to be powerful against the subset of alternatives H_1, instead of the more general set $\{F_1 \neq F_j$, some $i \neq j\}$.

Different tests arise from requiring power against specific elements or subsets of this rather extensive set of alternatives.

The most popular trend test in toxicology is currently that presented by Tarone in 1975 because it is that used by the NCI in the analysis of carcinogenicity data. A simple, but efficient alternative is the Cox and Stuart (1955) test, which is a modification of the sign test. For each point at which we have a measure (such as the indidence of animals observed with tumors) we form a pair of observations—one from each of the groups we wish to compare. In a traditional NCI bioassay this would mean pairing control with low dose and low dose with high dose (to explore a dose-related trend) or each time period observation in a dose group (except the first) with its predecessor (to evaluate time-related trend). When the second observation in a pair exceeds the earlier observation, we record a plus sign for that pair. When the first observation is greater than the second, we record a minus sign for that pair. A preponderance of plus signs suggests a downward trend while an excess of minus signs suggests an upward trend. A formal test at a preselected confidence level can then be performed.

More formally put, after having defined what trend we want to test for, we first match pairs as $(X_1 - X_1 + c)$, $(X_2, X_2 + c)$, … $(X_{n'-c}, X_{n'})$, where $c = n'/2$ when n' is even and $c = (n' + 1)/2$ when n' is odd (where n' is the number of observations in a set). The hypothesis is then tested by comparing the resulting number of excess positive or negative signs against a sign test table such as are found in Beyer (1976a,b).

We can, of course, combine a number of observations to allow ourselves to actively test for a set of trends, such as the existence of a trend of increasing difference between two groups of animals over a period of time.

ASSUMPTIONS AND LIMITATIONS

1. Trend tests seek to evaluate whether there is a monotonic tendency in response to a change in treatment. That is, the dose–response direction is absolute—as dose goes up, the incidence of tumors increases. Thus the test loses power rapidly in response to the occurrences of "reversals"—for example, a low-dose group with a decreased tumor incidence. There are methods (Dykstra and Robertson, 1983) which "smooth the bumps" of reversals in long data series. In toxicology, however, most data series are short (i.e., there are only a few dose levels).

Tarone's trend test is most powerful at detecting dose-related trends when tumor onset hazard functions are proportional to each other. For more power against other dose-related group differences, weighted versions of the statistic are also available (Breslow, 1984; Crowley and Breslow, 1984).

In 1985, the U.S. *Federal Register* recommended that the analysis of tumor incidence data is carried out with a Cochran–Armitage (Armitage, 1955; Cochran, 1954) trend test. The test statistic of the Cochran–Armitage test is defined as

$$T_{CA} = \sqrt{\frac{N}{(N-r)r}}, \frac{\sum_{i=0}^{k}[R_1 - (n_1/N)r]d_1}{\sqrt{\sum_{i=0}^{k}(n_i/N)d_i^2 - \left(\sum_{i=0}^{k}(n_i/N)d_1\right)^2}}$$

with dose scores d_i. Armitage's test statistic is the square of this term (T_{CA}^2). As one-sided tests are carried out for an increase of tumor rates, the square is not considered. Instead, the above-mentioned test statistic, which is presented by Portier and Hoel (1984), is used. This test statistic is asymptotically standard normally distributed. The Cochran–Armitage test is asymptotically efficient for all monotone alternatives (Tarone, 1975), but this result only holds asymptotically. And tumors are rare events, so the binominal proportions are small. In this situation approximations may become unreliable.

Therefore, exact tests which can be performed using two different approaches—conditional and unconditional—are considered. In the first case, the total number of tumors r is regarded as fixed. As a result the null distribution of the test statistic is independent of the common probability p. The exact conditional null distribution is a multivariate hypergeometric distribution.

The unconditional model treats the sum of all tumors as a random variable. Then the exact unconditional null distribution is a multivariate binomial distribution. The distribution depends on the unknown probability.

25.8 METHODS FOR REDUCTION OF DIMENSIONALITY

Techniques for the reduction of dimensionality are those that simplify the understanding of data, either visually or numerically, while causing only minimal reductions in the amount of information present. These techniques operate primarily by pooling or combining groups of variables into single variables but may also entail the identification and elimination of low-information-content (or irrelevant) variables.

Descriptive statistics (calculations of means, standard deviations, etc.) are the simplest and most familiar form of reduction of dimensionality. Here we first need to address classification, which provides the general conceptual tools for identifying and quantifying similarities and differences between groups of things which have more than a single linear scale of measurement in common (e.g., which have both been determined to have or lack a number of enzyme activities). Then we will consider two collections of methodologies which combine graphic and computational methods, multidimensional/nonmetric scaling, and cluster analysis. Multidimensional scaling (MDS) is a set of techniques for quantitatively analyzing similarities, dissimilarities, and distances between data in a displaylike manner. Nonmetric scaling is an analogous set of methods for displaying and relating data when measurements are nonquantitative (the data are described by attributes or ranks). Cluster analysis is a

collection of graphic and numerical methodologies for classifying things based on the relationships between the values of the variables that they share.

The final pair of methods for reduction of dimensionality which will be tackled in this chapter are Fourier analysis and life table analysis. Fourier analysis seeks to identify cyclic patterns in data and can analyze the patterns or the residuals after the patterns are taken out. Life table analysis techniques are directed to identifying and quantitating the time course of risks (such as death or the occurrence of tumors).

25.8.1 Classification

Classification is both a basic concept and a collection of techniques which are necessary prerequisites for further analysis of data when the members of a set of data are (or can be) each described by several variables. At least some degree of classification (which is broadly defined as the dividing of the members of a group into smaller groups in accordance with a set of decision rules) is necessary prior to any data collection. Whether formally or informally, an investigator has to decide which things are similar enough to be counted as the same and develop rules for governing collection procedures. Such rules can be as simple as "measure and record body weights only of live animals on study" or as complex as that demonstrated by the expanded weighting classification procedure demonstrated below. Such a classification also demonstrates that the selection of which variables to measure will determine the final classification of data.

EXPANDED WEIGHTING PROCEDURE

I.	Is animal of desired species?	Yes/no
II.	Is animal member of study group?	Yes/no
III.	Is animal alive?	Yes/no
IV.	Which group does animal belong to?	
	A. Control	
	B. Low dose	
	C. Intermediate dose	
	D. High dose	
V.	What sex is animal?	Male/female
VI.	Is the measured weight in acceptable range?	Yes/no

Classifications of data have two purposes (Hartigan, 1983; Gordon, 1981): data simplification (also called a descriptive function) and prediction. Simplification is necessary because there is a limit to both the volume and complexity of data that the human mind can comprehend and deal with conceptually. Classification allows us to attach a label (or name) to each group of data, to summarize the data (i.e., assign individual elements of data to groups and to characterize the population of the group), and to define the relationships between groups (i.e., develop a taxonomy).

Prediction, meanwhile, is the use of summaries of data and knowledge of the relationships between groups to develop hypotheses as to what will happen when further data are collected (as when more animals or people are exposed to an agent under defined conditions) and as to the mechanisms which cause such relationships to develop. Indeed, classification is the prime device for the discovery of mechanisms in all of science. A classic example of this was Darwin's realization that there were reasons (the mechanisms of evolution) behind the differences and similarities in species which had caused Linaeus to earlier develop his initial modern classification scheme (or taxonomy) for animals.

To develop a classification, one first sets bounds wide enough to encompass the entire range of data to be considered that is not unnecessarily wide. This is typically done by selecting some global variables (variables every piece of data have in common) and limiting the range of each so that it just encompasses all the cases on hand. Then one selects a set of local variables (characteristics which only some of the cases have, say the occurrence of certain tumor types, enzyme activity levels, or dietary preferences) and which thus serve to differentiate between groups. Data are then collected, and a system for measuring differences and similarities is developed. Such measurements are based on some form of measurement of distance between two cases (x and y) in terms of each single-variable scale. If the variable is a continuous one, then the simplest measure of distance between two pieces of data is the Euclidean distance $d(x, y)$ defined as

$$d(x, y) = \sqrt{(x_i - y_i)^2}$$

For categorical or discontinuous data, the simplest distance measure is the matching distance, defined as

$$d(x, y) = \text{number of times } x_i \neq y_i$$

After we have developed a table of such distance measurements for each of the local variables, some weighting factor is assigned to each variable. A weighting factor seeks to give greater importance to those variables which are believed to have more relevance or predictive value. The weighted variables are then used to assign each piece of data to a group. The actual act of developing numerically based classifications and assigning data members to them is the realm of cluster analysis and will be discussed later in this chapter. Classification of biological data based on qualitative factors has been well discussed. Glass (1975) and Schaper et al. (1985) do an excellent job of introducing the entire field and mathematical concepts.

Relevant examples of the use of classification techniques range from the simple to the complex. Schaper et al. (1985) developed and used a very simple classification of response methodology to identify those airborne chemicals which alter the normal respiratory response induced by CO_2. At the other end of the spectrum, Kowalski and Bender (1972) developed a more mathemati-

cally based system to classify chemical data (a methodology they termed *pattern recognition*).

25.8.2 Statistical Graphics

The use of graphics in one form or another in statistics is the single most effective and robust statistical tool and at the same time one of the most poorly understood and improperly used.

Graphs are used for one of four major purposes. Each of the four is a variation on the central theme of making complex data easier to understand and use. These four major functions are exploration, analysis, communication and display of data, and graphical aids. Exploration (which may be simply summarizing data or trying to expose relationships between variables) is determining the characteristics of data sets and deciding on one or more appropriate forms of further analysis, such as the scatterplot. Analysis is the use of graphs to formally evaluate some aspect of the data, such as whether there are outliers present or if an underlying assumption of a population distribution is fulfilled. As long ago as 1960 (Anderson), some 18 graphical methods for analyzing multivariate data relationships were developed and proposed. Table 25.7 presents a summary of major graphical techniques that are available.

Communication and display of data are the most commonly used functions of statistical graphics in toxicology, whether used for internal reports, presentations at meetings, or formal publications in the literature. In communicating data, graphs should not be used to duplicate data that are presented in tables but rather should show important trends and/or relationships in the data. Though such communication is most common in a quantitative compilation of actual data, it can also be used to summarize and present the results of statistical analysis. The fourth and final function of graphics is one that is largely becoming outdated as microcomputers become more widely available. Graphical aids to calculation include nomograms [the classic example in toxicology of a nomogram is that presented by Litchfield and Wilcoxon (1949) for determining median effective doses] and extrapolating and interpolating data graphically based on plotted data.

There are many forms of statistical graphics (a partial list, classified by function, is presented in Table 25.7), and a number of these (such as scatterplots and histograms) can be used for each of a number of possible functions. Most of these plots are based on a Cartesian system (i.e., they use a set of rectangular coordinates), and our review of construction and use will focus on these forms of graphs.

Construction of a rectangular graph of any form starts with the selection of the appropriate form of graph followed by the laying out of the coordinates (or axes). Even graphs which are going to encompass multivariate data (i.e., more than two variables) generally have as their starting point two major coordinates. The vertical axis, or ordinate (also called the Y axis), is used to present an independent variable. Each of these axes is scaled in the units of

TABLE 25.7 Forms of Statistical Graphics (by Function)

Data Summary	Two Variables	Three or More Variables
	Exploration	
Box and whisker plot	Autocorrelation plot	Biplot
Histogram[a]	Cross-correlation plot	Cluster trees[a]
Dot array diagram	Scatterplot[a]	Labeled scatterplot[a]
Frequency polygon	Sequence plot	Glyphs and metroglyphs
Ogive		Face plots
Stem-and-leaf diagram		Fourier plots
		Similarity and preference maps
		Multidimensional scaling displays
		Weathervane plot
Distribution Assessment	Model Evaluation and Assumption Verification	Decision Making
	Analysis	
Probability plot	Average versus standard deviation	Control chart
Q–Q plot	Component-plus-residual plot	Custom chart
P–P plot	Partial-residual plot	Half-normal plot
Hanging histogram	Residual plots	Ridge trace
Rootagram		Youden plot
Poissonness plot		
Quantitative Graphics	Summary of Statistical Analyses	Graphical Aids
	Communication and Display of Data	
Line chart	Means plot	Confidence limits
Pictogram	Sliding reference distribution	Graph paper
Pie chart	Notched box plot	Power curves
Contour plot	Factor space/response	Nomographs
Stereogram	Interaction plot	Sample size curves
Color map	Contour plot	Trilinear coordinates
Histogram	Predicted response plot	
	Confidence region plot	

[a]Reviewed in the text of this chapter.

measure which will most clearly present the trends of interest in the data. The range covered by the scale of each axis is selected to cover the entire region for which data are presented. The actual demarking of the measurement scale along an axis should allow for easy and accurate assessment of the coordinates of any data point yet should not be cluttered.

Actual data points should be presented by symbols which present the appropriate indicators of location, and if they represent summaries of data from a normal data population, it would be appropriate to present a symbol for the mean and some indication of the variability (or error) associated with

that population, commonly by using "error bars" which present the standard deviation (or standard error) from the mean. If, however, the data are not normal or continuous, it would be more appropriate to indicate location by the median and present the range or semiquartile distance for variability estimates. The symbols which are used to present data points can also be used to present a significant amount of additional information. At the simplest level a set of clearly distinct symbols (circles, triangles, squares, etc.) is very commonly used to provide a third dimension of data (most commonly treatment group). But by clever use of symbols, all sorts of additional information can be presented. Using a method such as Chernoff's (1973) faces, in which faces are used as symbols of the data points (and various aspects of the faces present additional data, such as the presence or absence of eyes denoting presence or absence of a secondary pathological condition), it is possible to present a large number of different variables on a single graph.

The three other forms of graphs that are commonly used are histograms, pie charts, and contour plots.

Histograms are graphs of simple frequency distribution. Commonly, the abscissa is the variable of interest (such as life span or litter size) and is generally shown as classes or intervals or measurements (such as age ranges of 0–10 weeks, 10–20 weeks, etc.). The ordinate, meanwhile, is the incidence or frequency of observations. The result is a set of vertical bars, each of which represents the incidence of a particular set of observations. Measures of error or variability about each incidence are reflected by some form of error bar on top of or in the frequency bars, as shown in Figure 25.8. The size of class intervals may be unequal (in effect, one can combine or pool several small class intervals), but it is proper in such cases to vary the width of the bars to indicate differences in interval size.

Pie charts are the only common form of quantitative graphic technique which is not rectangular. Rather, the figure is presented as a circle out of which several "slices" are delimited. The only major use of the pie chart is in presenting a breakdown of the components of a group. Typically the entire set of data under consideration (such as total body weight) constitutes the pie while each slice represents a percentage of the whole (such as the percentages represented by each of several organs). The total number of slices in a pie should be small for the presentation to be effective. Variability or error can be readily presented by having a subslice of each sector shaded and labeled accordingly.

Finally, there is the contour plot, which is used to depict the relationships in a three-variable, continuous data system. That is, a contour plot visually portrays each contour as a locus of the values of two variables associated with a constant value of the third variable. An example would be a relief map that gives both latitude and longitude of constant altitude using contour lines.

The most common misuse of graphs is to either conceal or exaggerate the extent of the difference by using inappropriately scaled or ranged axis. Tufte (1983) has termed a statistic for evaluating the appropriateness of scale size, the *lie factor*, calculated as the ratio of the shown effect size to the range of potential change or effect. An acceptable range for the lie factor is from 0.95

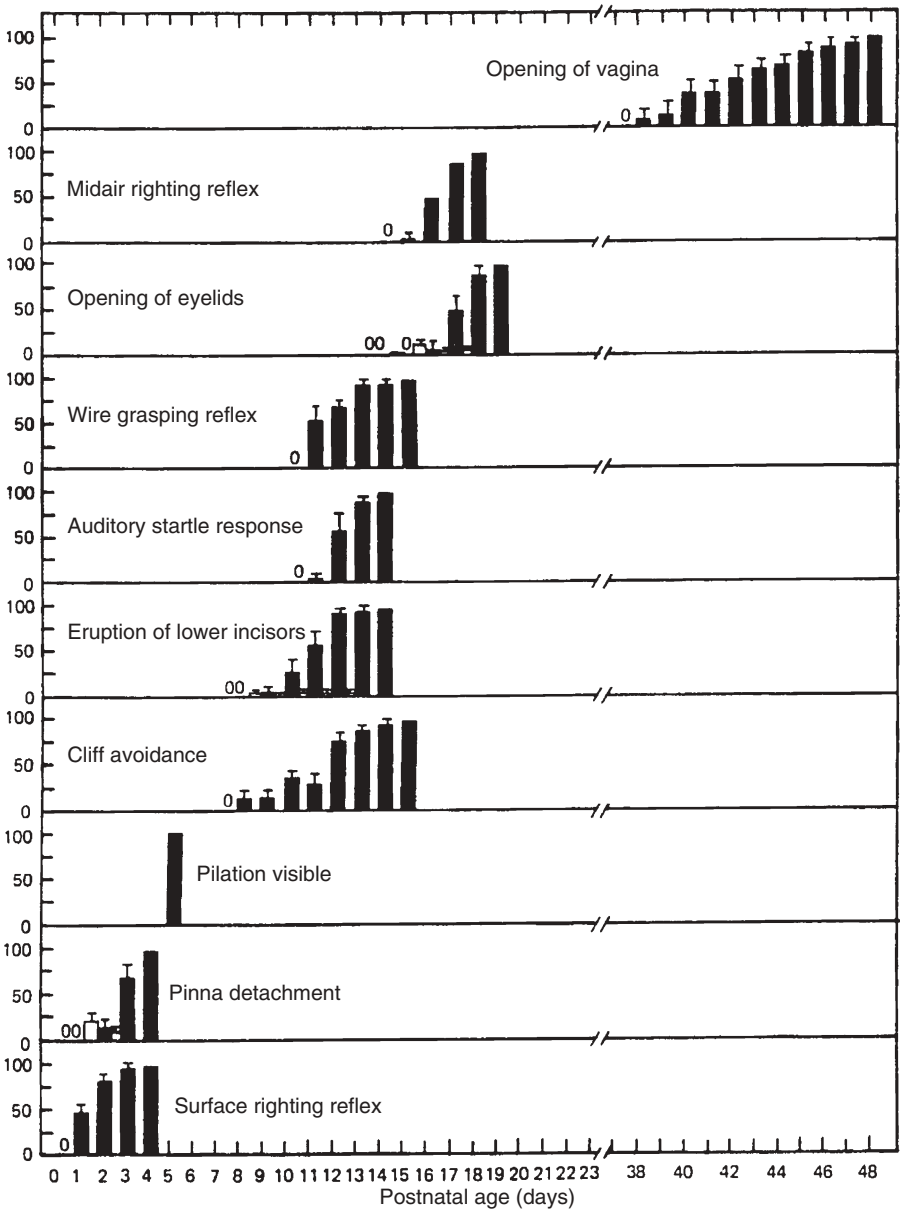


Figure 25.8 *Acquisitions of Post-Natal Development Landmarks in Rats.*

to 1.05. Less means the size of an effect is being understated, more that the effect is being exaggerated.

There are a number of excellent references available for those who would like to pursue statistical graphics more. Anscombe (1973) presents an excellent short overview, while others (Tufte, 1983, 1990, 1997; Schmid, 1983; Young, 1985) provide a wealth of information.

25.8.3 Multidimensional and Nonmetric Scaling

Multidimensional scaling (MDS) is a collection of analysis methods for data sets which have three or more variables making up each data point. MDS displays the relationships of three- or more-dimensional extension of the methods of statistical graphics.

MDS presents the structure of a set of objects from data that approximate the distances between pairs of the objects. The data, called similarities, dissimilarities, distances, or proximities, must be in such a form that the degree of similarities and differences between the pairs of the objects (each of which represents a real-life data point) can be measured and handled as a distance (remember the discussion of measures of distances under classifications). Similarity is a matter of degree—small differences between objects cause them to be similar (a high degree of similarity) while large differences cause them to be considered dissimilar (a small degree of similarity).

In addition to the traditional human conceptual or subjective judgments or similarity, data can be an "objective" similarity measure (the difference in weight between a pair of animals) or an index calculated from multivariate data (the proportion of agreement in the results of a number of carcinogenicity studies). However, the data must always represent the degree of similarity of pairs of objects.

Each object or data point is represented by a point in a multidimensional space. These plots or projected points are arranged in this space so that the distances between pairs of points have the strongest possible relation to the degree of similarity among the pairs of objects. That is, two similar objects are represented by two points that are close together, and two dissimilar objects are represented by a pair of points that are far apart. The space is usually a two- or three-dimensional Euclidean space but may be non-Euclidean and may have more dimensions.

MDS is a general term which includes a number of different types of techniques. However, all seek to allow geometric analysis of multivariate data. The forms of MDS can be classified (Young, 1985) according to the nature of the similarities in the data. It can be qualitative (nonmetric) or quantitative (metric MDS). The types can also be classified by the number of variables involved and by the nature of the model used, for example, classical MDS (there is only one data matrix and no weighting factors are used on the data), replicated MDS (more than one matrix and no weighting), and weighted MDS (more than one matrix and at least some of the data being weighted).

MDS can be used in toxicology to analyze the similarities and differences between effects produced by different agents in an attempt to use an understanding of the mechanism underlying the actions of one agent to determine the mechanisms of the other agents. Actual algorithms and a good intermediate-level presentation of MDS can be found in Davison (1983).

Nonmetric scaling is a set of graphic techniques closely related to MDS and is definitely useful for the reduction of dimensionality. Its major objective is to arrange a set of objects (each object, for our purposes, consisting of a number of related observations) graphically in a few dimensions while retaining the maximum possible fidelity to the original relationships between members (i.e., values which are most different are portrayed as most distant). It is not a linear technique, it does not preserve linear relationships (i.e., A is not shown as twice as far from C as B, even though its "value difference" may be twice as much). The spacings (interpoint distances) are kept such that if the distance of the original scale between members A and B is greater than that between C and D, the distances on the model scale shall likewise be greater between A and B than between C and D. Figure 25.5, presented earlier, uses a form of this technique in adding a third dimension by using letters to present degrees of effect on the skin.

This technique functions by taking observed measures of similarity or dissimilarity between every pair of M objects, then finding a representation of the objects as points in Euclidean space that the interpoint distances in some sense "match" the observed similarities or dissimilarities by means of weighting constants.

25.8.4 Cluster Analysis

Cluster analysis is a quantitative form of classification. It serves to help develop decision rules and then use these rules to assign a heterogeneous collection of objects to a series of sets. This is almost entirely an applied methodology (as opposed to theoretical). The final result of cluster analysis is one of several forms of graphic displays and a methodology (set of decision classifying rules) for the assignment of new members into the classifications.

The classification procedures used are based on either density of population or distance between members. These methods can serve to generate a basis for the classification of large numbers of dissimilar variables such as behavioral observations and compounds with distinct but related structures and mechanisms (Gad, 1984; Gad et al., 1985) or to separate tumor patterns caused by treatment from those caused by old age (Hammond et al., 1978).

There are five types of clustering techniques (Everitt, 1980; Romesburg, 1984):

(a) *Hierarchical Techniques* Classes are subclassified into groups, with the process being repeated at several levels to produce a tree which gives sufficient definition to groups.

(b) *Optimizing Techniques* Clusters are formed by optimization of a clustering criterion. The resulting classes are mutually exclusive; the objects are partitioned clearly into sets.

(c) *Density or Mode-Seeking Techniques* Clusters are identified and formed by locating regions in a graphic representation which contains concentrations of data points.

(d) *Clumping Techniques* A variation of density-seeking techniques in which assignment to a cluster is weighted on some variables so that clusters may overlap in graphic projections.

(e) *Others* Methods which do not clearly fall into classes (a)–(d).

Romesburg (1984) provides an excellent step-by-step guide to cluster analysis.

25.8.5 Fourier (Time) Analysis

Fourier analysis (Bloomfield, 1976) is most frequently a univariate method used for either simplifying data (which is the basis for its inclusion in this chapter) or modeling. It can, however, also be a multivariate technique for data analysis.

In a sense, it is like trend analysis; it looks at the relationship of sets of data from a different perspective. In the case of Fourier analysis, the approach is by resolving the time dimension variable in the data set. At the most simple level, it assumes that many events are periodic in nature, and if we can remove the variation in other variables because of this periodicity (by using Fourier transforms), we can better analyze the remaining variation from other variables. The complications to this are (a) there may be several overlying cyclic time-based periodicities and (b) we may be interested in the time cycle events for their own sake.

Fourier analysis allows one to identify, quantitate, and (if we wish) remove the time-based cycles in data (with their amplitudes, phases, and frequencies) by use of the Fourier transform:

$$nJ_i = x_i \exp(-iw_i t)$$

where n = length
J = discrete Fourier transform for that case
x = actual data
i = increment in series
w = frequency
t = time

25.8.6 Life Tables

Chronic in vivo toxicity studies are generally the most complex and expensive studies conducted by a toxicologist. Answers to a number of questions are

sought in such a study—notably if a material results in a significant increase in mortality or in the incidence of tumors in those animals exposed to it. But we are also interested in the time course of these adverse effects (or risks). The classic approach to assessing these age-specific hazard rates is by the use of life tables (also called survivorship tables).

It may readily be seen that during any selected period of time (t_i) we have a number of risks competing to affect an animal. There are risks of (a) "natural death," (b) death induced by a direct or indirect action of the test compound, and (c) death due to such occurrences of interest of tumors (Hammond et al., 1978; Salsburg, 1980). And we are indeed interested in determining if (and when) the last two of these risks become significantly different than the "natural" risks (defined as what is seen to happen in the control group). Life table methods enable us to make such determinations as the duration of survival (or time until tumors develop) and the probability of survival (or of developing a tumor) during any period of time.

We start by deciding the interval length (t_i) we wish to examine within the study. The information we gain becomes more exact as the interval is shortened. But as interval length is decreased, the number of intervals increases and calculations become more cumbersome and less indicative of time-related trends because random fluctuations become more apparent. For a two-year or lifetime rodent study, an interval length or a month is commonly employed. Some life table methods, such as the Kaplan–Meyer, have each new event (such as a death) define the start of a new interval.

Having established the interval length we can tabulate our data (Cutler and Ederer, 1958). We start by establishing the following columns in each table (a separate table being established for each group of animals—i.e., by sex and dose level):

(a) Interval of time selected (t_i)
(b) Number of animals in group that entered that interval of study alive (l_i)
(c) Number of animals withdrawn from study during interval (such as those taken for interim sacrifice or that may have been killed by technician error) (ω_i)
(d) Number of animals that died during interval (d_i)
(e) Number of animals at risk during interval, $l_i = l_i - \frac{1}{2}\omega_I$, or number on study at start of interval minus one-half number withdrawn during interval
(f) Proportion of animals that died, $=D_i = d_i/l_i$
(g) Cumulative probability of animal surviving until end of that interval of study, $P_i = 1 - D_i$, or 1 minus number of animals that died during interval divided by number of animals at risk
(h) Number of animals dying until that interval (M_i)
(i) Animals found to have died during interval (m_i)

(j) Probability of dying during interval of study $c_i = 1 - (M_i + m_i/l_i)$, or total number of animals dead until that interval plus animals discovered to have died during that interval divided by number of animals at risk through end of that interval

(k) Cumulative proportion surviving, p_i, equivalent to cumulative product of interval probabilities of survival (i.e., $p_i = p_1 \cdot p_2 \cdot p_3 \ldots p_x$)

(l) Cumulative probability of dying, C_i, equal to cumulative product of interval probabilities to that point (i.e., $C_i = c_1 \cdot c_2 \cdot c_3 \ldots c_i$).

