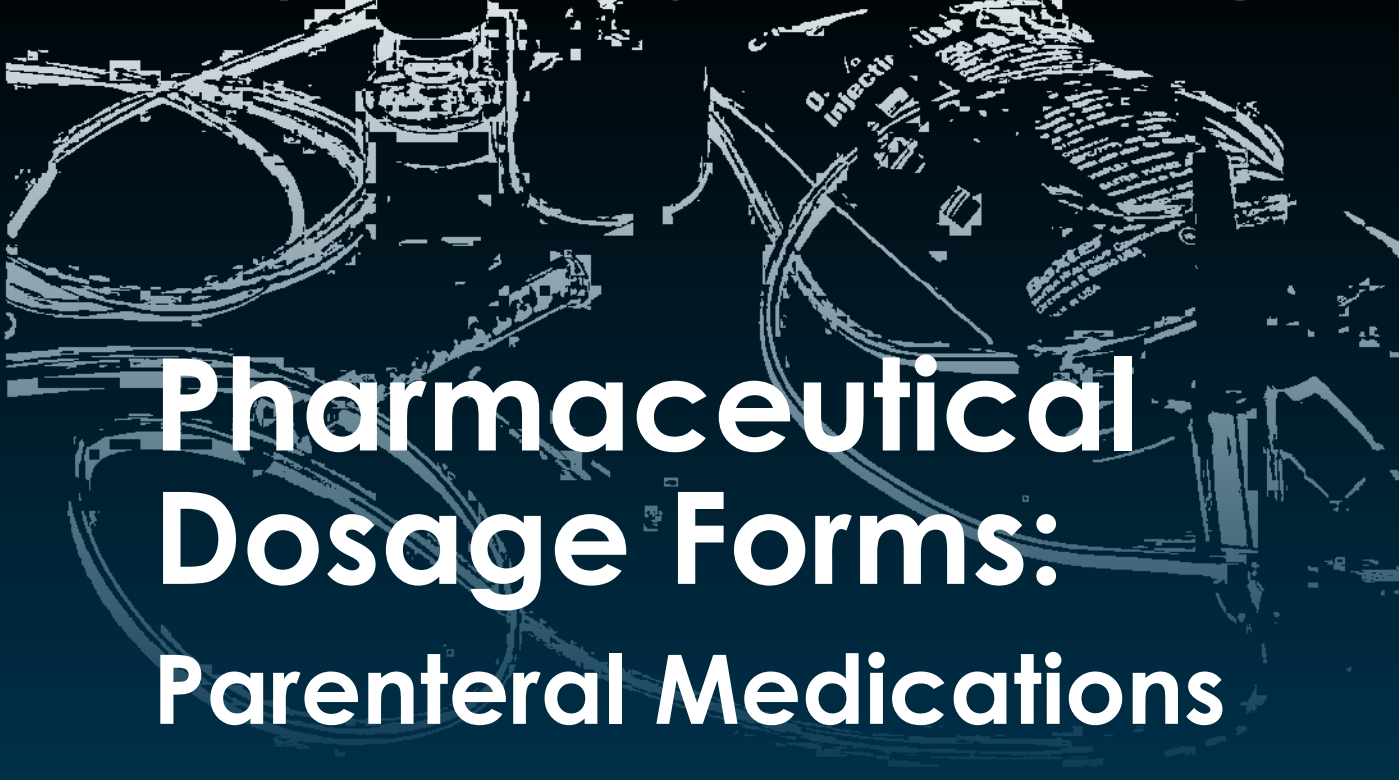

Pharmaceutical Dosage Forms:

Parenteral Medications

Third Edition

Volume 1: Formulation and Packaging

Edited by
Sandeep Nema
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Pharmaceutical Dosage Forms

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New York London

First published in 1984 by Marcel Dekker, Inc., New York, New York.
This edition published in 2010 by Informa Healthcare, Telephone House, 69-77 Paul Street, London EC2A 4LQ, UK.

Simultaneously published in the USA by Informa Healthcare, 52 Vanderbilt Avenue, 7th Floor, New York, NY 10017, USA.

Informa Healthcare is a trading division of Informa UK Ltd. Registered Office: 37–41 Mortimer Street, London W1T 3JH, UK. Registered in England and Wales number 1072954.

A CIP record for this book is available from the British Library.

Library of Congress Cataloging-in-Publication Data available on application

ISBN-13: 9781420086430
ISBN-13: 9781420086539 (three-volume set)

Orders may be sent to: Informa Healthcare, Sheepen Place, Colchester, Essex CO3 3LP, UK
Telephone: +44 (0)20 7017 5540
Email: CSDhealthcarebooks@informa.com
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Typeset by MPS Limited, A Macmillan Company
Printed and bound in India

We dedicate this work to those who have inspired us.
To my parents Walter and Ruth Ludwig and my wife Sue Ludwig
To my parents Hari and Pratibha Nema and my wife Tina Busch-Nema

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Foreword

I was a faculty member at the University of Tennessee and a colleague of Dr. Kenneth Avis when he conceived, organized, and edited (along with H.A. Lieberman and L. Lachman) the first edition of this book series that was published in 1984. It was so well received by the pharmaceutical science community that an expanded three-volume second edition was published in 1992. Dr. Avis did not survive long enough to oversee a third edition, and it was questionable whether a third edition would ever be published until two of his graduate students, Drs. Nema and Ludwig, took it upon themselves to carry on Dr. Avis' tradition.

Their oversight of this third edition is work that their mentor would be highly pleased and proud of. From 29 chapters in the second edition to 43 chapters in this new edition, this three-volume series comprehensively covers both the traditional subjects in parenteral science and technology as well as new and expanded subjects. For example, separate chapter topics in this edition not found in previous editions include solubility and solubilization, depot delivery systems, biophysical and biochemical characterization of peptides and proteins, container-closure integrity testing, water systems, endotoxin testing, focused chapters on different sterilization methods, risk assessment in aseptic processing, visual inspection, advances in injection devices, RNAi delivery, regulatory considerations for excipients, techniques to evaluate pain on injection, product specifications, extractables and leachables, process analytical technology, and quality by design.

The editors have done an outstanding job of convincing so many top experts in their fields to author these 43 chapters. The excellent reputations of the authors and editors of this book will guarantee superb content of each chapter. There is no other book in the world that covers the breadth and depth of parenteral science and technology better than this one. In my opinion, the editors have achieved their primary objectives—publishing a book that contains current and emerging sterile product development and manufacturing information, and maintaining the high standard of quality that readers would expect.

Michael J. Akers
Baxter BioPharma Solutions
Bloomington, Indiana, U.S.A.

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Preface

Pharmaceutical Dosage Forms: Parenteral Medications was originally published in 1984 and immediately accepted as a definitive reference in academic institutions and the pharmaceutical industry. The second edition was published in 1993. The ensuing years have produced incredible technological advancement. Classic small-molecule drugs are now complemented by complex molecules such as monoclonal antibodies, antibody fragments, aptamers, antisense, RNAi therapeutics, and DNA vaccines. There have been significant innovations in delivery devices, analytical techniques, in-silico modeling, and manufacturing and control technologies. In addition, the global regulatory environment has shifted toward greater emphasis on science-based risk assessment as evidenced by the evolving cGMPs, quality by design (QbD), process analytical technology (PAT), continuous processing, real time release, and other initiatives. The rapidly changing landscape in the parenteral field was the primary reason we undertook the challenging task of updating the three volumes. Our objectives were to (*i*) revise the text with current and emerging sterile product development and manufacturing science and (*ii*) maintain the high standard of quality the readers expect.

The third edition not only reflects enhanced content in all the chapters, but also more than half of the chapters are new underscoring the rapidly advancing technology. We have divided the volumes into logical subunits—volume 1 addresses formulation and packaging aspects; volume 2, facility design, sterilization and processing; and volume 3, regulations, validation and future directions. The authors invited to contribute chapters are established leaders with proven track records in their specialty areas. Hence, the textbook is authoritative and contains much of the collective experience gained in the (bio)pharmaceutical industry over the last two decades. *We are deeply grateful to all the authors who made this work possible.*

Volume 1 begins with a historical perspective of injectable drug therapy and common routes of administration. Formulation of small molecules and large molecules is presented in depth, including ophthalmic dosage forms. Parenteral packaging options are discussed relative to glass and plastic containers, as well as elastomeric closures. A definitive chapter is provided on container closure integrity.

Volume 2 presents chapters on facility design, cleanroom operations, and control of the environment. A chapter discussing pharmaceutical water systems is included. Key quality attributes of sterile dosage forms are discussed, including particulate matter, endotoxin, and sterility testing. The most widely used sterilization techniques as well as processing technologies are presented. Volume 2 concludes with an in-depth chapter on lyophilization.

Volume 3 focuses on regulatory requirements, risk-based process design, specifications, QbD, and extractables/leachables. In addition, we have included chapters on parenteral administration devices, siRNA delivery systems, injection site pain assessment, and control, PAT, and rapid microbiology test methods. Volume 3 concludes with a forward-looking chapter discussing the future of parenteral product manufacturing.

These three volumes differ from other textbooks in that they provide a learned review on developing parenteral dosage forms for *both* small molecules and biologics. Practical guidance is provided, in addition to theoretical aspects, for how to bring a drug candidate forward from discovery, through preclinical and clinical development, manufacturing, validation, and eventual registration.

The editors wish to thank Judy Clarkston and Lynn O'Toole-Bird (Pfizer, Inc.) for their invaluable assistance and organizational support during this project, and Sherri Niziolek and Bianca Turnbull (Informa Healthcare) for patiently leading us through the publishing process.

We also acknowledge the assistance of Pfizer, Inc. colleagues Lin Chen and Min Huang for reviewing several of the chapters.

We would like to express special gratitude to the late Kenneth E. Avis (University of Tennessee College of Pharmacy) for his dedication to teaching and sharing practical knowledge in the area of parenteral medications to so many students over the years, including us. Finally, we acknowledge the contributions of Dr Avis, Leon Lachman, and Herbert A. Lieberman who edited the earlier editions of this book series.

Sandeep Nema
John D. Ludwig

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1 Parenteral dosage forms: introduction and historical perspective

John D. Ludwig

INTRODUCTION

Parenteral dosage forms are those administered directly into body tissues rather than via the alimentary canal. "Parenteral" is derived from the Greek words *para* (beside) and *enteron* (the intestine) and most often refers to subcutaneous (SC), intramuscular (IM), or intravenous (IV) administration of drugs. Parenteral drug delivery can pose significant risk to the patient since the natural barriers of the body (gut, skin, and mucous membranes) are bypassed. The highest standards for quality and purity must be maintained throughout dosage form manufacture to protect the patient from physical, chemical, and microbial contaminants. A single contaminated vial out of a batch of thousands can seriously injure a patient (or worse). Further, if improper or poor aseptic technique is used while administering an injection the patient could be similarly harmed. The minimum quality standards for pharmaceutical manufacturers are expressed in the current good manufacturing practices (cGMPs), which are constantly evolving as technology advances. An equal burden of responsibility is placed on physicians, pharmacists, nurses, and other health professionals to follow strict good aseptic practices (GAPs) as they administer parenteral dosage forms to patients. Nosocomial infections associated with parenteral drug therapy remain a significant issue (1–4).

ADVANTAGES AND DISADVANTAGES OF PARENTERAL DRUG DELIVERY

Parenteral drug delivery provides a number of advantages for the patient. The parenteral route provides an effective way to dose patients who are unconscious or those who cannot or would not take oral medications. A drug administered parenterally generally produces an immediate therapeutic effect and is therefore desirable in emergency situations. Parenteral administration also provides a mechanism for dosing drugs that are not bioavailable via noninjectable routes such as many protein and peptide therapeutics. Total parenteral nutrition can be provided for seriously ill patients where tube feeding is not an alternative. In addition, large amounts of fluid and electrolytes can be given relatively quickly via the IV route to patients with serious fluid loss from dehydration or gastrointestinal infections.

A significant disadvantage of injectable drug administration is that once a drug has been dosed it is difficult to reverse its effect. For example, in the event of a dosing error (overdose) with an oral tablet, gastric lavage, induced emesis, or activated charcoal can be employed. The options for reversing an IV overdose are usually very limited. Secondly, the risk of infection is always present with parenteral dosing both in the hospital/clinic setting as well as home administration. Finally, the cost per dose of parenteral drugs is typically higher than for oral medications.

PARENTERAL DRUG DELIVERY ROUTES

Routes of parenteral drug delivery are summarized in Table 1. SC, IM, and IV are the most common modes of administration. The fastest onset of action is achieved via the IV route since the injection is directly into a vein. Relatively large amounts of fluid can be delivered quickly and efficiently using the IV route. Slower and more variable onset of action typically occurs following SC and IM administration since the drug must be absorbed into the bloodstream from the site of injection. The absorption step can be exploited for drugs requiring chronic administration. Formulations can be designed to provide sustained-release profiles therefore reducing the number of injections required and the associated risk. Examples of "depot" formulations include DEPO-PROVERA® Contraceptive Injection, which is administered deep IM every 13 weeks and depo-subQ provera 104™ which is administered SC in the anterior thigh or abdomen every 12 to 14 weeks. Intravitreal dosing has increased significantly in recent

Table 1 Parenteral Drug Delivery Routes

Route	Administration volume
Subcutaneous (SC)	Low, generally <2 mL
Intramuscular (IM)	Medium, 2 mL–5 mL
Intravenous (IV)	High
Intravitreal	Low, generally <0.1 mL
Intradermal (ID)	Low, 0.1 mL
Intra-articular	Medium
Intrathecal	Low
Intraepidural	Low
Intracisternal	Medium
Intra-arterial	High
Intracardiac	Medium
Intrapleural	Medium
Intraperitoneal	High
Intraosseous	Medium

years because of new treatments for neovascular wet age-related macular degeneration (AMD) such as Lucentis® (ranibizumb injection) and Macugen® (pegaptanid sodium injection). The intradermal (ID) route is commonly used for very small volume injections (0.1 mL) such as the tuberculosis skin test [or tuberculin purified protein derivative (PPD) test]. Intra-articular injections directly into joint synovial fluid are routinely used to administer corticosteroids or hyaluronic acid derivatives to relieve the symptoms of osteoarthritis. Intrathecal (intraspinal) and intraepidural injections are used to deliver anesthesia, analgesics, anti-infectives, and some cancer therapies. Intracisternal administration is used to deliver critical therapeutics directly to the caudal region of the brain. Less common parenteral routes include intra-arterial, intracardiac (e.g., epinephrine for cardiac resuscitation), intrapleural, intraperitoneal, and intraosseous (bone) (5,6).

QUALITY ATTRIBUTES OF PARENTERAL DOSAGE FORMS

Quality attributes specific to parenteral dosage forms are shown in Table 2. Injectable products must be manufactured using the highest quality active drug substance and excipients. The regulatory review process requires that each ingredient in the formulation must be justified as

Table 2 Quality Aspects of Parenteral Dosage Forms

Attribute	Comment
Highest level of purity for the active drug substance and excipients	Highly purified "parenteral grade" excipients are available.
Formulation containing the fewest number and the simplest excipients possible	The presence and amount of each excipient must be justified in regulatory filings.
Physical and chemical stability	Minimal degradation during shelf-life.
Container-closure system with low extractable/ leachable profile	Minimize the impact of the container on product purity and stability.
Sterile	Sterility assurance is critical for patient safety.
Pyrogen free	Pyrogens cause febrile response. The most potent pyrogens are bacterial endotoxins.
Free from visible particulate matter	Subvisible particulate matter must be excluded as much as possible as defined by compendial requirements.
Container-closure integrity	Product container maintains microbiological integrity during shelf-life.
Injection site tolerability	Formulation does not cause significant injection site irritation or tissue damage. Products are frequently formulated as isotonic solutions.
Detailed dosing and administration instructions including evaluation of compatibility with coadministered drugs	In clinical practice, multiple drugs are frequently administered through the same IV line to avoid the risk of an additional venipuncture.

to why it was included and the relative amount. As a general rule, formulations with the fewest excipients and simplest composition are highly desired. The quality and robustness of the container-closure system must also be described and justified relative to extractables/leachables, container integrity (microbiological, oxygen transmission, moisture transmission), and intended clinical use. Parenteral products must be sterile, pyrogen-free, and free from visible particulate matter and remain so throughout shelf-life. Adverse injection site events are widely reported and can cause significant tissue damage. Often, the formulation can be modified to increase injection site tolerability, for example, by changing buffers and/or decreasing buffer concentration as well as rendering the dosing solution isotonic. The compatibility of the formulation should be assessed with the most likely drugs that will be coadministered with the new product. Compatibility results are generally included in the approved dosing instructions to assist pharmacists, nurses, and other health care providers.