With such tables established for each group in a study, we may now proceed to test the hypothesis that each of the treated groups has a significantly shorter duration of survival or that each of the treated groups died more quickly (note that plots of total animals dead and total animals surviving will give one an appreciation of the data but can lead to no statistical conclusions).

There are a multiplicity of methods for testing significance in life tables, with (as is often the case) the power of the tests increasing as does the difficulty of computation (Salsburg, 1980; Cox, 1972; Haseman, 1977; Tarone, 1975).

We begin our method of statistical comparison of survival at any point in the study by determining the standard error of the K-interval survival rate as (Garrett, 1947)

$$S_K = P_k \sqrt{\sum_1^k \left(\frac{D_i}{l'_x - d_x} \right)}$$

We may also determine the effective sample size (l_1) in accordance with

$$l_1 = \frac{P(1-P)}{S^2}$$

We may now compute the standard error of difference for any two groups (1 and 2) as

$$S_D = \sqrt{S_1^2 + S_2^2}$$

The difference in survival probabilities for the two groups is then calculated as

$$P_D = P_1 - P_2$$

We can then calculate a test statistic as

$$t' = \frac{P_D}{S_D}$$

This is then compared to the z-distribution table. If $t' > z$ at the desired probability level, it is significant at that level. With increasing recognition of the effects of time (both as age and length of exposure to unmeasured background risks), life table analysis has become a mainstay in chronic toxicology. An example is the reassessment of the ED_{01} study (SOT, 1981) which radically changed interpretation of the results and understanding of underlying methods when adjustment for time on study was made.

The increased importance and interest in the analysis of survival data has not been restricted to toxicology but rather has encompassed all the life sciences. Those with further interest should consult Lee (1980) or Elandt-Johnson and Johnson (1980), both general in their approach to the subject.

25.9 META-ANALYSIS

Meta-analysis (meaning "analysis among") is being used increasingly in biomedical research to try to obtain a qualitative or quantitative synthesis of the research literature on a particular issue. The technique is usually applied to the synthesis of several separate but comparable studies.

25.9.1 Selection of Studies to Be Analyzed

The issue of study selection is perhaps the most troublesome issue for those doing meta-analysis. Several questions need to be addressed.

1. Should studies be limited to those which are published? It is well known that negative studies that report little or no benefit from following a particular course of action are less likely to be published than are positive studies. Therefore, the published literature may be biased toward studies with positive results, and a synthesis of these studies would give a biased estimate of the impact of pursuing some courses of action. Unpublished studies, however, may be of lower quality than the published studies, and poor research methods often produce an underestimate of impact. Moreover, the unpublished studies may be difficult to discover.

2. Should studies be limited to those which appear in peer-reviewed publications? Peer review is considered the primary method for quality control in scientific publishing. Some investigators recommend that only those studies which are published in peer-reviewed publications be considered in meta-analysis. Although this may seem an attractive option, it might produce an even more highly biased selection of studies.

3. Should studies be limited to those which meet additional quality control criteria? If investigators impose an additional set of criteria before including a study in meta-analysis, this may further improve the average quality of the studies used, but it introduces still greater concerns about selection bias. More-

over, different investigators might use different criteria for a "valid" study and therefore select a different group of studies for meta-analysis.

4. Should studies be limited to randomized controlled studies? This is a variant of the above question concerning quality control. At one time, rigid quality standards were more likely to be met by randomized controlled studies than by observational studies. Increasingly, however, observational methods have been used to evaluate certain kinds of effects, particularly those that are uncommon. A larger issue may well be that of combining data from studies performed in different laboratories and, even more so, using different strains of a single animal species.

5. Should studies be limited to those using identical methods? For practical purposes, this would mean using only separately published studies from the same lab in a limited time frame for which the methods were the same for all and the similarity of methods was monitored. This criterion is very difficult to achieve.

25.9.2 Pooled (Quantitative) Analysis

Usually, the main purpose of meta-analysis is quantitative. The goal is to develop better overall estimates of the degree of benefit achieved by specific exposure and dosing techniques based on the combining (pooling) of estimates found in the existing studies of the interventions. This type of meta-analysis is sometimes called a pooled analysis (Gerbarg and Horwitz, 1988) because the analysts pool the observations of many studies and then calculate parameters such as risk ratios or odds ratios from the pooled data.

Because of the many decisions regarding inclusion or exclusion of studies, different meta-analyses might reach very different conclusions on the same topic. Even after the studies are chosen, there are many other methodologic issues in choosing how to combine means and variances (e.g., what weighting methods should be used). Pooled analysis should report both relative risks and risk reductions as well as absolute risks and risk reductions (Sinclair and Bracken, 1994).

25.9.3 Methodologic (Qualitative) Analysis

Sometimes the question to be answered is not how much toxicity is induced by the use of a particular exposure but whether there is any biologically significant toxicity at all. In this case, a qualitative meta-analysis may be done in which the quality of the research is scored according to a list of objective criteria. The meta-analyst then examines the methodologically superior studies to determine whether or not the question of toxicity is answered consistently by them. This qualitative approach has been called methodologic analysis (Gerbarg and Horwitz, 1988) or quality scores analysis (Greenland, 1994). In some cases, the methodologically strongest studies agree with one another and

disagree with the weaker studies, which may or may not be consistent with one another.

25.10 BAYESIAN INFERENCE

It is useful to know the sensitivity and specificity of a test. Once a researcher decides to use a certain test, two important questions require answers: If the test results are positive, what is the probability that the researcher has the condition of interest? If the test results are negative, what is the probability that the patient does not have the disease? Bayes's theorem provides a way to answer these questions.

Bayes's theorem, which was first described centuries ago by the English clergyman after whom it is named, is one of the most imposing statistical formulas in biomedical sciences (Lindley, 1971). Put in symbols more meaningful for researchers such as pathologists, the formula is as follows:

$$P(D|T^+) = \frac{p(T^+|D^+)\,p(D^+)}{[p(T^+|D^+)\,p(D^+)]+[p(T^+|D^-)\,p(D^-)]}$$

where p denotes probability, D^+ means that the animal has the effect in question, D^- means that the animal does not have the effect, T^+ means that a certain diagnostic test for the effect is positive, T^- means that the test is negative, and the vertical line (|) means "conditional upon" what immediately follows.

Most researchers, even those who can deal with sensitivity, specificity, and predictive values, throw in the towel when it comes to Bayes's theorem. This is odd because a close look at the above equation reveals that Bayes's theorem is merely the formula for the positive predictive value (Box and Tiao, 1973).

The numerator of Bayes's theorem merely describes cell *a* (the true-positive results). The probability of being in cell *a* is equal to the prevalence times the sensitivity, where $p(D^+)$ is the prevalence (the probability of being in the effected column) and where $p(T^+|D^+)$ is the sensitivity (the probability of being in the top row *given the fact of being in the effected column*). The denominator of Bayes's theorem consists of two terms, the first of which once again describes cell *a* (the true-positive results) and the second of which describes cell *b* (the false-positive error rate, or $p(T^+|D^-)$, and is multiplied by the prevalence of nonaffected animals, or $p(D^-)$. The true-positive results (a) divided by the true-positive plus false-positive results ($a + b$) gives $a/(a + b)$, which is the positive predictive value.

In genetics, an even simpler appearing formula for Bayes's theorem is sometimes used. The numerator is the same but the denominator is $p(T^+)$. This makes sense because the denominator in $a/(a + b)$ is equal to all those that have positive test results, whether they are true-positive or false-positive results.

25.10.1 Bayes's Theorem and Evaluation of Safety Assessment Studies

In a population with a low prevalence of a particular toxicity, most of the positive results in a screening program for that lesion or effect would be falsely positive. Although this does not automatically invalidate a study or assessment program, it raises some concerns about cost-effectiveness, and these can be explored using Bayes's theorem (Racine et al., 1986).

A program employing an immunochemical-stain-based test to screen tissues for a specific effect will be discussed as an example. This test uses small amounts of antibody tissues for a specific effect, and the presence of an immunologically bound stain is considered a positive result. If the sensitivity and specificity of the test and the prevalence of biochemical effect are known, Bayes's theorem can be used to predict what proportion of the tissues with positive test results will have true-positive results (actually be affected).

Example 25.3 shows how the calculations are made. If the test has a sensitivity of 96% and if the true prevalence is 1%, only 13.9% of tissues with a positive test result are predicted to actually be affected.

Pathologist and toxicologists can quickly develop a table that lists different levels of test sensitivity, test specificity, and effect prevalence and shows how these levels affect the proportion of positive results that are likely to be true-positive results. Although this calculation is fairly straightforward and is extremely useful, it has seldom been used in the early stages of planning for large studies or safety assessment programs.

25.10.2 Bayes's Theorem and Individual Animal Evaluation

Suppose a pathologist is uncertain about an animal's cause of death and obtains a positive test result for a certain pathology. Even if the pathologist knows the sensitivity and specificity of the test, that does not solve the problem, because to calculate the positive predictive value, it is necessary to know the prevalence of the particular tissue/effect that the test is designed to detect. The prevalence is thought of as the expected prevalence in the population from which the animal comes. The actual prevalence is usually not known, but often a reasonable estimate can be made.

Say, for example, a pathologist evaluates a male primate that was observed to have easy fatigability and has signs of kidney stones but has no other symptoms or signs of parathyroid disease on physical examination. The pathologist considers the probability of hyperparathyroidism and decides that it is now perhaps 2% (reflecting that in 100 such primates probably only 2 of them would have the disease). This probability is called the prior probability, reflecting the fact that it is estimated prior to the performance of laboratory tests and is based on the estimated prevalence of a particular pathology among primates with similar signs and symptoms. Although the pathologist believes that the probability of hyperparathyroidism is low, he or she considers the

Example 25.3 Use of Bayes's Theorem or 2 × 2 Table to Determine Positive Predictive Value of Hypothetical Tuberculin Screening Program

Part 1. Beginning Data

Sensitivity of immunological stain	96% = 0.96
False-negative error rate of test	4% = 0.04
Specificity of test	94% = 0.94
False-positive error rate of test	6% = 0.06]
Prevalence of effect in tissues	1% = 0.01

Part 2. Use of Bayes's Theorem

$$p(D^+|T^+) = \frac{p(T^+|D^+ p(D^+)}{[p(T^+|D^+)\,p(D^+)]+[PT^+|D^-)\,p(D^-)]}$$

$$= \frac{(\text{sensitivity})(\text{prevalence})}{(\text{sensitivity})(\text{prevalence})+(\text{false}-\text{postive error rate})(1-\text{prevalence})}$$

$$= \frac{(0.96)(0.01)}{(0.96)(0.01)+(0.06)(0.99)} = \frac{0.0096}{0.0096+0.0594} = \frac{0.0096}{0.0690}$$

$$= 0.139 - \mathbf{13.9\%}$$

Part 3. Use of 2 × 2 Table with Numbers Based on Assumption That 10,000 Tissues are in Study

	True Disease Status					
	Diseased		Nondeseased		Total	
	Number	Percentage	Number	Percentage	Number	Percentage
Test result						
Positive	96	96	594	6	690	7
Negative	4	4	9,306	94	9,310	93
Total	100	100	9,900	100	10,000	100

Positive predictive value = 96/690 = 0.139 = **13.9%**.

results of the serum calcium test to "rule" out the diagnosis. Somewhat to the pathologist's surprise, the results of the test were positive, with an elevated level of 12.2 mg dL^{-1}. He or she could order more special tests or stains for parathyroid disease, but some test results might come back positive and some negative.

Under the circumstances, Bayes's theorem could be used to make a second estimate of probability, which is called the posterior probability, reflecting the fact that it is made after the test results are known. Calculation of the posterior probability is based on the sensitivity and specificity of the test that was performed, which in this case was the serum calcium test, and on the prior prob-

ability, which in this case was 2%. If the serum calcium test had a 90% sensitivity and a 95% specificity, that means it had a false-positve error rate of 5% (specificity plus the false-positive error rate equals 100%). When this information is used in the Bayes equation, as shown in Example 25.4, the result is a posterior probability of 27%. This means that the patient is now in a group of primates with a significant possibility of parathyroid disease. In Example 25.4, note that the result is the same (i.e., 27%) when a 2×2 table is used. This is true because, as discussed above, the probability based on the Bayes theorem is identical to the positive predictive value.

In light of the 27% posterior probability, the pathologist decides to order a parathyroid hormone radioimmunoassay, even though this test is expensive. If the radioimmunoassay had a sensitivity of 95% and a specificity of 98% and the results turned out to be positive, the Bayes theorem could again be used to calculate the probability of parathyroid disease. This time, however, the

Example 25.4 Use of Bayes's Theorem or 2×2 Table to Determine Posterior Probability and Positive Predictive Value

Part 1. Beginning Data

Sensitivity of first test	90% = 0.90
Specificity of first test	95% = 0.95
Prior probability of disease	2% = 0.02

Part 2. Use of Bayes's Theorem

$$\begin{aligned} p(D^+|T^+) &= \frac{p(T^+|D^+)\,p(D^+)}{[p(T^+|D^+)\,p(D^+)]+[p(T^+|D^-)\,p(D^-]} \\ &= \frac{(0.90)(0.02)}{(0.90)(0.02)+(0.05)(0.98)} \\ &= \frac{0.018}{0.018+0.049} = \frac{0.018}{0.067} = 0.269 = 27\% \end{aligned}$$

Part 3. Use of 2×2 Table

	True Disease Status					
	Diseased		Nondiseased		Total	
	Number	Percentage	Number	Percentage	Number	Percentage
Test result						
Positive	18	90	49	5	67	6.7
Negative	2	10	931	95	933	93.3
Total	20	100	980	100	1000	100.0

Positive predictive value = 18/67 = 0.269 = 27%.

posterior probability for the first test (27%) would be used as the prior probability for the second test. The result of the calculation, as shown in Example 25.5, is a new probability of 94%. Thus, the primate in all probability did have hyperparathyroidism.

Why did the posterior probability increase so much the second time? One reason was that the prior probability was considerably higher in the second calculation than in the first (27% vs. 2%), based on the fact that the first test yielded positive results. Another reason was that the specificity of the second test was quite high (98%), which markedly reduced the false-positive error rate and therefore increased the positive predictive value.

Example 25.5 Use of Bayes's Theorem or 2 × 2 Table to Determine Second Posterior Probability and Second Positive Predictive Value

Part 1. Beginning Data

Sensitivity of first test	95% = 0.95
Specificity of first test	98% = 0.98
Prior probability of disease	27% = 0.27

Part 2. Use of Bayes's Theorem

$$\boldsymbol{p}(D^+|T^+) = \frac{p(T^+|D^+)\,p(D^+)}{[p(T^+|D^+)\,p(D^+)]+[p(T^+|D^-)\,p(D^-)]}$$
$$= \frac{(0.95)(0.27)}{(0.95)(0.27)+(0.02)(0.73)}$$
$$= \frac{0.257}{0.257+0.0146} = \frac{0.257}{0.272} = 0.9449^* = 94\%$$

Part 3. Use of 2 × 2 Table

	True Disease Status					
	Diseased		Nondiseased		Total	
	Number	Percentage	Number	Percentage	Number	Percentage
Test result						
Positive	256	95	15	2	271	27.1
Negative	13	5	716	98	729	72.9
Total	269	100	731	100	1000	100.0

Positive predictive value = 256/271 = 0.9446* = **94%**.
*The slight difference in the results for the two approaches is due to rounding errors. It is not important biologically.

25.11 DATA ANALYSIS APPLICATIONS IN SAFETY ASSESSMENT STUDIES

Having reviewed basic principles and provided a set of methods for statistical handling of data, the remainder of this chapter will address the practical aspects and difficulties encountered in working safety assessment.

There are now common practices in the analysis of safety data, though they are not necessarily the best. These are discussed in the remainder of this chapter, which seeks to review statistical methods on a use-by-use basis and to provide a foundation for the selection of alternatives in specific situations. Some of the newer available methodologies (meta-analysis and Bayesian approachs) should be kept in mind, however.

25.11.1 Body and Organ Weights

Among the sets of data commonly collected in studies where animals are dosed with (or exposed to) a chemical are body weight and the weights of selected organs. In fact, body weight is frequently the most sensitive indication of an adverse effect. How to best analyze this and in what form to analyze the organ weight data (as absolute weights, weight changes, or percentages of body weight) have been the subject of a number of articles (Jackson, 1962; Weil, 1962; Weil and Gad, 1980).

Both absolute body weights and rates of body weight change (calculated as changes from a baseline measurement value which is traditionally the animal's weight immediately prior to the first dosing with or exposure to test material) are almost universally best analyzed by ANOVA followed, if called for, by a post hoc test. Even if the groups were randomized properly at the beginning of a study (no group being significantly different in mean body weight from any other group, and all animals in all groups within two standard deviations of the overall mean body weight), there is an advantage to performing the computationally slightly more cumbersome (compared to absolute body weight) changes in body weight analysis. The advantage is an increase in sensitivity because the adjustment of starting points (the setting of initial weights as a "zero" value) acts to reduce the amount of initial variability. In this case, Bartlett's test is first performed to ensure homogeneity of variance and the appropriate sequence of analysis follows.

With smaller sample sizes, the normality of the data becomes increasingly uncertain and nonparametric methods such as Kruskal–Wallis may be more appropriate (Zar, 1974).

The analysis of relative (to body weight) organ weights is a valuable tool for identifying possible target organs (Lee, 1999; Bickis, 1990). How to perform this analysis is still a matter of some disagreement, however. Weil (1962) presented evidence that organ weight data expressed as percentages of body weight should be analyzed separately for each sex. Furthermore, since the conclusions from organ weight data of males differed so often from those of

females, data from animals of each sex should be used in this measurement. Others (Grubbs, 1969; Weil, 1973; Boyd and Knight, 1963; Boyd, 1972) have discussed in detail other factors which influence organ weights and must be taken into account.

The two competing approaches to analyzing relative organ weights call for either

1. calculating organ weights as a percentage of total body weight (at the time of necropsy) and analyzing the results by ANOVA or
2. analyzing results by ANCOVA, with body weights as the covariates, as discussed previously by the author (Weil and Gad, 1980).

A number of considerations should be kept in mind when these questions are addressed. First, one must keep a firm grasp on the difference between biological significance and statistical significance. In this particular case, we are especially interested in examining organ weights when an organ weight change is not proportional to changes in whole-body weights. Second, we are now required to detect smaller and smaller changes while still retaining a similar sensitivity (i.e., $p < 0.05$ level).

There are several devices to attain the desired increase in power. One is to use larger and larger sample sizes (number of animals) and the other is to utilize the most powerful test we can. However, the use of even currently employed numbers of animals is being vigorously questioned and the power of statistical tests must therefore now assume an increased importance in our considerations.

The biological rationale behind analyzing both absolute body weight and the organ weight–body weight ratio (this latter as opposed to a covariance analysis of organ weights) is that in the majority of cases, except for the brain, the organs of interest in the body change weight (except in extreme cases of obesity or starvation) in proportion to total body weight. We are particularly interested in detecting cases where this is not so. Analysis of actual data from several hundred studies (unpublished data) has shown no significant difference in rates of weight change of target organs (other than the brain) compared to total body weight for healthy animals in those species commonly used for repeated-dose studies (rats, mice, rabbits, and dogs). Furthermore, it should be noted that ANOVA is of questionable validity in analyzing body weight and related organ weight changes, since a primary assumption is the independence of treatment—that the relationship of the two variables is the same for all treatments (Ridgemen, 1975). Plainly, in toxicology this is not true.

In cases where the differences between the error mean squares are much greater, the F ratios will diverge in precision from the result of the efficiency of covariance adjustment. These cases are where either sample sizes are much larger or the differences between means themselves are much larger. This latter case is one which does not occur in the designs under discussion in any manner that would leave ANOVA as a valid approach because group means

start out being very similar and cannot diverge markedly unless there is a treatment effect. As we have discussed earlier, a treatment effect invalidates a prime underpinning assumption of ANOVA.

25.11.2 Clinical Chemistry

A number of clinical chemistry parameters are commonly determined on the blood and urine collected from animals in chronic, subchronic, and occasionally acute toxicity studies. In the past (and still in some places), the accepted practice has been to evaluate these data using univariate–parametric methods (primarily *t* tests and/or ANOVA). However, this can be shown to not be the best approach on a number of grounds.

First, such biochemical parameters are rarely independent of each other. Neither is our interest often focused on just one of parameters. Rather, there are batteries of parameters associated with toxic actions at particular target organs. For example, increases in creatinine phosphokinase (CPK), γ-hydroxybutyrate dehydrogenase (γ-HBDH), and lactate dehydrogenase (LDH) occurring together are strongly indicative of myocardial damage. In such cases, we are interested in not just a significant increase in one of these but in all three. Detailed coverage of the interpretation of such clinical laboratory tests can be found in other references (Martin et al., 1975; Harris, 1978; Gad and Chengelis, 1992; Loeb and Quimby, 1999) or elsewhere in this text.

Similarly, the serum electrolytes (sodium, potassium, and calcium) interact with each other; a decrease in one is frequently tied, for instance, to an increase in one of the others. Furthermore, the nature of the data (in the case of some parameters), either because of the biological nature of the parameter or the way in which it is measured, frequently is either not normally distributed (particularly because of being markedly skewed) or not continuous in nature. This can be seen in some of the reference data for experimental animals in Mitruka and Rawnsley (1977) or Weil (1982) in, for example, creatinine, sodium, potassium, chloride, calcium, and blood urea nitrogen.

25.11.3 Hematology

Much of what we said about clinical chemistry parameters is also true for the hematological measurements made in toxicology studies. Which test to perform should be evaluated by use of a decision tree until one becomes confident as to the most appropriate methods. Keep in mind that sets of values and (in some cases) population distribution vary not only between species but also between the commonly used strains of species and that "control" or "standard" values will "drift" over the course of only a few years.

Again, the majority of these parameters are interrelated and highly dependent on the method used to determine them. Red blood cell (RBC) count, platelet counts, and mean corpuscular volume (MCV) may be determined using a device such as a Coulter counter to take direct measurements, and the

resulting data are usually stable for parametric methods. The hematocrit, however, may actually be a value calculated from the RBC count and MCV values and, if so, is dependent on them. If the hematocrit is measured directly, instead of being calculated from the RBC count and MCV, it may be compared by parametric methods.

Hemoglobin is directly measured and is an independent and continuous variable. However, and probably because at any one time a number of forms and conformations (oxyhemoglobin, deoxyhemoglobin, methemoglobin, etc.) of hemoglobin are actually present, the distribution seen is not typically a normal one but rather may be a multimodal one. Here a nonparametric technique such as the Wilcoxon or multiple rank sum is called for.

Consideration of the white blood cell (WBC) and differential counts leads to another problem. The WBC count is, typically, a normal population amenable to parametric analysis, but differential counts are normally determined by counting, manually, one or more sets of 100 cells each. The resulting relative percentages of neutrophils are then reported as either percentages or are multiplied by the total WBC count with the resulting "count" being reported as the "absolute" differential WBC. Such data, particularly in the case of eosinophils (where the distribution does not approach normality), should usually be analyzed by nonparametric methods. It is widely believed that "relative" (%) differential data should not be reported because they are likely to be misleading.

Lastly, it should always be kept in mind that it is rare for a change in any single hematological parameter to be meaningful. Rather, because these parameters are so interrelated, patterns of changes in parameters should be expected if a real effect is present, and analysis and interpretation of results should focus on such patterns of changes. Classification analysis techniques often provide the basis for a useful approach to such problems.

25.11.4 Histopathological Lesion Incidence

The last 20 years have seen increasing emphasis placed on histopathological examination of tissues collected from animals in subchronic and chronic toxicity studies. While it is not true that only those lesions which occur at a statistically significantly increased rate in treated/exposed animals are of concern (for there are cases where a lesion may be of such a rare type that the occurrence of only one or a few such in treated animals "raises a flag"), it is true that, in most cases, a statistical evaluation is the only way to determine if what we see in treated animals is significantly worse than what has been seen in control animals. And although cancer is not our only concern, this category of lesions is that of greatest interest.

Typically, comparison of incidences of any one type of lesion between controls and treated animals are made using the multiple 2×2 chi-square test or Fisher's exact test with a modification of the numbers of animals as the denominators. Too often, experimenters exclude from consideration all those animals

(in both groups) which died prior to the first animals being found with a lesion at that site. The special case of carcinogenicity bioassays will be discussed in detail in Section 25.11.5.

An option which should be kept in mind is that, frequently, a pathologist can not only identify a lesion as present but also grade those present as to severity. This represents a significant increase in the information content of the data which should not be given up by performing an analysis based only on the perceived quantal nature (present/absent) of the data. Quantal data, analyzed by chi-square or Fisher's exact tests, are a subset (the 2×2 case) of categorical or contingency table data. In this case it also becomes ranked (or "ordinal") data—the categories are naturally ordered (e.g., no effect < mild lesion < moderate lesion < severe lesion). This gives a $2 \times R$ table if there are only one treatment and one control group or an $N \times R$ ("multiway") table if there are three or more groups of animals.

The traditional method of analyzing multiple, cross-classified data has been to collapse the $N \times R$ contingency table over all but two of the variables and to follow this with the computation of some measure of association between these variables. For an N-dimensional table this results in $N\,(N-1)/2$ separate analyses. The result is crude, "giving away" information and even (by inappropriate pooling of data) yielding a faulty understanding of the meaning of data. Though computationally more laborious, a multiway ($N \times R$ table) analysis should be utilized.

25.11.5 Carcinogenesis

In the experimental evaluation of substances for carcinogenesis based on experimental results in a nonhuman species at some relatively high dose or exposure level, an attempt is made to predict the occurrence and level of tumorogenesis in humans at much lower levels. An entire chapter could be devoted to examining the assumptions involved in this undertaking and review of the aspects of design and interpretation of animal carcinogenicity studies, and to special cases such as skin painting carcinogenicity studies (Wilson, 1982). Such is beyond the scope of this effort. The reader is referred to Gad (1998) for such an examination.

The single most important statistical consideration in the design of carcinogenicity bioassays in the past was based on the point of view that what was being observed and evaluated was a simple quantal response (cancer occurred or it did not) and that a sufficient number of animals needed to be used to have reasonable expectations of detecting such an effect. Though the single fact of whether or not the simple incidence of neoplastic tumors is increased due to an agent of concern is of interest, a much more complex model must now be considered. The time to tumor, patterns of tumor incidence, effects on survival rate, and age at first tumor all must now be included in a model.

The rationale behind this assumption is that, though humans may be exposed at very low levels, detecting the resulting small increase (over back-

ground) in the incidence of tumors would require the use of an impractically large number of test animals per group. This point was illustrated by Table 25.1, where, for instance, while only 46 animals (per group) are needed to show a 10% increase over a zero background (i.e., a rarely occurring tumor type), 770,000 animals (per group) would be needed to detect a tenth of a percent increase above a 5% background. As we increase dose, however, the incidence of tumors (the response) will also increase until it reaches the point where a modest increase (say 10% over a reasonably small background level (say 1%) could be detected using an acceptably small sized group of test animals (in Table 25.8 we see that 51 animals would be needed for this example case). There are, however, at least two real limitations to the highest dose level. First, the test rodent population must have a sufficient survival rate after receiving a lifetime (or two years) of regular doses to allow for meaningful statistical analysis. Second, we really want the metabolism and mechanism of action of the chemical at the highest level tested to be the same as at the low levels where human exposure would occur. Unfortunately, toxicologists usually must select the high dose level based only on the information provided by a sub-chronic or range-finding study (usually 90 days in length), but selection of either too low or too high a dose will make the study invalid for detection of carcinogenicity and may seriously impair the use of the results for risk assessment.