MILESTONES IN PARENTERAL DRUG THERAPY

Various scholars have summarized the development of parenteral drug therapy (7–13). A compiled historical timeline is presented in Table 3. The reader should be aware there is disagreement in the literature about exact dates as well as who was "first," particularly for

Table 3 Historical Milestones in Parenteral Drug Delivery

Year	Milestone
1616	William Harvey described the circulation of blood. His findings were published in 1628.
1656	Christopher Wren infused dogs with opiates and alcoholic beverages using a sharpened quill and animal bladder.
1665	Johannes Escholtz described techniques for IV infusion of drugs into humans.
1796	Edward Jenner vaccinated children against smallpox using intradermal administration with cowpox virus.
1818	James Blundell performed a successful blood transfusion following postpartum hemorrhage.
1831	William O'Shaughnessy studied the blood of cholera patients and developed the concepts for IV water and electrolyte replacement therapy.
1832	Thomas Latta established the first clinical practice of IV infusions of water and salts to treat cholera patients, based on O'Shaughnessy's work.
1855	Alexander Wood developed the first modern hypodermic syringe with a steel barrel and hollow steel needle.
1867	Joseph Lister developed the concepts of antisepsis using carbolic acid (phenol) solutions to sanitize hands, instruments, and wounds to reduce postsurgery infections.
1860s–1880s	Louis Pasteur confirmed the germ theory of disease, discovered techniques for pasteurization of milk, and developed vaccinations against chicken cholera, bovine anthrax, and rabies.
1879	Charles Chamberland invented the autoclave.
1884	Charles Chamberland invented the "Chamberland filter" (porcelain) that removed bacteria from solutions prior to dosing.
1891	R.M. Matas demonstrated the effective use of IV saline solutions to treat shock.
1912	Using a rabbit model, E.C. Hort and W.J. Penfold determined the pyrogenic response following many IV injections was caused by a substance produced by gram-negative bacterial contamination of the solution (14–16).
1918	Richard Zsigmondy and W. Bachman developed technology to manufacture microporous membrane filters from cellulose esters (nitrocellulose, acetyl cellulose, cellulose acetate).
1923	Florence Siebert and L.B. Mendel developed a definitive rabbit pyrogen test model and showed that endotoxin from gram-negative bacteria was the substance responsible for the pyrogenic response following injection with sterile solutions (17–19,20).
1923	Frederick Banting and J.J.R. Macleod share the Nobel Prize in Physiology or Medicine for the extraction of insulin and demonstration of clinical efficacy.
1923	Purified insulin product marketed (Iletin®).
1924	R.M. Matas demonstrates continuous IV "drip" (21).
1933	L. Rademaker reported that after installation of a distilled water system for pharmaceutical production, pyrogenic reactions by surgery patients to parenteral injections dropped from 30% to 4% (22).
1938	Lloyd A. Hall and Carroll L. Griffith patented the use of ethylene oxide to sterilize and preserve spices. This technology was applied to sterile pharmaceutical product manufacturing during the 1940s.

(*Continued*)

Table 3 Historical Milestones in Parenteral Drug Delivery (*Continued*)

Year	Milestone
1942	Rabbit pyrogen test (Seibert and Mendel) published in the U.S. Pharmacopeia.
1940s	High Efficiency Particulate Air (HEPA) filters designed and installed for clean air supply in rudimentary cleanrooms at Manhattan project sites and biological weapons research laboratories at Fort Detrick, Maryland (10,23,24).
1946	Parenteral Drug Association founded.
1950s	Cleanrooms with HEPA filtered air supply widely used for pharmaceutical fill/finish (10,23,24).
1961	Willis J. Whitfield pioneered the concept of laminar air flow and constructed the first modern cleanroom at Sandia Corporation in Albuquerque, New Mexico (10,23,24).
1961	Arvid Wretlind and O. Schuberth formulated the first lipid emulsion, Intralipid®, suitable for IV infusion (7,25).
1964	Arvid Wretlind developed a total parenteral nutrition (TPN) program providing half of the calories from lipid and half from glucose. Recognized as the father of TPN (7,25).
1967	Stanley J. Dudrick reported comprehensive technique to provide long-term total parenteral nutrition (TPN) (7,25).
1969	DW Wilmore and Stanley J Dudrick used an in-line filter to reduce the risk of IV infusions (7, 25).
1971	James F. Cooper, Jack Levin, and H.N. Wagner Jr. pioneered use of the limulus amebocyte lysate test for screening parenteral drug products for endotoxin contamination (26).
1973	Infusion Nurses Society founded.
1976	Food and Drug Administration publishes *Current Good Manufacturing Practice in the Manufacture, Processing, Packing, or Holding of Large Volume Parenterals* (never formally adopted).
1978–1979	Human insulin cloned. Human growth hormone cloned.
1980s	First steps toward barrier isolator technology for aseptic fill/finish operations—gray side maintenance (24).
1980s	Sterilizable isolators introduced for compendial sterility testing (27).
1982	Humulin® (human insulin recombinant) marketed.
1985	Protropin® (somatrem for injection) and Somatonorm® (somatrem) marketed. (methionyl human somatropin).
1986	Orthoclone® OTK3 marketed to treat the rejection of transplanted organs.
1987	FDA publishes *Industry Guideline on Sterile Drug Products Produced by Aseptic Processing and Guideline on General Principles of Process Validation*.
1987	Humatrope® (somatropin recombinant) and Genotropin® [somatropin (rDNA) for injection] marketed.
1987	First dual chamber pen injector launched (KabiPen®).
1990s	Barrier isolator technology for fill/finish operations—Restricted Access Barrier Systems (RABS) and Isolators (24).
1992	The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) is established.
1994	FDA publishes *Guidance for Industry for the Submission Documentation for Sterilization Process Validation in Applications for Human and Veterinary Drug Products*.
1996	*Note for Guidance on Manufacture of the Finished Dosage Form* issued by the Committee For Proprietary Medicinal Products (CPMP), CPMP/QWP/486/95.
1997	First monoclonal antibody to treat cancer approved Rituxan® (rituximab).
1999	*Decision Trees for the Selection of Sterilization Methods* finalized by the CPMP, CPMP/QWP/054/98.
2003	Pharmaceutical Compounding—Sterile Preparations <797> became official in the U.S. Pharmacopeia.
2003	European Commission: Ad Hoc GMP Inspections Services Group, EC Guide to Good Manufacturing Practice Revision to Annex 1, Title: *Manufacture of Sterile Medicinal Products*.
2004	FDA publishes *Guidance for Industry Sterile Drug Products Produced by Aseptic Processing—Current Good Manufacturing Practice* (replaces 1987 version).
2006	Infusion Nurses Society publishes updated *Infusion Nursing Standards of Practice* (28).
2008	Heparin recalls due to intentional contamination during production of active pharmaceutical ingredient.
2009	European Commission: EudraLex—The Rules Governing Medicinal Products in the European Union, Volume 4, EU Guidelines to Good Manufacturing Practice, Medicinal Products for Human and Veterinary Use, Annex 1, *Manufacture of Sterile Medicinal Products* (replaces 2003 version).

Abbreviation: IV, intravenous.

discoveries prior to the 20th century. Therefore, the author attempted to arrive at reasonable dates after consulting multiple sources. It is clear early scientific findings were not disseminated quickly because of lack of modern communication tools, and scientists were often working without knowledge of similar research occurring in other laboratories. In addition, advancements were occasionally "forgotten" only to be rediscovered independently a century later, all adding to the fascinating history of medicines and health care. Specific references have been included in Table 3 for recent advances and milestones.

CONCLUSION

The advent of safe, effective parenteral therapy has resulted in tremendous improvement in the quality of medical care around the world. Those of us fortunate enough to work in this exciting area whether in research, dosage form development, manufacturing, or clinical practice share a common goal of providing the highest standard of care. To do so requires diligence at each step in the process, be it synthesis of the active ingredient and excipients, production of the container and closure, compounding of the formulation, or aseptic fill/finish of the final product. The minimum quality standards are provided in the cGMPs, but regulatory and ethical expectations go well beyond the written requirements. Providing the highest standard of care also requires strict adherence to GAPs as the health care professional or family member is preparing and administering the dose to the patient. The risk of introducing infection and causing harm is ever present. Maxine B. Perdue of the Infusion Nurses Society summarized these sentiments as follows (29):

> "My word for competency is *excellence*. Excellence is not perfection; it is stellar performance. It is keeping current and complying with evidence-based practice standards. It is not accepting the status quo, rather, being visionary and innovative and a catalyst for research. It is sharing information with others by writing articles. . .and speaking at meetings. Each day is an opportunity to step outside the box and look at how we practice infusion therapy and to focus on each aspect of what we do as a chance to improve infusion care."

The constant pursuit of *excellence* is what drives us to the highest standard of care. Our patients deserve nothing less.

ACKNOWLEDGMENT

The author wishes to dedicate this chapter to Kenneth E. Avis, major professor and friend, who passed away in January 1999, and to Michael J. Akers for many years of mentoring and collaboration.

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2 Parenteral drug administration: routes of administration and devices

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INTRODUCTION

The word Parenteral is coined from the greek words "*para enteron*" meaning "to avoid the intestines." Drugs administered via any route other than oral or rectal routes, are considered to be parenteral. However, common usage more closely associates the term as being synonymous with "injectable." These include drugs that are topically administered to the eye, ear, and skin or even inhaled may be considered broadly as parenterals. It is estimated that 40% of all drugs administered in hospitals are in the form of an injection. In some institutional settings, the percentage of injectables is greater than 40%. However, medical and pharmacy practioners of today generally limit the classification of parenterals to include only those drugs administered directly into tissues, tissue spaces, or compartments by injection or infusion.

Injectable products are sterile products and may require special handling and administration. Estimates indicate that over one billion disposable plastic syringes are used annually in American hospitals. With increasing complexity of the drugs being administered by the parenteral route, significant development with respect to techniques for parenteral administration have evolved in recent years and continue to do so. Moreover, development of site specific, efficacious, safe, and reproducible administration techniques have led to the development of highly advanced stand alone drug delivery devices. Some of these developments have addressed significant safety and efficacy concerns but the area of drug delivery device research is an active field of study. This chapter is an attempt to review and to update the current usage of parenteral drugs and their routes of administration. Additionally, this chapter will address currently available parenteral drug delivery devices and the trends of existing technology in the field.

PARENTERAL ADMINISTRATIONS CONCEPTS

Although oral administration is more prevalent in the current market place, parenteral administration of drugs has a number of distinct advantages over the former. Increasing complexity of new drug entities (e.g., biomolecules) and treatment regimens to treat life threatening diseases have led many formulation groups utilize parenteral routes. In some instances, parenteral administration is essential for the drug to be absorbed in active form. For example, almost all protein drugs are administered by injection, rather than administration by the oral route, because protein drugs are broken down by stomach acid and digestive enzymes. Absorption through the parenteral route is usually more rapid and predictable than when a drug is administered orally. Because of its predictable rate of absorption and bio-availability, parenteral drugs are routinely used in emergency therapy. If a patient is unconscious, uncooperative, or unable to retain anything administered orally, parenteral therapy may become a necessity.

Parenteral dosage forms ensure delivery of therapeutic concentrations of drug/s to its desired site/s of action (diseased tissues or target areas of the body). This factor becomes more significant especially when inadequate or marginal transport of drug/s into the tissues or target areas occurs or is anticipated. One such example is a direct intra-articular injection of drugs, (e.g. anti-inflammatory drugs such as the steroids) which exhibit poor transport characteristics into the synovial spaces between joints, may be used to reduce inflammation. Additionally, injectable drugs allow researcher to exert direct control over pharmacological parameters, such as the time of drug onset, serum peak and trough levels, tissue distribution, clearance and rates of elimination of the drug from the body; for example, sustained or prolonged action of intramuscular (IM) insulin administration. Parenteral administration of drugs, in some cases may aid in decreased side effects of the drug by avoiding the traditional

oral route. Methotrexate, an antimetabolite, used for blood malignancies, exhibits varied physiological side effects when administered via the intravenous (IV) route and shows poor blood-brain barrier (BBB) penetration. However in patients suffering from acute lymphoblastic leukemina (ALL) (1), methotraxate can be administered intrathecally to avoid systemic side effects. In a clinical setting, parenterally administered drugs are commonly employed for immediate correction of electrolyte or fluid imbalance, for example, dehydration or excessive blood loss due to trauma. Patients who require hyper alimentation can also be administered total parenteral nutrition consisting of minerals, amino acids, vitamins, and carbohydrates via the IV route.

Although parenterally administered drugs have a number of advantages they do suffer from certain shortcomings. One of the major disadvantages is the possiblity for infections resulting from inadequate aseptic technique during product administration. Asepsis must be maintained to avoid infection, particularly for an intravascular or intraventricular injection. Apart from infections, other life threatening conditions like AIDS (2) and hepatitis C (3) can be attributed to improper use of parenteral devices. Disinfection of the patient's skin with an antibacterial solution or rubbing alcohol before injection and using a new syringes and needles for each administration is considered a best practice. Since injecting a needle into vascular compartments or body cavities can be considered as invasive processes, pain may be an additional factor. This is especially a significant factor for patients who perform self-administration (e.g., insulin, human growth hormone). Many of the products in the current market are highly specialized drug products and expense is still a major consideration.

Although in many instances precaution are unique to the route to be utilized, several factors need to be emphasized. Needless to say good aseptic technique and sterile practices is an absolute necessity. The health practitioner should always examine the product carefully before administration to identify potential or real contamination by microorganisms or particulate matter unless the product is supplied as a suspension or emulsion. Adequate attention should be given to details with respect to dosage, mixing, potential drug interaction, and storage. Informed actions and precautions should be taken during handling of accessory or delivery devices necessary to accomplish the task of injection or infusion or to monitor the patient's conditions. Selection of correct equipment for administration of the drug product, careful assessment of the patient history, evaluation of risk factors (e.g., bleeding diathesis, previous drug interactions, predisposition to infection, etc.) and a careful observation of the patient during and after parenteral administration are recommended.

The need for good practices in storage and handling of parenteral drugs or infusions is also an important factor and should be appropriately emphasized. From the moment a parenteral drug product is manufactured, its purity and sterility are constantly threatened by handling or storage errors. Such problems are not unique to manufacturers but extend throughout the life of the product in all areas of delivery, receiving, and distribution. Difficulties encountered may range from inadequate temperature control of storage temperatures, to outdated shelf lives, to defective containers and closures (4). On the other hand errors encountered during handling or compounding usually occur at the hospital pharmacy or at bedside. Past attempts by hospital pharmacies emphasizing a "central additive programs" as a method of reducing such errors have led to reduced admixing errors (4). In such a setting sterile parenteral product received from the manufacturer is mixed in a central location (usually in the pharmacy) with specific agents or fluid formulas that physicians may have prescribed. The central location is isolated and compounding is performed aspectically under a laminar flow hood. Complex formulas are often generated in these specialized units to satisfy the therapeutic needs of an extremely difficult medical or surgical problem (e.g. hyperalimentation). Upon compounding, the product/s is shipped to the hospital ward for administration to the patient. Newer infusion devices like the "smart pumps" or "intelligent pumps" are now available that have shown to significantly reduce compounding errors related to dose accuracy (5). Central additive programs reduce the high risk of compounding and contaminating errors which may occur because of personnel variability.

In addition to these problems, difficulties exist in securing properly trained, highly intelligent, motivated health care personnel to employ correctly and responsibly the complicated methods often utilized in the modern hospital or clinic setting. Such personnel,

in addition to being expensive and scarce, must be constantly educated on new techniques and problems (continual education). Similarly, some of the devices employed in administration are not only expensive but also highly advanced, and in some instances possess inherent or generated problems too difficult to identify with 100% assurance with even the best quality control techniques. The actively engaged personnel or administrator must able to identify real and potential dangers associated with such delivery systems.

General hazards or complications are at risk of occurring regardless of the agent or class of drugs being administered, whereas specific hazards or complications are unique or peculiar to certain agents and methods of administration. An important fact to remember about all parenteral injections is that if a reaction or adverse side effect of any sort occurs, it is usually impossible to retrieve or locally neutralize the offending agent, whereas with oral agents, recovery or expulsion of the medication is possible.

ROUTES OF ADMINISTRATION

The major routes of parenteral administration are IV, subcutaneous (SC), and IM. These three routes satisfy to a large extent the four principal reasons for administering parenterals: (1) for therapy (definitive or palliative), (2) for prevention, (3) for diagnosis, and (4) for temporarily altering tissue function(s) to facilitate other forms of therapy. Besides these three primary routes, additional ones are utilized under special circumstances: for example, intrathecal, subconjunctival, intraocular, intrathecal, intra-articular, and so on. A comprehensive description of the most commonly used routes of administration is discussed in the following section.

Intravenous Route

Injections or infusions directly into a vein are termed as IV administration (Fig. 1). Such administrations of true solution drug products is considered to be 100%. Drug absorption and factors concerning absorption are circumvented by IV injection of drugs in aqueous solution. At the desired concentration of a drug in the blood an accurate and immediate action is obtained that is not always possible by other procedures. It is of the most common parenteral routes employed in hospitals for drugs, fluids, and/or electrolytes. It offers a convenient route for rapidly infusing large volumes of fluid. If the dose is administered over a few minutes, it is

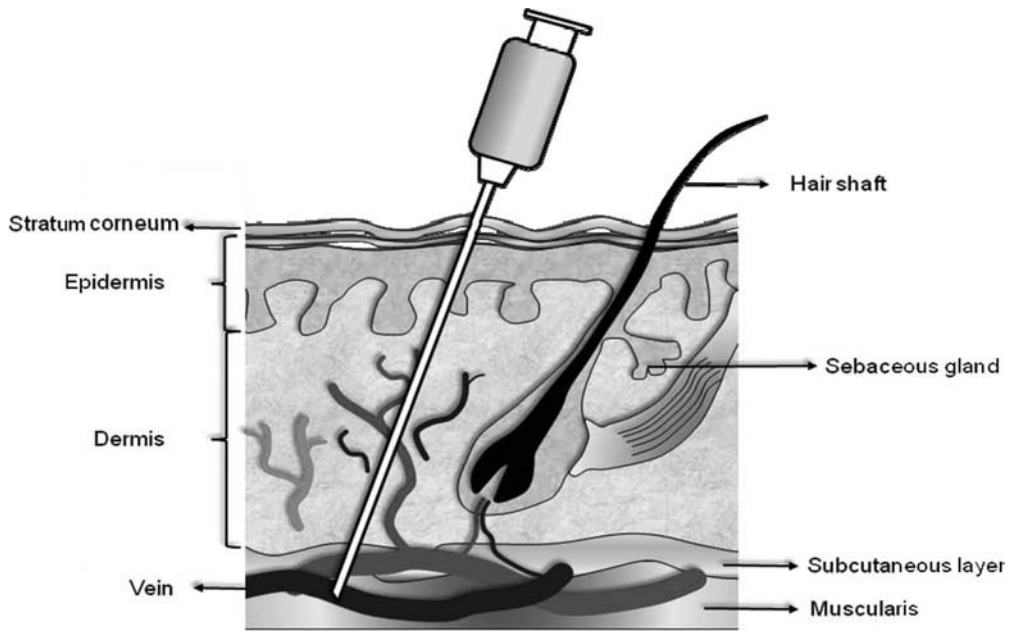

Figure 1 Schematic representation of an intravenous administration.

called a bolus dose and is primarily administered by a syringe directly into the vein. If the drug product is administered over hours from an infusion bag, it is termed an IV drip or infusion. Unfavorable reactions are prone to occur, since high concentrations of drug may be attained rapidly in both plasma and tissues. Repeated IV injections are dependent on the ability to maintain a patent vein. For prolonged IV use, flexible plastic catheters are better than sharp metal needles that may puncture through the other side of the vein.

Examples of drugs that are commonly administered by the IV route are analgesics, general anesthetics, antiviral agents, antibiotics, immunosuppressive agents, antifungal agents, antibacterial agents, antihypertensive agents, vasodilators, antiarrhythmic drugs, and chemotherapeutic agents. The preferred route for strong analgesics is a continuous IV infusion, because it produces less fluctuation in serum concentrations of the drug than do intermittent IM injections. Today, many IV drips are made in the pharmacy or by a special team rather than bedside preparations to insure accuracy of the drug product being administered.

The most common indication for use of this route are: (1) to guarantee delivery and distribution when hypotension or shock exists; (2) to restore rapidly electrolyte and fluid balance; (3) to achieve an immediate pharmacological effect, especially in emergencies, such as the treatment of certain arrhythmias or of seizures; (4) to treat serious, life threatening infections or conditions; (5) to provide continuous nutrition (hyperalimentation) when patients are unable to be fed by mouth; and (6) to avoid complications which might result if other administration routes are employed (e.g., hematomas at the site of IM injections in a patient with a bleeding diathesis). In addition, the IV route may be used for a variety of other purposes, such as plasmapheresis, blood transfusion, and hemodynamic monitoring, among others. Patient-controlled analgesia (PCA) is another unique mode of IV administration and is designed to deliver IV bolus doses in addition to a slow, continuous IV by this route for narcotic analgesics such as fentanyl, methadone, and morphine (6). Programmable infusion pumps with limited patient controls are often used for this type of administration and only allow the patient to receive an additional dose within limited time periods (7).

The IV route is not without adverse effects. Generally IV injections are administered directly into the venous circulation, and hence highly vascular and perfused organs, such as the heart, lungs, liver, and kidney, rapidly acquire the drug. However, a sudden increase in serum drug concentration may lead to toxicity and adversely affect the vital organs. This can be prevented by giving a slow IV bolus injection or controlling an IV drip. Some drugs with poor aqueous solubility may precipitate from solution and produce an embolism, for example, phenytoin IV injection. Hence, in such instances, it is important that proper selection of the diluent and slow IV administration be carried out; the latter allows for proper mixing of the drug into the circulation. Some vehicles may cause adverse effects in pediatric patients. For example, phenobarbital sodium when dissolved in propylene glycol may cause hyperosmolality in infants. In addition, because the alcohol and aldehyde dehydrogenase pathway that metabolizes propylene glycol is not well developed in infants and children younger than four years, repeated use of IV injections containing propylene glycol can lead to toxicity (8). Some lipid-soluble drugs, like diazepam, can cross the BBB and are effective when given by the IV route. Thus, lipid-soluble drugs, especially central nervous system (CNS) active drug, for example, sedatives, depressants, etc., often need to be administered by specialized routes of delivery that bypass the BBB. Other complications that may occur using the IV route are as follows: (1) thrombosis with or without complicating infection at the site of injection or infusion; (2) injection of microorganisms, toxins, particulate matter, or air; (3) the occurrence of physical or chemical incompatibilities between agents prior to or at the time of injection; (4) uncontrolled or excessive administration of drugs or fluids; and (5) extravasation of injections or infusions at the site of administration. When indwelling catheters are utilized, rarely the catheter tip may break off and lodge in a major vessel, in the heart, or in the lung and can cause fatalities.

To administer drugs through the IV route the upper extremities are chosen whenever possible for the site of injection or infusion. The most peripheral veins (e.g., over the hand) are selected for initial use. When arm sites are no longer available, the leg veins (femoral and saphenous) or dorsal foot veins may be utilized; and in small children the scalp veins. A recent improved in locating veins in pediatric and geriatric population is being used in clinical trials and is based on noninvasive infrared technology (Fig. 2). This unique device captures a near

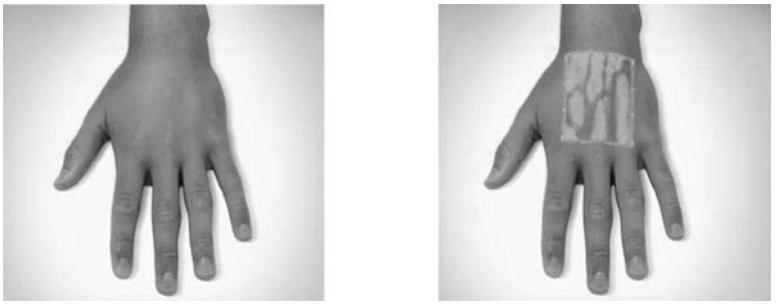

Figure 2 Visualization of veins using the proprietary VeinViewer® instrument form LuminetX, LLC.

infrared vein image, processes it, and projects it onto the skin using green light thus aiding phlebotomy (9). Selection of a vein depends on the size of the needle intended for use, type of fluids to be infused, flow rate anticipated, volume to be received, concomitant medications to be given, degree of patient mobility desired, and of course the skill of the person performing the venipuncture or catheterization. The veins in the antecubital fossa are among the most commonly chosen, because they are large and readily punctured. Other veins utilized commonly are basilic, cephalic, radial at the wrist, and the metacarpal and dorsal venous plexuses. Needles are generally preferred to indwelling IV catheters, as the risk of infection is believed to be less. Even after apparent exhaustion of all available venous sites, surgical cut downs of deep veins with insertion of catheters may be performed. When long-term, repeated usage is expected or when prolonged infusion is anticipated, the subclavian or internal jugular in the upper chest may be utilized. For peripheral veins and single or short-term usage, a 1 to 2 inch long, beveled, 18- to 22-gauge, stainless steel needle is commonly used.

For long-term and/or repeated IV administration, a sterile plastic catheter may be inserted into the vein percutaneously through or over the needle that was used for the initial puncture. The needle is then removed and the catheter is left in place. The indwelling needle or catheter, whichever is utilized, is anchored to the extremity or body by means of appropriate, sterile occlusive or nonocclusive dressings, often impregnated with an antibiotic ointment. Indwelling catheters may contain a heparin lock to ensure against clotting and loss of patency through venous thrombosis.

Intramuscular Route

An IM injection is defined as an injection directly into the body of a relaxed muscle (Fig. 3). The IM route is one of the most popular and convenient routes available, both for the administrator and for the patient, and a route of choice especially for pediatric subjects. Therefore, whenever it is possible and practicable, the IM route is used. The IM route provides a means for prolonged release of drugs formulated as aqueous or oily solutions or suspensions.

The IM route is preferred over the SC route when a rapid rate of absorption is desired for certain life threatening conditions. For example, administration of epinephrine via the IM route causes a higher peak plasma concentration compared with the SC route (10). However the rate of absorption is slow when compared with the IV route. One reason for using the IM route is because of the inability to administer the drug directly into the vascular compartment. Drugs commonly injected by IM administration include lidocaine, cephalosporins, aminoglycosides, diazepam, phenytoin, insoluble salts of penicillin G (procaine penicillin G), corticosteroids, narcotics, narcotic antagonists, and contraceptive steroids.

Although IM injections are much easier to administer than other injections, the main precaution is to avoid entering a blood vessel (especially an artery), which might lead to infusion of a toxic agent or a toxic vehicle directly to an organ or tissue. This can be prevented usually by pulling back on the plunger of the syringe; if blood does not appear, the needle is probably not in a vessel. Also, the accidental striking of or injection into a peripheral nerve may result in a peripheral nerve palsy with or without sensory damage. Occasionally, when a large bolus of drug is injected into the muscle, local damage or muscle infarction may result,

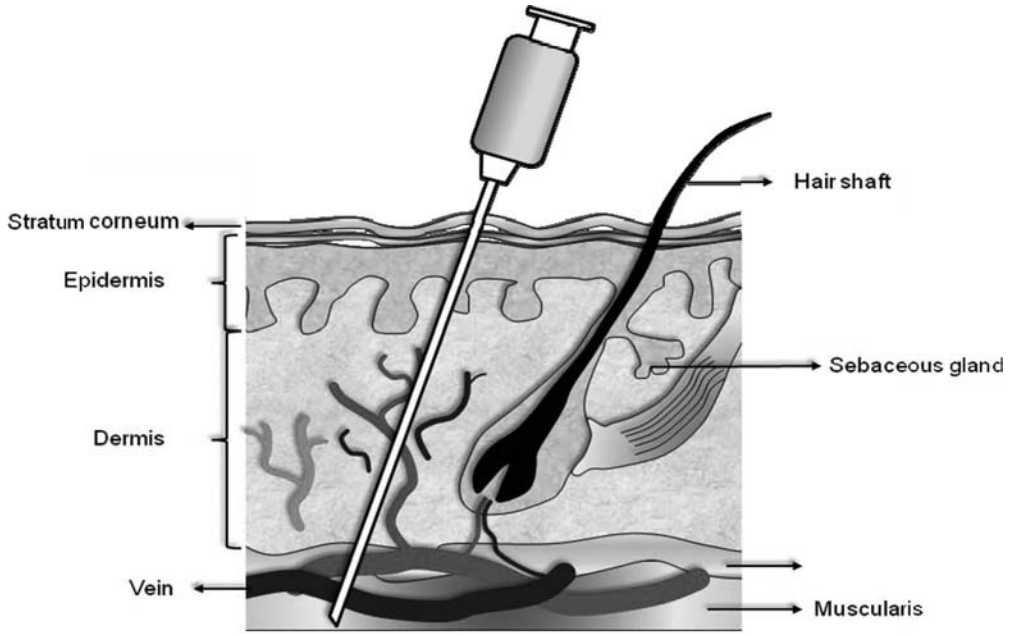

Figure 3 Schematic representation of an intramuscular administration.

leading to a sterile abscess or elevation of serum levels of muscle enzymes. The latter complication may present confusing diagnostic problems, especially in patients under suspicion of having a myocardial infarction or hepatitis.

If materials contaminated with microorganisms are injected, a septic abscess may result. Therefore, appropriate precautions must be taken to ensure sterility prior to injection. In patients with poor hygiene or skin care, microorganisms from the skin flora may be punched in by the needle at the time of injection, resulting in staphylococcal or streptococcal abscesses and rarely gas gangrene (11) or tetanus (12). An important note of caution: the IM route should never be employed in patients with significant heart failure or shock, where uptake into the vascular compartment may be expectantly poor. This caution should be followed especially if immediately high serum or plasma concentrations of the drug are desired or if rapid distribution to a distal organ is mandatory.

Various muscle sites are available for delivery, including the gluteal, deltoid, triceps, pectoral, and vastus lateralis muscles. In adults the site of choice often is the gluteal muscle, because large volumes of drug may be injected and tolerated. However, the vastus lateralis of the thigh may also be used because it not only tolerates large volumes of medication, but it is also away from any major vessels or nerves. For rapid absorption and small volumes ($<$2 mL), the deltoid muscle is preferred, as some studies suggest that blood flow in the deltoid muscle is 7% greater than that of the vastus lateralis and 17% greater than that of the gluteus maximus (4). In infants and small children, the vastus lateralis of the thigh is often preferred because it is better developed than other muscle groups.