There are several solutions to this problem. One of these has been the rather simplistic approach of the NTP Bioassay Program, which is to conduct a three-month range-finding study with sufficient dose levels to establish a level which significantly (10%) decreases the rate of body weight gain. This dose is defined as the maximum tolerated dose (MTD) and is selected as the highest dose. Two other levels, generally one-half MTD and one-quarter MTD, are selected for testing at the intermediate and low dose levels. In many earlier NCI studies, only one other level was used.

TABLE 25.8 Average Number of Animals Needed to Detect Significant Increase in Incidence of Event (Tumors, Anomalies, etc.) Over Background Incidence (Control) at Several Expected Incidence Levels Using Fisher Exact Probability Test ($p = 0.05$)

	Expected Increase in Incidence, %					
Background Incidence, %	0.01	0.1	1	3	5	10
0	46,000,000[a]	460,000	4,600	511	164	46
0.01	46,000,000	460,000	4,600	511	164	46
0.1	47,000,000	470,000	4,700	520	168	47
1	51,000,000	510,000	5,100	570	204	51
5	77,000,000	770,000	7,700	856	304	77
10	100,000,000	1,000,000	10,000	1,100	400	100
20	148,000,000	1,480,000	14,800	1,644	592	148
25	160,000,000	1,600,000	16,000	1,840	664	166

[a]Number of animals needed in each group—controls as well as treated.

The dose range-finding study is necessary in most cases, but the suppression of body weight gain is a scientifically questionable benchmark when dealing with establishment of safety factors. Physiological, pharmacological, or metabolic markers generally serve as better indicators of systemic response than body weight. A series of well-defined acute and subchronic studies designed to determine the "chronicity factor" and to study onset of pathology can be more predictive for dose setting than body weight suppression.

Also, the NTP's MTD may well be at a level where the metabolic mechanisms for handling a compound at real-life exposure levels have been saturated or overwhelmed, bringing into play entirely artifactual metabolic and physiological mechanisms (Gehring and Blau, 1977). The regulatory response to questioning the appropriateness of the MTD as a high dose level (Haseman, 1985) has been to acknowledge that occasionally an excessively high dose is selected but to counter by saying that using lower doses would seriously decrease the sensitivity of detection.

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26

Combination Products: Drugs and Devices

26.1 INTRODUCTION

Recent years have seen a vast increase in the number of new therapeutic products which are not purely drug, device, or biological, but rather a combination of two or more of these. Classical examples are implanted drug delivery systems (whose primary function is drug delivery) and drug-impregnated devices (in which drug delivery is an adjunct to the device function). Congress first acknowledged the need for specific regulation of such combination products in the 1990 Safe Medical Device Act.

26.2 HISTORICAL BACKGROUND

The history of this category includes a variety of product types dating at least from the perfection of the hypodermic needle (1855). There are many modern examples of implanted delivery systems, such as the insulin pump (1980). One fundamental driving force for delivery systems has been the growth of new pharmaceutical products, especially since the dramatic expansion of drug research after 1945.

That research has led to the synthesis and testing of millions of compounds for pharmacological and antimicrobial properties. Indeed, today much of that development is performed in automated computer-controlled systems, leading

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to an even greater acceleration of the process. The continued emergence of a stream of novel and more complex combination products has blurred any distinguishing lines of regulatory authority and has complicated product designation and regulation. The issue of products combing a device and a drug, such as an asthma inhaler, has received considerable scrutiny over the past several years. But products combining a device and a biological, such as organ replacement or assist devices, have received less attention. Recent trends, however, suggest that device and biological combination products are quickly moving into the spotlight. A 1998 survey conducted by the U.S. Food and Drug Administration (FDA) identified hardware and tissue-engineered combination products as a rapidly growing trend in medical device technology (Herman et al., 1998).

Even less than drug and device combinations, device and biological products—which include, among other things, cellular and tissue implants, infused or encapsulated cells, artificial and replacement organs, heart valves and pumps, and cardiac, neural, and neuromuscular stimulation devices—do not fit neatly into existing regulatory paradigms (see Table 26.3 for a longer list of such products). For example, as part of the question of regulation, the FDA must take into account the possibility of tissue contamination and other hazards involved in using animal-derived tissues.

What has resulted to date is still a developing regulatory process. The written guidelines are fixed, but the day-to-day process is in flux (Merrill, 1994; March, 1998; Segal, 1999; Gopalaswamy and Gopalaswamy, 2008). It starts with determination of a principal mode of action (PMOA) which governs which center of the FDA will have the primary regulatory oversight. More recently, the FDA [Center for Drug Evaluation and Research (CDER), 2005] has promulgated a guidance on the nonclinical safety evaluation of drug–drug combinations. Such combinations are now becoming more frequent, most commonly by the 505(b)(2) route for approval. If the combination is of two approved and marketed products, the primary safety concern is for international (pharmacokinetics and pharmacodynamics). As such, the primary nonclinical safety studies that must be conducted are systemic toxicity studies of the fixed combination itself with toxicokinetic sampling components. Genetic toxicity and safety pharmacology studies of the combination are usually not required.

26.3 FUTURE TRENDS

Table 26.1 presents anticipated developments in the device combination product category between now and 2020 which lead to new clinical products. Three types of developments are generally expected. First, additional products designed for implanted delivery of insulin and other drugs. These include implanted pumps, possibly intelligent devices with improved biosensors to monitor concentrations in body fluids and make dynamic adjustments in delivery rates. Also there is the likely development of new polymeric timed-release

TABLE 26.1 Likelihood New Combination Device Drug Technologies

Biosensors	Biosensors (for glucose, implantation, and systemic infection markers), genetic diagnostics, laser diagnosis and treatment, minimally invasive devices
Blood vessel prosthetics	Genetic therapy, tissue-engineered vessels, nerves, and devices
Bone prosthetics/growth	Artificial organs, tissue-engineered devices
Cardiac stimulation	Intelligent devices, microminiaturized devices
Cartilage prosthetics	Tissue-engineered device
Computer-aided clinical labs	Computer-aided diagnosis, networks of devices
Drug-impregnated devices	Device/drug/biological products
Endoscopy	Minimally invasive devices, telemedicine, virtual reality diagnostics
Genetics—cancer	Genetic diagnostics, genetic therapy
Hearing aids	Intelligent devices, microminiaturized devices, nonimplanted sensory aids
Heart pumps	Artificial organs
Heart valves	Artificial organs, tissue-engineered devices, device/drug/biological products
Home diagnostics	Home/self-monitoring and diagnosis
Image contrast agents	Medical imaging
Imaging: functional, content	Medical imaging, minimally invasive devices, networks of devices
Implanted drug	Biosensors, device/drug/biological products, delivery systems, home/self-therapy, intelligent devices, robotic devices
Integrated patient medical info systems	Computer-aided diagnosis, networks of devices, telemedicine
Kidney prosthetics	Artificial organs, home/self-therapy, tissue-engineered devices
Laser surgery	Laser diagnosis and treatment
Liver prosthetics	Artificial organs, tissue-engineered devices
Minimum invasive cardiology	Minimally invasive devices, vascular surgery
Minimum invasive neurosurgery	Minimally invasive devices
Magnetic resonance imaging	Greater resolution imaging
Nanotechnology	Microminiaturized devices
Nerve regeneration	Tissue-engineered devices
Neural stimulation	Artificial organs, electrical stimulation, intelligent devices
Neuromuscular stimulation	Electrical stimulation, home/self-therapy
Ocular prosthetics	Artificial organs, electrical stimulation, intelligent devices
Pancreas prosthetics	Artificial organs, tissue-engineered devices
Patient smart cards	Computer-aided diagnosis, networks of devices, telemedicine
Positron emission tomography (PET) imaging	Combined PET and computer-aided tomography imaging
Robotic surgery	Microminiaturized devices, robotic devices
Skin prosthetics	Tissue-engineered devices
Telemedicine—home use	Home/self-monitoring and testing, diagnosis, telemedicine
Telemedicine—radiology	Telemedicine

devices which could improve the delivery of long-acting pharmaceuticals at optimized locations and rates.

Second, new developments in drug-impregnated devices are expected. Examples included new types of cardiac implants with antithrombogenic and anti-infective drugs as well as orthopedic implants with bacteriostatic coatings.

Finally, under development are new developments in drug delivery systems to simplify reliable use by unsophisticated patients in home settings, including the increasing elderly population. Examples included nasal and inhalation products.

Device regulation designation is by PMOA, which is generally straightforward but can become less clear as precedents accumulate and technology becomes more complex.

Although both extracorporeal and peritoneal dialysis systems are regulated as devices, dialysate concentrate for use with the former is a device but prepackaged dialysate for use with the latter is a drug. Sometimes consistency was elusive even when there was no combination, but just a single product. For example, in vitro diagnostics for detecting antibodies to HIV are regulated as biologicals when they are used for screening the blood supply but as medical devices when used for diagnostic or other screening purposes. When the FDA decides quickly and unequivocally on the regulatory status of a product, whether it was deemed a single product or was in combination with another product, there was relatively little opportunity for objection to the agency's decisions about how to regulate combination products and products whose status was uncertain. In the case of blood devices, the European Union (EU) has affirmed this process (Anonymous, 2000).

In the Safe Medical Devices Act of 1990 (SMDA), Congress took these issues in hand and amended the Federal Food, Drug and Cosmetic Act (FDCA) to make it easier for the FDA to regulate combination products in a rational fashion. The new provisions altered the substantive provisions of the FDCA only in minor respects. The main thrust of the new law was managerial, directing the FDA to make decisions about which center would have "primary jurisdiction" over a combination product based on the agency's understanding of the primary mode of action of the product.

For these products, the center's jurisdiction turns on the PMOA. If the primary mode of action is that of a drug, then the CDER has primary jurisdiction; if it is that of a device, jurisdiction is with the Center for Devices and Radiological Health (CDRH); if that of a biological product, the Center for Biologics Evaluation and Research (CBER) has this jurisdiction. As the statute prescribed, the regulations go on to state that the center with primary jurisdiction may consult with other agency components.

Although neither the statute nor the regulations explain what "primary jurisdiction" means, it seems clear that the FDA intends it to mean that the center that has primary jurisdiction will review the combination product and ordinarily give it just one approval, that is, a new drug application (NDA),

PMA (Product Marketing Application) or biological license application (BLA) as appropriate. Section 3.4(b) makes it clear, however, that the FDA's designation of one agency component as having primary jurisdiction does not preclude, in appropriate cases, the requirement for separate application, for example, a 510(k) and a BLA. When separate applications are required, both can be reviewed by the lead center, but "exceptional" cases may involve a second application to be reviewed by a different center. To facilitate this, the agency published new delegations giving officials in each of the three centers the authority to clear devices and to approve devices, drugs, biologicals, or any combination of two or more of them (FDA, 1991).

Contemporaneous with publication of the new regulations, the FDA made public three new intercenter agreements between CDRH and CBER, CDRH and CDER, and CDER and CBER. They describe the allocations of responsibility for numerous categories of specific products, both combination and noncombination. According to the regulations, these intercenter agreements are not binding; they are intended to "provide useful guidance to the public" and, as a practical matter, to FDA staff as well.

The intercenter agreements are a treasure trove of information. In addition to explicit guidance about which center has the lead with respect to particular products and whether one center or two will work on particular issues, they contain information and hints about whether the FDA believes it can regulate certain products at all, and if so, how (Pilot and Waldeermann 1998; Adams et al., 1997).

The regulations and intercenter agreements, however, do not answer every question, and the regulations recognize a role for the sponsor in cases of uncertainty. When the identity of the center with primary jurisdiction is unclear or in dispute or a sponsor believes its combination product is not covered by the intercenter agreements, a sponsor can request a designation from the FDA's product jurisdiction officer. A sponsor "should" file a request for designation with the product jurisdiction officer before submitting its application for marketing approval or an investigational notice. In practice, though, disputes or lack of clarity may not become evident until well into the review process, and it seems likely that the FDA would, if necessary, entertain requests for designation submitted at a later time.

Section 3.7(c) of the regulations lists the information to be included in the request, all of which must fit on 15 pages or less, including the identity of the sponsor, detailed information on the product, where the developmental work stands, the product's known modes of action and its primary mode of action, and, importantly, the sponsor's recommendation for which center should have primary jurisdiction and the reasons for the recommendation.

The FDA promises to check the request for designation for completeness within 5 working days of receipt and to issue a letter of designation within 60 days of receipt of a complete request. If the FDA does not meet the 60-day time limit, then the sponsor's recommendation for the appropriate lead center is honored.

The agency's letter of designation can be changed only with the sponsor's written consent or, if the sponsor does not consent, "to protect the public health or for other compelling reasons" (Gopalaswamy and Gopalaswamy, 2008). A sponsor must be given prior notice of any proposed nonconsensual change and must be given an opportunity to object in writing and at a "timely" meeting with the product jurisdiction officer and appropriate center officials.

The CDRH is designated the center for major policy development and for the promulgation and interpretation of procedural regulations for medical devices under the act. The CDRH regulates all medical devices inclusive of radiation-related device that are not assigned categorically or specifically to CDER. In addition, CDRH will independently administer the following activities (references to sections are the provisions of the act):

1. A. Small business assistance programs under Section 10 of the amendments [see Public Law (PL) 94–295]. Both CDER and CDRH will identify any unique problems relating to medical device regulation for small business.
   B. Registration and listing under Section 510 including some CDER-administered device applications. The CDRH will receive printouts and other assistance as requested.
   C. Color additives under Section 706, with review by CDER, as appropriate.
   D. Good Manufacturing Practices (GMPs) Advisory Committee. Under Section 520(f)(3), CDER will regularly receive notices of all meetings, with participation by CDER, as appropriate.
   E. Medical device reporting. The manufacturers, distributors, importers, and users of all devices, including those regulated by CDER, shall report to CDRH under Section 519 of the act as required. The CDRH will provide monthly reports and special reports as needed to CDER for investigation and follow-up of those medical devices regulated by CDER.

Table 26.2 presents the primary product responsibilities of CDER and CBER.

26.4 DEVICE PROGRAMS THAT CDER AND CDRH EACH WILL ADMINISTER

Both CDER and CDRH will administer and, as appropriate, enforce the following activities for medical devices assigned to their respective centers (references to sections are the provisions of the act):

1. A. Surveillance and compliance actions involving general controls violations, such as misbranded or adulterated devices under Sections 301, 501, and 502
   B. Warning letters, seizures, injunctions, and prosecutions under Sections 302, 303, and 304

TABLE 26.2 Product Class Review Responsibilities

Center for Drug Evaluation and Review
Natural products purified from plant or mineral sources
Products produced from solid tissue sources (excluding procoagulants, venoms, blood products, etc.)
Antibiotics, regardless of method of manufacture
Certain substances produced by fermentation
Disaccharidase inhibitors
HMG–CoA inhibitors
Synthetic chemicals
Traditional chemical synthesis
Synthesized mononuclear or polynuclear products including antisense chemicals
Hormone products
Center for Biologics Evaluation and Review
Vaccines, regardless of manufacturing method
In vivo diagnostic allergenic products
Human blood products
Protein, peptide, and/or carbohydrate products produced by cell culture (other than antibiotics and hormones)
Immunoglobulin products
Products containing intact cells or microorganisms
Proteins secreted into fluids by transgenic animals
Animal venoms
Synthetic allergens
Blood banking and infusion adjuncts

C. Civil penalties under Section 303(f) and administrative restraint under Section 304(g)
D. Nonregulatory activities, such as educational programs directed at users, participation in voluntary standards organizations, and so on
E. Promulgation of performance standards and applications of special controls under Section 514
F. Premarket notification, investigational device exemptions including humanitarian exemptions, premarket approval, product development protocols, classification, device tracking, petitions for reclassification, postmarket surveillance under Sections 510(k), 513, 515, 519, 520(g) and (m), and 522, and the advisory committees necessary to support these activities
G. Banned devices under Section 516
H. FDA-requested and firm-initiated recalls whether under Section 518 or another authority and other Section 518 remedies such as recall orders
I. Exemptions, variances, and applications of current GMP (CGMP) regulations under Section 520(f)
J. Governmentwide quality assurance program
K. Requests for export approval under Sections 801(e) and 802

TABLE 26.3 Examples of Existing Device–Drug Combination Products

Cardiac output catheter	Heparin	As device in U.K.
Extracorporeal sets	Heparin	As device in U.K.
Viscose/rayon dressings	Povidone iodine	As drug in U.K.
Cardiovascular oxygenator	Heparin	As drug in U.K. defoamer reservoir
Paste bandages	Clioquinol, coal tar, calamine	As drug in U.K. (if they have antiinfective ancillary action)
Medicated tulle dressings	Chlorhexidine	As drug in U.K.
Antimicrobial drape	Iodophore	As device in U.K.
Antiseptic wipes	Chlorhexidine, centrimide, alcohol	As drug in U.K.
Cardiovascular guidewires	Heparin	As device in Spain
Guidewires	Heparin	As device in Spain, Switzerland, U.K.
Antibiotic bone cement	Antibiotic (e.g., gentamicin sulfate), colistin sulfomethate, sodium, erythromycin	As drug (but soon to be regulated as device)
Extracorporeal cardiotomy reservoirs and filters	Heparin	As devices in Spain, Benelux, Italy
Extracorporeal venous reservoirs and filters	Heparin	As devices in Spain, Benelux, Italy
Bacteriostatic urological catheters	Silver	As devices in three Benelux countries
Antiseptic island dressing	Chlorhexidine digluconate	As device in Italy
Spermicidal condoms	Nonoxynol-9	As device in Germany
Pacemaker lead with porous tip (seulte)	Dexamethasone	As device
Pacemaker lead with protector mannitol capsule (Sweet Tip)	Mannitol	As device
Biomedicus centrifugal pump	Heparin	Not applicable
Peripheral vascular cannulae	Heparin	Not applicable
Surgical gauzes or nonwoven fabrics impregnated with iodophore	Iodophore	Not applicable
Surgical gauzes or nonwoven fabrics impregnated with alignates and Clioquinol	Clioquionol (NaCa alignates, clauden powder)	As device in Germany
Vascular prosthesis	Collagen, albumen	As devices in U.K.

26.5 COORDINATION

The centers will coordinate their activities in order to assure that manufacturers do not have to independently secure authorization to market their product from both centers unless this requirement is specified in Section VII.

26.6 SUBMISSIONS

Submissions should be made to the appropriate center, as specified herein, at the following addresses: Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Central Document Room (CDR), 5901-B Ammendale Road, Beltsville, Maryland 20705-1266 or Food and Drug Administration, Center for Devices and Radiological Health, Document Mail Center (HFZ-401), 9200 Corporate Blvd., Rockville, MD 20850.

For submissions involving medical devices and/or drugs that are not clearly addressed in this agreement, sponsors are referred to the product jurisdiction regulations (21 CFR Part 3). These regulations have been promulgated to facilitate the determination of regulatory jurisdiction but do not exclude the possibility for a collaborative review between the centers.

26.7 CENTER JURISDICTION

The following subsections provide details concerning status, market approval authority, special label/regulatory considerations, investigational options, and intercenter consultations for the categories of products specified. Section VII provides the general criteria that CDRH and CDER will apply in reaching decisions as to which center will regulate a product.

A. 1. (a) Device with primary purpose of delivering or aiding in the delivery of a drug that is distributed without a drug (i.e., unfilled)

Examples

Devices that calculate drug dosages

Drug delivery pump and/or catheter infusion pump for implantation iontophoresis device

Medical or surgical kit (e.g., tray) with reference in instructions for use with specific drug (e.g., local anesthetic)

Nebulizer

Small-particle aerosol generator (SPAG) for administering drug to ventilated patient

Splitter block for mixing nitrous oxide and oxygen

Syringe, jet injector, storage and dispensing equipment

Status Device and drug as separate entities

Market Approval Authority CDRH and CDER, respectively, unless the intended use of the two products, through labeling, creates a combination product

Special Label/Regulatory Considerations The following specific procedures will apply depending on the status of the drug delivery device and drugs that will be delivered with the device:

(i) It may be determined during the design or conduct of clinical trials for a new drug that it is not possible to develop adequate performance specifications data on those characteristics of the device that are required for the safe and effective use of the drug. If this is the case, then drug labeling cannot be written to contain information that makes it possible for the user to substitute a generic, marketed device for the device used during development to use with the marketed drug. In these situations, CDER will be the lead center for regulation of the device under the device authorities.

(ii) For a device intended for use with a category of drugs that are on the market, CDRH will be the lead center for regulation for the device under the device authorities. The effects of the device use on drug stability must be addressed in the device submission, when relevant. An additional showing of clinical effectiveness of the drug when delivered by the specific device will generally not be required. The device and drug labeling must be mutually conforming with respect to indication, general mode of delivery (e.g., topical, IV), and drug dosage/schedule equivalents.

(iii) For a drug delivery device and drug that are developed for marketing to be used together as a system, a lead center will be designated to be the contact point with the manufacturer(s). If a drug has been developed and marketed and the development and study of device technology predominate, the principal mode of action will be deemed to be that of the device, and CDRH would have the lead. If a device has been developed and marketed and the development and study of drug predominate, then, correspondingly, CDER would have the lead. If neither the drug nor the device is on the market, the lead center will be determined on a case-by-case basis.

Investigation Options IDE (Investigational Device Exemption) or IND as appropriate

Intercenter Consultation CDER, when the lead center, will consult with CDRH if CDER determines that a specific device is required as part of the NDA process. CDRH as lead center will consult with CDER if the device is intended for use with a marketed drug and the device creates a significant change in the intended use, mode of delivery (e.g., topical, IV), or dose/schedule of the drug.

1. Device with primary purpose of delivering or aiding in the delivery of a drug and distributed containing a drug (i.e., "pre-filled delivery system")

Examples
Nebulizer
Oxygen tank for therapy and over-the-counter (OTC) emergency use
Prefilled syringe

Transdermal patch

Status Combination product

Market Approval Authority CDER using drug authorities and device authorities as necessary

Special Label/Regulatory Considerations None

Investigation Options IND

Intercenter Consultations Optional

2. Device incorporating a drug component with the combination product having the primary intended purpose of fulfilling a device function

Examples

Bone cement containing antimicrobial agent
Cardiac pacemaker lead with steroid-coated tip
Condom, diaphragm, or cervical cap with contraceptive or antimicrobial agent (including virucidal) agent
Dental device with fluoride
Dental wood wedge with hemostatic agent
Percutaneous cuff (e.g., for a catheter or orthopedic pin) coated/impregnated with antimicrobial agent
Skin closure or bandage with antimicrobial agent
Surgical or barrier drape with antimicrobial agent
Tissue graft with antimicrobial or other drug agent
Urinary and vascular catheter coated/impregnated with antimicrobial agent
Wound dressing with antimicrobial agent

Status Combination product

Market Approval Authority CDRH using device authorities

Special Label/Regulatory Considerations These products have a drug component that is present to augment the safety and/or efficacy of the device.

Investigation Options IDE

Intercenter Consultation Required if a drug or the chemical form of the drug has not been legally marketed in the United States as a human drug for the intended effect

3. Drug incorporating a device component with the combination product having the primary intended purpose of fulfilling a drug function

Examples

Skin-prep pads with antimicrobial agent
Surgical scrub brush with antimicrobial agent

Status Combination product

Market Approval Authority CDER using drug authorities and, as necessary, device authorities

Special Label/Regulatory Considerations Marketing of such a device requires a submission of an NDA with safety and efficacy data on the drug component or that it meet monograph specifications as generally

recognized as safe (GRAS) and generally recognized as effective (GRAE). Drug requirements (e.g., CGMPs, registration and listing, experience reporting) apply to products.

Investigation Options IND

Intercenter Consultation Optional

4. (a) Device used in the production of a drug either to deliver directly to a patient or for use in the producing medical facility (excluding use in a registered drug manufacturing facility)

Examples
Oxygen concentrators (home or hospital)
Oxygen generator (chemical)
Ozone generator

Status Device

Market Approval Authority CDER, applying both drug and device authorities

Special Label/Regulatory Consideration May also require an NDA if the drug produced is a new drug. Device requirements (e.g., CGMPs, registration and listing, experience reporting) will apply to products.

Investigation Options IDA or NDA as appropriate

Intercenter Consultation Optional

(b) Drug/device combination product intended to process a drug into a finished package form

Examples
Device that uses drug concentrates to prepare large-volume parenterals
Oxygen concentrator (hospital) output used to fill oxygen tanks for use within that medical facility

Status Combination product

Market Approval Authority CDER, applying both drug and device authorities

Special Label/Regulatory Considerations Respective drug and device requirements (e.g., CGMPs, registration and listing, experience reporting) will apply.

Investigation Options IDE or IND as appropriate

Intercenter Consultation Optional but will be routinely obtained

B. Device used concomitantly with a drug to directly activate or to augment drug effectiveness.

Examples
Biliary lithotriptor used in conjunction with dissolution agent
Cancer hyperthermia used in conjunction with chemotherapy

Current generator used in conjunction with an implanted silver electrode (drug) that produces silver ions for an antimicrobial purpose

Materials for blocking blood flow temporarily to restrict chemotherapy drug to the intended site of action

Ultraviolet and/or laser activation of oxsoralen for psoriasis or cutaneous T-cell lymphoma

Status Device and drug as separate entities

Market Approval Authority CDRH and CDER, respectively

Special Label/Regulatory Considerations The device and drug labeling must be mutually conforming with respect to indications, general mode of delivery (e.g., topical, IV), and drug dosage/schedule equivalence. A lead center will be designated to be the contact point with the manufacturer. If a drug has been developed and approved for another use and development and study of device technology predominate, then CDRH would have the lead. If a device has been developed and marketed for another use and development and study of drug action predominate, then CDER would have the lead. If neither the drug nor the device is on the market, the lead center will be determined on a case-by-case basis. If the labeling of the drug and device create a combination product, as defined in the combination product regulations, then the designation of the lead center for both applications will be based upon a determination of the product's primary mode of action.

Investigation Options IDE or IND as appropriate.

Intercenter Consultations Required

2. Device kits labeled for use with drugs that include both device(s) and drug(s) as separate entities in one package with the overall primary intended purpose of the kit fulfilling a device function

   Example

   Medical or surgical kit (e.g., tray) with drug component

Status Combination product

Market Approval Authority CDRH, using device authorities, is responsible for the kit if the manufacturer is repackaging a market drug. Responsibility for overall packaging resides with CDRH. CDER will be consulted as necessary on the use of drug authorities for the repackaged drug component.

Special Label/Regulatory Consideration Device requirements (e.g., CGMPs, registration and listing, experience reporting) apply to kits. Device manufacturers must assure that manufacturing steps do not adversely affect drug components of the kit. If the manufacturing steps do affect the marketed drug (e.g., the kit is sterilized by irradiation), an ANDA or NDA would also be required with CDRH as the lead center.