With IM injections a beveled, 19- to 22-gauge, 1 to 2 inch long, stainless steel needle is used and no more than 5 mL of fluid is injected, depending on the site selected. The skin is first cleaned with alcohol or a suitable disinfectant, and the plunger on the syringe is always retracted prior to injection to be sure that the needle is not in a vessel. For deep IM injections, as might be used for irritating medications such as iron preparations, a "z-track" injection method is employed (4).

Subcutaneous Route

A SC injection (abbreviated as SC, SQ, sub-cu, sub-Q or subcut) is administered as a bolus into the subcutis, the layer of skin directly below the dermis and epidermis, collectively referred to

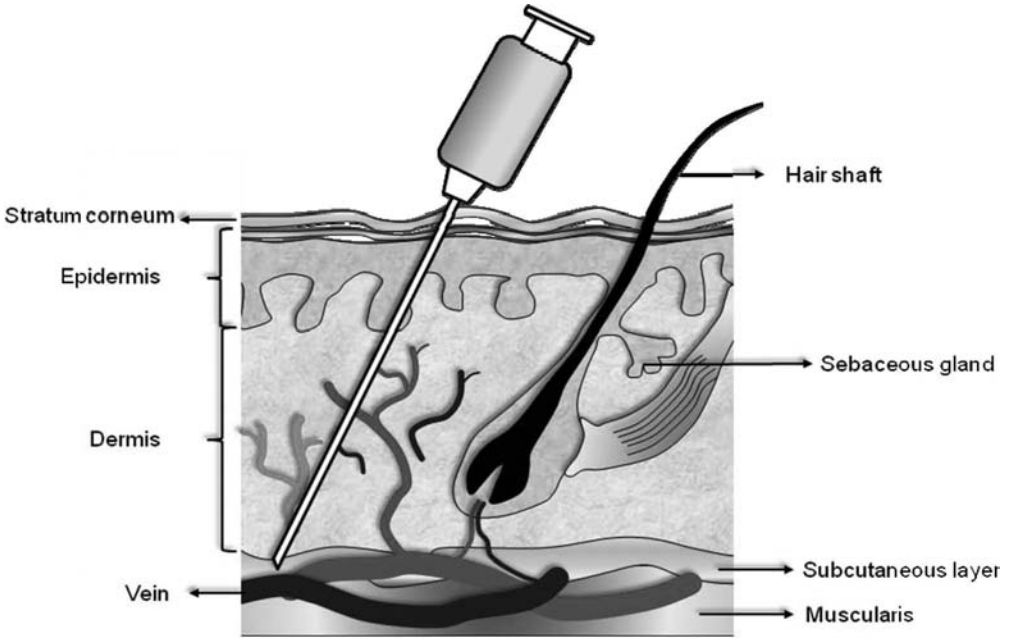

Figure 4 Schematic representation of a subcutaneous administration.

as the cutis (Fig. 4). SC injections are highly effective in administering vaccines and such medications as insulin, morphine, diacetylmorphine or goserelin. This route may be utilized if drugs cannot be administered orally because of lack of absorption from or inactivation by the contents of the gastrointestinal tract, if the patient is unable to ingest medications by mouth or if self-medication of parenterals (e.g., insulin) is desired. Drugs are more rapidly and more predictably absorbed by this route than by the oral route. However absorption of drugs via this route is slower and less predictable compared to the IM route and this effect can be attributed to the difference in vascularity of the muscle and dermis. Medications commonly administered subcutaneously include insulin, vaccines, narcotics, epinephrine, and vitamin B12. As with the IM route, if heart failure, shock, or vascular collapse exists, this route should not be depended on. Hypodermoclysis is a special form of SC administration, namely, the infusion of large amounts of fluid into the SC tissues when IV sites are not available. This form of administration is rarely (if ever) used today but in the recent past was a common mode of replenishment of fluid and electrolytes in infants and elderly patients.

Medications that are highly acidic, alkaline, or irritating, causing the production of pain, inflammation, and/or necrosis of tissues, should not be administered by this route. Infection, as with all parenteral injections, may occur, particularly in a patient with poor skin hygiene and particularly in situations where self-administration is practiced. Generally, a beveled, 24- to 25-gauge, 0.25 to 0.625 inch long, stainless steel needle is utilized. The volume injected generally does not exceed 0.5 to 1. 5 mL. Injection sites include the abdomen at the level of the umbilicus, the upper back, the upper arms, and the upper hip. The skin over the site of administration should be disinfected prior to injection with a sterile alcohol sponge. Prior to injection, aspiration should be attempted to be certain that the needle has not inadvertently entered a vessel. If blood does not appear in the syringe when the plunger is retracted, then the product is not injected.

It is advisable that the area of injection must be rotated for long-term therapies like administration of insulin or human growth hormone. Changing the injection site keeps lumps or small dents called lipodystrophies from forming in the skin. However, patients should try to use the same body area for injections that are given at the same time each day. Using the same body area for these routine injections lessens the possibility of changes in the timing and action of drugs like insulin.

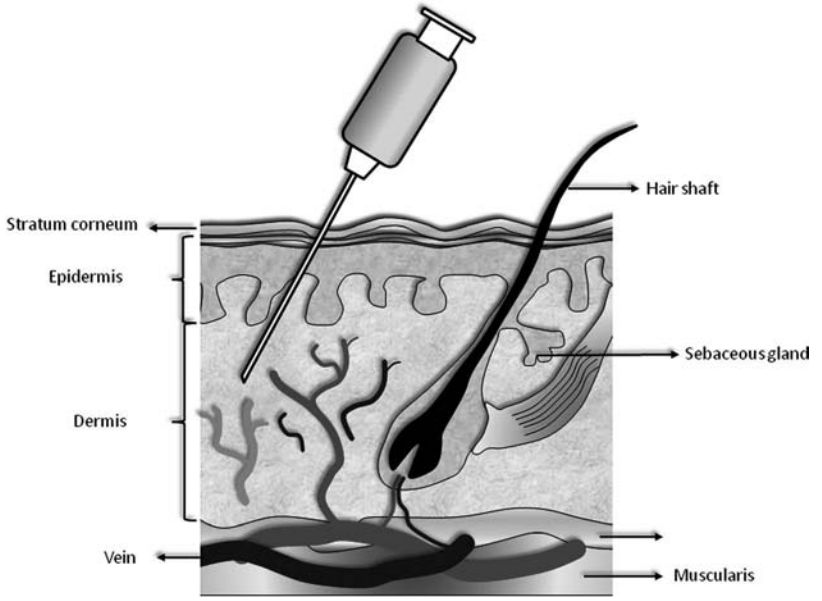

Figure 5 Schematic representation of an intradermal administration.

Intradermal Route

Injection into the dermis, located just beneath and adjacent to the epidermis is called an intradermal injection (Fig. 5). A number of diagnostic agents, antigens (e.g., tuberculin) and vaccines (e.g., smallpox) are administered by this route. The volume of fluid injected generally does not exceed 0.1 mL. Absorption by the intradermal route is very fast compared with the SC or IM route.

Generally a beveled, 26- or 30-gauge, 0.375 inch long, stainless steel needle is utilized. The skin at the site of administration should be cleaned prior to injection with 70% alcohol. Certainty of intradermal injection is evident by the appearance of a localized swelling of the skin, giving the appearance of an orange peel. The most common mistakes in intradermal injections are injecting beneath the skin rather than into it or permitting materials to leak out of the needle tip if it is not inserted completely into the skin.

Intra-arterial Route

The intra-arterial route is infrequently used route. Injection of a drug into an artery terminates in a target area, which may be an organ. Almost every artery is approachable by arterial catheterization and none are inaccessible to the skilled surgeon or radiologist.

The nature of the drug and the physiology of the circulatory system require IV injection to be diluted in the blood rather than going directly to an organ or tissue where the effects will be localized. The intra-arterial route is employed generally for diagnostic purposes, such as injecting radiopaque substances for roentgenographic studies of the vascular supply of various organs or tissues (e.g., coronary, cerebral, pulmonary, renal, enteric, or peripheral arteries). The usual reason for using the intra-arterial route is to introduce radiopaque materials for diagnostic purposes, such as for arteriograms. This route can be extremely hazardous, because products administered intra-arterially are not adequately diluted nor are they filtered by the lungs, liver, or kidneys before contact with peripheral tissue/s or vital organs nourished by the artery. Products contaminated with microorganisms, endotoxin, and/or particulate matter may result in serious complications or reactions, such as infection (either intra-arterial or extra-arterial) or arterial thromboembolism or vasospasm. This may result in ischemia, infarction, or gangrene of the tissues or organs supplied. In addition, if the technique of entry is faulty, damage to the arterial intima and vessel wall may occur resulting in serious hemorrhagic

extravagation or a dissecting aneurysm. If air is infused accidentally, air embolism with consequent ischemia and/or infarction of the tissue may occur; an event which usually does not occur when small amounts of air are infused into the venous system.

Usage of the intra-arterial route for treatment purposes is infrequent and limited generally to organ-specific chemotherapy, such as treating certain localized cancers (e.g., malignant melanomas of the lower extremities), where regional perfusion with high concentrations of toxic drugs (which when given intravenously may be associated with serious systemic reactions) can be achieved. Arterial spasm and subsequent gangrene present problems that make the intra-arterial route hazardous.

Either a suitably sized, smooth-bore, stainless steel needle or a short, flexible, plastic catheter is surgically inserted into the desired artery or a lengthy catheter is guided over a stylet or needle through a percutaneous entry site (sometimes under fluoroscopy) until the desired artery, organ, or tissue is reached; or the skin over the artery may be punctured directly, and the needle then inserted into the artery. Also, an open operative incision through the skin may be made (a "cut-down"), by which the artery is surgically exposed and under direct visualization is entered; a catheter is then inserted into the artery and sewn in place. Regardless of the method used, strict aseptic technique is practiced and appropriate occlusive or nonocclusive dressings are employed.

Intracisternal Route

Administration of drug products directly into the cisternal space surrounding the base of the brain is called as intracisternal injection. This route is employed mainly for diagnostic purposes. Additionally this route is used to decrease elevated intracranial pressures and reduce the risk of herniation of the brain if fluid is removed from the lumbar sac. Diseases involving the cisterns generally extend to nearby, contiguous structures are treated by utilizing the intraventricular route. Rarely, in order to locate and define a particular disease process; especially a spinal tumor or abscess, various contrast materials are injected into the cisterns. Intrathecal or intracisteral injections do not result in distribution of the drug into the ventricular space; thus disease within the ventricles would not be treated by these routes.

Many of the precautions concerning the use of the intraventricular route are applicable to the use of the intracisternal route, particularly as regards to aseptic practices and the threat of physicochemical irritation of the substances injected. One very serious drawback to the use of this route is the danger of producing permanent, serious, neurological injury or death due to possible damage to the midbrain. The space entered is relatively small, and insertion of a needle into it should be attempted only when other routes may not be used and only by the most experienced personnel. For intracisternal puncture the patient is placed in a head-down position and the entry approach is posterior between the occiput and the first cervical vertebrae. The cisterna magnum is punctured and extreme care is exercised to continue aspirating with a syringe while inserting the needle.

Intraventricular Route

Here the drug product is injected or infused directly into the lateral ventricles of the brain. This route is employed mainly in the treatment of infections (such as bacterial or fungal meningitis and/or ventriculitis) or of malignancies (such as leukemic infiltrates of the meninges or carcinomatoses) involving the membranes and cerebrospinal fluid surrounding the CNS. It is used especially in situations where the drugs involved are known to diffuse or pass poorly from the vascular compartment into the ventricles and subarachnoid space and/or where reduction of systemic side effects from a particular agent are desired. One such example is the treatment of fungal meningitis with amphotericin B (13) or in the therapy of leukemic infiltrates with methotrexate (14). Often, therapy via this route is complemented by the IV administration of the same agent which has been injected into the ventricles.

In the treatment of diseases of these areas, the intraventricular route often is preferred over the intracisternal or intrathecal. This is because the flow of cerebrospinal fluid is unidirectional and originates principally in the choroid plexus of the lateral ventricles and pursues a path through the third and fourth ventricles out the foramina of Luschka and

Magendie into the posterior fossa at the level of the pons, down over the spinal cord, and then finally reversing itself to flow up over the cerebral hemispheres. In addition, the ventricle provides a large fluid space in which to inject drugs, thereby diluting such drugs in a large volume of cerebrospinal fluid, thus minimizing potential, localized physicochemical irritation to the cells lining the ventricle and subsequent damage from a host reaction. In addition, if intracranial pressures are excessive, the risk of brain stem herniation may be avoided, a known risk factor for intracisternal route. Radiopaque tracers, radiolabeled, or dyes may be injected into the intraventricular space for studies of either the anatomy or patency of the system or for studies of the flow of cerebrospinal fluid.

Since cerebrospinal fluid bathes such critical organs as the brain and spinal cord and since one of its functions is believed to be a protective or cushioning fluid for these organs, any disturbance of this fluid or the membranes containing it may be deleterious and possibly lethal. Any foreign material, chemical or biological, when injected into the system may precipitate an inflammatory response anywhere or everywhere within the system. Strict aseptic techniques should be adhered to when entering the ventricles to prevent iatrogenic infections, and care should be exercised to be certain that the substances injected or infused are not irritating to the cells lining the ventricular or subarachnoid spaces. If irritating drugs are injected, ventriculitis or myelitis may result (sometimes progressive), producing obstruction of the system (hydrocephalus) or permanent neurological injury.

The vehicles employed for intraventricular injection should have physical characteristics as close to the cerebrospinal fluid as possible. If the ventricles are small or almost closed because of intracerebral edema, these spaces may be difficult to locate, and undesirable intracerebral injection of the drug with subsequent neurological injury may result. In addition, hemorrhages in the subdural, epidural, intraventricular, or intracerebral regions may occur. If the ventricular needle is inserted too far, passing through the ventricles, damage to the basal ganglia, thalamus, or other vital structures may occur. The procedure should be carried out only by experienced personnel.

To administer drug products via this route a 3.5 inch long, smooth-bore, 18-gauge, stainless steel, blunt-ended ventricular needle is used. The patient's skin is prepared as in any surgical procedure, taking extreme care to maintain strict aseptic technique. A twist drill puncture of the cranium is first performed, generally over the coronal suture about 2 cm from the midline and in line with the ipsilateral pupil. The needle, which is a special blunt, open-ended needle, is passed through the frontal lobe into the lateral ventricle. When repeated injections or infusions are required, use of an Ommaya (15) or Rickam (16) reservoir or similar silicone, elastomer, SC reservoir is recommended. Surgical placement of the reservoir may be accomplished in a variety of ways. Often with these devices no local anesthetic is required for reinjection, and the system may be sampled and injected repeatedly with minimum disturbance to the patient and with reduced risk of infection.

Intrathecal Route

Intrathecal (Latin *intra* "inside," Greek *theka* "capsule," "hull") is an adjective that refers to events that happen inside the spinal canal. An intrathecal injection (often simply called "intrathecal") is an injection into the spinal canal (intrathecal space surrounding the spinal cord), as in a spinal anesthesia or in chemotherapy or pain management applications (Fig. 6). This route is also used for some infections, particularly postneurosurgical. Drugs given intrathecally often have to be made up specially by a pharmacist or technician because they cannot contain any preservative or other potentially harmful inactive ingredients that are sometimes found in standard injectable drug preparations.