Investigation Options IDA or IND as appropriate

Intercenter Consultation Optional if ANDA or NDA not required

C. Liquids, gases, or solids intended for use as devices (e.g., implanted, or components, parts, or accessories to devices)

Examples
Dye for tissues used in conjunction with laser surgery to enhance absorption of laser light in target tissue
Gas mixtures for pulmonary function-testing devices
Gases used to provide "physical effects"
Hemodialysis fluids
Hemostatic devices and dressings
Injectable silicon, collagen, and Teflon
Liquids functioning through physical action applied to the body to cool or freeze tissues for therapeutic purposes
Liquids intended to inflate, flush, or moisten (lubricate) indwelling device (in or on the body)
Lubricants and lubricating jellies
Ophthalmic solutions for contact lenses
Organ/tissue transport and/or perfusion fluid with antimicrobial or other drug agent, that is, preservation solutions
Powders for lubricating surgical gloves
Sodium hyaluronate or hyaluronic acid for use as a surgical aid
Solution for use with dental "chemical drill"
Spray-on dressings not containing a drug component

Status Device

Market Approval Authority CDRH

Special Label/Regulatory Considerations None

Investigation Options IDE

Intercenter Consultation Required if the device has direct contact with the body and the drug or the chemical form of the drug has not been legally marketed as a human drug

D. Products regulated as drugs

Examples
Irrigation solutions
Purified water or saline in prefilled nebulizers for use in inhalation therapy
Skin protectants (intended for use on intact skin)
Sun screens
Topical/internal analgesic-antipyretic

Status Drug

Market Approval Authority CDER

Special Label/Regulatory Considerations None

Investigation Options IND

Intercenter Consultations Optional

E. Ad hoc jurisdictional decisions
Examples

	Status	Center
Motility marker constructed of radiopaque plastic	Device	CDRH
Brachytherapy capsules, needles, etc., that are radioactive and may be removed from the body after radiation therapy has been administered	Device	CDRH
Skin markers	Device	CDRH

Status Device or drug
Market Approval Authority CDRH or CDER as indicated
Special Label/Regulatory Considerations None
Investigation Options IDE or IND as appropriate
Intercenter Consultation Required to assure agreement on drug/device status

26.8 GENERAL CRITERIA AFFECTING DRUG/DEVICE DETERMINATION

The following represent the general criteria that will apply in making device/drug determinations:

A. *Device Criteria*
1. A liquid, powder, or other similar formulation intended only to serve as a component, part, or accessory to a device with a primary mode of action that is physical in nature will be regulated as a device by CDRH.
2. A product that has the physical attributes described in 201(h) (e.g., instrument, apparatus) of the act and does not achieve its primary intended purpose through chemical action within or on the body or by being metabolized will be regulated as a device by CDRH.
3. The phrase "within or on the body" as used in 201(h) of the act does not include extra corporeal systems or the solutions used in conjunction with such equipment. Such equipment and solutions will be regulated as devices by CDRH.
4. An implant, including an injectable material, placed in the body for primarily a structural purpose even though such an implant may be absorbed or metabolized by the body after it has achieved its primary purpose will be regulated as a device by CDRH.
5. A device containing a drug substance as a component with the primary purpose of the combination being to fulfill a device function is a combination product and will be regulated as a device by CDRH.

6. A device (e.g., machine or equipment) marketed to the user, pharmacy, or licensed practitioner that produces a drug will be regulated as a device or combination product by CDER. This does not include equipment marketed to a registered drug manufacturer.
7. A device whose labeling or promotional materials make reference to a specific drug or generic class of drugs unless it is prefilled with a drug ordinarily remains a device regulated by CDRH. It may, however, also be subject to the combination products regulation.

B. *Drug Criteria*
1. A liquid, powder, tablet, or other similar formulation that achieves its primary intended purpose through chemical action within or on the body or by being metabolized, unless it meets one of the specified device criteria, will be regulated as a drug by CDER.
2. A device that serves as a container for a drug or a device that is a drug delivery system attached to the drug container where the drug is present in the container is a combination product that will be regulated as a drug by CDER.
3. A device containing a drug substance as a component with the primary purpose of the combination product being to fulfill a drug purpose is a combination product and will be regulated as a drug by CDER.
4. A drug whose labeling or promotional materials make reference to a specific device or generic class of devices ordinarily remains a drug regulated by CDER. It may, however, also be subject to the combination products regulation.

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27

Qualification of Impurities, Degradants, Residual Solvents, and Leachables in Pharmaceuticals

Impurities (from either materials intended to be part of the process of making a drug or formulating it), degradants (unintentionally formed by unintended reactions after the drug substance or product is produced and prone to increase in quantity over time due to instability of the product under conditions of storage), and residual solvents (purposely added to the synthesis product to facilitate synthesis, formulation, or dosage form production—always liquids of some degree of volatility) can become part of a drug product or substance in multiple ways. But the extent of their presence is now strictly governed by a series of International Conference on Harmonisation (ICH), U.S. Food and Drug Administration (FDA), and European Medicines Agency (EMEA) guidelines. These ICH guidelines call for these materials to be present at levels no greater than in product specifications and (because they serve no functional purpose in the drug) are both to be kept to a practical minimum and must be qualified for safety at the highest specification levels under the assumption of maximum potential patent use of the drug (and, therefore, exposure to the

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unintended substances). Contaminants coming from the vessels and machinery used to manufacture a drug but not intended to be present and leachables/extractants are materials which transfer to a drug product from packaging or delivery systems. Undesired chemical substances which end up in pharmaceutical products may have a range of sources, but all of these must be evaluated for potential risks to patients. Depending on the source of the undesired substance, it is categorized and regulated somewhat differently. Categories of such substances include impurities, degradants, residual solvents, contaminants, leachables, and extractables. Each of these will be considered in this chapter.

Biologicals and biotechnology products have both similar and unique process impurity issues. The process needs to ensure that there are no residual cellular components in the biological product. Biotechnology processing needs to avoid causing structural deformities to the protein. In all cases, the process must be scrutinized closely. Checking for impurities at various steps throughout the manufacturing phase may help to pinpoint where the impurities are produced.

27.1 IMPURITIES

The ICH (2003a) "Guidance for Industry, Q3A: Impurities in New Drug Substances," is intended to "provide guidance for registration applications on the content and qualification of impurities in new drug substances produced by chemical syntheses and not previously registered in a region or member state" (p. 1). A new drug substance is not the final marketed product but the active ingredient used in the marketed product. Impurities in new drug substances are addressed from both a chemistry and safety perspective.

The guidance is not intended to apply to new drug substances during the clinical research stage of development (though such drugs in development must have consideration of meeting these requirements at the time of marketing approval) but rather addresses safety concerns associated with such substances during development. Nor does it cover natural product or biological process–produced drugs or extraneous contaminants that should not occur in new drug substances and are more appropriately addressed as good manufacturing practice (GMP) issues. The guidance further describes the circumstances in which impurities need to be reported, identified, and qualified.

The rationale for the reporting and control, identification, and qualification of impurities is discussed in the guidance. Organic impurities need to be summarized based on the actual and potential impurities most likely to arise during the synthesis, purification, and storage of a new drug substance. This discussion can be limited to those impurities that might reasonably be expected based on knowledge of the chemical reactions and conditions involved.

Studies conducted to characterize the structure of impurities present in a new drug substance at a level greater than the identification threshold (Table 27.1) should be described and any impurity from any batch or degradation

TABLE 27.1 Thresholds for Action on Impurities in Drug Product

Maximum Daily Dose[a]	Reporting Threshold[b,c]	Identification Threshold[c]	Qualification Threshold[c]
≤2 g day^{-1}	0.05%	0.10% or 1.0 mg day^{-1} intake (whichever is lower)	0.15% or 1.0 mg day^{-1} intake (whichever is lower)
>2 g day^{-1}	0.03%	0.05%	0.05%

[a]The amount of drug substance administered per day.
[b]Higher reporting thresholds should be scientifically justified.
[c]Lower thresholds can be appropriate if the impurity is unusually toxic.

product from stability studies should be identified. If identification of an impurity or degradant is not feasible, a summary of the laboratory studies demonstrating the unsuccessful effort should be included in the application. If an impurity is pharmacologically or toxicologically active, identification of the compound should be conducted even if the impurity level is below the identification threshold.

The guidance also states:

> Qualification is the process of acquiring and evaluating data that establishes the biological safety of an individual impurity or a given impurity profile at the level(s) specified. The applicant should provide a rationale for establishing impurity acceptance criteria that includes safety considerations. The level of any impurity that is present in a new drug substance that has been adequately tested in safety and/or clinical studies would be considered qualified. Impurities that are also significant metabolites present in animal and/or human studies are generally considered qualified. A level of a qualified impurity higher than that present in a new drug substance can also be justified based on an analysis of the actual amount of impurity administered in previous relevant safety studies. If data are unavailable to qualify the proposed acceptance criterion of an impurity, safety studies to obtain such data can be appropriate when the usual qualification thresholds are exceeded.

ICH (2003b) Q3B(R) describes considerations for the qualification of impurities when thresholds are exceeded. If the level of impurity cannot be decreased to below the threshold or if adequate data is not available in the scientific literature to justify safety, then additional safety testing should be considered. The studies considered appropriate to qualify an impurity will depend on a number of factors, including the patient population, daily dose, and route and duration of administration. Toxicology studies are discussed briefly later in this chapter and in more detail in other chapters in this volume. Such studies can be conducted on the new drug substance containing the impurities to be controlled, although studies using isolated impurities can sometimes be appropriate.

ICH Q3A states that "safety assessment studies to qualify an impurity should compare the new drug substance containing a representative amount

of the new impurity with previously qualified material. Safety assessment studies using a sample of the isolated impurity can also be considered." The latter is especially important to consider for genetic toxicology studies and the importance of testing the isolated impurity is discussed in more detail at the end of this chapter.

Therefore, according to the guidance, if the maximum daily dose of the drug is less than 2 g day^{-1} and the impurity intake is more than 0.15% or 1.0 mg day^{-1}, the qualification threshold has been reached, meaning safety studies will need to be performed. Lower thresholds can be appropriate if the impurity is unusually toxic. In addition, the impurity will need to be reported and identified. These studies include general and genetic toxicology studies, and possibly other specific toxicology endpoints, as appropriate. Discussion of specific toxicity testing with the relevant FDA division is recommended.

If considered desirable, a minimum screen (e.g., genotoxic potential) should be conducted. A study to detect point mutations and one to detect chromosomal aberrations, both in vitro, are considered an appropriate minimum screen.

Qualification studies for impurities are essentially bridging studies. If general toxicity studies are desirable, one or more studies should be designed to allow comparison of unqualified to qualified material. The study duration should be based on available relevant information and performed in the species most likely to maximize the potential to detect the toxicity of a degradation product. On a case-by-case basis, single-dose studies can be appropriate, especially for single-dose drugs. In general, a minimum duration of 14 days and a maximum duration of 90 days would be considered appropriate.

The genetic toxicology studies can include a minimum screen (a study to detect point mutations and one to detect chromosome aberrations, both in vitro). The general toxicology studies should include one or more studies designed to allow comparison of unqualified to qualified material. The study duration should be based on available relevant information and performed in the species most likely to maximize the potential to detect the toxicity of an impurity. On a case-by-case basis, single-dose studies can be appropriate, especially for single-dose drugs. In general, a minimum duration of 14 days and a maximum duration of 90 days would be considered appropriate.

Inorganic impurities are normally detected and quantified using pharmacopeial or other appropriate procedures. The need for inclusion or exclusion of inorganic impurities in a new drug substance specification should be discussed. Acceptance criteria should be based on pharmacopeia standards or known safety data. The control of residues of the solvents used in the manufacturing process for a new drug substance should be discussed and presented according to ICH (2003c) Q3C.

A registration application should include documented evidence that the analytical procedures are validated and suitable for the detection and quantification of impurities. Organic impurity levels can be measured by a variety of techniques, including those that compare an analytical response for an

TABLE 27.2 Threshold for Degradation Products in New Drug Products

Maximum Daily Dose[a]	Threshold[b,c]
Reporting Thresholds	
≤1 g	0.1%
>1 g	0.05%
Identification Thresholds	
<1 mg	1.0% or 5 μg TDI, whichever is lower
1–10 mg	0.5% or 20 μg TDI, whichever is lower
>10 mg–2 g	0.2% or 2 mg TDI, whichever is lower
>2 mg	0.10%
Qualification Thresholds	
<10 mg	1.0% or 50 μg TDI, whichever is lower
10–100 mg	0.5% or 200 μg TDI, whichever is lower
>100 mg–2 g	0.2% or 3 mg TDI, whichever is lower
>2 g	0.15%

[a]The amount of drug substance administered per day.
[b]Thresholds for degradation products are expressed either as a percentage of the drug substance or as total daily intake (TDI) of the degradation product. Lower thresholds can be appropriate if the degradation product is unusually toxic.
[c]Higher thresholds should be scientifically justified.

impurity to that of an appropriate reference standard or to the response of the new drug substance itself. Differences in the analytical procedures used during development and those proposed for the commercial product should be discussed in the registration application. Analytical results should be provided in an application for all batches of a new drug substance used for clinical, safety, and stability testing as well as for batches representative of the proposed commercial process. The application should also contain a table that links the specific new drug substance batch to each safety study and each clinical study in which the new drug substance has been used. Any impurity at a level greater than the reporting threshold (Table 27.1) and total impurities observed in these batches of the new drug substance should be reported with the analytical procedures indicated. Table 27.2 is an illustration of reporting impurity results for identification and qualification in an application.

The guidance also states that when analytical procedures change results provided in the application should be linked to the procedure used, with appropriate validation information provided, including representative chromatograms of representative batches. The applicant should ensure that complete impurity profiles (e.g., chromatograms) of individual batches are available, if requested.

The ICH Q3A guidance also states that the specification for a new drug substance should include a list of impurities. Individual impurities with specific

acceptance criteria included in the specification for a new drug substance are referred to as specified impurities. Specified impurities can be identified or unidentified. A rationale for the inclusion or exclusion of impurities in a specification should be presented.

> According to ICH (2000): Acceptance criteria should be set no higher than the level that can be justified by safety data and should be consistent with the level achievable by the manufacturing process and the analytical capability. Where there is no safety concern, impurity acceptance criteria should be based on data generated on batches of a new drug substance manufactured by the proposed commercial process, allowing sufficient latitude to deal with normal manufacturing and analytical variation and the stability characteristics of the new drug substance. Although normal manufacturing variations are expected, significant variation in batch-to-batch impurity levels can indicate that the manufacturing process of the new drug substance is not adequately controlled and validated.

ICH "Q3B(R): Impurities in New Drug Products" was published November 2003 and is intended to provide guidance for registration applications on the content and qualification of impurities in new drug products produced from chemically synthesized new drug substances not previously registered in a region or member state. A new drug product is a finished dosage form, for example, a tablet, capsule, or solution, that contains a drug substance generally but not necessarily in association with one or more other ingredients. The Q3B(R) guidance complements the ICH guidance "Q3A: Impurities in New Drug Substances," which should be consulted for basic principles along with ICH "Q3C: Impurities: Residual Solvents" when appropriate.

Q3A addresses only those impurities in new drug products classified as degradation products of the drug substance or reaction products of the drug substance with an excipient and/or immediate container closure system (collectively referred to as *degradation products*). Generally, impurities present in a new drug substance need not be monitored or specified in a new drug product unless they are also degradation products. This guidance does not address impurities arising from excipients present in a new drug product or extracted or leached from the container closure system. This guidance also does not apply to new drug products used during the clinical research stages of development. It also does not cover the same types of products as in ICH Q3A(R): biological/biotechnological, peptides, oligonucleotides, radiopharmaceuticals, fermentation products and associated semisynthetic products, herbal products, and crude products of animal or plant origin. Also excluded from this guidance are extraneous contaminants that should not occur in new drug products and are more appropriately addressed as GMP issues, polymorphic forms, and enantiomeric impurities.

Qualification of an impurity for a new drug substance has similar concerns as Q3A. The main differences are the reporting, identification, and qualification thresholds (Table 27.2). The thresholds are basically higher than they were in Q3A; however, there are more categories for dosages. If the qualification

TABLE 27.3 Illustration of Reporting Degradation Product Results for Identification and Qualification in an Application

Raw Result (%)	Reported Result (%)	Total Daily Intake (TDI) of Degradation Product	Action	
			Identification Threshold	Qualification Threshold
		50 mg Maximum Daily Dose[a]		
0.04	Not reported	20	None	None
0.2143	0.2	100	None	None
0.349	0.3	150	Yes	None
0.550	0.6	300	Yes	Yes
		1.9 g Maximum Daily Dose[b]		
0.049	Not reported	1	None	None
0.079	0.08	2	None	None
0.183	0.18	3	Yes	None[c,d]
0.192	0.19	4	Yes	Yes[c]

[a]Reporting threshold 0.1%; TDI rounded result in-micrograms; identification threshold 0.2%; qualification threshold, TDI equivalent to 0.4%.

[b]Reporting threshold 0.05%; TDI rounded result in millingrams; identification threshold 2 mg TDI (equivalent to 0.11%); qualification threshold 3 mg TDI (equivalent to 0.16%).

[c]After identification, if the response factor is determined to differ significantly from the original assumptions, it can be appropriate to remeasure the actual amount of the degradation product present and reevaluate against the qualification threshold.

[d]Although the reported result of 0.18% exceeds the calculated threshold value of 0.16%, in this case the action is acceptable since the TDI (when rounded) does not exceed 3 mg. Chromatograms with peaks labeled (or equivalent data if other analytical procedures are used) from representative batches, including chromatograms from analytical procedure validation studies and from long-term and accelerated stability studies, should be provided. The applicant should ensure that complete degradation product profiles (e.g., chromatograms) of individual batches are available, if requested.

thresholds given in Table 27.3 are exceeded and data are unavailable to qualify the proposed acceptance criterion of a degradation product, additional studies to obtain such data may be appropriate.

U.S. FDA (CDER) "Guidance for Industry, NDAs: Impurities in Drug Substances" was published in February 2000. The guidance refers applicants to ICH "Q3A: Impurities in New Drug Substances" when seeking guidance on identification, qualification, and reporting of impurities in drug substances that are not considered new drug substances. Q3A was developed by the ICH to provide guidance on the information that should be provided in a new drug application (NDA) in support of impurities in new drug substances that are produced by chemical syntheses. The FDA believes that such guidance on identification, qualification, and reporting of impurities should also be considered when evaluating impurities in drug substances produced by chemical syntheses that are not considered new drug substances. ICH Q3A defines a new drug substance (also referred to as a new molecular entity or new chemical entity) as a designated therapeutic moiety that has not been

TABLE 27.4 Amount of Residual Solvent per Day at Maximum Clinical Dose (mg day^{-1}) of Drug

Residual Solvent	Concentration in Drug Product	Potential Maximum Clinical exposure (mg day^{-1})	Reporting Threshold (0.05%)	Qualification Threshold (0.5%)
Name	In ppm		In mg day^{-1}	in mg day^{-1}

[a]Based on the determined highest allowable specification level of solvent.

previously registered in a region or member state. The definition also states that a new drug substance may be a complex, a simple ester, or a salt of a previously approved drug substance.

27.2 RESIDUAL SOLVENTS

ICH Q3C is intended to provide guidance for recommending acceptable amounts for residual solvents in pharmaceuticals for the safety of the patient. The guidance recommends use of less toxic solvents and describes levels considered to be toxicologically acceptable for some residual solvents. A complete list of the solvents included in this guidance is provided in a companion document entitled "ICH Q3C—Tables and List" which can be found at the ICH or FDA website. The list is not exhaustive, and other solvents may be used and later added to the list.

Residual solvents in pharmaceuticals are defined here as organic volatile chemicals that are used or produced in the manufacture of drug substances or excipients or in the preparation of drug products. The solvents are not completely removed by practical manufacturing techniques. Appropriate selection of the solvent for the synthesis of drug substance may enhance the yield or determine characteristics such as crystal form, purity, and solubility. Therefore, the solvent may sometimes be a critical parameter in the synthetic process. This guidance does not address solvents deliberately used as excipients nor does it address solvates. However, the content of solvents in such products should be evaluated and justified.

As there are no therapeutic benefits from residual solvents, all residual solvents should be removed to the extent possible to meet product specifications, EMPs, or other quality-based requirements. Drug products should contain no higher levels of residual solvents than can be supported by safety data. Some solvents that are known to cause unacceptable toxicities (carcinogens), such as benzene and carbon tetrachloride (class 1, see Table 1 in ICH, 1997), should be avoided in the production of drug substances, excipients, or drug products unless their use can be strongly justified in a risk–benefit assessment. Some solvents associated with less severe toxicity (nongenotoxic animal carcinogens or possible causative agents of other irreversible toxicity such as neurotoxicity or teratogenicity), such as acetonitrile and chlorobenzene (class 2), should be limited in order to protect patients from potential adverse effects. Ideally, less toxic solvents, such as acetic acid and acetone (class 3), should be

used where practical. Table 27.4 presents a format for a residual solvent qualification table.

This guidance does not apply to potential new drug substances, excipients, or drug products used during the clinical research stages of development nor does it apply to previously existing marketed drug products.

The guidance applies to all dosage forms and routes of administration. Higher levels of residual solvents may be acceptable in certain cases such as short-term (30 days or less) or topical application. Justification for these levels should be made on a case-by-case basis and discussed with the appropriate FDA division.

The limits of residual solvents may include a value for the permitted daily exposure (PDE), which is the maximum acceptable intake per day of residual solvent in pharmaceutical products. These limits vary depending on the class.

For solvents where quantities are limited to set values in pharmaceutical products because of their inherent toxicity, class 2 (Table 2) should be consulted. PDEs are given to the nearest $0.1\,\text{mg}\,\text{day}^{-1}$, and concentrations are given to the nearest 10 ppm.

For solvents with low toxic potential, solvents in class 3 (Table 3) may be regarded as less toxic and of lower risk to human health. Class 3 includes no solvent known as a human health hazard at levels normally accepted in pharmaceuticals. However, there are no long-term toxicity or carcinogenicity studies for many of the solvents in class 3. Available data indicate that they are less toxic in acute or short-term studies and negative in genotoxicity studies. It is considered that amounts of these residual solvents of $50\,\text{mg}\,\text{day}^{-1}$ or less (corresponding to 5000 ppm or 0.5% under option 1) would be acceptable without justification. Higher amounts may also be acceptable provided they are realistic in relation to manufacturing capability and GMPs.

For solvents for which no adequate toxicological data were found, the solvents listed (Table 4, ICH, 1997) may also be of interest to manufacturers of excipients, drug substances, or drug products. However, no adequate toxicological data on which to base a PDE were found. Manufacturers should supply justification for residual levels of these solvents in pharmaceutical products.

The Gaylor–Kodell (1980) method of risk assessment is appropriate for class 1 carcinogenic solvents. Only in cases where reliable carcinogenicity data are available should extrapolation by the use of mathematical models be applied to setting exposure limits. Exposure limits for class 1 solvents could be determined with the use of a large safety factor (i.e., 10,000–100,000) with respect to the no-observable-effect level (NOEL). Detection and quantitation of these solvents should be by state-of-the-art analytical techniques.

Acceptable exposure levels in this guidance for class 2 solvents were established by calculation of PDE values according to the procedures for setting exposure limits in pharmaceuticals (*Pharmacopeial Forum*, Nov.–Dec. 1989) and the method adopted by IPCS (International Program on Chemical Safety) for Assessing Human Health Risk of Chemicals [EHC (environmental health criteria) 170, World Health Organization (WHO), 1994]. These methods are similar to those used by the U.S. EPA [IRIS (Integrated Risk Information System)] and the U.S. FDA (*Red Book*) and others. The method is outlined here to give a

better understanding of the origin of the PDE values. It is not necessary to perform these calculations in order to use the PDE values tabulated in Section 4 of this document.

The PDE is derived from the NOEL or the lowest observable effect level (LOEL) in the most relevant animal study as follows:

$$\text{PDE} = \frac{\text{NOEL} \times \text{weight adjustment}}{F_1 \times F_2 \times F_3 \times F_4 \times F_5} \quad (1)$$

The PDE is derived preferably from a NOEL. If no NOEL is obtained, the LOEL may be used. Modifying factors proposed here for relating the data to humans are the same kind of *uncertainty factors* used in EHC 170 (WHO, Geneva, 1994) and *modifying factors* or *safety factors* in *Pharmacopeial Forum*. The assumption of 100% percent systemic exposure is used in all calculations regardless of route of administration.

The modifying factors are as follows:

- F_1 = Factor to account for extrapolation between species
  F_1 = 5 for extrapolation from rats to humans
  F_1 = 12 for extrapolation from mice to humans
  F_1 = 2 for extrapolation from dogs to humans
  F_1 = 2.5 for extrapolation from rabbits to humans
  F_1 = 3 for extrapolation from monkeys to humans
  F_1 = 10 for extrapolation from other animals to humans

Factor F_1 takes into account the comparative surface area–body weight ratios for the species concerned and for humans. Surface area (S) is calculated as:

$$S = kM \times 0.67 \quad (2)$$

Where M is body mass and the constant k has been taken to be 10. The body weights used in the equation are those shown below.

- F_2 = Factor of 10 to account for variability between individuals. A factor of 10 is generally given for all organic solvents and 10 is used consistently in this guidance:
- F_3 = Variable factor to account for toxicity studies of short-term exposure:
  F_3 = 1 for studies that last at least one half-lifetime (1 year for rodents or rabbits; 7 years for cats, dogs, and monkeys)
  F_3 = 1 for reproductive studies in which whole period of organogenesis is covered
  F_3 = 2 for 6-month study in rodents or 3.5-year study in nonrodents
  F_3 = 5 for 3-month study in rodents or 2-year study in nonrodents
  F_3 = 10 for studies of shorter duration

In all cases, the higher factor has been used for study durations between the time points (e.g., a factor of 2 for a 9-month rodent study).

- F_4 = Factor that may be applied in cases of severe toxicity (e.g., nongenotoxic carcinogenicity, neurotoxicity, or teratogenicity). In studies of reproductive toxicity, the following factors are used:
  F_4 = 1 for fetal toxicity associated with maternal toxicity
  F_4 = 5 for fetal toxicity without maternal toxicity
  F_4 = 5 for a teratogenic effect with maternal toxicity
  F_4 = 10 for teratogenic effect without maternal toxicity
- F_5 = Variable factor that may be applied if NOEL was not established. When only an LOEL is available, a factor of up to 10 could be used depending on the severity of the toxicity.

The weight adjustment assumes an arbitrary adult human body weight for either sex of 50 kg. This relatively low weight provides an additional safety factor against the standard weights of 60 or 70 kg that are often used in this type of calculation. It is recognized that some adult patients weigh less than 50 kg; these patients are considered to be accommodated by the built-in safety factors used to determine a PDE. If the solvent was present in a formulation specifically intended for pediatric use, an adjustment for a lower body weight would be appropriate.

As an example of the application of this equation, consider a toxicity study of acetonitrile in mice that is summarized in *Pharmeuropa*, Vol. 9, No. 1, Supplement, April 1997, page S24. The NOEL is calculated to be 50.7 mg kg^{-1} day^{-1}. The PDE for acetonitrile in this study is calculated as

$$\text{PDE} = \frac{50.7\,\text{mg kg}^{-1}\,\text{day}^{-1} \times 50\,\text{kg}}{12 \times 10 \times 5 \times 1 \times 1} = 4.22\,\text{mg day}^{-1}$$

In this example,

F_1 = 12 to account for extrapolation from mice to humans
F_2 = 10 to account for differences between individual humans
F_3 = 5 because duration of study was only 13 weeks
F_4 = 1 because no severe toxicity was encountered
F_5 = 1 because NOEL was determined

27.3 EXTRACTABLES AND LEACHABLES

Leachables are chemical entities, either organic or inorganic, that migrate from pharmaceutical container closure system components into a drug product

formulation. Since patients can be exposed to leachables during normal use of a drug product, leachables are of potential safety concern. Extractables are compounds that are forced out of container closure system materials and components under laboratory experimental conditions. All extractables from a given pharmaceutical container closure system and its components are, therefore, potential leachables in a drug product incorporating the same container closure system components. Regulatory concern for regarding leachables and extractables is directly related to the potential for contamination and/or interaction of the drug product formulation with the container closer system, with the greatest concern focused on orally inhaled and nasal drug products (OINDP), which include metered-dose inhalers (MDIs), dry-powder inhalers (DPIs), inhalation solutions, suspensions and sprays, and nasal sprays (Norwood et al., 2007).

> Controlled extraction studies are an extremely important part of the pharmaceutical development process for OINDP and should be performed on critical components as identified by the manufacturer and regulatory authority. As stated in the PQRI L&E Recommendations: A controlled extraction study is a laboratory investigation into the qualitative and quantitative nature of extractables profiles of critical components of an OINDP container closure system. The purpose of a controlled extraction study is to systemically and rationally identify and quantify potential leachables, i.e., extractables, to the extent practicable, and within certain defined analytical threshold parameters.

Controlled extraction studies result in extractables profiles of OINDP components. Extractables profiles contain information which allows the identification, to the extent possible, and quantitation of individual extractables from a given component and therefore are an early indication of potential leachables of concern. Controlled extraction studies generally establish a basis for the development and validation of routine quality control methods for drug product leachables and, finally, allow for the correlation of extractables and leachables profiles. Although information on component composition from suppliers is very useful, helping to inform component selection and guide controlled extraction studies, such knowledge does not provide a complete extractables profile and therefore does not alleviate the requirement for controlled extraction studies no matter how "complete" the information might appear to be.

It is therefore critical that controlled extraction studies be performed properly and thoroughly. Specific expectations for "proper and thorough" controlled extraction studies will ultimately depend on the nature of the OINDP being developed. However, the PQRI L&E Working Group was able to establish some general best practice recommendations for OINDP controlled extraction studies based on the data that the group generated by conducting its own controlled extraction studies on the elastomer and polymer test articles. These recommendations are:

- Controlled extraction studies should employ vigorous extraction with multiple solvents of varying polarity.
- Controlled extraction studies should incorporate multiple extraction techniques.
- Controlled extraction studies should include careful sample preparation based on knowledge of analytical techniques used.
- Controlled extraction studies should employ multiple analytical techniques.
- Controlled extraction studies should include a defined and systematic process for identification of individual extractables.
- Controlled extraction study "definitive" extraction techniques and methods should be optimized.
- During the controlled extraction studies, sponsors should revisit supplier information describing component information.
- Controlled extraction studies should be guided by analytical evaluation thresholds (AETs) that are based on an accepted safety concern threshold.
- Qualitative and quantitative extractables profiles should be discussed with and reviewed by toxicologists so that any potential safety concerns regarding individual extractables, that is, potential leachables, are identified early in the development process.
- Polynuclear aromatics (PNAs), *N*-nitrosamines, and 2-mercaptobenzothiazole (MBT) are "special-case" compounds requiring evaluation by specific analytical techniques and technology-defined thresholds.

The characterization and control of leachables and extractables represent possibly the most significant challenges facing a pharmaceutical development team responsible for the development, registration, and manufacture of an OINDP. Indeed, detecting, identifying, and quantifying organic leachables is a formidable task. In contrast to drug substance or excipient-related impurities, organic leachables can represent a diversity of chemical structures and compound classes and are potentially present at widely varying concentrations in any particular OINDP. Additionally, the information available to a pharmaceutical development team on container closure system component composition and processing, which is provided by the component supplier, is often incomplete. In some cases, the supplier may provide no information. Thus, when an extractables study is first undertaken, the development team may only have a limited idea of what to look for and what extraction techniques and analytical methods to use for identification and assessment of potential leachables.

27.4 RESIDUAL METALS AND METAL CATALYSTS

In early 2008, the EMEA promulgated a standard for metals as impurities in pharmaceuticals. They organized metals of concern into categories, as presented in Table 27.5.

TABLE 27.5 Class Exposure and Concentration Limits for Individual Metal Catalysts and Metal Reagents

	Oral Exposure		Parenteral Exposure		Inhalation Exposure[a]
Classification	PDE ($\mu g\,day^{-1}$)	Concentration (ppm)	PDE ($\mu g\,day^{-1}$)	Concentration (ppm)	PDE ($ng\,day^{-1}$)
Class 1A: Pt, Pd	100	10	10	1	Pt: 70[a]
Class 1B: Ir, Rh, Ru, Os	100[b]	10[b]	10[b]	1[b]	
Class 1C: Mo, Ni, Cr, V; metals of significant safety concern	250	25	25	2.5	Ni: 100 Cr (VI): 10
Class 2: Cu, Mu, metals with low safety concern	2,500	250	250	25	
Class 3: Fe, Zu; metals with minimal safety concern	13,000	1,300	1,300	130	

[a]Pt as hexachloroplatinic acid.
[b]Subclass limit: the total amount of listed metals should not exceed the indicated limit.

If synthetic processes of pharmaceutical substances are known or suspected to lead to the presence of metal residues due to the use of a specific metal catalyst or metal reagent, a concentration limit and validated test for residues of each specific metal should be set. All concentration limits should be realistic in relation to analytical precision, manufacturing capability, and reasonable variation in the manufacturing process. Since the use of metal catalysts or metal reagents during synthesis is restricted to validated and controlled chemical reactions, limitation of their residues in pharmaceutical substances itself will normally be sufficient. A limit for a metal residue in the pharmaceutical substance may however be replaced by a limit for that metal residue in the final medicinal product, as described below.

For pharmaceutical products administered via the oral, parenteral, or inhalation route of administration, two options are available when setting a concentration limit for a metal residue:

Option 1: For each metal, the concentration limit in parts per million (ppm) as stated in Table 27.5 can be used. The concentration limits in Table 1 have been calculated using equation (3) below by assuming a daily dose of 10 g of the drug product:

$$\text{Concentration ppm} = \frac{\text{PDE } \mu g\,day^{-1}}{\text{daily dose } g\,day^{-1}} \tag{3}$$

If all pharmaceutical substances in a drug product meet the option 1 concentration limit for all metals potentially present, then all these substances may be used in any proportion in the drug product as long as the daily dose of the drug product does not exceed 10 g day^{-1}. When the daily dose of the drug product is greater than 10 g day^{-1}, option 2 should be applied.

Option 2a: The PDE in terms of micrograms per day as stated in Table 1 can be used together with the actual daily dose of a pharmaceutical substance in the drug product to calculate the concentration of residual metal allowed in that pharmaceutical substance.

Option 2b: Alternatively, it is not considered necessary for each pharmaceutical substance to comply with the limits given in option 1 or the calculated limits using option 2a.

The PDE in terms of micrograms per day as stated in Table 1 can also be used with the known maximum daily dose of the drug product to determine the concentration of a metal residue originating from any of the pharmaceutical substances in the drug product (not the substance). This approach is considered acceptable provided that it has been demonstrated that the metal residue has been reduced to the practical minimum in every substance. This approach implies that the maximum levels of a metal in certain substances may be higher than the option 1 or option 2a limit but that this should then be compensated for by lower maximum levels in the other substances.

For pharmaceutical products applied via other routes of administration, the concentration limits should be set in consideration of the route of administration.

Without proper justification, parenteral limits/PDEs should be used for pharmaceutical substances that are administered by other routes of administration, including inhalation. Oral limits/PDEs may be applied if the absorption by other routes of administration is not likely to exceed the absorption following oral administration. For example, for cutaneous administration, oral concentration limits/PDEs are considered acceptable.

Platinum salts have been shown to be allergenic, with hexachloroplatinic acid being clearly the most allergenic (Malo, 2005). Consequently a specific limit for inhalation exposure for this molecule has been set at 70 ng day^{-1} (see monograph). Chromium VI and nickel, when inhaled, have been associated with carcinogenicity. Therefore specific limits for inhalation exposure have been set for chromium VI at 10 ng day^{-1} and for nickel at 100 ng day^{-1} (see respective monographs).

For pharmaceutical products used for short-term and for life-saving indications, as the PDEs and concentration limits mentioned in this guideline are based on chronic use, higher PDEs and concentration limits may be acceptable in cases of short-term use (30 days or less). For instance, this may be applicable to contrasting agents, antidotes, or products for diagnostic use. This may however only be applied if neither an option 1 nor an option 2 limit is feasible.

Specific risk–benefit considerations, such as for compounds used for life-saving indications, may also warrant the use of higher limits. Justifications should be made on a case-by-case basis.

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U.S. Food and Drug Administration (FDA) (2005): http://www.fda.gov/cder/guidance/cGMPs/packaging.htm.

Appendix A

Selected Regulatory and Toxicological Acronyms

510(k)	Premarket notification for change in a device
AALAS	American Association Laboratory Animal Science
AAMI	Association for the Advancement of Medical Instrumentation
ABT	American Board of Toxicology
ACGIH	American Conference of Governmental Industrial Hygienists
ACT	American College of Toxicology
ADE	Adverse drug event (of drug substances)
ADI	Allowable daily intake
AIDS	Acquired immunodeficiency syndrome
AIMD	Active implantable medical device
ANSI	American National Standards Institute
APHIS	Animal and Plant Health Inspection Service
ASTM	American Society for Testing and Materials
CAS	Chemical Abstract Service
CBER	Center for Biologics Evaluation and Research (FDA)
CDER	Center for Drug Evaluation and Research (FDA)
CDRH	Center for Devices and Radiological Health (FDA)
CFAN	Center for Food and Nutrition (FDA)
CFR	Code of Federal Regulations
CIIT	Chemical Industries Institute of Toxicology
CPMP	Committee on Proprietary Medicinal Products (U.K.)

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CSE	Control standard endotoxin
CSM	Committee on Safety of Medicines (U.K.)
CTC	Clinical trial certificate (U.K.)
CTX	Clinical trial certificate exemption (U.K.)
CVM	Center for Veterinary Medicine (FDA)
DART	Development and reproduction toxicology
DHHS	Department of Health and Human Services
DIA	Drug Information Associates
DMF	Drug (or device) master file
DSHEA	Dietrary Supplement Health and Education Act
EEC	European Economic Community
EFPIA	European Federation of Pharmaceutical Industries Association
EM	Electron microscopy
EPA	Environmental Protection Agency
EU	European Union
FCA	Freund's complete adjuvant
FDA	Food and Drug Administration
FDCA	Food, Drug and Cosmetic Act
FDLI	Food and Drug Law Institute
FIFRA	Federal Insecticides, Fungicides and Rodenticides Act
GCP	Good clinical practices
GLP	Good laboratory practices
GMP	Good manufacturing practices
GPMT	Guinea pig maximization test
HEW	Department of Health, Education and Welfare (no longer existent)
HIMA	Health Industry Manufacturer's Association
HSDB	Hazardous Substances Data Bank
IARC	International Agency for Research on Cancer
ICH	International Conference on Harmonisation
ID	Intradermal
IDE	Investigational device exemption
IND(A)	Investigational new drug application
INN	International nonproprietary names
IP	Intraperitoneal
IRAG	Interagency Regulatory Alternatives Group
IRB	Institutional review board
IRLG	Interagency Regulatory Liaison Group
ISO	International Standards Organization
IUD	Intrauterine device
IV	Intravenous
JECFA	Joint Expert Committee for Food Additives
JMAFF	Japanese Ministry of Agriculture, Forestry, and Fishery
JPMA	Japanese Pharmaceutical Manufacturers Association
LA	Licensing Authority (U.K.)

LAL	*Limulus* amebocyte lysate
LD_{50}	Lethal dose 50: dose calculated to kill 50% of subject population, median lethal dose
LOEL	Lowest observed effect level
MAA	Marketing authorization application (EEC)
MCA	Medicines Control Agency
MD	Medical device
MedDRA	Medical Dictionary for Regulatory Activities
MHW	Ministry of Health & Welfare (Japan)
MID	Maximum implantable dose
MOE	Margin of exposure
MOU	Memorandum of understanding
MRL	Maximum residue limits
MSDS	Material safety data sheet
MTD	Maximum tolerated dose
NAS	National Academy of Science
NCTR	National Center for Toxicological Research
NDA	New drug application
NIH	National Institutes of Health
NIOSH	National Institute of Occupational Safety and Health
NK	Natural killer
NLM	National Library of Medicine
NOEL	No-observable-effect level
NTP	National Toxicology Program
ODE	Office of Device Evaluation
OECD	Organization for Economic Co-operation and Development
PDI	Primary dermal irritancy
PDN	Product development notification
PEL	Permissible exposure limit
PhRMA	Pharmaceutical Research and Manufacturers Association
PL	Produce license (U.K.)
PLA	Produce license application
PMA	Premarket approval application
PO	Per os (orally)
PTC	Points to consider
QAU	Quality assurance unit
RAC	Recombinant DNA Advisory Committee
RCRA	Resources Conservation and Recovery Act
RTECS	Registry of Toxic Effects of Chemical Substances
SARA	Superfund/Amendments and Reauthorization Act
SC	Subcutaneous
SCE	Sister chromatic exchange
SNUR	Significant new use regulations
SOP	Standard operating procedure
SOT	Society of Toxicology

SRM	Standard Reference Materials (Japan)
STEL	Short-term exposure limit
TLV	Threshold limit value
USAN	United States Adopted Name Council
USDA	United States Department of Agriculture
USP	United States Pharmacopeia
VAERS	Vaccine adverse-event reporting system
VSD	Vaccine safety data link
WHO	World Health Organization

Appendix B

Definition of Terms and Lexicon of Clinical Observations in Nonclinical (Animal) Studies

MOVEMENT

Anesthetized The absence of or reduced response to external stimuli accompanied with a loss of righting reflex.

Ataxia Incoordination of muscular action involving locomotion, including loss of coordination and unsteady gait.

Catalepsy Condition characterized by waxy rigidity of muscles such that animal tends to remain in any position in which it is placed.

Hyperactivity Abnormally high level of motor activity.

Hypersensitivity Abnormally strong reaction to external stimuli such as noise or touch.

Lethargy State of deep and prolonged depression stupor from which it is possible to be aroused followed by immediate relapse.

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Low carriage Animal's torso is carried very close to the ground during movement.

Paralysis Inhibition or loss of motor function; may be characterized by affected portion of body.

Prostrate Animal assumes a recumbent position due to loss of strength or exhaustion and may slow intermittent uncoordinated movements.

Righting reflex Ability of an animal, when placed on its back, to regain a position on all fours.

Unsteady gait Erratic manner or style of walking.

RESPIRATION

Audible respiration Abnormal respiratory sound heard while listening to breathing of animal (e.g., wheezing and rales).

Bradypnea Abnormal slowness of respiration rate.

Cheyne–Stokes respiration Breathing characterized by rhythmic waning and waxing of depth of respiration, with regularly recurring periods of apnea: seen especially in coma resulting from affection of nervous centers.

Dyspnea "Shortness of breath"; difficult or labored breathing.

Gasping Spasmodic breathing with the mouth open or laborious respiration with breath caught convulsively.

Hyperpnea Deep and rapid breathing.

Hypopnea Shallow and slow breathing.

Irregular respiration No definite cycle or rate of breathing.

Labored respiration Forced or difficult, usually irregular breathing.

Tachypnea Excessive rapidity of respiration rate.

CONDITION OF SKIN AND FUR

Alopecia Deficiency of hair (baldness).

Cyanosis Visible skin and/or mucous membranes turn dusky blue due to lack of oxygenation of blood.

Necrosis Actual tissue destruction, masses of dead/destroyed tissue.

UROGENITAL REGION

Anuria Absence of or sharp decline in urine excretion.

Diarrhea Abnormal frequency and liquidity of fecal discharge.

Polyuria Abnormally sharp increase in amount of urine excretion.

CONVULSIONS AND TREMORS

Clonic Often seen as a "paddling" motion of forelegs of animal.

Convulsions Transient, self-sustaining electrical dysrhythmias which have tendency to recur. Convulsions are generally associated with a finite period of unconsciousness and have a muscular involvement manifested as disorganized limb movements.

Fasciculation Rapid, often continuous contraction of bundle of skeletal muscle fibers which does not produce purposeful movement (twitching).

Tonic Muscular contraction, keeping limbs in fixed position, generally extended to rear.

Torsion Postural incoordination or rolling. Generally associated with vestibular (ear canal) system.

Tremor Fine oscillating muscular movements which may or may not be rhythmic.

CONDITION OF EYES

Blepharospasm Twitching or spasmodic contraction of orbicularis oculi muscle.

Chemosis Edema of conjunctiva(e)—conjunctival tissue responds to noxious stimuli by swelling.

Chromodacryorrhea Response of reddish conjunctival exudate; no blood cells present in exudate (i.e., not true "bloddy tears").

Conjunctivitis Inflammation of conjunctiva (mucous membrane which lines eyelids and is reflected into eyeball).

Exophthalmos Abnormal protrusion of eyeball from orbit.

Lacrimation Secretion of tears.

Miosis Constriction of pupil.

Mydriasis Dilation of pupil.

Nystagmus Abnormal involuntary movement of eyes. It may be rotational or horizontal or vertical plane.

Ocular exudate Secretion (usually transparent and yellow) directly from eye.

Opacity Loss of transparency of eyeball.

Pinpoint pupils Ultimate state of miosis.

Ptosis Refers to dropping of upper eyelid, thought to be due to impaired conduction in third cranial nerve.

MISCELLANEOUS

Analgesia Absence of (or reduced response to) painful stimuli.

Hunched posture Drawing-in of both ends of body and extremities with sharp arching of back.

Kyphosis Humpback—abnormal curvature and dorsal prominence of vertebrae column.

Nasal discharge Fluid secretion from nostrils.

Piloerection Body hair stands on end; dilation of pupils usually accompanies piloerection.

Salivation Excessive secretion of saliva from mouth.

Straub tail Condition, especially in mice, in which animal carries its tail in erect (vertical or nearly vertical) position. This sign is commonly associated with chemicals (e.g., morphine) that bind to opiate receptors.

REFLEXES

Corneal reflex Closure of eyelids in response to corneal touch (e.g., with soft brush bristle).

Grip strength (or screen grip) Measure of grip strength of forelimbs or hindlimbs; may be evaluated quantitatively or by subjective estimate or impairment (rodents only).

Pinna reflex Twitch of outer ear in response to gentle touch.

Preyer's reflex (auditory startle response) Involuntary movement of outer ears produced by auditory stimulus (especially in rats).

Pupillary reflex Contraction of pupil in response to light stimulation of retina.

Righting reflex Ability to land on (when dropped) or regain normal stance on all four limbs.

Startle reflex Response to sharp sound, touch, or other startling stimulus; response may range from "absent," to "normal," to "hyperreactive," including exaggerated jerking, jumping, frantic attempts to escape, and even convulsion.

Appendix C

Notable Regulatory Internet Addresses

Organization or Publication	Web Address (URL)	Sample Main Topics
ABPI	http://www.abpi.org.uk/	
Adverse Reactions Bulletin	http://www.thomsonscience.co	
Agency for Toxic Substances and Disease Registry	www.atsdr.cdc.gov	
Association of Clinical Biochemists	http://www.leeds.ac.uk/acb/	Items of general medical interest and an assay finder to help researcher find methods or labs to measure a wide variety of hormones, metals, enzymes, and drugs in body fluids
Australian Therapeutic Goods Administration	http://www.health.gov.au/tga	Medical devices; GMP codes; Parliamentary Secretary's Working; status document; party on complementary medicines; medical releases; publications; site map; related sites

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Organization or Publication	Web Address (URL)	Sample Main Topics
BioMedNet	http://www.cursci.co.uk/BioMedNet/biomed.html/ or http://www.BioMedNet.com	World Wide Web club for biological and medical community (free membership)
Canadian Health Protection Board	http://www.hwc.ca/hpb	
Canadian Health Protection Branch	http://www.hc-sc.gc.ca/hpb	Medical devices; Chemical hazards; food; product safety; science advisory board; diseases; radiation protection; drugs; HPB transition policy, planning, and coordination
Centre for Medicines Research	http://www.cmr.org/	
ChemInfo	www.indiana.edu/~cheminfo/ca_csti.html	SirCH: Chemical Safety or Toxicology Information
Clinical Pharmacology Drug Monograph Service	http://www.cponline.gsm.com	
Clinician's Computer-Assisted Guide to the Choice of Instruments for Quality of Life Assessment in Medicine	http://www.glamm.com/ql/guide.htm	Contains hypertext with references to QoL measurements divided into (a) general diseases, (b) specific diseases and therapies, (c) health organizations, and (d) bibliography
ClinWeb	http://www.ohsu.edu/clinweb	Oregon Health Sciences University
CNN Interactive (Health)	http://www.cnn.com/HEALTH/index.html	Up-to-date information on health issues including drug safety concerns and withdrawals
Code of Federal Regulations	http://www.access.gpo.gov/nara/cfr/index.html or http://www.access.gpo.gov/su_docs/aces/aces140.html	For proposed rules and regulations
	http://www.access.gpo.gov/nara/cfr/cfr-table-search.html	NARA code sections
Committee on Safety of Medicines (CSM)	http://www.open.gov.uk/mca/csmhome.htm	
Cornell Legal Library	http://www.law.cornell.edu	Code of Federal Regulations; Supreme Court Decisions; U.S. Code; Circuit Courts of Appeal
Current problems in pharmacovigilance	http://www.opwn.gov.uk/mca/mcahome.htm	

Organization or Publication	Web Address (URL)	Sample Main Topics
Cutaneous drug reactions	http://triz.dermatology.uiowa.edu/home.html	
DIA home page	http://www.diahome.org	Home page of Drug Information Association
Doctor's Guide to the Internet	http://www.psigroup.com	
Documents for Clinical Research	http://www.ams.med.unigoettingen.de/~rhilger/Document.html	*Declaration of Helsinki*, other documents and collection of related sites
Druginfonet	http://www.druginfonet.com	
EC DGXIII Telecommunications	http://www.ispo.cec.be/	Information
EMBASE	http://www.healthgate.com/healthGate/price/embase.html	
European Protection Agency (EPA)	www.epa.gov	
Eudra Net: Network Services for the European Union Pharmaceutical Regulatory Sector	http://www.eudra.org	Includes information on European Agency for the Evaluation of Medicinal Products.
EMEA	http://www.eudra.org/emea.html	
Europa	http://www.cec.lu	Official website of European Union
European Agency for the Evaluation of Medicinal Products	http://www.eudra.org/en_home.htm	What's new; documents forum; Other sites
European Sites	http://www.eucomed.be/eucomed/links/links.htm	European institutions; related sites
European Pharmacovigilance Research Group	http://www.ncl.ac.uk/~neprg/	
Food and Drug Administration (FDA)	www.fda.gov	Foods; human drugs; biologics; animal drugs; cosmetics; medical devices/radiological health
FDA CBER: Center for Biologics Evaluation and Research	http://www.fda.gov/cber	
CBER What's New	http://www.fda.gov/cber/whatsnew.htm	
FDA CDER: Center for Drug Evaluation and Research	http://www.fda.gov/cder	
FDA Adverse Events Database	http://www.fda.gov/cder/adr	
CDER What's New	http://www.fda.gov/cder/whatsnew.htm	

Organization or Publication	Web Address (URL)	Sample Main Topics
FDA CDRH	www.fda.gov/cdrh/index.html	Home page
Search site	www.fda.gov/cdrh/search.html	Search CDRH site
Comment	www.fda.gov/cdrh/comment4.html	Comment on CDRH site
Device advice	www.fda.gov/cdrh/devadvice/32.html	
PDF reader	www.fda.gov/cdrh/acrobat.html	
FDA CFSAN: Center for Food Safety and Applied Nutrition	http://vm.cfsan.fda.gov	
FDA Center for Toxicological Research	http://www.fda.gov/nctr	
FDA CVM: Center for Veterinary Medicine	http://www.fda.gov/cvm	
FDA—Bioengineered food	http:www.fda.gov/oc/biotech/default.htm	
FDA—Breast Implants	http:www.fda.gov/cdrh/breastimplants/index.html	
FDA—Cosmetics	http://vm.cfsan.fda.gov//~lrd/cosmetm.html	
FDA—Dietary supplements	http:vm.cfsan.fda.gov/~dms/supplmt.html	
FDA's Electronic Freedom of Information Act	http:www.fda.gov/foi/foia2.htm	
FDA—Field Operations	www.fda.gov/ora/	What's new; import program; inspectional, science and compliance references; federal/state relations
Common Technical Document for the Registration of Pharmaceuticals for Human use: 08-24-00	http://www.fda.gov/cder/guidance/4022dfts.htm	
Design Controls	www.fda.gov/ora/inspect_ref/qsreq/dcrpgd.html	Design control report and guidance text
	www.fda.gov/ora/inspect_ref/igs/elec_med_dev/emcl.html	Guide to inspections of electromagnetic compatibility aspects of medical device quality systems text
Guide to Inspections of Quality Systems	www.fda.gov/ora/inspect_ref/igs/qsit/qsitguide.htm	QSIT inspection handbook text
Guide to Inspections of Quality Systems	www.fda.gov/ora/inspect_ref/igs/qsit/QSITGUIDE.PDF	PDF version of QSIT inspection handbook text
Photosafety Testing 07-05-00	http://www.fda.gov/cder/guidance/3281dft.htm	
Skin Irritation and Sensitization Testing of Generic Transdermal Drug Products 06:01:00	http://www.fda.gov/cder/guidance/2887fnl.htm	

Organization or Publication	Web Address (URL)	Sample Main Topics
FDA—MedWatch	http://www.fda.gov/medwatch/	FDA drug adverse-event reporting system
FDA—Tampons	http://www.fda.gov/oc/opacpm/topicindexes/tampons.html	
Food and Drug Law Institute	http://www.fdli.org	Special interest; publications; multimedia; order products; academic programs; directory of lawyers and consultants; contact us
Health Industry and Manufacturers Association (HIMA)	http://www.himanet.com	About HIMA; newsletter; HIMA calendar; industry resources; business opportunities; FDA/EPA/OSHA; reimbursement/payment; global year 2000; government relations; public relations; small company; diagnostics
Health on the Net	http://www.hon.ch	
Health information on Internet	http://www.wellcome.ac.uk.healthinfo/	New bimonthly newsletter from the Wellcome Trust and the RSM
Hyppos Project	http://ifinet.it/hypposnet	Information in Italian and English about Hyppos Project, which has led to development of QoL tool for measurement of hypertensive patients in Italy. It contains a description of the project, the tool, publications about the development of the tool and its application, plus general references to QoL and hypertension.
International Classification of Disease (ICD)-10	http://www.cihi.ca.newinit/scope.htm	
International Conference on Harmonisation (ICH) 3 Home Page	http://cc.umin.u-tokyo.ac.jp/ich/ich3.html	Official ICH website with documents (needs password)
ICH documents	http://www.pharmweb.net/pwmirror/pw9/ifpma/ich1/html	
International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human use	http://www.ifpma.org/ich1.html	