This route is a very popular for a single 24-hour dose of analgesia (opioid with local anesthetic). However extreme control had to be employed during dosing as most narcotic pain medications can cause a late onset respiratory depression when administered through this route. Often reserved for spastic cerebral palsy, intrathecally-administered baclofen is done through a intrathecal pump implanted just below the skin of the stomach with a tube connected directly to the base of the spine, where it bathes the appropriate nerves using low dose baclofen (17). Intrathecal baclofen also carries none of the side effects, such as sedation, that typically occur with oral baclofen. It is the preferred route for long-term management of

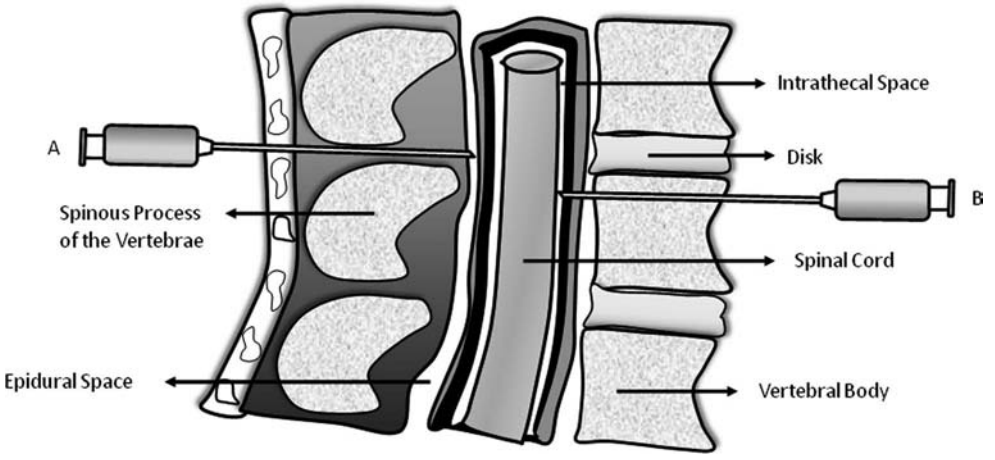

Figure 6 Schematic representation of an intrathecal administration (A) epidural route; (B) intrathecal route.

spasticity in people with cerebral palsy for whom other procedures, such as rhizotomy or orthopedic surgery, are inappropriate.

The same precautions required for intraventricular administration apply to use of the intrathecal route. In addition, a real threat of tonsilar or brain stem herniation (and possibly death) exists if this procedure is performed while intracranial pressure is elevated. Great care must be exercised to avoid this complication, which usually occurs one to two hours or sooner after removal of fluid. To administer via this route a 3.5 inch long, smooth-bore, beveled, 20- to 22-gauge stainless steel spinal needle is used for adults. The patient's skin is prepared as in any surgical procedure, taking the greatest caution to use aseptic technique. The needle is inserted posteriorly at the midline into any space below the third lumbar spinal process. The patient is in the lateral decubitus position with head, back, and thighs flexed. If intracranial pressure is diffusely elevated, the special precautions outlined above should be taken, but if intracranial masses are suspected, this procedure should not be done.

Epidural Route

The epidural space (or extradural space or peridural space) is a part of the human spine. It is the space inside the bony spinal canal but outside the membrane called the dura mater (Fig. 5). In contact with the inner surface of the dura is another membrane called the arachnoid matter. The arachnoid encompasses the cerebrospinal fluid that surrounds the spinal cord. The term epidural is often synonymous with epidural anesthesia, is a form of regional anesthesia involving injection of drugs through a catheter placed into the epidural space. The injection can cause both a loss of sensation and analgesia, by blocking the transmission of signals through nerves in or near the spinal cord.

Injecting medication into the epidural space is primarily performed for analgesia (18). This may be performed using a number of different techniques and for a variety of reasons. A patient receiving an epidural for pain relief typically receives a combination of local anesthetics and opioids (19). This combination works better than either type of drug used alone. Common local anesthetics include lidocaine, bupivacaine, ropivacaine, and chloroprocaine. Common opioids include morphine, fentanyl, sufentanil, and meperidine in the United States. These are injected in relatively small doses. Occasionally, other agents may be used, such as clonidine or ketamine.

When a catheter is placed into the epidural space, a continuous infusion can be maintained for several days, if needed. Epidural analgesia may be used for the following: (*i*) Analgesia alone especially where surgery is not contemplated. An epidural for pain relief (e.g., in childbirth) is unlikely to cause loss of muscle power, but is not usually sufficient for

surgery. (*ii*) An adjunct to general anesthesia. The anesthetist may use epidural analgesia in addition to general anesthesia. This may reduce the patient's requirement for opioid analgesics. This is suitable for a wide variety of surgery, for example, gynecological surgery (e.g., hysterectomy), orthopedic surgery (e.g., hip replacement), general surgery (e.g., laparotomy) and vascular surgery (e.g., open aortic aneurysm repair). (*iii*) As a sole technique for surgical anesthesia. Some operations, most frequently cesarean section, may be performed using an epidural anesthetic as the sole technique. Typically the patient would remain awake during the operation. The dose required for anesthesia is much higher than that required for analgesia. (*iv*) For postoperative analgesia, in either of the two situations above. Analgesics are given into the epidural space for a few days after surgery, provided a catheter has been inserted. Through the use of a patient-controlled epidural analgesia (PCEA) infusion pump (20), a patient may be given the ability to control postsurgical pain medications administered through the epidural. (*v*) For the treatment of back pain. Injection of analgesics and steroids into the epidural space may improve some forms of back pain. (*vi*) For the treatment of chronic pain or palliation of symptoms in terminal care, usually in the short or medium term. The epidural space is more difficult and risky to access as one ascends the spine, so epidural techniques are most suitable for analgesia for the chest, abdomen, pelvis or legs. They are much less suitable for analgesia for the neck, or arms and are not possible for the head.

There are certain instances where the risks of an epidural are higher than normal. Anatomical abnormalities, such as spina bifida, meningomyelocele or scoliosis could be a major limiting factor for using this route. If the patient has previous history of spinal surgery, which can lead to scar tissue, can potentially cause disruption in the distribution of the medication. Use of this route is not recommended for patient suffering from certain CNS disorders like multiple sclerosis. Certain heart-valve problems such as aortic stenosis, where the vasodilation induced by the anesthetic may impair blood supply to the thickened heart muscle, may be fatal.

A particular type of needle known as a *Tuohy* needle is used. This needle is specially designed for locating the epidural space safely, and has several specific features. The needle is inserted to the ligamentum flavum and a loss of resistance to injection technique is used to identify the epidural space. This technique works because the ligamentum flavum is extremely dense, and injection into it is almost impossible. The anesthesiologist attaches a syringe to the Tuohy needle and advances it slowly. The syringe may contain air or saline. The principles are the same, but the specifics of the technique are different because of the greater compressibility of air with respect to saline. When the tip of the needle enters a space of negative or neutral pressure (such as the epidural space), there occurs a "loss of resistance" and is possible to inject through the syringe (21).

Traditionally anesthesiologists have used either air or saline for identifying the epidural space, depending on their personal preference. However, evidence is accumulating that saline may result in more rapid and satisfactory quality of analgesia (22,23). In addition to the loss of resistance technique, real-time observation of the advancing needle is becoming more common. This may be done using a portable ultrasound scanner, fluoroscopy or real-time X-ray (1).

Intra-articular Route

Injection or infusion into the synovial sacs of accessible joints is termed as an intra-articular injection (Fig. 7). Antibiotics, lidocaine, and antiinlammatory drugs, like corticosteroid, may be administered into joints for the treatment of infections, pain, inflammation, or other problems resulting from inflammatory diseases (e.g., rheumatoid arthritis or trauma). Some agents are administered in single injections and some (e.g., antibiotics) via continuous infusion and "bathing" of the joint.

Intra-articular injections are easily accomplished in the knee, ankle, wrist, elbow, shoulder, phalangeal, sternoclavicular, and acromioclavicular joints. Joints deformed by any disease process (e.g., rheumatoid arthritis or trauma) may be more difficult to enter and inject. Usually, the intra-articular approach is utilized when no more than one or two joints are involved. Often it supplements systemic therapy since; when the synovium is inflamed it is

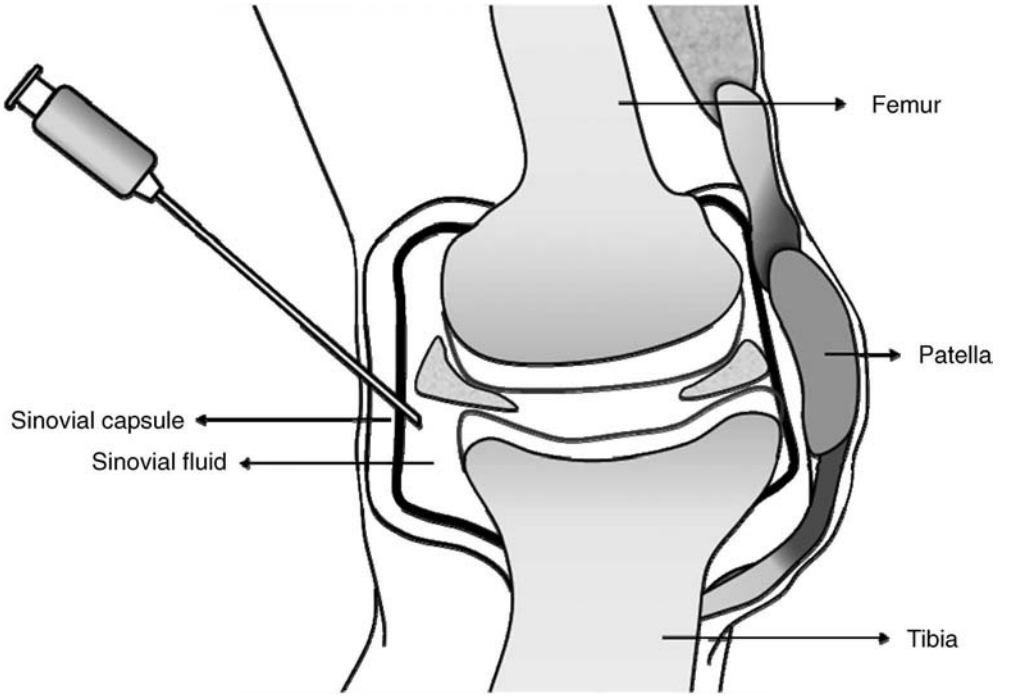

Figure 7 Schematic representation of an intra-articular administration.

often highly vascularized, permitting a multitude of agents to enter with ease from the intravascular compartment.

Iatrogenic infection is always a threat following intra-articular injection. The consequences of such infection may result in destruction of the joint. Administration of corticosteroids is particularly troublesome because if serious infection does occur, recognition may be delayed because of suppression of the local inflammatory response; thus destruction of the joint and the cartilage may occur before the identification of a complicating infection. Severe, recurrent, intra-articular hemorrhage may be produced if a bleeding diathesis, such as hemophilia or severe hypoprothrombinemia, is present. Ordinarily, such blood is resorbed, but with recurrent hemorrhage eventual destruction of weight-bearing joints may occur. If the therapist is inexperienced, tendons may be ruptured if appropriate administration technique is not employed.

The anatomy of the joint to be treated should be studied by X-ray or imaging techniques prior to injection. Entry should be at the point where the synovial cavity is most superficial and free of large vessels and nerves. The site of skin entry is cleaned and prepared as with any surgical procedure; strict aseptic technique is mandatory. A sterile, 19- to 22-gauge, stainless steel needle attached to a syringe is inserted into the synovial cavity. The synovial fluid should be first aspirated to ensure that the needle is within the joint space. The syringe is changed, and one containing the drugs to be injected is attached and administered.

Intra-abdominal Route

This route is also known as the intraperitoneal route. An injection or infusion directly into the peritoneal cavity via a needle or indwelling catheter or directly into an abdominal organ, such as the liver, kidney, or bladder is defined as a intra-abdominal injection. The intra-abdominal route may be employed to treat local or widespread intra-abdominal disease due to microbial infection or tumor. The route is also employed to dialyze (peritoneal dialysis) various toxic substances from the abdomen when severe renal failure prohibits excretion. Another

application of this route is to determine the patency, as well as the structure, of various vascular or lymphatic systems employing radio opaque agents.

The intra-abdominal route of administration can cause serious abdominal infection (peritonitis) and hemorrhage. The source of infection may be extrinsic (e.g., from skin or contaminated drugs or infusates) or intrinsic (e.g., from puncture of the bowel). The risk of infection is enhanced if an indwelling catheter, rather than a single injection using a sterile needle, is utilized. Such infections are particularly difficult to treat, especially in the presence of ascites; thus every precaution should be taken to prevent them. In addition, an aseptic peritonitis may be induced if the agent or fluid injected is highly irritable or contains endotoxin. The chance of inducing hemorrhage is related generally to the size of the needle employed, the anatomical site selected for injection, the skill of the technician, and any tendencies of the patient to bleed (i. e., coagulation problems). If hemorrhage is induced, it may be difficult to control and may require surgical intervention and repair.

Drugs injected into the intraperitoneal space are usually absorbed into the vascular compartment, and under certain pathological conditions this can be unpredictable. This can result in an uncontrolled risk of toxicity or therapeutic failure. To administer a drug intraperitonially, suitable aseptic preparation of the skin should be carried out. A 16- or 18-gauge, stainless steel needle is then inserted through the anterior abdominal wall just lateral to the rectus muscles. If ascites is present, there is little risk of bowel puncture; however, if the peritoneal cavity is "dry," puncture of the bowel may occur (indicated by aspiration of fecal contents). Bowel puncture may be avoided by shallow punctures and withdrawing on the plunger while advancing the needle.

Intracardiac Route

An injection directly into chambers of the heart or the cardiac muscle is called as an intracardiac injection. The use of this route is not common for delivery of drugs. Nevertheless, under unusual circumstances and in certain emergency situations, such as cardiac arrest, in which drugs may have to reach the myocardium immediately, intracardiac injections may be employed.

One of the major risk factors is the damage inflicted on the heart muscle, coronary arteries, or the conducting system due to trauma of an injecting needle or by the drug injected. Occasionally, hemorrhage into the myocardium or pericardium may result, leading to infarction or pericardial tamponade. If extracardiac structures such as the lung are inadvertently punctured, a pneumothorax may result and breathing may be impaired.

Selection of the route may be influenced by the presence of left or right ventricular hypertrophy, the former being better suited for the anterolateral approach and the latter being better suited for the medial approach, or any anatomical derangements of the chest which may exist. Generally, a beveled, 18- to 21-gauge, 4 to 6 inch long, stainless steel needle is used.

Intraocular Route

Injection of drug products directly into the various chambers of the eye is collectively termed as intraocular injection (Fig. 8). Four types of intraocular injections are utilized. These include (*i*) anterior chamber: injection or irrigation directly into the anterior chamber of the eye; (*ii*) intravitreal: injection directly into the vitreous cavity of the eye; (*iii*) retrobulbar: injection around the posterior segment of the globe; and (*iv*) subconjunctival (4). Although included under this heading, subconjunctival (and retrobulbar) injections are not intraocular (Fig. 9). Instead, such injections are adminstered beneath the conjunctiva, so that medication diffuses through the limbus and sclera into the eye. This route is generally used in the treatment of infections and inflammatory diseases of the eye which are not treated effectively by topical or systemic drug administration for anesthesia of the globe (retrobulbar) and occasionally for pupillary dilation with cycloplegics and mydriatics. Absorption of drugs into the eye is challenging, as intraocular transport and diffusion are poor. Intraocular injections are complemented frequently by IV infusions of the therapeutic drugs employed. Selection of the type of intraocular injection depends on the disease present and the precise location of that disease within the eye.