Organization or Publication	Web Address (URL)	Sample Main Topics
International Federation of Pharmaceutical Manufacturers	http://www.ifpharma.com	ICH documents and postings; international pharmaceutical issues
International Regulatory Monitor (Monitor)	http://www.gonsi.com/pubs	Editorial portion of newsletter
International Society of Pharmacoepidemiology	http://www.pharmacoepi.org	
Internet Grateful Med	www.igm.nlm.nih.gov	
InterPharma	http://www.interpharma.co.uk	The latter are vast sites with links to other databases for pharmaceutical support sites—http://www.MedsiteNavigator.com
JAMA	http://www.amahyphen;assn.org/jama	Gives many other useful U.S. sites
Japanese Ministry of Health and Welfare	http://www.mhw.go.jp/english/index.html	Organization; Y2K problem; statistics; white paper; related sites
Library of Congress	http://thomas.loc.gov	Searchable database of federal legislation, Congressional Record, and committee information
Market and Exploitation of Research	http://www.cordis.lu	
Medical Device Link	http://www.devicelink.com	News; consultants; bookstore; links; discussion; magazines (MDDI; MPMN; IVD Technology)
Medicines Control Agency (MCA)	http://www.opengov.uk/mcahome.htm	
Medical Matrix	http://www.medmatrix.org	
Medical Research Council	http://nimr.mcr.ac.uk/MRC/	
MEDLINE (free)	http://www.ncbi.nlm.nih.gov/PubMed or http://www.medmatrix.org/Spages/medline.asp	List of free sites
MEDLINE	http://www.medmatrix.org/SPAges/medline.asp or http://www.medsitenavigator.com/medline/medline.html	Metasite with full and changing MEDLINE search engines; list of free sites
Medscape	http://www.medscape.com	
Multilingual glossary of medical terms	http://allserv.rug.ac.be/~rvdstich/eugloss/welcome.html	
National Archives and Public Records Administration	http://www.access.gpo.gov/su_docs/aces/aces140.html	Code of Federal Regulations; *Federal Register*; laws; U.S. Congress information

Organization or Publication	Web Address (URL)	Sample Main Topics
National Institutes of Health (U.S.)	http://www.nih.gov	
National Library Network	www.toxnet.nlm.nih.gov	TOXNET: Toxicology Data Network, a cluster of databases on toxicology, hazardous chemicals, and related areas
National Toxicology Program	http://ntp-server.niehs.nih.gov/	
New Quality System (QS) Regulation	www.fda.gov/bbs/topics/ANSWERS/ANS00763.html	FDA talk paper announcing GMP final rule text
Organised Medical Network Information	http://www.omni.ac.uk	
Pharmaceutical and Medical Safety Bureau—Japan	http://www.mhlw.go.jp/english	
PharminfoNet	http://www.pharminfo.com or http://www.pharminfo.com/phrmlink.html	Independent assessment of therapeutics and advances in new drug development
Pharmweb	http://www.pharmweb.net	Information resource for pharmaceutical and health-related information
Quality of Life	http://www.glamm.com/ql/guide.htm	Choice of instrument
Quality of Life Assessment in Medicine	http://www.glamm.com/q1/ursl.htm	Contains hypertext with references to QoL measurements divided into (a) assessment tools, (b) reference organizations and groups, (c) diseases, symptoms, and specific populations, (d) the top-10 journals that publish articles of interest to QoL assessment in medicine, (e) methodology, and (f) bibliographical research.
Regulatory Affairs Professionals Society (RAPS)	http://www.raps.org	Certificates; resource center; publications; chapters; related links; contacting RAPS
Reuters Health Information Services	http://www.reutershealth.com	
SCRIP: World Pharmaceutical News	http://www.pjbpubs.co.uk/scrip	
SNOMED	http://snomed.org	Systemised Nomenclature of Human and Veterinary Medicines

Organization or Publication	Web Address (URL)	Sample Main Topics
Swedish Medical Products Agency	http://www.mpa.Se	
U.S. Department of Agriculture (USDA)	http://www.usda.gov	
Food Safety	http://www.foodsafety.gov/	
USDA FMS: Farm Service Agency	http://www.fsa.usda.gov/pas/default.asp	
USDA FSA: Food and Nutrition Service	http://www.fns.usda.gov/fns/	
USDA FSIS: Food Safety and Inspection Service	http://www.usda.gov/fsis	
U.S. Department of Commerce	http://204.193.246.62	Bureau of Export Administration; International Trade Association; patent and trademark; National Institute of Standards and Technology
U.S. Pharmacopeia	www.usp.org/prn	
University of Pittsburgh	www.pitt.edu	
World Health Organization	http://www.who.int	Governance; health topics; information sources; reports; directorgeneral; about WHO; International Digest of Health; legislation (http://www.who.int/pub/dig.html)
WHO Collaborating Centre for International Drug Monitoring	http://www.who.ch/ or http://www.who.pharmasoft.se	

Appendix D

Glossary of Terms Used in Clinical Evaluation of Therapeutic Agents

Abnormality Sign, symptom, or laboratory result not characteristic of normal individuals.

Adverse event Unwanted effects that occur and are detected in populations. The term is used whether there is or is not any attribution to a medicine or other cause. Adverse events may be known parts of a disease that are observed to occur within a period of observation, and they may be analyzed to test for their frequency in a given population or trial. This is done to determine if there is an unexpectedly increased frequency resulting from nondisease factors such as medicine treatment. The term adverse event or adverse experience is used to encompass adverse reactions plus any injury, toxicity, or hypersensitivity that may be medicine related as well as any medical events that are apparently unrelated to medicine that occur during the study (e.g., surgery, illness, and trauma). See definition of *adverse reaction*.

Adverse experience See *adverse event*.

Adverse reaction Unwanted effect(s) (i.e., physical and psychological symptoms and signs) resulting from treatment. A less rigid definition of adverse reaction includes the previous definition plus any undesirable effect or problem that is present during the period of treatment and may or may not be a well-known or obvious complication of the disease itself. Thus, many

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common personality, physical, psychological, and behavioral characteristics that are observed in medicine studies are sometimes characterized as adverse reactions even if they were present during baseline.

Synonyms of adverse reactions generally include adverse medical effects, untoward effects, side effects, adverse drug experiences, and adverse drug reactions. Specific distinctions among some of these terms may be defined operationally. For example, the term adverse reaction is used to denote those signs and symptoms at least possibly related to a medicine, whereas the term adverse experiences is used to include nonmedicine-related medical problems in a trial such as those emanating from trauma or concurrent illness. Distinctions among side effects, adverse events, and adverse reactions are illustrated in the definitions of the two former terms.

Bias (1) Point of view that prevents impartial judgment on issues relating to that point of view. Clinical trials attempt to control this through double blinding. (2) Any tendency for a value to deviate in one direction from the true value. Statisticians attempt to prevent this type of bias by various techniques, including randomization.

Clinical significance Quality of a study's outcome that convinces physicians to modify or maintain their current practice of medicine. The greater the clinical significance, the greater is the influence on the practice of medicine. The assessment of clinical significance is usually based on the magnitude of the effect observed, the quality of the study that yielded the data, and the probability that the effect is a true one. Although this operational definition is presented from the physician's perspective, the term could operationally be defined from the patient's perspective. Patients are primarily concerned with results that will lead to an improved quality of life or a lengthening of their life. In addition, clinical significance may be applied to either positive data or efficacy or negative safety data such as for adverse reactions. Synonyms include *clinical importance*, *clinical relevance*, and *clinical meaningfulness*.

Clinical studies Class of all scientific approaches to evaluate medical disease preventions, diagnostic techniques, and treatments. Investigational and marketed prescription medicine evaluations plus over-the-counter medicines are included.

Clinical trials Subset of those clinical studies that evaluates investigational medicines in phases I, II, and III. Phase IV evaluations of marketed medicines in formal clinical trials using the same or similar types of protocols to those used in phases I and III are also referred to as clinical trials.

Compliance (1) Adherence of patients to following medical advice and prescriptions. Primarily applied to taking medicine as directed, but also applies to following advice on diet, exercise, or other aspects of patient's life. (2) Adherence of investigators to following a protocol and related administrative and regulatory responsibilities. (3) Adherence of sponsors to following

regulatory, legal, and other responsibilities and requirements relating to clinical trial.

Compound Chemical synthesized or prepared from natural sources that is evaluated for its biological activities in preclinical tests.

Development of medicines Term *development* as applied to medicines is used in several different contexts, even within the pharmaceutical industry. This often leads to confusion and misunderstanding. No single definition is preferred, but the particular meaning intended should be made clear by all people using the term. Three operational definitions are presented, from the broadest to the narrowest:

1. All stages and processes involved in discovering, evaluating, and formulating a new medicine until it reaches the market (i.e., commercial sale).
2. All stages involving the evaluation and formulation of a new medicine (after the medicine has been discovered and has gone through preclinical testing) until it reaches the market.
3. Those stages after the preclinical discovery and evaluation that involve technical development. These processes include formulation work, stability testing, scaling up the compound for larger scale synthesis, and providing analytical support. Clinical trials are not included in this definition.

Disease Disorders (e.g., anxiety disorders, seizure disorders), conditions (e.g., obesity, menopause), syndromes, specific illnesses, and other medical problems that are an acquired morbid change in a tissue, organ, or organism. Synonyms are *illness* and *sickness*.

Dosage regimen (1) Number of doses per given time period (usually days), (2) time that elapses between doses (e.g., dose to be given every 6 h) or the time that the doses are to be given (e.g., dose to be given at 8 AM, noon, and 4 PM each day), or (3) quantity of a medicine (e.g., numbe of tablets, capsules) that are given at each specific time of dosing.

Efficacy Relative concept referring to the ability of a medicine to elicit a beneficial clinical effect. This may be measured or evaluated using objective or subjective parameters and in terms ranging from global impressions to highly precise measurements. Efficacy is assessed at one or more levels of organization (e.g., subcellular, cellular, tissue, organ, whole body) and may be extrapolated to other levels.

Endpoint Indicator measured in a patient or biological sample to assess safety, efficacy, or another trial objective. Some endpoints are derived from primary endpoints (e.g., cardiac output is derived from stroke volume and heart rate). Synonyms include outcome, variable, parameter, marker, and measure. See surrogate endpoint in the text. Also defined as the final trial objective by some authors.

Incidence rate Rate of occurrence of new cases of a disease, adverse reaction, or other event in a given population at risk (e.g., the incidence of disease *X* is *Y* patients per year per 100,000 population).

Interpretation Process whereby one determines the clinical meaning or significance of data after the relevant statistical analyses have been performed. These processes often involve developing an explanation of the data that are being evaluated.

Medicine When a compound or substance is tested for biological and clinical activity in humans, it is considered to be a medicine. Some individuals prefer to define a medicine as a compound that has demonstrated clinically useful properties in patients. This definition, however, would restrict the term to use sometime during or after phase II. Others use the term loosely and apply it to compounds with biological properties during the preclinical period that suggest medical usefulness in humans. The author has adopted the first definition for use in this book.

Patient Used almost exclusively throughout this book in preference to *subject* or *volunteer*. Patient is used to cover those cases in which the term *volunteer* would be appropriate.

Pharmacodynamics Processes of responses resulting from treatment with a medicine or compound. The processes include pharmacological, biochemical, physiological, and therapeutic effects. The pharmacodynamics of a response to treatment are presented with the scientific and/or clinical language of the disciplines involved in detecting, measuring, and describing the effects.

Pharmacokinetics Processes of absorption, distribution, metabolism, and excretion of compounds and medicines.

Phases of clinical trials and medicine development Four phases of clinical trials and medicine development exist and are defined below. Each of these definitions is a functional one and the terms are not defined on a strict chronological bases. An investigational medicine is often evaluated in two or more phases simultaneously in different clinical trials. Also, some clinical trials may overlap two different phases.

Phase I Initial safety trials on a new medicine, usually conducted in normal volunteers. An attempt is made to establish the dose range tolerated by volunteers for single and for multiple doses. Phase I trials are sometimes conducted in severely ill patients (e.g., in the field of cancer) or in less ill patients when pharmacokinetic issues are addressed (e.g., metabolism of a new antiepileptic medicine in stable epileptic patients whose microsomal liver enzymes have been induced by other antiepileptic medicines). Pharmacokinetic trials are usually considered phase I trials regardless of when they are conducted during a medicine's development.

Phase IIa Pilot clinical trials to evaluate efficacy (and safety) in selected populations of patients with the disease or condition to be treated, diagnosed, or prevented. Objectives may focus on dose–response, type of patient, frequency of dosing, or numerous other characteristics of safety and efficacy.

Phase IIb Well-controlled trials to evaluate efficacy (and safety) in patients with the disease or condition to be treated, diagnosed, or prevented. These clinical trials usually represent the most rigorous demonstration of a medicine's efficacy. Sometimes referred to as pivotal trials.

Phase IIIa Trials conducted after efficacy of the medicine is demonstrated but prior to regulatory submission of a new drug application (NDA) or other dossier. These clinical trials are conducted in patient populations for which the medicine is eventually intended. Phase IIIa clinical trials generate additional data on both safety and efficacy in relatively large numbers of patients in both controlled and uncontrolled trials. Clinical trials are also conducted in special groups of patients (e.g., renal failure patients) or under special conditions dictated by the nature of the medicine and disease. These trials often provide much of the information needed for the packaging insert and labeling of the medicine.

Phase IIIb Clinical trials conducted after regulatory submission of an NDA or other dossier but prior to the medicine's approval and launch. These trials may supplement earlier trials, complete earlier trials, or be directed toward new types of trials (e.g., quality of life, marketing) or phase IV evaluations. This is the period between submission and approval of a regulatory dossier for marketing authorization.

Phase IV Studies or trials conducted after a medicine is marketed to provide additional details about the medicine's efficacy or safety profile. Different formulations, dosages, durations of treatment, medicine interactions, and other medicine comparisons may be evaluated. New age groups, races, and other types of patients can be studied. Detection and definition of previously unknown or inadequately quantified adverse reactions and related risk factors are an important aspect of many phase IV studies. If a marketed medicine is to be evaluated for another (i.e., new) indication, then those clinical trials are considered phase II clinical trials. The term *postmarketing surveillance* is frequently used to describe those clinical studies in phase IV (i.e., the period following marketing) that are primarily observational or nonexperimental in nature to distinguish them from well-controlled phase IV clinical trials or marketing studies.

Prevalence Total number of people in a population that are effected with a particular disease at a given time. This term is expressed as the rate of all cases (e.g., the prevalence of disease *X* is *Y* patients per 100,000 population) at a given point or period of time.

Research (on medicines) Numerous definitions of research are used both in the literature and among scientists. In the broadest sense, research in the pharmaceutical industry includes all processes of medicine discovery, preclinical and clinical evaluation, and technical development. In a more restricted sense, research concentrates on the preclinical discovery phase, where the basic characteristics of a new medicine are determined. Once a decision is reached to study the medicine in humans to evaluate its

therapeutic potential, the compound passes from the research to the development phase.

Research and development When research and development are used together, it refers to the broadest definition for research (see above). Some people use the term research colloquially to include most or all of the scientific and medical areas (discovery, evaluation, and development) covered by the single term research and development. *Medicine development* has several definitions and, in its broadest definition, is exactly the same as the broad definition of research.

Risk Measure of (1) the probability of occurrence of harm to human health or (2) the severity of harm that may occur. Such a measure includes judgment of the acceptability of risk. Assessment of safety involves judgment, and there are numerous perspectives (e.g., patients, physicians, company, regulatory authorities) used for judging it.

Safety Relative concept referring to the freedom from harm or damage resulting from adverse reactions or physical, psychological, or behavioral abnormalities that occur as a result of medicine or nonmedicine use. Safety is usually measured with one or more of the following: physical examination (e.g., vital signs, neurological, ophthalmological, general physical), laboratory evaluations of biological samples (e.g., hematology, clinical chemistry, urinalysis), special tests and procedures (e.g., electrocardiogram, pulmonary function tests), psychiatric tests and evaluations, and determination of clinical signs and symptoms.

Serious adverse reactions Multiple definitions are possible and no single one is correct in all situations. In general usage referring to patients in clinical trials, a serious adverse reaction may be (1) any bad adverse reaction that is observed, (2) any bad adverse reaction that one does not expect to observe, (3) any bad adverse reaction that one does not expect to observe and is not in the label, or (4) any bad adverse reaction that has not been reported with standard therapy. Definitions also may be based on the degree to which an adverse reaction compromises a patient's function or requires treatment.

Side effect Any effect other than the primary intended effect(s) resulting from medicine or nonmedicine treatment or intervention. Side effects may be negative (i.e., an adverse reaction), neutral, or positive (i.e., a beneficial effect) for the patient. This term therefore includes all adverse reactions plus other effects of treatment. See *adverse reaction*.

Site Place where a clinical trial is conducted. A physician who has offices and sees patients in three separate locations is viewed as having one site. A physician who is on the staff of four hospitals could be viewed as having one or four sites, depending on how similar or different the patient populations are and whether the data from these four locations will be pooled and considered a single site. For example, a single physician who enrolls groups of patients at a university hospital, private clinic, community hospital, and

Veterans Administration Hospital should generally be viewed as having four sites, since the patient populations would be expected to differ at each site.

Statistical significance Probability that an event or difference occurred by chance alone. Thus, it is a measure of whether a difference is likely to be real, but it does not indicate whether the difference is small or large, important or trivial. The level of statistical significance depends on the number of patients studied or observations made as well as the magnitude of differences observed.

Therapeutic window Difference between the minimum and maximum doses that may be given patients to obtain an adequate clinical response and avoid intolerable toxic effects. The greater the value calculated for the therapeutic window, the greater a medicine's margin of safety. Synonyms are *therapeutic ratio* and *therapeutic index*.

Volunteer Normal individual who participates in a clinical trial for reasons other than medical need and who does not receive any direct medical benefit from participating in the trial.

Appendix E

Common Vehicles for Nonclinical Evaluation of Therapeutic Agents

TABLE 1 Acacia

	Route	Duration	Dose	Comments
Rat	*Oral*	30 days	500 mg/kg	Well tolerated
		90 days	10 mL/kg	As 20% of formulation; well tolerated
Primate	*Oral*	90 days	100 mg/kg	Well tolerated, but with some reduction in food intake

TABLE 2 Acetate Sodium

	Route	Duration	Dose	Comments
Rat	*Intravenous*	1 month	1 mL/kg	Well tolerated as 5 mM solution in saline

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TABLE 3 Acetic Acid

	Route	Duration	Dose	Comments
Rat	*Oral*	90 days	5 mL/kg	Well tolerated (gavage), 3% solution
		1 month	10 mL/kg	Well tolerated, 20% solution
	Intravenous	1 month	As pH buffer	Well tolerated
Mouse	*Oral*	90 days	5 mL/kg	Well tolerated (gavage), 3% solution

TABLE 4 Acetone (2-Propanone)

	Route	Duration	Dose	Comments
Rat	*Oral*	2 weeks	5 mL/kg	Higher doses cause acidosis; transitory neurobehavorial effects at this dose
	Dermal	30 days	5 mL/kg	Well tolerated
Mouse	*Oral*	2 weeks	3 mL/kg	Higher doses cause acidosis; transitory neurobehavorial effects at this dose
	Dermal	2 years	0.5 mL	Well tolerated
Guinea pig	*Dermal*	1 month	1 mL	Well tolerated
Rabbit	*Dermal*	90 days	1 mL	Defatting of application site

TABLE 5 Alginic Acid

	Route	Duration	Dose	Comments
Rat	*Intraperitoneal*	1 month	100 mg/kg	Well tolerated

TABLE 6 Anecortave Acetate

	Route	Duration	Dose	Comments
Rat	*Subcutaneous*	4 doses	2 mL/kg	Well tolerated

TABLE 7 Benzoic Acid

	Route	Duration	Dose	Comments
Rat	*Oral*	N/A	100 mg	Well tolerated

TABLE 8 -Cyclodextrin

	Route	Duration	Dose	Comments
Rat	*Oral*	12 months	500 g/kg	Hepatitis, nephrosis, acute tubular necrosis at dose levels above 20 g/kg
	Intravenous	—	—	Tubular hypertrophy at doses above 100 mg/kg/day at 3 months or longer
Primate	*Oral*	12 months	—	Tubular hypertrophy at doses above 100 mg/kg/day at 3 months or longer

TABLE 9 Canola Oil

	Route	Duration	Dose	Comments
Dog	*Oral*	1 month	2 mL/kg	Well tolerated

TABLE 10 Capryol 90

	Route	Duration	Dose	Comments
Dog	*Oral*	28 days	1000 mg/kg	Well tolerated
			2500 mg/kg	Well tolerated
Rat	*Oral*	Acute		Well tolerated; $LD_{50} > 5$ g/kg
		28 days	500, 1500, 2500 mg/kg	NOAEL of 2500 mg/kg
		7 days	300, 1000, 2500 mg/kg	Well tolerated
Rabbit	*Cutaneous*	Acute	No dilution	Mildly irritant
	Ocular	Acute	No dilution	Moderately irritant

TABLE 11 Captisol

	Route	Duration	Dose	Comments
Rat	*Oral*	1 month	10 mL/kg	12% solution, well tolerated
	Intravenous	1 month	4 mL/kg	12% solution, well tolerated
Primate	*Oral*	9 months	1 g/kg	10% solution, well tolerated
	Subcutaneous	12 months with 3 weekly administrations	120 mg/kg	Well tolerated
Mouse	*Oral*	1 month	500 mg/kg	10% solution, well tolerated
	Subcutaneous	6 months	1200 mg/kg	NOAEL
		90 days	1200 mg/kg	NOEL

TABLE 12 Carboxymethyl Cellulose (CMC)

	Route	Duration	Dose	Comments
Primate	*Oral*	30 days	5% in water	Well tolerated
	Subcutaneous	Acute	10 mL/kg	Well tolerated
Rat	*Oral*	1 year	5% in water	Well tolerated

TABLE 13 Carboxymethyl Cellulose Calcium

	Route	Duration	Dose	Comments
Dog	*Oral*	90 days	1 mL/kg	Well tolerated; 1% solution

TABLE 14 Carboxymethyl Cellulose Sodium

	Route	Duration	Dose	Comments
Rabbit	*Oral*	1 month	0.5 mL/kg	Well tolerated; 1% solution

TABLE 15 Cetyl Alcohol

	Route	Duration	Dose	Comments
Mouse	*Intraperitoneal*	1 month	100 mg/kg	Well tolerated

TABLE 16 Citrate Buffer

	Route	Duration	Dose	Comments
Dog	*Intravenous*	8 doses	30 mL/kg/day	Well tolerated
	Subcutaneous	30 days	10 mL/kg/day	Well tolerated
Rat	*Oral*	2 weeks	15 mL/kg	Well tolerated (50 mM)
			10 mL/kg	Well tolerated (50 mM)

TABLE 17 Citric Acid Buffer

	Route	Duration	Dose	Comments
Rat	*Oral*	2 weeks	15 mL/kg	Well tolerated (50 mM)
			10 mL/kg	Well tolerated (50 mM)

TABLE 18 Collagen Matrix

	Route	Duration	Dose	Comments
Primate	*Implantation in humerus bone*	6 months	Two strips/site (humerus right and left)	Well tolerated
Rabbit	*Implantation*	6 months	Single application, 5 mL/kg	Well tolerated

TABLE 19 Corn Oil

	Route	Duration	Dose	Comments
Dog	*Oral*	1 month	3.0 mL/kg	Well tolerated
Rat	*Oral*	20 doses	5 mL/kg	Well tolerated
		1 dose	10 mL/kg	Well tolerated
Mouse	*Oral*	1 month	2.5 mL/kg	Well tolerated
Rabbit	*Oral*	1 month	1 mL/kg	Well tolerated
Chick embryo	*Oral*			
	Injection into egg	Once	0.1 μL/g	Less mortality than 1.0 μL/g egg
			1 μL/g	Increase in mortality, decreased activity during righting reflex, running time, visual discrimination, and olfactory aversion test

TABLE 20 Cremophore EL

	Route	Duration	Dose	Comments
Dog	*Intravenous*	1 month	2 mL/kg	Well tolerated
Rat	*Oral*	1 month	100 mg/kg	Well tolerated

TABLE 21 Cyclohexane

	Route	Duration	Dose	Comments
Rat	*Oral*	4 weeks	5 mL/kg/day	Clinical signs: intermittent convulsive after dosing, piloerection round back and emaciated appearance
	Dermal	30 days	1 mL/kg/day	Well tolerated
Rabbit	*Oral*	30 days	0.5 mL/kg/day	Well tolerated

TABLE 22 D-Glucose anhydrous 30%–PEG 70% (V/V)

	Route	Duration	Dose	Comments
Dog	*Oral*	2 weeks	0.32 mL/kg	Well tolerated
	Intravenous	2 weeks	Bolus 0.24–0.33 mL/kg infusion, 0.08–0.11 mL/kg/h	Well tolerated
Rat	*Intravenous*	3 weeks	Bolus 0.8–1.07 mL/kg infusion, 0.266–0.356 mL/kg intravenous injection (into tail vein) followed by an intravenous injection for 6 h	Well tolerated

TABLE 23 Dextrose (0.5%)

	Route	Duration	Dose	Comments
Dog	*Intravenous*	90 days	150 mL/h	Well tolerated
Rat	*Intravenous*	1 dose	1.4 mL per animal	Well tolerated

TABLE 24 Diethyleneglycol-Monoethylether

	Route	Duration	Dose	Comments
Primate	*Intravenous*	1 month	0.355 mL/kg	Well tolerated

TABLE 25 Dimethylsulfoxide

	Route	Duration	Dose	Comments
Dog	*Intravenous*	1 month	1.25 mL/kg	Well tolerated
Rat	*Oral*	7 days	5 mL/kg	Well tolerated
		4 weeks	5 mL/kg	Well tolerated
	Intravenous	1 month	200 mg/kg	Well tolerated
	Intraperitoneal	1 month	5 mL/kg	Well tolerated
Guinea pig	*Intravenous*	1 month	0.1 mL/kg	Well tolerated
Primate	*Oral*	Efficacity	3 mL/kg/day	Well tolerated
Mouse	*Oral*	4 weeks	5 mL/kg	Well tolerated
	Intraperitoneal	1 month	100 mg/kg	Well tolerated
		3 days	10 mL/kg	Well tolerated
Rabbit	*Subcutaneous*	1 month	1 mL/kg	Erythema, inflammation

TABLE 26 Dulbecco's Modified PBS

	Route	Duration	Dose	Comments
Rat	*Oral*	4 weeks	0.1, 0.8 and 1.2 mg/kg	Well tolerated
	Intravenous	1 month	1 mL/kg/day	Well tolerated

TABLE 27 Ethanol

	Route	Duration	Dose	Comments
Dog	*Oral*	6 months	400 mL/kg	Hepatopathy, myopathy, CNS changes
		90 days	5 mL/kg	5% solution; well tolerated
		1 month	5 mL/kg	7.5% solution; well tolerated
	Intravenous	Once	1 mL/kg	30% solution; CNS depression, ataxia
Rat	*Oral*		5 mL/kg	Depression
		1 month	175 g/kg	Depression, decreased RBCs
		12 months	1000 mg/kg	Fatty liver
		7 days	10 mL/kg	10% solution; well tolerated
		4 weeks	2 mL/kg	70% solution; hypokinesia, dyspnea, regurgitation, distended lungs/ileum, and swollen abdomen
		90 days	8 mL/kg	10% solution; well tolerated
	Intravenous	12 months	250 g/kg	Nephrosis, ATN, bladder changes, weight loss
Primate	*Oral*	9 months	250 mg/kg	Behavioral changes
Mouse	*Oral*	6 months	2500 mg/kg	Well tolerated
		1 month	2.5 mL/kg	5% solution; well tolerated
	Intraperitoneal	Acute	5 mL/kg	5% solution; well tolerated
	Cutaneous	13 weeks	100 μL/animal/day	70% solution; well tolerated

TABLE 28 Gelucire 44/14

	Route	Duration	Dose	Comments
Rabbit	*Cutaneous*	Acute	0.5 mL	Not irritant
	Ocular	Acute	0.1 mL	Slight irritant
Rat	*Oral*	28 days	600, 1500, 2400 mg/kg/day	NOEL: 2400 mg/kg/day
		7 days	600, 1500, 2400 mg/kg/day	NOEL: 2400 mg/kg/day
		Acute	No dilution	LD_{50}: >2004 mg/kg/day
Dog	*Oral*	3 months	400, 1000, 2500 mg/kg/day	NOAEL: >2500 mg/kg/day
		14 days	400, 1000, 2500 mg/kg/day	

TABLE 29 Glucose

	Route	Duration[a]	Dose	Comments
Dog	*Oral*	ADME	2/10 mL/kg/day	5% solution; well tolerated
Rat	*Oral*	26 weeks	0.71–8.6 mL/kg	10% solution; well tolerated
		Prelim	5 mL/kg	5% solution; well tolerated
	Subcutaneous	2 weeks	0.75 mL/kg	5% solution; well tolerated
Primate	*Oral*	13 weeks	0.78–9.3 mL/kg	10% solution; well tolerated
		ADME card. vas.	5 mL/kg	5% solution; well tolerated

[a]ADME: Absorption, distribution, metabolism, and excretion.