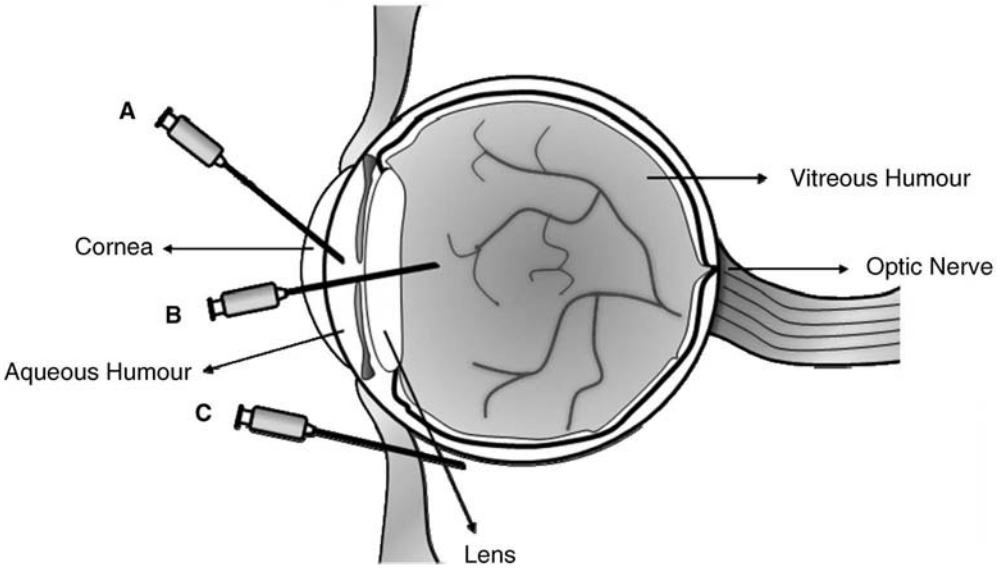

Figure 8 Schematic representation of an intraocular administration: (**A**) anterior chamber injection, (**B**) intravitreal injection, and (**C**) retrobulbar injection.

Extreme care and precise technique are required to minimize or prevent damage to the eye, especially to the corneal endothelium. Complications that can occur, depending on the route selected, are optic nerve damage, hemorrhage, retinal detachment, retinal necrosis, cataracts, and injection of the drug directly into the circulation with consequent systemic effects. Infection is always a threat and must be avoided as such infections may result in rapid destruction of the eye and/or blindness. The volume of solution that may be injected into the eye is severely restricted, generally to not more than 0.1 to 0.2 mL. Since an excellent knowledge of the anatomy and function of the eye is required, only an ophthalmologist should attempt these procedures.

The anterior chamber (containing the aqueous humor) is entered at a point located on the edge of the cornea (the limbus) with a 25-gauge or smaller, stainless steel needle, withdrawing a volume of fluid prior to injection equal to that to be instilled. For intraocular injections excluding the anterior chamber, a drop of 1:100,000 dilution epinephrine may be placed on the iris to dilate the pupil. Great care must be taken not to inject or damage the lens, as this may result in cataract formation.

Entry into the vitreous humor is accomplished by injection through the pars plana (junction of retina and ciliary body) with a 25-gauge stainless steel needle. The vitreous appears to be an inert fluid which is not replaced once removed. During injection, great care must be taken not to detach the retina. Again, a volume of fluid equal to that to be injected must be removed before instillation. Generally, not more than 0.1 mL may be injected. Injection of steroids into this chamber can be dangerous, resulting in destruction of the retina (retinal necrosis).

Entering the retrobulbar space involves insertion of the needle at the junction of the lateral and medial third of the orbital rim and then advancing the needle toward the apex of the orbit. Care must be taken not to inject the optic nerve directly. A 1 to 0.5 inch long, 25-gauge stainless steel needle is generally employed. Subconjunctival injections generally do not exceed volumes of 0.5 mL. This route is especially used in treating corneal abscesses. Injection of the sub-Tenon fascia is utilized for the treatment of uveitis (e.g., secondary to localized sarcoidosis) or chronic cyclitis. Again, care must be taken not to inject or nick the orbit.

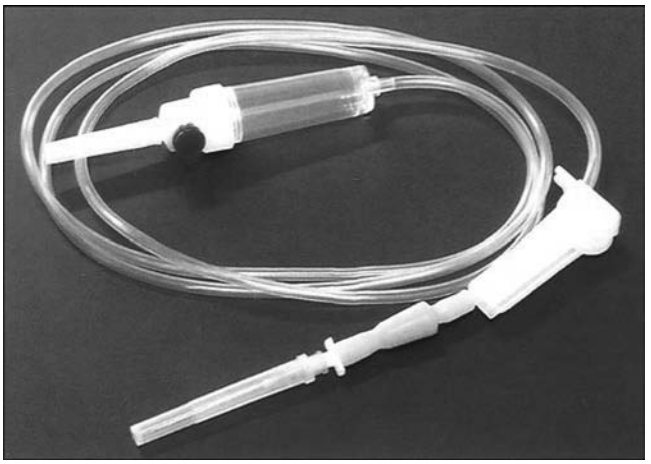

Figure 9 Picture of a standard infusion set indicating its components: a piercing spike; a vent; a drop chamber; a connection tubing; a roller clamp; a luer fitting; and a protective cap on the spike.

PARENTERAL DRUG ADMINISTRATION: METHODS AND DEVICES

This section describes the factors which determine the necessity of exact dosage as well as those which affect the flow of the infusion. Various infusion techniques such as gravity infusion, positive pressure infusion as well as other highly specialized types of infusion equipment will be discussed. Related information about their function and areas of application will be provided.

GENERAL CONCEPTS

Venous or arterial administration of a liquid into the circulatory system requires an accurate dosage and the infusion technique employed determines the accuracy of the dosage. The required dosage accuracy is generally dependent on the patient's status as well as on the type and amount of fluid to be infused, and the infusion equipment used. The flow of the infusion is affected by a range of factors including resistance in the channel of the piercing spike; resistance in the tubing and in the connector pieces; speed of drop formation; variability of the delivery pressure; and physicochemical characteristics of the solution.

GRAVITY INFUSION

The technique is the most frequently used one comprising of more than 80% of all infusions performed. The accuracy of the dosage and the infusion rate requirements are low for this type of infusion (±50%). The volume administered is based on the hydrostatic pressure differential between the patient and the infusion container. The rate of fluid administration can only be accelerated through compression of the container or by increasing the internal pressure of the container. Over the years, a standardised infusion set (Fig. 9) has been developed. Components used for this type of infusion are; a piercing spike; a vent; a drop chamber; a connection tubing; a roller clamp; a luer fitting; and a protective cap on the spike.

Depending on the type of container to be used with, the piercing spike is sharp for rubber stoppers or rounded and blunt for bag insertion sites. The infusion bag contains one channel for fluid and optionally a second channel for venting with a cap or stopper. Upon opening of a cap or stopper air flows into the container. The vent usually is equipped with a bacterial filter. A drop generator is located at the top of the drop chamber, which produces drops of a certain size. The chamber is partially filled with liquid to prevent air bubbles from entering the tubing. A particle filter is often located at the bottom outlet of the chamber. The connecting tube is usually 150 cm long and made of PVC. These are also available in other lengths and materials for special applications. The roller clamp supplied within the connecting tube is used to

regulate the flow rate of infusion by controlled compression of the tubing. The Luer fittings at the end of the line, guarantees a secure connection to all other products by means of the standardized Luer cone. In the lock version the lock connection is further secured against jerks and pressure by means of a screw thread. This prevents damage to the packaging and thus loss of sterility. The standardised infusion set is connected to a infusion container (bottle, bag) using the spike.

The rate of the infusion is a critical factor for gravity infusion and is mainly regulated by means of the roller clamp in most of the hospital settings. The roller clamp is positioned on the infusion tubing of the infusion set in such a way that the lumen of the infusion tubing is compressed from outside. With respect to gravity infusion the rate of infusion is calculated on the basis of number of drops/min. Most standard infusion sets are designed to deliver approximately 20 drops/min (equivalent to 1 mL/min). Specialized roller clamps are available that allow for drop rates of 60 drops/min. However, even with higher drop rates, the microdroppers (e.g., Dosifix® from B. Braun) still delivers only 1 mL/min; that is, 60 drops = 1 mL/min.

Another type of flow regulator is the tubing independent flow regulators that can replace the traditional roller clamp for improved control of dosage accuracy. The flow rate is controlled by varying the size of an accurately designed flow channel and flow rates can range from 3–200 mL/hr. These units are used for infusion solutions which are carrier solutions for drugs that need to be administered at a specific concentration for longer duration. It is important to note that an ideal flow regulator is the one that can maintain the desired flow rate irrespective of changes in the infusion height and patient activities.

PRESSURE INFUSION

In certain instances during IV administrations using infusion or transfusion bags, a pressure infusion may be performed. For this purpose a pressure cuff is used which is pumped up with an inflation bulb in a similar manner as with a blood pressure measurement instrument, thus exerting pressure on the container. A pressure of up to a maximum of 300 mmHg can be exerted on a regular infusion bag. Other types of positive pressure infusion equipments are available and employed for such infusions. They are especially used when the dosage accuracy is required or increased rate of infusion is needed or when a constant rate of delivery during long-term infusions is desired. The infusion equipment used should meet certain and the important criteria: (*i*) requirement-based infusion rate, (*ii*) exact dosage, (*iii*) robustness of equipment, (*iv*) quick functional readiness, (*v*) simple and safe operation, (*vi*) alarms for interruption of infusion or in the event of danger, (*vii*) mains-independent operation, and (*viii*) easy cleaning.

Depending of different applications and administrations to be performed, the required infusion rates extend over a wide range. Pressured infusion rates may vary from 1 mL/hr and $>$ 1000 mL/hr (e.g., shock therapy) for adult patients. Such a type of infusion is generally used in an intensive care medicine scenario. Cost of equipment for pressured infusion can also be a limiting factor for many settings. The degree of accuracy of dosage depends on the status of the patient, the solution to be infused and other factors. Also, the degree of accuracy a dosage can have is determined by the kind of infusion technique that is employed.

With regard to these techniques, distinctions are made between gravity infusion, pressure infusion and the use of infusion equipment. Additional infusion equipment is required when the dosage accuracy should be increased, the rate of infusion should be raised or when a constant rate of delivery during long-term infusions should be achieved. In equipment-supported infusion techniques, distinctions are made between infusion regulators, that is, electronic medical devices without a delivery drive, infusion pumps and syringe pumps. In contrast to the infusion regulators, infusion pumps have their own delivery drives. Depending on the type of drive, there is a distinction between roller pumps, peristaltic pumps and plunger or syringe pumps. The accuracy of the dosage mainly depends on how the pumps are regulated. Syringe pumps are pressure infusion devices which administer the content of one or more syringes simultaneously using a precision linear drive. This form of infusion is particularly suited for an exact administration of drugs.

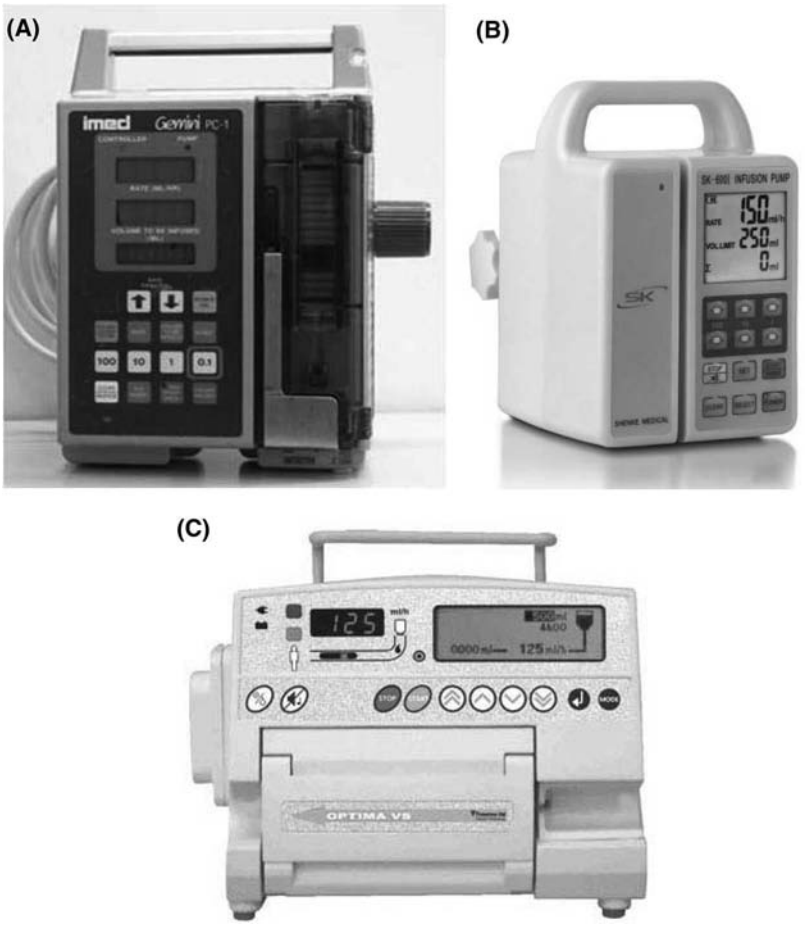

Figure 10 Examples of different types of infusion pumps: (**A**) a roller infusion pump, (**B**) a syringe-driven pump, and (**C**) a peristaltic infusion pump.

Types of Equipment

Over the recent years significant advances have occurred in the area of pressure infusion or positive pressure infusion. Most of the infusion systems available in today's market are highly sophisticated, precise, and electronically advanced requiring specialized training. They can be broadly classified into three distinct classes: (*i*) infusion regulators, (*ii*) infusion pumps, and (*iii*) syringe pumps. Other infusion devices like the disposable infusion pumps, smart pumps, and associated accessories are regularly employed in different medical settings (Figure 10).

a. *Infusion regulators*: Infusion regulators are electronic medical devices which do not have their own delivery drive. They regulate and monitor the supply of fluid in the flow process. Simply stated, they are mechanized roller clamps. The dosage accuracy is often sufficient for everyday clinical purposes and ranges between $\pm 10\%$ and 20%.
b. *Infusion pumps*: In contrast to the regulators, infusion pumps are equipped with their own delivery drive. Depending on the type of drive, it can be classified as roller pumps, peristaltic pumps and piston pumps (Fig. 10). The main purpose of an infusion pump is to deliver medication(s) at a regulated rate and thereby in a regulated dose. Control of infusion pumps can either be drop based or volume based. The basic design of infusion pumps comprise of a delivery drive, a control or regulating system, and an infusion set. The dosage mainly depends on how the

pump is regulated. Roller pumps and peristaltic pumps are examples of volume-based pumps. The delivery principle of a roller pump is based on the rollers bringing a set amount of fluid into the tubing which is then transported by help of rotation in the flow direction. On the other hand, delivery principle of a peristaltic pump depend on the successive compression of the tubing by the individual fingers, makes the fluid be advanced forward.

In the case of the drop regulated infusion pumps, the dosage accuracy of these pumps relates to the number of drops (per minute) and depends on the volume of the drops. The drop accuracy is subject to several important conditions such as the viscosity of a solution, the solution's surface tension and the flow behavior resulting from these factors. Dosage accuracy is ±1 0%.

Accurate fluid infusion and drug administration is crucial for the optimum management of a critically ill patient. Continuous and controlled IV delivery of common medications, such as inotropic agents, vasodilators, aminophylline, insulin, heparin, etc., via infusion pump is the preferred mode of therapy in acute care. This is especially true for drugs with short half lives, so as to maintain a desirable constant serum concentration and in situations when constant infusion of glucose is needed. Patients with compromised renal, cardiac or pulmonary function have limited fluid tolerance and hence it is essential to use infusion pumps so as to prevent inadvertent volume overload. For intensive care, more than one infusion pump is often used when drug dosage, concentration, interaction and fluid volume require separate infusion rates. The use of infusion pumps has been advocated over manual flow control system on the basis of assuring precise and accurate delivery of prescribed fluid volumes over a specified time and to help in better nursing management.

The performance of infusion pumps is generally acceptable for clinical use, but the volume that may be infused is limited by the syringe capacity and infusion must be stopped whenever it is necessary to replace or refill the syringe. The largest syringe accepted by these pumps accommodates 100 mL of drug product. The small weight and no interference of gravity and positioning makes these syringe pumps suitable for transport. These pumps can be mounted on an IV pole or on the operating table. In addition these are small and light weight and have an occlusion alarm pressure of 570 mmHg.

Recently introduced modern infusion pumps incorporate a soft key interface by which a range of body weight and drug concentrations can be entered. Bolus doses can be easily and rapidly administered at any time during the infusion. These systems are also modifiable to accept all syringe sizes from 10-100 mL and have two independent microprocessors to monitor and control infusion processes for consistent delivery.

c. *Syringe pumps*: The syringe pump has been defined as a power driven device for pushing the plunger of a syringe forward at an accurately controlled rate. These are pressure infusion devices which supply the content of one or more syringes simultaneously by means of a precision linear drive. The dosage accuracy with these pumps is ±2% since a precise syringe volume is delivered through these pumps and all the error sources involved in drop regulation do not apply. This form of infusion is particularly suited for an exact administration of drugs with a dosage rate of 0.1 to 200 mL/hr. Special syringes of 10, 20, and 50/60 mL are commercially available. Because infusion pumps work with a maximum pressure of 1 bar, all tubings connected with such pumps need to be pressure resistant for safety reasons.

Previous research has demonstrated that variation occurs when different types of syringes are used with electronic syringe drivers (Medical Device Amendment (MDA), 2003). For example, it has been reported that there is a difference in the amount of drug delivered and the occlusion to alarm time in two different types of syringe (24). Similar findings are associated with spring devices (25). Luer-lock syringes are commonly recommended to avoid separation of the syringe and infusion set. This is particularly important for subjects who may be restless or lack of

understanding about the importance of protecting the device. Clearly, the type of syringe should be standardized to avoid variation in infusion rate and ensuing symptom control. The MDA (2003) recommends using specific types of syringes as indicated by the manufacturer of the pump used.

Nonelectronic spring driven devices work on the principle that the syringe compresses the spring and the flow of liquid from the syringe is controlled by tubing with a restrictive narrow bore (Springfusor®). Such devices are reported to be advantageous in comparison with electronic devices in terms of cost and simplicity of use. A number of researchers have compared the two in terms of accuracy and reliability. One disadvantage of the Springfusor is that it is calibrated at 25°C and is affected by temperature variation. When the temperature rises, for example, if the device is close to the skin or under the bed clothes, the flow rate increases. Although this is not expected to cause clinical effects in adults, it may well have implications for children in terms of over-infusion (26).

SMART PUMPS

Studies indicate that although 38% of errors occur at the time of drug administration, only 2% are actually caught (27). Roughly 35% to 60% of all harmful IV medication errors can be directly associated with the use of an infusion pump device (28). Because many of these harmful errors occur with drugs that are classified as high-alert medications it is not a surprise that safety-minded organizations are choosing to convert their infusion pumps to the newest form of "smart infusion devices." The term "smart" or "intelligent" is used to describe this pump technology because these infusion devices contain error reduction software with the ability to store organization-specific dosing guidelines, and they produce real-time alerts for practitioners when attempts are made to program doses outside of the established safe range. Smart pumps are computerized infusion devices with dose-error reduction software designed to help avert IV programming errors, as well as other errors associated with infusions (29). Smart pumps differ from older pumps because they can be programmed to include facility customized drug libraries—lists of IV medications and their concentrations. Software provides point-of-care decision support for high or low infusion rates. The device prompts the user to choose a medication from the library, confirm the selection, input a volume to be infused, and input an infusion rate or dose. For all medications selected from the library, the keypad entry of an infusion rate in milliliters will automatically calculate the equivalent dose in units, milligrams or micrograms (5).

PATIENT-CONTROLLED ANALGESIA

One of the most common methods for providing postoperative analgesia is via patient-controlled analgesia (PCA). Although the typical approach is to administer opioids via a programmable infusion pump, other drugs and other modes of administration are available. There are several advantages of using a PCA (30). It reduces the time between when the patient feels pain and/or the need to receive analgesia and when it is administered (activation automatically pumps the dose into a preexisting IV line into the patient). It also reduces the workload of the nursing staff (an amount of the prescribed analgesic is preloaded into the PCA, enough for multiple doses) and the chances for medication errors. The PCA is programmed per the physician's order for amount and interval between doses and "locks out" the patient if he or she attempts excessive self-administeration. Patients can receive medicine when they need it, instead of having to wait for nurse practitioner or caretaker. Patients who use PCAs report better analgesia and lower pain scores than those patients who have to request analgesia from the nursing staff when they are in pain. Additionally careful examination of the syringes in a PCA provides a measurement of how much pain an individual patient is experiencing from one day to the next. It involves patients in their own care, giving them control and ultimately rendering better patient outcomes.

PCAs do suffer from certain disadvantages. Patients may be unwilling to use the PCA or be physically or mentally unable to. However, PCA pumps are rated among the world's most accessible pieces of equipment since all manufacturers must have alternative switch access

built into their PCA pumps. Most companies employ a TASH (The Association for Persons with Severe Handicaps) approved switch interface connection as TASH is one of the industry standards in accessibility switches (31). The pumps are often expensive and may malfunction.

DISPOSABLE INFUSION PUMPS

All nonelectric disposable pumps exploit the same physical principle: mechanical restriction within the flow path determines the speed of pressurized fluid. The pressure on the fluid is generated by a variety of mechanisms using nonelectric power, including a stretched elastomer or compressed spring, pressure generated during a chemical reaction (32), and pressure supplied from a cartridge of pressurized gas. The restriction of flow in all disposable pumps is caused by narrow-bore tubing. Tubing diameter has a determining influence on the device's flow rate. Therefore, flow restrictors are usually made of materials whose dimensions change little with temperature to maintain accuracy. Glass capillary-flow restrictors are typically used for devices infusing at a rate of 0.5–10 mL/hr; plastic is typically used for flow restrictors of pumps infusing at rates of 50–250 mL/hr. The flow restrictor is always integral to the administration set. The administration set can be integrated within or can be detachable from the pump reservoir.

Elastomeric infusion pumps are disposable devices, in which the pressure on the fluid is generated by the force of a stretched elastomer. Elastomeric disposable pumps consist of an elastomeric membrane, which contains the drug that is contained within an outer protective shell. The outer protective shell can either be a conformable elastomer (e.g., Homepump Eclipse®, BBraun) or a more rigid plastic (e.g., Infusor®). A soft elastomeric outer shell offers less protection against sharps puncture but requires less storage and disposal space. The membranes of elastomeric pumps are made of various elastomers, both natural and synthetic (e.g., isoprene rubber, latex, and silicon), and can be made of a single or multiple layers. The type of elastomer and the geometry of the elastomeric balloon determine the pressure generated on the fluid when the balloon is stretched (33). Multiple-layer elastomeric membranes can generate higher pressures than the single-layer membranes. Elastomeric pumps operate with a driving pressure of 260–520 mmHg and infuse at rates of 0.5–500 mL/hr.

Another type of disposable pump used is negative-pressure pumps. With negative-pressure pumps, a driving force is generated from the pressure difference across two sides of the pump's low-pressure chamber wall, with one side being at very low pressure (inside a vacuum chamber) and another side being at atmospheric pressure. The very low pressure in the vacuum chamber is created by the user while filling the device. Expansion of the drug reservoir, caused by the addition of fluid to the drug-containing reservoir, causes simultaneous expansion of the reduced pressure chamber, thus creating a significant vacuum. During infusion delivery, pressure on the movable wall plunger is generated by the large pressure difference between its two sides, causing it to move and compress the fluid in the drug-containing chamber.

SUMMARY

Although over the years the different routes of administration used for parenteral medications has remained the same, the science behind the design, development, and delivery of parenteral dosage forms have become complex. With continued and ever increasing need for superior dosage administration control, accuracy, and efficacy the development of newer dosage forms as well as parenteral drug delivery devices have become highly sophisticated. Additionally, new as well as older highly potent and difficult to formulate drug molecules are being rescrutinized and drugs once thought to be not viable because of poor oral bio-availability are seeing a comeback as parenteral dosage forms. These potent drug entities require accurate control of dose and higher safety margins. The advent of smarter and sleeker electronics and computers have helped to achieve this and also helped in the development of "error proof" infusion systems that have increased patient compliance and have lead to improved therapeutic outcomes. Some of these systems have considerably reduced the risks involved with parenteral administration of drugs and others show promise for safe and efficacious administration of drugs via this route.

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3 Biopharmaceutics of NCEs and NBEs

Balaji Agoram, Kazuko Sagawa, Ravi M. Shanker, and Satish K. Singh

INTRODUCTION

The term Biopharmaceutics is the study of the influence of formulation on the biological activity of a drug product, including its in vitro properties such as its physicochemical characteristics, formulation, and delivery technology (1). Pharmacokinetics (PK) is used to define the science of in vivo performance of a drug such as its bioavailability/absorption and systemic disposition, and is an important marker of the likely intensity and duration of the biological activity of the drug. Therefore, an understanding of the underlying processes governing drug absorption and disposition within the human body, methods of analyzing the characterizing the concentration-time profile, and the temporal relation between the measured concentration-time profile and the efficacy and safety time profiles are all critical elements in the design of appropriate dosage forms. This chapter has been designed to provide an overview of these topics.

The first part of this chapter focuses on the physicochemical properties of small-molecule drugs that influence their absorption by the parenteral route. An increasingly important category of injectable drugs now also includes biotherapeutics. Biotherapeutics (also called biologicals, biologics, or biopharmaceuticals) are compounds that are biologically produced as opposed to chemically synthesized. Some common examples of biotherapeutics are peptides, proteins and monoclonal antibodies. Most biotherapeutics are large hydrophilic molecules with complex tertiary structures. While the biopharmaceutical properties of small-molecule therapeutics have been extensively studied, the number of corresponding publications on injected biologics molecules is relatively rare (2). Therefore, biotherpeutics have also been considered in this chapter. However, many of the discussions on the basics of exposure (PK) and exposure-response (pharmacodynamics) analysis in this chapter are applicable to both biotherapeutics and small molecules. The impact of key physiological and physicochemical parameters on PK is also discussed in this chapter. A key biopharmaceutical aspect unique to biotherapeutics is their potential to cause immunological reactions, which can affect both PK and safety/efficacy profile. Immunogenicity, and the impact of formulation changes on immunogenicity is therefore covered in this chapter. Finally, the concept of comparability for biotherapeutics is discussed from the bioequivalence and PK perspective.

PHYSICOCHEMICAL PROPERTIES OF SMALL-MOLECULE DRUGS AFFECTING ABSORPTION BY THE PARENTERAL ROUTE

Takeru Higuchi, known as the "father of physical pharmacy" is credited with the introduction of many of the basic principles of physical chemistry that are known to influence the absorption, distribution, metabolism and excretion of drugs from the body. Although much of the literature on factors influencing absorption of drugs has focused on gaining detailed understanding after oral administration (3), the same physicochemical properties of molecules are important for absorption after administration via subcutaneous (SC), intramuscular (IM), intraperitoneal, and other extravascular routes delivered via injection. On the other hand, when a drug is injected directly into the vascular system, that is, via intravenous (IV) route then there are no physicochemical factors that affect absorption. Figure 1 provides a simplified, schematic overview of the relationships of administered dose of an injectable drug to the elicitation of the pharmacological effect, which includes therapeutic benefits as well as undesirable side effects. In the case of direct vascular injection via a bolus dose or as an infusion, the drug must be dissolved prior to administration to avoid the risks of causing

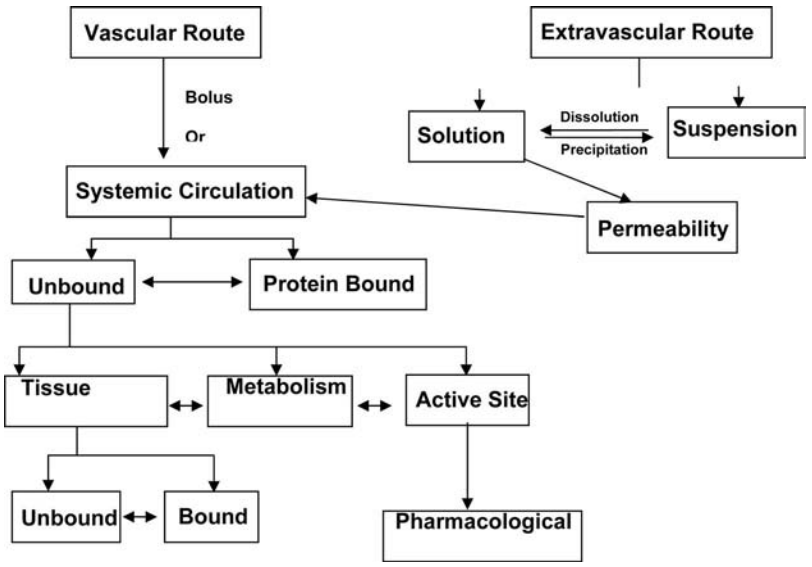

Figure 1 A schematic overview of fundamental relationships between routes of administration of injectable drugs to their ability to elicit pharmacological response.

blockage of capillaries that can affect the safety of the patient. However, injections through the extravascular route may be administered as either solutions or as suspensions of particles in aqueous or oil-based vehicles. Ultimately, for the drug to reach its intended target of the diseased tissue to elicit a pharmacological response, it is imperative that the drug must dissolve in the aqueous environment of the interstitial fluid and in the blood. Similarly, for the drug to reach the site of action from extravascular sites, it must have the ability to diffuse through cell membranes. These two essential properties of drug molecules: dissolution in aqueous and biological environment, and diffusing to reach the site of action, are governed by a multitude of physicochemical properties. The aim of this section is to provide the formulator of injectable drugs, a basic understanding of physicochemical properties of drugs that influence their PK as well as pharmacodynamics to assist with the design of drug products that can utilize these properties; to help identify formulation approaches to overcome limitations presented by any of these properties and also to assist in troubleshooting suboptimal performance of either novel or purportedly equivalent injectable drug products.

To consider the physicochemical properties of drugs that influence their absorption, distribution, metabolism and excretion it is essential to consider the anatomical and physiological characteristics of the vascular and extravascular injection sites. Detailed discussion of these factors and their impact on the design consideration of injectable dosage forms have been provided in preceding chapters. Similarly detailed discussions of preformulation and formulation approaches to quantitiatively understand the solubility and stability of a variety of injectable dosage forms are covered in various chapters. Factors such as the pH of blood, intracellular and extracellular fluid; the nature of ions and ionic strength of these physiological fluids; blood flow as well as number of capillaries at extravascular sites; the presence of lymphatic network; muscle movement; body temperature; nature of disease state; and, age of the patient are important considerations in understanding the PK and pharmacodynamics of drugs. The physicochemical properties of drugs may be broadly classified into two categories: (*i*) intrinsic properties and (*ii*) adjustable or changeable properties. Examples of intrinsic properties are molecular structure, functional groups, the ionization constant (pK_a—the negative logarithm of the ionization constant) of the functional groups, partition coefficient ($\log P$), melting point, and intrinsic aqueous solubility (of the unionized form of the drug). Examples of properties that can either adjusted or selected by the

formulator include salt forms of ionizable drugs, particle size, degree of crystallinity, amorphous form and solubilization via selection of excipients that can alter the solubility of the drug.

Ionization (pK_a)

A molecule or an atom group in a molecule may lose or gain a proton when the molecule is placed in an aqueous solution. The symbol K_a is used to describe the tendency of compounds to accept protons and is called the ionization constant. Expressed in mathematical terms, the negative logarithm ($-\log_{10}$) of the ionization constant (K_a) is defined as pK_a. Since pH is the negative logarithm of the hydrogen ion concentration ($-\log_{10}$ [H^+]), the relationship between pH and pK_a for an acidic drug can be expressed as follows:

$$\mathrm{pH} = \mathrm{p}K_a - \log \frac{[\text{Unionized}]}{[\text{Ionized}]}$$

This mathematical relationship provides an ability to calculate the fraction or percentage of ionized and unionized species of a drug in the pH of physiological interest by knowing the pK_a of the drug. This understanding of distribution of species is extremely important in predicting and quantifying the solubility, distribution coefficient (log$D$) and thus the drug's PK (ADME) and pharmcodynamics. The unionized form is the only species that diffuses through cell membranes; however, it is also the form that has the lowest aqueous solubility. Therefore, an injectable drug product when formulated at a pH to take advantage of its increased solubility in the ionized state stands the risk of precipitation of drug upon encountering physiological pH at the site of injection. This phenomenon of precipitation of drug can result in phlebitis as well as significant pain at the site of injection (4,5).