TABLE 30 Glycerol

	Route	Duration	Dose	Comments
Rat	*Oral*		1000 mg/kg	Well tolerated
		1 month	15 g/kg	Reduced adrenal weights
		1 month	1000 mg/kg	Well tolerated
	Subcutaneous		10 mg/kg	Well tolerated
Guinea pig	*Oral*	1 month	500 mg/kg	Well tolerated
Mouse	*Oral*	90 days	500 mg/kg	Depression and reduced respiration
	Intravenous	1 month	100 mg/kg	Well tolerated
	Subcutaneous	Acute	10 mg/kg	Well tolerated
	Intraperitoneal	1 month	250 mg/kg	Well tolerated
Rabbit	*Intravenous*		10 mg/kg	Well tolerated

TABLE 31 Gum Tragacanth

	Route	Duration	Dose	Comments
Mouse	*Oral*	2 weeks	10 mL/kg	0.5% solution; Well tolerated

TABLE 32 Hydroxypropyl Betacyclodextrin

	Route	Duration	Dose	Comments
Dog	*Intravenous*	1 month	10 mL/kg	40% solution; well tolerated
Rat	*Intravenous*	1 month	10 mL/kg	40% solution; well tolerated

TABLE 33 Hydroxypropyl Cellulose

	Route	Duration	Dose	Comments
Rat	*Oral*	90 days	1000 mg/kg	Well tolerated

TABLE 34 Hydroxypropyl Methylcellulose

	Route	Duration	Dose	Comments
Dog	*Intraperitoneal*	28 days	200 mg/kg	Well tolerated
Rat	*Oral*	One dose	10 mL/kg	0.2%; well tolerated
		One dose	10 mL/kg	0.5%; well tolerated
			10 mL/kg	0.5%; well tolerated
	Intraperitoneal	One dose	5 mL/kg	0.5%; well tolerated
Mouse	*Oral*	10 doses	10 mL/kg	0.2%; well tolerated
		One dose	10 mL/kg	0.5%; well tolerated
	Intraperitoneal	Acute	50 mg/kg	Well tolerated
		Acute	5 mL/kg	0.5%; well tolerated

TABLE 35 Isopropyl Alcohol

	Route	Duration	Dose	Comments
Rabbit	*Dermal*	1 month	1000 mg/kg	Well tolerated

TABLE 36 Isopropyl Myristate

	Route	Duration	Dose	Comments
Rabbit	*Dermal*	1 month	500 mg/kg	Well tolerated

TABLE 37 Labrafil MI944

	Route	Duration	Dose	Comments
Dog	*Oral*	1 month	2 mg/kg	Well tolerated

TABLE 38 Labrasol

	Route	Duration	Dose	Comments
Rat	*Oral*	Acute	20, 22.4, 25.1, 28.21, and 31.60 g/kg	LD_{50} = 22 g/kg; nontoxic
		ADME	10 and 150 mg/kg/day	
		Segment II: embryofetal development	1000, 2000, or 3000 mg/kg/day	NOEL: 3000 mg/kg/day with no indication of teratogenicity
		14 days	100, 300, 1000 and 3000 mg/kg/day	NOAEL: 3000 mg/kg/day
		6 months	300, 1000 and 3000 mg/kg/day	NOEL: 300 mg/kg/day; NOAEL: 3000 mg/kg/day
	Intravenous	28 days	10 mg/kg/day	
	Cutaneous	Patch test	0.02 mL/animal	Well tolerated
		Acute		Very well tolerated
	Ocular			Slight irritant
Dog	*Oral*	13 weeks	0, 300, 1000, and 3000 mg/kg/day	NOEL: 1000 mg/kg/day; NOAEL: 3000 mg/kg/day
		14 days	100, 300, 1000, and 3000 mg/kg/day	In high-dose group, moderate suppurative inflammation of lungs; no adverse effects on survival and clinical observations
		3 months	300, 1000 and 3000 mg/kg/day	NOEL: 1000 mg/kg/day; NOAEL: 3000 mg/kg/day
Rabbit	*Cutaneous*	Patch test	0.5 mL	Well tolerated
	Ocular	Acute	0.1 mL	Slight irritant

TABLE 39 Lactose

	Route	Duration	Dose	Comments
Primate	*Inhalation*	2 weeks	1 L/min/animal	Well tolerated

TABLE 40 Lanolin

	Route	Duration	Dose	Comments
Rabbit	*Dermal*	90 days	1000 mg/kg	Well tolerated

TABLE 41 L-Ascorbic Acid

	Route	Duration	Dose	Comments
Rat	*Oral*	90 days	500 mg/kg	Hematological changes, weight loss

TABLE 42 Lauroglycol

	Route	Duration	Dose	Comments
Rabbit	*Cutaneous*	Acute	No dilution	Moderately irritant
	Ocular	Acute	No dilution	Slightly irritant
Rat	*Oral*	Acute		LD_{50}: >2003 mg/kg/day

TABLE 43 Maltitol Solution

	Route	Duration	Dose	Comments
Rat	*Intraperitoneal*	1 month	500 mg/kg	Well tolerated

TABLE 44 Maltol

	Route	Duration	Dose	Comments
Guinea pig	*Oral*	1 month	75 mg/kg	Well tolerated
Rabbit	*Oral*	1 month	100 mg/kg	Well tolerated

TABLE 45 Mannitol

	Route	Duration	Dose	Comments
Primate	*Oral*	2 months	10 mL/kg	Well tolerated

TABLE 46 Methyl Cellulose

	Route	Duration	Dose	Comments
Rat	*Oral*	1 month	10 mL/kg	0.5%; well tolerated
		1 month	5 mL/kg	0.5%; well tolerated
		14 doses	10 mL/kg	1%; well tolerated
		1 dose	10 mL/kg	2%; well tolerated
Guinea pig	*Oral*	12 doses	4 mL/kg	0.5%; well tolerated
Primate	*Oral*	1 month	10 mL/kg	0.1%; well tolerated
		1 month	10 mL/kg	0.5%; well tolerated
		2 weeks	5 mL/kg	0.5%; well tolerated
		1 month	5 mL/kg	1%; well tolerated
Mouse	*Oral*	90 days	10 mL/kg	0.5%; well tolerated
Rabbit	*Oral*	1 month	4 mL/kg	0.5%; well tolerated
Dog	*Oral*	2 weeks	10 mL/kg	0.5%; well tolerated

TABLE 47 Mineral Oil

	Route	Duration	Dose	Comments
Rat	*Oral*	1 month	5 mL/kg	Well tolerated
Mouse	*Oral*	1 month	250 mg/kg	Well tolerated
Dog	*Oral*	1 month	2.5 mL/kg	Well tolerated

TABLE 48 Phosphate-Buffered Saline

	Route	Duration	Dose	Comments
Rat	*Oral*	1 month	10 mL/kg	Well tolerated
	Intravenous	1 dose	1 mL/kg	Well tolerated
	Subcutaneous	1 month	1 mL/kg	Well tolerated
	Slow bolus	11 doses	1 mL/kg	Well tolerated
Mouse	*Subcutaneous*	6 months	10 mL/kg	Well tolerated
Primate	*Oral*	2 weeks	10 mL/kg	Well tolerated
		2 weeks	1.6 mL/kg	Well tolerated
	Subcutaneous	1 week	0.2 mL/kg	Well tolerated
		9 months	1 mL/kg	Well tolerated

TABLE 49 Peanut Oil

	Route	Duration	Dose	Comments
Rat	*Oral*	1 month	10 g/kg	Well tolerated
		12 months	10 g/kg	Well tolerated
		90 days	10 g/kg	Well tolerated

TABLE 50 PEG 300

	Route	Duration	Dose	Comments
Guinea pig	*Intravenous*	1 month	1 mL/kg	Well tolerated
Mouse	*Oral*	ADME	10 mL/kg/day	Well tolerated
Rabbit	*Oral*	1 month	500 mg/kg	Well tolerated

TABLE 51 PEG 400

	Route	Duration	Dose	Comments
Guinea pig	*Oral*	1 month	1000 mg/kg	Well tolerated
Mouse	*Oral*	4 weeks	10 mL/kg/day	Well tolerated
	Intraperitoneal	1 month	500 mg/kg	Well tolerated
		3 days	10 mL/kg	35%; well tolerated
		1 month	2.5 mL/kg	40%; well tolerated
Rat	*Oral*	10 doses	1.67 mg/kg	Well tolerated
		1 dose	2 mL/kg	Well tolerated
		1 dose	5 mL/kg	Well tolerated
		4 weeks	5 mL/kg/day	Well tolerated
		1 month	5 mL/kg	Well tolerated
	Intravenous	1 dose	0.5 mL/kg	Well tolerated
		4 weeks	0.5 mL/kg	Well tolerated
	Intraperitoneal	1 month	5 mL/kg	35%; well tolerated
	Cutaneous	13 weeks	2.5 mL/kg	Well tolerated
		104 weeks	2.5 mL/kg	Well tolerated
Minipig	*Cutaneous*	2 weeks	2.5 mL/kg	Well tolerated

TABLE 52 Petrolatum

	Route	Duration	Dose	Comments
Rabbit	*Dermal*	1 month	1 mg/kg	Well tolerated

TABLE 53 Poloxamer

	Route	Duration	Dose	Comments
Rat	*Oral*	1 month	10 mL/kg	7.5%; well tolerated
Mouse	*Oral*	1 month	10 mL/kg	Well tolerated

TABLE 54 Povidone

	Route	Duration	Dose	Comments
Rat	*Intramuscular*	90 days	1 mL/kg	1%; well tolerated

TABLE 55 Propylene Glycol

	Route	Duration	Dose	Comments
Rat	*Oral*	1 month	2.5 mL/kg	Well tolerated
		2 weeks	2 mL/kg	Well tolerated
	Subcutaneous	4 weeks	2.5 mL/kg	Well tolerated
Minipig	*Cutaneous*	26 weeks	2.5 mL/kg	Well tolerated
Mouse	*Oral*	1 month	10 mL/kg	50%; well tolerated
	Intraperitoneal	1 month	2.5 mL/kg	40%; well tolerated
Dog	*Oral*	1 month	2.5 mL/kg	Well tolerated
		13 weeks	2 mL/kg	Well tolerated

TABLE 56 Rameb 7.5%

	Route	Duration	Dose	Comments
Primate	*Intranasal*	1 month, 3 doses per day	82.8 mg/mL (with treatment), 74.7 mg/mL (as placebo)	Well tolerated

TABLE 57 Sesame Oil

	Route	Duration	Dose	Comments
Rat	*Oral*	1 month	1 mL/kg	Well tolerated
Mouse	*Oral*	1 month	0.25 mL/kg	Well tolerated
Rabbit	*Oral*	1 month	0.5 mL/kg	Well tolerated
Dog	*Oral*	1 month	1 mL/kg	Well tolerated

TABLE 58 Sodium Acetate Trihydrate Buffer

	Route	Duration	Dose	Comments
Primate	*Intravenous*	2 weeks	1 mL/kg	Well tolerated

TABLE 59 Sodium Chloride 0.9%

	Route	Duration	Dose	Comments
Rat	*Intravenous*	7 doses	1 mL/kg	Well tolerated
		1 dose	1 mL/kg	Well tolerated
		14 doses	10 mL/kg	Well tolerated
		3 doses	4 mL/kg	Well tolerated
	Subcutaneous	1 dose	0.1–0.4 mL	Well tolerated
		1 month	4 mL/kg	Well tolerated
		56 doses	2 mL/kg	Well tolerated
	Slow bolus	1 dose	1 mL/kg	Well tolerated
		1 dose	10 mL/kg	Well tolerated
Mouse	*Intravenous*	1 dose	10 mL/kg	Well tolerated
	Subcutaneous	1 dose	10 mL/kg	Well tolerated
Rabbit	*Intravenous*	1 dose	0.1 mL/kg	Well tolerated
	Intraperitoneal	1 dose	0.1 mL/kg	Well tolerated
Dog	*Oral*	1 dose	0.282 mL/kg	Well tolerated
	Intravenous	1 dose	10 mL/kg	Well tolerated
		1 dose	2 mL/kg	Well tolerated
		2 weeks	5 mL/kg	Well tolerated
	Subcutaneous	1 month	0.025 mL	Well tolerated
	Slow bolus	1 dose	0.3 mL/kg	Well tolerated
Primate	*Subcutaneous*	1 month	0.67 mL/kg	Well tolerated
		56 doses	0.5 mL/kg	Well tolerated

TABLE 60 Sodium Phosphate

	Route	Duration	Dose	Comments
Dog	*Oral*	2 weeks	10 mL/kg	70 mM; well tolerated
Rat	*Oral*	2 weeks	10 mL/kg	70 mM; well tolerated

TABLE 61 Tartaric Acid

	Route	Duration	Dose	Comments
Rat	*Oral*	39 weeks	0.5 mL/kg	Well tolerated
		2 weeks	3 mL/kg	Well tolerated
Rabbit	*Oral*	Preliminary segment II	3 mL/kg/day	Well tolerated
		Segment II	3 mL/kg/day	Well tolerated

TABLE 62 Transcutol

	Route	Duration	Dose	Comments
Cat	*Intravenous*	1 month	2 mL/kg	Well tolerated
Rabbit	*Dermal*	Skin irritation	0.5 mL over 2-cm^2 area	50%; nonirritant
		28 days	0, 300, 1000, 3000 mg/kg/day	Undiluted; NOEL >1000 mg/kg/day
	Ocular	Eye irritation	0.1 mL	30%; slight irritation
		Eye irritation	0.1 mL	Undiluted; slight irritation
Rat	*Oral*	90 days	0, 0.25, 1, and 5%	NOEL is 1%
		Acute	5.0 g/kg	LD_{50} > 5000 mg/kg
		Fertility and embryotoxicity range-finding study	500, 1000, 2000, 4000 mg/kg/day	NOEL > 500 mg/kg/day
Mouse	*Oral*	Acute	—	6.6 g/kg tested toxic
	Oral	Chronic (12 months)	—	NOEL: 850–1000 mg/kg
Dog	*Oral*	90 days	—	NOAEL: 1000 mg/kg

TABLE 63 Trisodium Citrate Dihydrate

	Route	Duration	Dose	Comments
Dog	*Oral*	52 weeks	10 mL/kg/day	Well tolerated
Rat	*Oral*	Segment III	10 mL/kg/day	Well tolerated
		39 weeks	10 mL/kg/day	Well tolerated
		4 weeks	10 mL/kg/day	Well tolerated
Mouse	*Intravenous*	13 weeks	10 mL/kg/day	Well tolerated

TABLE 64 Tween 20

	Route	Duration	Dose	Comments
Rat	*Oral*	1 month	250 mg/kg	Well tolerated
		90 days	500 mg/kg	Diarrhea
Mouse	*Oral*	1 month	10 mg/kg	Well tolerated

TABLE 65 Tween 80

	Route	Duration	Dose	Comments
Dog	*Oral*	90 days	5 mL/kg	As 1% of formulation; well tolerated
Rat	*Oral*		350 mg/kg	Well tolerated
		4 weeks	5 mL/kg	1%; well tolerated
		7 days	10 mL/kg	1%; well tolerated
	Intravenous		100 mg/kg	Well tolerated
Mouse	*Intraperitoneal*	1 month	10 mL/kg	2%; well tolerated
	Intranasal	3 days	10 μL/nostril	2%; well tolerated
Primate	*Oral*	90 days	5 mL/kg	1%; well tolerated

TABLE 66 Xylitol

	Route	Duration	Dose	Comments
Primate	*Intranasal*	1 month	1200 μL/day for control and high concentration; 200 and 400 μL/day for respectively low and intermediate dose level	Well tolerated

TABLE 67 Summary of Vehicle Information

Excipient/Vehicle	Data in Table Number	CAS Number	Chemical Name	Formula	Key Toxicity Review Articles[a]	Animal Species Evaluated
2-hydroxypropyl-β-cyclodextrin	NA	128446-35-5			Gould, S. (2005)	Rat, primate, mouse, rabbit, dog
Acacia	3	9000-01-5	Acaciae gummi		TOXSYS; Anderson (1986); Bachmann, E. (1978)	Rat, primate
Acetate, sodium	4	127-09-3	Acetic acid sodium salt	$C_2H_3NaO_2$	TOXSYS	Rat
Acetic acid	5	64-19-7	Ethanolic acid	$C_2H_4O_2$	Cragg, S.T. (2001); Schonwald, S. (2004), "Irrigating Solutions"	Rat, mouse
Acetone	6	67-64-1	2-Propanone	CH_3COCH_3	Meditext, 2006	Rat, mouse, guinea pig, rabbit
Acetylmethylamine in water	NA	79-16-3	N-Methylacetamide	C_3H_7NO	NA	
Alginic acid	7	9005-32-7	Norgine	$(C_6H_3O_6)_n$	JECFA, 49th (1997)	Rat
Anecortave acetate	8	7753-60-8		$C_{23}H_{30}O_5$	Jockovich (2006); Talsma (2004)	Rat
Benzoic acid	9	65-85-0	Benzoic acid	$C_7H_6O_2$	TOXSYS; Nair, B. (2001); David, R. M., et al. (2001)	Rat
β-Cyclodextrin	10	7585-39-9	β-Dextrin	$C_{42}H_{70}O_{35}$	TOXSYS; Toyoda, K. (1997); Waner, T. (1995); Marttin, E. (1998); Challa, R. (2005)	Rat, primate
BHT	NA	128-37-0	Butylated hydroxytoluene	$C_{15}H_{24}O$	Lanigan, R. S. (2002); Nakagawa, Y. (1984); Briggs, D. (1989)	

Canola oil	11	120962-03-0	Canbra oil		Evangelista, C. M. (2004)	Dog
Capryol 90	12	31565-12-5	Propylene glycol monocaprylate	$C_{11}H_{22}O_3$	Li (2005); Cho (2004)	Rat, dog, rabbit
Captisol	13	182410-00-0	β-Cyclodextrin sulfobutyl ether, sodium salt (CDSBE)	$C_{42}H_{70}$-*n*-O_{35}* $(C_4H_8SO_3Na)_n$	TOXSYS	Rat, primate, mouse
Carboxymethylcellulose (CMC)	14	9000-11-7	Acetic acid; 2,3,4,5,6-pentahydroxyhexanal	$C_8H_{16}O_8$	Mehlman, M. A. (2001), "Carboxymethylcellulose"	Primate, rat
Carboxymethylcellulose calcium	15	9050-04-8	Calcium CMC		TOXSYS	Dog
Carboxymethylcellulose sodium	16	9004-32-4	Carmellose sodium		Bar, A. (1995); Freeman, C. (2003); Cavender, F. L. (2001), "Sodium Carboxymethyl Cellulose"; Bachmann, E. (1978)	Rabbit
Cavasol W7	NA	128446-35-5	2-Hydroxypropylcycloheptaamylose		Wacker Fine Chemicals (2006)	
Cetyl alcohol	17	36653-82-4	Hexadecan-1-ol	$C_{16}H_{34}O_1$	TOXSYS; Bevans, C. (2001), p. 494.	Mouse
Citrate buffer	18	77-92-9	Sodium citrate–citric acid buffer		Schonwald, S. (2004), "Acidifying and Alkalinizing Agents"	Dog, rat
Citric acid buffer	19	77-92-9		$C_6H_8O_7$*H_2O	Cragg, S. T. (2001), "Citric Acid"	Rat
CMC with dimethicone	NA	9004-32-4 9006-65-9	Carboxymethylcellulose sodium Trimethyl-trimethylsilyloxysilane	$C_6H_{18}OSi_2$	NA	
Coconut oil	NA	8001-31-8	NA		Shadnia, S. (2005); National Toxicology Program (2001)	
Collagen matrix	20	9007-34-5	Collagen human		McCarthy, D. M. (2002); Clark, D. P. (1989)	Primate, rabbit
Corn oil	21	8001-30-7	Corn germ oil, glyceridic		TOXSYS; Wu, B. (2004); Dupont, J. (1990); DeWitt, J. C. (2005)	Dog, rat, mouse, rabbit, chick embryo
Cremophore EL	22	61791-12-6	Polyoxyl castor oil		TOXSYS; Gelderblom, H. (2001); Ramadan, L. A. (2001); Lorenz, W. (1977)	Dog, rat

TABLE 67 ***Continued***

Excipient/Vehicle	Data in Table Number	CAS Number	Chemical Name	Formula	Key Toxicity Review Articles[a]	Animal Species Evaluated
Cyclohexane	23	110-82-7	Hexahydrobenzene; hexamethylene; hexanaphthene	C_6H_{12}	Kreckmann, K. H. (2000); Malley, L. A. (2000); Gad, S. E. (2005), "Cyclohexane"	Rat, rabbit
DAM PEG (polyethylene glycol)	24				NA	Dog, rat
Dextrose	25	50-99-7	D-Glucose, anhydrous; dextrosol	$C_6H_{12}O_6$	Buard, A. (2003)	Dog, rat
Diethyleneglycolmonoethylether	26	111-90-0			Hardin, B. D. (1983); Hardin, B. D. (1984)	Primate
DMSO	27	67-68-5	Dimethylsulfoxide	C_2H_6OS	TOXSYS; White, C. W. (1983); Augustine, K. A. (2000); Ali, B. H. (2001); Schonwald, S. (2004), "Irrigating Solutions"	Dog, rat, guinea pig, primate, mouse, rabbit
Dulbecco's modified PBS	28				NA	Rat
EDTA	NA	60-00-4	Ethylenediaminetetraacetic acid	$C_{10}H_{16}N_2O_8$	TOXSYS; Heimbach, J. (2000); Lanigan, R. S. (2002); Cavender, F. L. (2001), "Ethylenediamine Tetraacetic Acid"	
Ethanol	29	64-17/5	Ethyl alcohol	C_2H_6O	TOXSYS; Church, A. S. (1997); Bevan, C. (2001), "Ethanol"	Dog, rat, primate, mouse
Gelucire 44/14	30	121548-04-7	PEG-32 glyceryl laurate		Yan (2005); Kawakami (2004)	Rabbit, rat, dog
Gelucire 50/13	NA	121548-05-8	G-50-13		Sharma (2006)	
Glucose	31	50-99-7	Dextrose	$C_6H_{12}O_6$	Robertson (2003)	Dog, rat, primate
Glycerol	32	56-81-5	Glycerine	$C_3H_8O_3$	TOXSYS; Anderson, R. C.; Final report (2004) Glycerols	Rat, guinea pig, mouse, rabbit
Gum tragacanth	33	9000-65-1			Anderson, D. M. (1989); Bachmann, E. (1978); Hagiwara, A. (1991)	Mouse

Gum xanthane	NA	11138-66-2		$(C_{35}H_{49}O_{29})_n$	NA	
Hydroxypropyl β-cyclodextrin	34	94035-02-6		$C_{42}H_{70}$–*n*-O_{35}	Gerloczy, A. (1994)	Dog, rat
Hydroxypropyl cellulose	35	9004-64-2	Methocel		TOXSYS; Cavender, F. L. (2001), "Hydroxypropyl Cellulose"	Rat
Hydroxypropyl methylcellulose	36	9004-65-3	Benecel MHPC, Hypromellose		TOXSYS; Geerling, G. (2001); Maki, K. C. (2000); Rosen, P. A. (1987); Mehlman, M. A. (2001), "Hydroxypropyl Methylcellulose"; Obara, S. (1992)	Dog, rat, mouse
Isopropyl alcohol	37	67-63-0	*sec*-Propyl alcohol	C_3H_8O	TOXSYS; Tyl, R. W. (1994); Bevan, C. (2001), "Isopropanol" ; Sivilotti, M. L. A. (2004); Burleigh-Flayer, H. (1998); Church, A. S. (1997); Allen, B. (1998)	Rabbit
Isopropyl myristate	38	110-27-0	Crodamol IPM	$C_{17}H_{34}O_2$	TOXSYS; Komatsu, H. (1979); Campbell, R. L. (1981)	Rabbit
Labrafil M1944	39	62563-68-2	Labrafil		TOXSYS; Beckwith-Hall, B. M. (2002)	Dog
Labrasol	40	85536-07-8	Polyglycolyzed Glycerides		Hu, Z. (2001)	Rat, dog, rabbit
Lactose	41	63-42-3 (anhy)	*O*-β-D-Galactopyranosyl-(1->4)-α - D-glucopyranose	$C_{12}H_{22}O_{11}$ (anhy)	TOXSYS; Baldrick, P. (1997); Ahmad, S. K. (2004)	Primate
Lanolin	42	8006-54-0	Lanolin		Kligman, A. M. (1983)	Rabbit
L-Ascorbic acid	43	50-81-7	Cevatine, Cevex, Cevital	$C_6H_8O_6$	Bendich, A. (1990); Dykes, M. H. (1975); Temple, B. R. (2004)	Rat
Lauroglycol	44	27194-74-7	Lauric acid, monoester with propane-1,2-diol		Liu (2006)	Rabbit, rat

TABLE 67 ***Continued***

Excipient/Vehicle	Data in Table Number	CAS Number	Chemical Name	Formula	Key Toxicity Review Articles[a]	Animal Species Evaluated
Maltitol solution	45	9053-46-7	Liquid Maltitol	$C_{12}H_{24}O_{11}$ + $C_6H_{14}O_6$	Walker, R. (1978); Modderman, J. P. (1993)	Rat
Maltol	46	118-71-8	3-Hydroxy-2-methyl-4*H*-pyran-4-one	$C_6H_6O_3$	Hironishi, M. (1996); Murakami, K. (2006)	Guinea pig, rabbit
Mannitol	47	69-65-8	D-Mannitol	$C_6H_{14}O_6$	TOXSYS; Horvath, I. P. (1982); Lina, B. A. (1996)	Primate
Methane sulfonic acid	NA	75-75-2	Methylsulfonic acid	CH_4O_3S	TOXSYS; Shertzer, H. G. (2001)	
Methyl cellulose	48	9004-67-5	Cellulose methyl ester		TOXSYS; Mehlman, M. A. (2001), "Methylcellulose"; Bachmann, E. (1978); Sellers, R. S. (2005)	Rat, guinea pig, primate, mouse, rabbit, dog
Miglyol 810	NA	85409-09-2	Caprylic, capric triglycerides		TOXSYS; Traul, K. A. (2000); Sellers, R. S. (2005)	
Mineral oil	49	8012-95-1	Liquid paraffin		TOXSYS; Dalbey, W. E. (2003); Trimmer, G. W. (2004); Nash, J. F. (1996)	Rat, mouse, dog
Neobee 1053	NA	73398-61-5	Medium–chain triglycerides		NA	
N-Methylpyrrolidone (Pharmasolv)	NA	872-50-4	1-Methyl-2-pyrrolidinone	C_5H_9NO	TOXSYS; Solomon, G. M. (1996); Trochimowicz, H. J. (2001); Lee, K. P. (1987)	
PBS (phosphate-buffered saline)	50				NA	Rat, primate, mouse
Peanut oil	51	8002-03-7	Arachis oil, Fletcher's		TOXSYS; "Final Report on the Safety Assessment of Peanut …" (2001)	Rat

PEG 300	52	25322-68-3	Polyethylene glycol #300	$H_0CH_2(CH_2OCH_2)_mCH_2OH$	TOXSYS; Cavender, F. L. (2001), "Polyethylene Glycols"; Patel, M. (2005)	Guinea pig, mouse, rabbit
PEG 400	53	25322-68-3	Polyethylene glycol #400	$(C_2H_4O)_nH_2O$	TOXSYS; Hermansky, S. J. (1995); Patel, M. (2005)	Rat, minipig, guinea pig, mouse
Petrolatum	54	8009-03-8	Yellow soft paraffin		TOXSYS	Rabbit
Poloxamer	55	9003-11-6	Lutrol	$HO(C_2H_4O)$-*a*(C_3H_6O)-*b*-H	Frim, D. M. (2004); TOXSYS; Grindel, J. M. (2002)	Rat, mouse
Polysorbate 80	NA	9005-65-6	Polyoxyethylene (20) sorbitan monooleate		TOXSYS; (1992) NTP Toxicology and Carcinogenesis Studies	
Povidone	56	9080-59-5	2-Methoxy-6-methyl-phenol	$C_8H_{10}O_2$	Beji, S. (2006)	Rat
Propylene glycol	57	57-55-6	1,2-Dihydroxypropane	$C_3H_8O_2$	TOXSYS; (1999) Final Report on the Safety Assessment of Propylene Glycol …; Cavender, F. L. (2001), "Propylene Glycol"	Rat, minipig, mouse, dog
Rameb 7.5%	58		Randomly methylated-β-cyclodextrins		Challa, R. (2005)	Primate
Sesame oil	59	8008-74-0	Sesame oil		TOXSYS; Farber, T. M. (1976); Genovese, R. F. (1999)	Rat, mouse, rabbit, dog
Sodium acetate trihydrate buffer	60	6131-90-4			NA	Primate
Sodium chloride	61	7647-14-5	Salt, Halite	NaCl	Meneely, G. R. (1953); Meneely, G. R. (1958); Teitelbaum (2001); Caraccio, T. R. (2004), "Over the Counter Products"	Dog, rat, primate, mouse, rabbit

TABLE 67 ***Continued***

Excipient/Vehicle	Data in Table Number	CAS Number	Chemical Name	Formula	Key Toxicity Review Articles[a]	Animal Species Evaluated
Sodium phosphate	62	7558-80-7			Moore, G. L. (1988); Pierce, S. W. (2001)	Dog, rat
Solutol HS15/purified water	NA	70142-34-6	Polyethylene glycol-15-hydroxystearate		Coon (1991); Ruchatz (1998)	
Succinate, sodium	NA	150-90-3	Succinic acid sodium salt	$C_4H_4Na_2O_4$	TOXSYS; MSDS; Cragg, S. T. (2001), "Succinic Acid"	
Tartaric acid	63	87-69-4	*d*-Tartaric acid; 2,3-dihydroxybutanedioic acid	$HOOC(CH_2O)_2COOH$	Sourkes, T. L. (1950); Cragg, S. T. (2001), "Tartaric Acid"	Rat, rabbit
Transcutol	64	111-90-0	2-(2-Ethoxyethoxy)ethanol	$C_6H_{14}O_3$	Liu, Z. (2006)	Cat, rabbit, rat
Trisodium citrate dihydrate	65	6132043		$C_6H_5Na_3O_7 \cdot 2H_2O$	NA	Dog, rat, mouse
Tween 20	66	9005-64-5	Polysorbate 20 NF		TOXSYS; Bartsch, W. (1976)	Rat, mouse
Tween 80	67	9005-65-6	Armotan pmo-20, Tween(R) 80		TOXSYS; Daher, C. F. (2003); Fisherman, E. W. (1974); Sellers, R. S. (2005)	Rat, primate, mouse, dog
Xylitol	68	87-99-0	Xylite	$C_5H_{12}O_5$	TOXSYS; Takahashi, K. (1999)	Primate

Note: NA, not currently available.

[a]See Gad, S. C., Cassidy, C., Aubert, N., Spainhour, B., and Robbe, H. (2006). Nonclinical vehicle use in studies by multiple routes in multiple species. *Int. J. Toxicol.* 25:499–522.

Appendix F

Global Directory of Contract Toxicology Laboratories

Drug Safety Evaluation, Second Edition, by Shayne Cox Gad

Laboratory	Location	Phone Number	Website	Additional Services[a]	Rat	Rabbits	Dog	Carcinogenicity	DART	Inhalation	Primate	Pig	IV Infusion	Genotoxicity	Devices	Metabolism	Analytical	Special Studies[b]
ABC Labs.	Missouri, IA; North Ireland; United Kingdom	(573) 474-8579, 44 (0) 2870 320 639	www.abclabs.com	Bioanalysis, methods development radioactivity	X											X	X	
ADMETRx	Kalamazoo	(269) 372-3272	www.admetrx.com	Yes														
Applied Preclinical Services	Oxford, NJ	(908) 637-4427	NO		X		X			X		X				X		
Apptec/Wuxi	St. Paul, MN; Philadelphia, PA; Atlanta, GA	(888) 794-0077 (800) 622-8820 (888) 847-6633	www.apptec-usa.com	Biotech, biocomp./ med. devices, microbiology	X	X	X					X		X	X		X	
Aptuit	Ledbury, India, United Kingdom and United States	44 (0) 131 451 2560, (877) 988-2100	www.aptuit.com/	Yes, clinical, development, +	X	X	X	X	X			X	X	X	X	X	X	SP
Austrian Research Center	Seibersdorf, Austria	43(0) 50550-0	www.arcs.ac.at/		X	X			X		X							SP
Azopharma & Aniclin	Miramar, FL; Oxford, NJ	(954) 604-9879	www.Aniclin.com	Implant/surgical	X	X	X	X	X	X	X	X	X			X	X	X
BAS	West Lafayette, IN	(800) 845-4246	www.bioanalytical.com	Yes	X	X	X	X	X			X	X	X	X	X	X	
Battelle	Columbus, OH Richland, WA	(800) 201-2011	www.battelle.org	Yes	X X	X	X X	X X	X	X X	X	X X	X			X	X	
BEC Labs	Toledo, OH	(419) 693-5307, (888) BEC-LABS	www.beclabs.com	Yes											X			
Bioagri	Piracicaba, Campinas, and Sao Paulo, BRAZIL	+55-19-3429-7720	www.bioagri.com.br		X	X	X							X			X	
Biocon	Farmington, MN	(651) 460-3330			X	X												
Bio-Life	Neillsville, WI	(715) 743-3171	No	Yes											X			WL

Bio-Quant	San Diego, CA	(858) 450-0048	www.bio-quant.com	Tumor models, disease models	X		X					X				X		
BioReliance	Rockville, MD	(800) 738-1000	www.bioreliance.com	Full-range genotoxicity,[c] transgenic mouse CA models, p53	X	X							X	X			X	
Biotechnics	Hillsborough, NC	(919) 245-3114	www.biotechnics-inc.com	Yes	X	X	X							X				
BRI	Vancouver, BC	(604) 432-9237	www.bripharm.com	Yes, pharmaceutical, development, +	X									X		X	X	
BRIDGE	Beijing, China; Gaithersburg, MD	+650-859-3853	www.bridgepharmaceuticals.com	Regulatory, consulting	X		X				X		X	X		X	X	
BRT	Raleigh, NC	(919) 851-4499		*Specialty immunotox	X	X									X			
BTC	Irvine, CA	(949) 660-3185	www.biologicaltestcenter.com	Yes, pediatrics, +	X	X	X					X	X	X	X	X	X	
Calvert	Olyphant, PA	(570) 586-2411	www.calvertlabs.com	Yes	X	X	X	X	X		X	X	X		X	X	X	SP
CEDRA	Austin, TX	(512) 834-7766	www.cedracorp.com	Yes													X	
Celsis	St. Louis, MO	(314) 487-6776	www.celsislabs.com	Yes, stability, methods	X			X	X	X				X	X	X	X	X
Central Toxicology Labs. (Sygenta)	Alderley Park, Macclesfield SK104TJ United Kingdom	+44 16255 15852, +44 16255 17314 (fax)	www.syngenta.com	Yes	X	X	X	X	X	X	X		X	X		X	X	SP
Cerep	Redmond, WA	(425) 895-8666	www.cerep.com	Chemistry, in vitro														SP
CeeTox Inc.	Kalamazoo, MI	269.353.5555	www.ceetox.com	Chemistry, in vivo, cell culture												X	X	X
Chantest	Cleveland, OH	(216) 332-1665, (216) 332-1706 (fax)	www.chantest.com	HERG, ion channels														
Charles River	Worchester, MA	(978) 658-6000	www.criver.com	Tumor models, implant studies	X	X	X				X	X	X		X	X	X	SP
	Sparks, NV	(775) 331-2201		Primates, immunotox[c]	X						X		X					
	Horsham, PA[d]	(215) 443-8710		Phototoxicity,[d] ferret emesis[d]					X									
	Spencerville, OH	(419) 647-4196			X	X	X	X										

Laboratory	Location	Phone Number	Website	Additional Services[a]	Rat	Rabbits	Dog	Carcinogenicity	DART	Inhalation	Primate	Pig	IV Infusion	Genotoxicity	Devices	Metabolism	Analytical	Special Studies[b]
	Tranent, Scotland (Inveresk)	44 (0) 1875 614555		Clinical, ferret emesis	X	X	X	X	X	X	X	X		X		X	X	SP
	Montreal, Quebec (CTBR)	(514) 630-8200		Clinical, ferret emesis	X	X	X	X	X	X	X	X	X			X	X	SP
Cirion	Canada	(450) 688-6445	www.cirion.ca	Yes, clinical	X	X	X				X	X		X	X	X		
CIT—Centre Int. de Toxicologie	Cedex, France	+33 2 32 292626, +33 2 32 678705 (fax)	www.citox.com		X	X	X	X	X		X	X	X	X		X	X	SP
Clintox Bioservices	Hyderabad, India	91(40) 55153222	www.clintoxbio.com	Phototox, hamsters	X	X										X		SP
Commonwealth Biotechnologies, Inc.	Richmond, VA	(804) 648-3820	www.cbi-biotech.com	Genomics		X												
Comparative Biosciences	Sunnyvale, CA	(408) 504-8871, (408) 738-9278	www.compbio.com	Special discovery,[c] models	X	X							X		X	X	X	
CorDynamics	Chicago, IL	(312) 421-8876	www.cordynamics.com	CV safety only														
Covance	Vienna, VA	(888) COVANCE, (888) 541-LABS	www.covance.com	Clinical, consulting	X	X	X	X	X		X	X	X	X	X	X		SP
	Madison, WI															X	X	SP
	Harrogete, United Kingdom																	
	Muenster, FRG																	
CPTC	Fairfield, NJ	(973) 808-7111	www.cptclabs.com	Yes, clinical +	X	X									X		X	
CXR	Dundee, United Kingdom	44-1382-432163	www.cxrbiosciences.com	Humanized mouse models														
Dermtech	San Diego, CA	(800) 808-2774	www.dermtechintl.com	Yes, consulting						X	X							
Enviro-Bio-Tech-Ltd.	Bernville, PA	(610) 488-7664	No	Chicken, cattle, food animals													X	WL
Experimur	Chicago, IL	(312) 842-9700	http://www.experimur.com	PK	X	X	X	X	X		X	X	X					
Exygen	State College, PA	(800) 281-3219, (800) 272-1019 (fax)	www.exygen.com	Methods, development												X	X	
Fraunhofer ITEM	Nikolai-Fuchs Str., D-30625 Hanover, Germany	+49 511 5353 0, +49 511 5353 155 (fax)	www.item.fraunhofer.de		X	X	X	X	X	X				X		X	X	

Frontier Biosciences	China	U.S. Office, (301) 251-0231	www.frontierbsi.com		X	X	X	X	X		X			X		X		SP
Genelogic	Gaithersburg, MD	(301) 330-3733, (800) 436-3564	www.genelogic.com	Yes	X	X	X	X	X		X	X	X			X	X	SP
Gibraltar Laboratories, Inc.	Fairfield, NJ	(973) 227-6882, (973) 227-0812 (fax)	www.gibraltarlabsinc.com	Microbiology	X	X								X	X			
Gwathmey	Cambridge, MA	(617) 491-0022, (617) 492-5545 (fax)	www.gwathmey.com	Consulting, biology	X	X									X	X		
Harlan	Rehovot, Israel	972 (0) 8-9409451	None	Bioproducts, breeder	X		X									X	X	SP
Haskell Laboratory	Wilmington, DE	(306) 366-5200		Yes										X				
Huntingdon	East Millstone, NJ	(732) 873-2550	www.huntingdon.com	Discovery support	X	X	X	X	X	X	X	X	X			X	X	SP
	Cambridgeshire, United Kingdom	44 (0) 1480 892000		Radioactivity	X	X		X[e]	X	X				X			X	
Hurley Consulting Associates Ltd.	One Main Street, Chatham, NJ, 07928	1-770-529-1154, 1-770-529-3783 (fax)	www.hurleyconsulting.com	Statistical analysis, study design, preclinical assessments, data management, QA, regulatory affairs, pharmacovigilances, training, etc												X	X	
Ibex	Logan, UT	(435) 752-4448	www.ibexpreclinical.com	Cardio telem.	X			X	X						X	X		
IITRI	Chicago, IL; McLean, VA	(312) 567-4000, (703) 918-4480	www.iitri.com	Yes	X		X	X	X	X	X							
ILS	RTP, NC	(919) 544-5857	www.ils-limited.co.uk	Data analysis, ecotoxicology	X			X	X					X		X	X	SP
INA (Int. Non-clinical Assessment)	Nagano-ken, Japan	+81-265-72-6616, +63-49-541-2294 (fax)	www.ina-research.co.jp/en/	Pediatric tox	X		X	X	X		X			X				
Industrial Toxicology Research Center (ITRC)	Lucknow Nadu, India	+91-522-2211547, +91-522-2228227 (fax)	www.itrcindia.org/index.html	Irritation, behavioural				X	X					X				
Intox	Pune, India	+91 22923633	www.intoxlab.com/html/profile.htm	Irritation, Farm animals	X	X		X	X					X				

Laboratory	Location	Phone Number	Website	Additional Services[a]	Rat	Rabbits	Dog	Carcinogenicity	DART	Inhalation	Primate	Pig	IV Infusion	Genotoxicity	Devices	Metabolism	Analytical	Special Studies[b]
Inveresk	Tranent, Scotland	44 (0) 1875	www.inveresk.com	Clinical, ferret	X	X	X	X	X	X		X		X		X	X	SP
	Montreal, Quebec	614555 (514) 630-8200		emesis	X	X	X	X	X	X	X	X	X			X	X	SP
Irvine Analytical Labs, Inc.	Irvine, CA	(877) 445-6554	www.ialab.com	Yes						X							X	
Int. Inst. of Biotech and Toxicology	Tamil Nadu, India	+91-4111-274246 +91-4111-274455		Biotechnology, entomology, agronomy													X	
Int. Hungary Ltd.	Veszprem,Hungary	+36-1-4314840 fax +36-88-545-301	www.trc.hu	Yes, toxicology	X	X	X	X		X			X			X		
IPS Therapeutis	Sherbrooke, Quebec	819-564-5391, 819-820-6831 (fax)	www.ipstherapeutique.com/	hERG testing, CV, safety pharm														
ITL Labs Ltd.	Delhi, India	91-11-27915654	www.indiamart.com/itl/														X	
ITR	Montreal, Quebec	(514) 457-7400	www.itrlab.com	Affiliated labs	X	X	X	X	X	X	X		X	X		X	X	SP
ITRI	Albuquerque, NM	(408) 428-9988	www.itri.com	Yes	X			X		X							X	
Jai Research Foundation International	Gujarat, India	+ 44 1684 891519	www.jrfonline.com		X	X	X	X	X					X		X	X	
JSW Research	Austria		www.jswresearch.com	Yes	X			X	X		X			X		X	X	
KIT (Korean Institute of Technology)	Korea	+82-42-610-8105	www.kitox.re.kr/eng_about.html	Yes	X	X	X	X			X	X	X	X				
LABS	Laval, Canada	(450) 973-2240	www.preclin.com	Yes	X	X	X	X			X		X			X		
	Veszprem, HU	(416) 815-0700	www.trc.hu		X	X	X	X		X			X			X		
Liberty Research	Waverly, NY	(607) 565-8131 (p) (607) 565-7420 (f)	NO	Cats, ferrets			X											
Lovelace	Albuquerque, NM	(505) 348-9456	www.lrri.org	Clinical						X								
LPT	Hamburg, GE	40-70-20-20 40-70-20-22-99	www.LPT-pharm-tox.de		X	X	X	X	X	X	X	X	X	X	X	X	X	SP

LyChron	Mountain View, CA	650-938-3675	www.lychron.com	Pig device implant studies								X						
Maccine	Singapore	+65-6778-7305	www.maccine.com	Yes	X								X			X	X	
MB Research	Spinnerstown, PA	(215) 536-4110	www.mbresearch.com	In vitro and alternative models	X	X	X								X			
MDS Pharma Services	Lincoln, NE; L'Arbresle, France; Geneva, Switzerland	(514) 333-0033	www.mdsps.com	Clinical, receptor screening	X	X	X	X	X		X			X	X	X	X	SP
														X	X	X	X	SP
															X			
Medicilon/MPI	Shanghai, China	8621-51-320228	www.medicilon.com		X		X				X			X		X	X	
MicaGenix	Greenfield, IN	(888)-779-5500	www.micagenix.com	Histology, image analysis, Non-GLP mice and rats												X		
Micro Test	Agawam, MA	(800) 631-1680	www.microtestlabs.com	Yes											X			
Midwest Bio Research	Evanston, IL	847-682-6141(p) 847-556-6560(f)	www.midwestbioresearch.com	Consultation										X		X		
MPI	Mattawan, MI	(269) 668-3336	www.mpiresearch.com	Yes, radioactivity	X	X	X	X	X		X	X	X		X	X	X	SP
MSI	Minato-ku, Tokyo, Japan	81 3 3454 7571	www.ankaken.co.jp	Yes				X		X	X					X		
MWRI	Kansas City, MO	(816) 753-7600	www.mriresearch.org	Yes	X			X										
nanoTox	Austin, TX; The Netherlands	(877) 804-2828	www.nanotox.com	Yes, risk assessment of nanomaterials; particle sizing, nanodose formulations and analysis													X	X
NAMSA	Toledo, OH	(419) 666-9455	www.namsa.com	Yes										X	X			
	Irvine, CA	(949) 951-3110												X	X			
	Kennesaw, GA	(770) 427-3101																
Nelson Labs	Salt Lake City, UT	(800) 826-2088	www.nelsonlabs.com	Yes											X		X	
Next Century, INC	Newark, DE	(302) 453-0571	www.nextcenturyinc.com	Yes	X	X	X	X		X	X	X	X		X			
Northern Biomedical Research	Muskegon, MI	(231) 759-2333		Yes, surgery, large animals	X	X	X				X	X	X			X		
Northview	Northbrook, IL	(847) 564-8181	www.northviewlabs.com	Yes	X	X	X	X	X					X	X	X	X	
	Hercules, CA	(510) 694-9000																
	Spartan, SC	(864) 574-7728																

Laboratory	Location	Phone Number	Website	Additional Services[a]	Rat	Rabbits	Dog	Carcinogenicity	DART	Inhalation	Primate	Pig	IV Infusion	Genotoxicity	Devices	Metabolism	Analytical	Special Studies[b]
NOTOX	s'Hertogenbosch, The Netherlands	31 73 640 67 00, 31 73 640 67 99 (fax)	www.notox.nl	Yes, birds, +	X	X	X	X	X			X	X	X	X	X		WL
Nucro-Technics	Ontario, Canada	(416) 438-6727, (416) 438-3463 (fax)	www.nucro-technics.com	Microbiology	X	X	X	X						X	X		X	
Pacific Biolabs	Hercules, CA	(510) 694-9000	http://www.pacificbiolabs.com/home.htm	Yes	X	X	X	X	X					X	X	X	X	
Pathology Experts	Basel, Switzerland Rye, NY	44-61-422-09-29 (888) 473-4554	www.pathexperts.com	Histology and histopathology														
Perry Scientific	San Diego, CA	(858) 560-9000, (858) 467-9608 (fax)	www.perryscientific.com	Yes; large animals	X	X	X	X	X			X	X		X	X	X	SP
Porsolt & Partners	Bouloungne-Billancourt, France	+33146109990	http://www.porsolt.com/	Yes	X	X	X				X		X			X	X	
Product Safety Labs	Dayton, NJ	(732) 438-5100, (732) 254-9200	www.productsafetylabs.com	Environmental, ferret emesis studies, radioactivity	X	X		X	X	X			X	X	X		X	WL
Provident Preclinical, Inc	Doylestown, PA	215-348-3868, 215-348-5081 (fax)	www.ppicro.com	Yes	X	X	X	X		X	X	X			X			
Quest	Newark, DE	(302) 369-5601	www.questpharm.com	Yes, radioactivity	X	X	X		X		X			X		X	X	
Rallis Research Center	Bangalore, India	+91-80-28394959, +91-80-28394015	www.rallis.co.in	Yes	X	X		X						X		X	X	
RCC Ltd.	Zelgliweg 1 CH-4452 Itegen, Switzerland	(410) 385-1666, US sales office, +41 61 975 1111	www.rcc.ch	Consulting, regulatory	X	X	X	X	X	X		X	X	X	X	X	X	SP
Ricerca	Concord, OH	(888) 763-4797	www.ricerca.com	Synthesis,[a] product development, radioactivity	X	X	X	X	X	X						X	X	
RTC S.p.A.	Via Tito Speri 12, I-40 Pomezia, Rome, Italy	+39 06 910 951, +39 06 910 5737 (fax)	www.rtc.it	Yes, consulting, regulatory	X	X	X		X									WL

RTI	RTP, NC	(919) 541-6000	www.rti.org	Yes	X			X	X						X	X	
SafePharm	Derby, United Kingdom	00 44 (0) 1332792896	www.safepharm.com	Yes, (fish+)	X			X	X	X			X	X	X	X	SP
SCANTOX	Hestehavevej 36a, DK-4623 Lille Skensved, Denmark	+45 56 82 11 00, +45 56 82 12 02 (fax)	www.scantox.com		X	X	X	X	X	X		X	X	X	X			
Sequani Limited	Bromyard Road, Herefordshire HR8 1LH, Ledbury, United Kingdom	+44 1531 634121, +44 1531 634753 (fax)	www.sequani.com		X	X	X	X	X			X	X	X	X			
SGS Life Sciences Division	Fairfield, NJ	(800) 747-8782	www.ustesting.sgsna.com	Implant, combustion, fish, industrial, consumer products	X	X		X					X	X		X	SP, WL
Sinclair	PO Box 658	(573) 387-4400	www.sinclairresearch.com	Radiolabeled; cats; other	X			X				X	X		X	X		
Sitek	Rockville, MD	(301) 926-4900	www.siteklabs.com	Yes	X								X	X	X			
Skeletech	Bothell, WA	(425) 424-2663	www.skeletech.com	Bone,[c] CNS	X	X	X				X	X	X		X			
SNBL	Kagoshima, Japan	81 (0) 99-294-2600, 81 (0) 99-294-3196 (fax)	www.snbl.com	Yes, clinical, +, Irritation	X	X	X	X	X	X	X	X	X	X				SP
SNBL USA Ltd.	6605 Merrill Creek Parkway, Everett, WA 98203	(425) 407 0121, (425) 407 8601 (fax)	http://www.snblusa.com/	Formulation	X				X		X		X				X	SP
Southern RI	Birmingham, AL	(888) 322-1166	www.southernreaserch.org	Yes, formulation, development, tumor models	X	X	X	X	X		X	X	X	X	X	X	X	SP
SP1-bio	France	33 (0) 169 53 14 00	www.spibio.com	Clinical	X											X	X	
Spring Valley Lab.	Woodbine, MD	(800) 864-1839	www.springvalleylabs.com	Ferrets	X	X		X	X						X			
SRI	Palo Alto, CA	(650) 859-2000, (866) 451-5998	www.sri.com	Formulation, CTM	X	X	X	X				X		X	X	X	X	
Stillmeadow Inc.	Sugarland, TX	(281) 240-8828	www.stillmeadow.com	Companion animal studies	X	X	X			X		X					X	
STS duo Tek	Rush, NY, Henrietta, NY	(716) 321-1130, (800) 836-4850	www.stsduotek.com	Yes, ocular	X	X							X	X	X			
Tandem Labs	Ewing, NJ	(609) 434-0044	www.tandemlabs.com	Yes													X	

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TNO Pharma	Utrechtseweg 48, NL-3700AJ Zeist, 3704 HE, The Netherlands	+31 30 694 4806, +31 30 694 4845 (fax)	www.voeding.tno.nl	Yes, packaging, food and nutrition, clinical	X	X	X	X	X	X	X	X	X	X	X	X	X	SP, WL
Torrent Pharmaceuticals Ltd.	Gujarat, India		www.torrentpharma.com					X	X					X				
TOXIKON Corp.	15 Wiggins Ave., Bedford, MA 01730	(781) 275 3330, (781) 271-1136 (fax)	www.toxikon.com	Yes	X	X	X	X	X			X	X	X	X	X		WL
UIC Tox Research Lab	Chicago, IL	(312) 996-2123	www.uic.edu/labs/tox	Yes, radioactivity	X	X	X		X	X	X		X			X	X	
Valley Biosystems	West Sacramento, CA	916-374-2735	www.valleybiosystems.com								X							
VIMTA	Hyderabad, India	+91-40-2726 4141	www.vimta.com	Clinical					X					X			X	
Vivo Bio Tech, Ltd.	Hyderabad, India	92-40-55784714, 91-40-55776112 (fax)	www.vivobio.com	Immunodeficient, Mice	X	X			X					X		X		
WIL Research Labs, Inc.	1407 George Rd., Ashland, OH 44805	(419) 289 8700, (419) 289 3650 (fax)	www.wilresearch.com	Yes, radioactivity	X	X	X	X	X	X	X	X	X			X	X	SP, WL
WuXi Pharmatech	288 FuTe ZhongLu WaiGaoQiao, Shanghai 200131, P.R. China		http://www.pharmatechs.com	Rodent tox. (oral, IV, and PK)	X								X	X				
XenoBiotics	Plainsboro, NJ	(609) 799-2295, (609) 799-7497	www.xbl.com	Clinical, radioactivity	X	X	X									X		
Xenotech	Lenexa, KS	(913) 438-7450	www.xenotech.com	Yes												X		

[a]A "yes" or "+" in this column indicates that there are more services available than just those listed.

[b]SP in this column indicates safety pharmacology, WL wildlife/environment testing is available and EN that environmental testing in available.

[c]Limited availability studies.

[d]Near unique capabilities.

[e]Hamster carcinogenicity is available.

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Note: Italicized letters *f*, and *t* following page numbers indicate figures and tables, respectively.

Drug Safety Evaluation, Second Edition, by Shayne Cox Gad