Partition Coefficient (log$P$ and log$D$)

Partition coefficient (P) of a drug is the ratio of its concentration in the two phases of a mixture of two immiscible solvents at equilibrium. Conventionally, one of the solvents chosen is water while the second is octanol (6). Logarithm of the partition coefficient is referred to as log$P$ as is defined as the ratio of the concentration of the unionized species in octanol divided by the concentration of unionized species in water.

$$P = \frac{[\text{Unionized Species}]_{\text{Octanol}}}{[\text{Unonized Species}]_{\text{Water}}}$$

Similarly, log$D$, refers to the logarithm of the distribution coefficient (D), which is defined as the ratio of the concentration of all the species, that is, unionized and ionized in octanol divided by the concentration of all species in water.

$$D = \frac{[\text{Unionized Species} + \text{Ionized Species}]_{\text{Octanol}}}{[\text{Unonized Species} + \text{Ionized Species}]_{\text{Water}}}$$

Since the fraction of unionized and ionized species in aqueous solution is governed by the pH of the solution and the pK_a of the molecule, therefore, the log$D$ or distribution of the drug is dependent on pH and pK_a. Since only the unionized molecule diffuses through biological membrane, therefore, the permeability of the drug is dependent on log$D$. The interrelationships between ionization, pH, and partitioning of the drug through biological membrane are often referred to as the "pH-partition hypothesis" (Fig. 2). These interrelations are summarized in Figure 3. The pH-partition hypothesis was first proposed to explain the influence of pH of the gastrointestinal tract on the oral absorption of drugs (7). The concept is extensively used for not only understanding oral absorption but also the toxicity of drug molecules as well as the accumulation of drugs in specific tissues. Therefore, the interrelationships between the degree of ionization, the pH of biological fluid and the distribution coefficient is important for understanding the biopharmaceutical aspects of drugs.

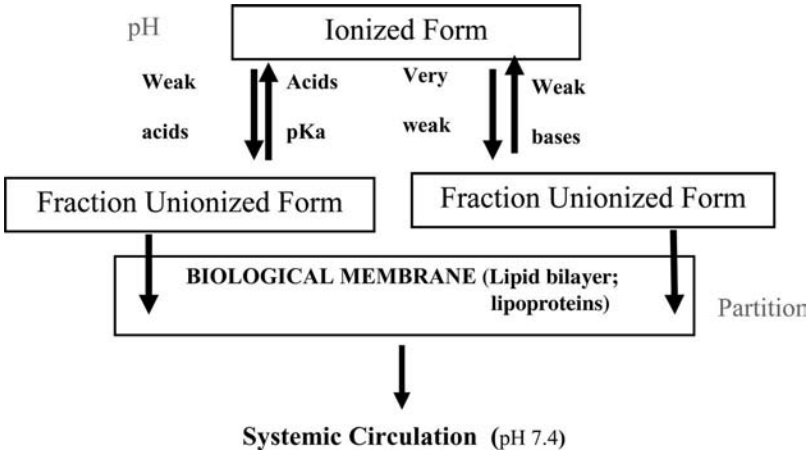

Figure 2 The pH-partition theory for the absorption of drugs across biological membrane from extravascular sites of administration of injections.

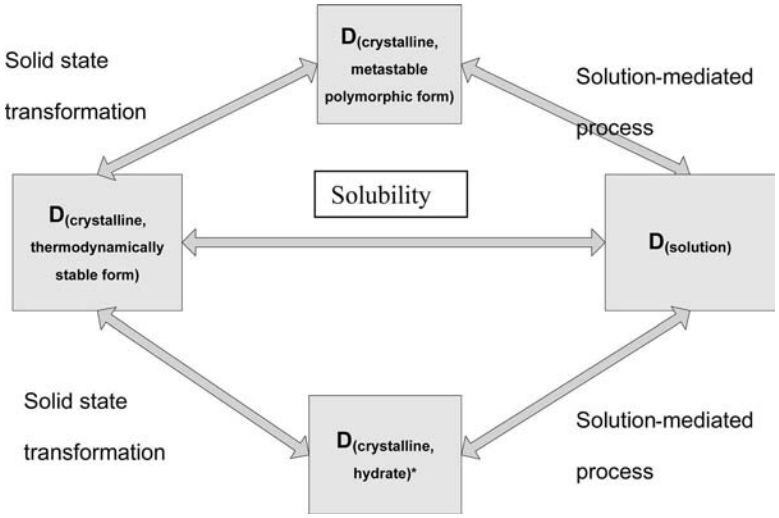

Figure 3 Potential pathways for transformation of solid form of drug (basic description for unionized drug since ionized form can undergo additional transformations to unionized form) during determination of equilibrium solubility or during transit through the body upon injection. Each arrow depicts a forward rate and a backward rate to maintain equilibrium.

Diffusion and Permeability (P_{app})

Molecular diffusion, often referred to as just diffusion is the physical phenomenon of transport of molecules via random molecular motion from a region of high concentration to one of low concentration. The phenomenon is typically described by Fick's laws of diffusion; the first law relates the diffusive flux to the concentration gradient and the second law predicts how the diffusion of molecules causes the concentration field to change with time. Mathematical expressions based on Fick's first law have been used to model transport processes in many systems including drugs across biological membranes. Fick's first law is expressed as follows:

$$J = -PA(C_2 - C_1)$$

where J is the diffusion flux in units of [(amount of substance) length^{-2} time^{-1}]; P is the permeability of the membrane (e.g., biological cell membrane) for a given molecule at a given

temperature; A is the surface area over which diffusion is taking place; and $C_2 - C_1$ is the difference in concentration or concentration gradient of the molecule across the membrane in the direction of flow of molecules ($C_1 - C_2$). Biological membranes consisting of lipid bilayer are semi-permeable in nature and are also known as selectively permeable membranes, that is, they allow certain molecules or ions to diffuse through. There are several factors that influence the permeability of organic molecules through biological, semipermeable membranes such as molecular size (molecular weight), charge on the molecule, lipohilicity (log$P$ or log$D$) of the molecule, polar surface area, number of rotatable bonds, etc. Although it is possible to utilize formulation factors to change the concentration gradient to influence the flux across the biological membrane, it is not possible to alter the intrinsic permeability of a compound using formulation approaches. Because of the challenges of experimentally determining the permeability of drugs across biological membranes, several in vitro approaches, based on cell cultures, have been utilized extensively to ascertain the apparent permeability (P_{app}). Understanding and predicting molecular descriptors that can influence permeability of drugs across biological membranes continues to be a matter of extensive fundamental and applied research (8,9).

Solubility, Dissolution, and Solubilization

The pharmaceutical literature in the past few decades has used multiple terms to describe solubility and dissolution of drugs which has often resulted in confusion and misunderstanding (10). Terms such as thermodynamic solubility, equilibrium solubility, intrinsic solubility, kinetic solubility, apparent solubility, intrinsic equilibrium solubility, dissolution rate, intrinsic dissolution rate, etc., have been used by researchers to describe different aspects of experimental observations. Aqueous solubility of solutes is a relatively straightforward thermodynamic concept, especially for crystalline drug molecules since solubility represents the concentration of drug in solution which is in an equilibrium two phase system consisting of the drug in the solid state and the solution state. This concept is often schematically illustrated by

$$\text{Solute}_{(\text{solid, crystalline, excess})} + \text{Solvent} \overset{K}{\Leftrightarrow} \text{Solute}_{(\text{solution})}$$

where K, the equilibrium constant, is the ratio of activity of solute in solution to that in the solid. Typically, when the solute concentrations are low then the solute activity coefficients are essentially unity. Since solubility is equilibrium constant, it is dependent on temperature and pressure. However, in the context of drug delivery and biopharmaceutics, pressure is not considered to be a variable. The above definition of solubility highlights the importance of characterizing the solid at equilibrium in addition to measuring the concentration of drug in solution. If the solid form undergoes a change in its solid state relative to the initial form that was used for experimentally assessing solubility then the equilibrium solubility is reflective of the new solid form (polymorph, hydrate, solvate, etc.) rather than the original form. Solid state transformations can either be mediated through solution or directly through the solid state. Figure 3 provides a schematic overview of potential transformations that can occur during experimental determination of solubility determination or during the time the dosage form is present in the body.

Since a change in drug's solid state (polymorph, hydrate, solvate, amorphous form, etc.) can result in significant change in its solubility as well as dissolution rate, such transformations as depicted in Figure 3 can have a direct impact on the biopharmaceutic performance of an injectable drug product. An additional aspect of understanding the equilibrium constant between the drug in solid state and drug in solution is the rates of the forward and backward processes.

If both the forward rate, that is, dissolution and backward rate, that is, crystallization as shown in Figure 3 were completely controlled by diffusion process, then these rates would be identical at equilibrium, which however is rarely the case. Crystallization is not merely based on diffusion but is known to be a stochastic (probability-driven) process that requires random collisions to form a critical size of nuclei before crystal growth can occur. Therefore, it is imperative to understand the impact of dilution and mixing of solubilizing excipients with biological fluids at the site of injection as the resulting decrease in solubility of the drug can

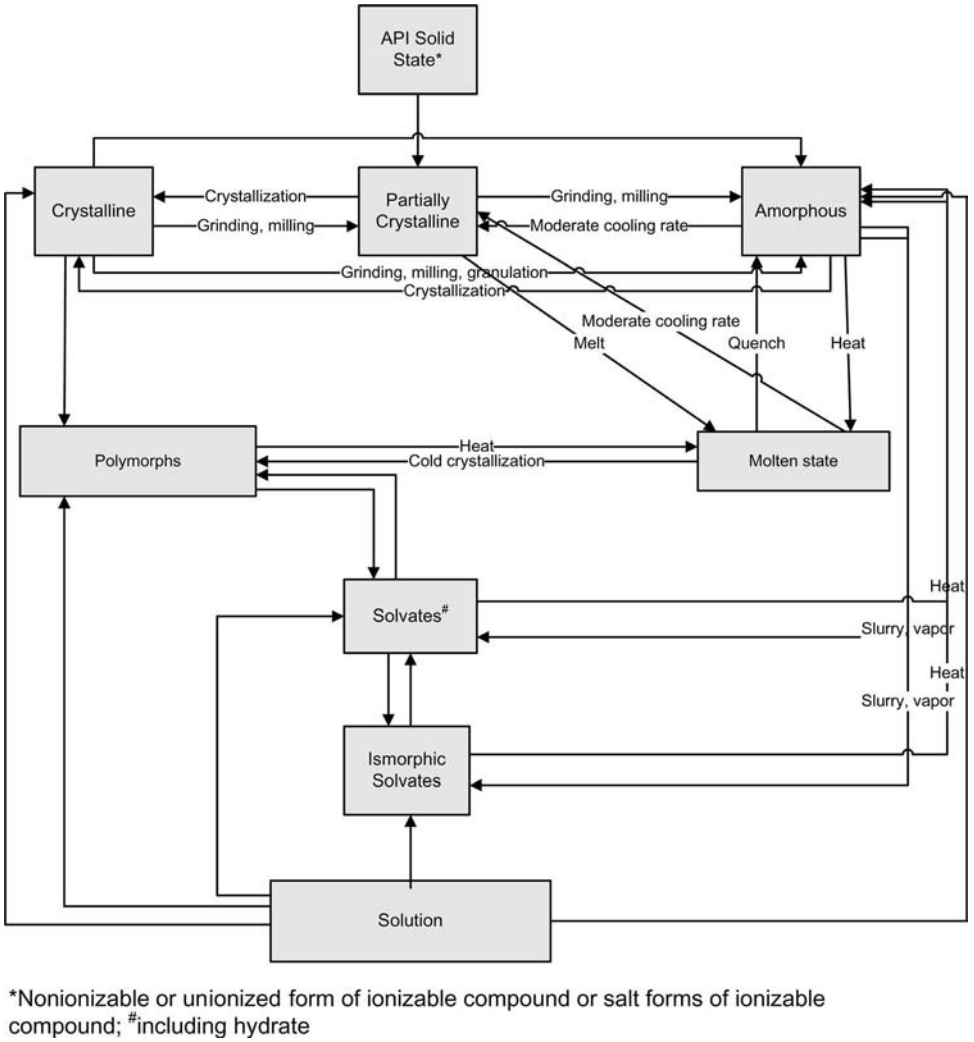

Figure 4 Processing options available for solid state transformation of drug to facilitate isolation of preferred API form for development of injectable formulations.

lead to precipitation of drug and consequently lead to decrease in available concentration of drug at the site, pain and phlebitis. Understanding of the thermodynamics and kinetics of interconversion of the solid state transformations can facilitate the development of processes that isolate the preferred stable form (thermodynamically or kinetically stable) for manufacture of dosage form. Figure 4 provides a schematic overview of the processing options available to the formulator.

In addition to equilibrium solubility, the time required to reach solubility, that is, dissolution is an extremely important phenomenon for the biopharmaceutics characteristics of an injectable suspension. There are several theories that model dissolution of solids to form solutions and the most frequently used relationship known as Nernst-Brunner equation, which was a modification of the original Noyes-Whitney equation (11). The Nernst-Brunner equation, shown below, is derived from Fick's law of diffusion and takes into account the presence of an aqueous diffusion boundary layer on the surface of the dissolving solid.

$$\text{Dissolution Rate} = -\frac{A_{\text{solid}(t)} D_{\text{Drug}}}{h_{(t)}} \left(S_{\text{Bulk}} - \frac{X_{\text{Solution}(t)}}{V_{\text{Bulk}}} \right)$$

where $A_{\text{solid}(t)}$ is the total surface area of the solid at time t; D_{Drug} is the diffusion coefficient of the drug; $h_{(t)}$ is the thickness of the diffusion layer at time t; S_{Bulk} is the solubility of the drug in the bulk liquid; $X_{\text{solution}(t)}$ is the amount of drug dissolved in bulk solution at time t; and V_{bulk} is the volume of the bulk solution. Although the Nernst-Brunner equation is useful, it is not always applicable for biopharmaceutical applications. Modeling of dissolution kinetics especially of powders is of significant practical importance especially for injectable drug products. To model dissolution of particles, knowledge of particle size distribution as well as an estimate of the thickness of the aqueous diffusion layer as a function of particle size are necessary. In terms of particle size, it is important to take into account the poydispersity of the particle size (12). Several mathematical relationships have been developed to model the dissolution of powders. However, it is important to note that the dissolution of particles from an extravascular site of injection does not follow these models adequately because of poor mixing and agitation at the site. Therefore, biopharmaceutical considerations of particle size-dependent dissolution for injectables requires the development of more complex mathematical models (13).

The influence of degree of ionization of an ionizable drug on its partitioning into biological membranes was discussed previously. Similarly, the degree of ionization greatly affects the solubility of the drug. The ionized form (either acid or base) has higher solubility than the unionized form. Theoretical pH-solubility profiles of ionizable drugs is given by the Henderson-Hasselbalch equation, which relates the solubility of the unionized form of the drug (S_0) to the dissociation constant (pK_a) to obtain the total solubility (S_T) of the drug.

The underlying assumption in these predictions is that the drug molecule does not self-associate in solution either in the unionized or ionized states. However, these relationships cannot predict the pH independent, limiting solubility of the salt forms of ionizable drugs. There are no theoretical methods available to predict the solubility product (K_{sp}) of a drug with a specific counterion. Therefore, it is essential to determine the K_{sp} experimentally. Although salt forms of ionizable drugs can provide wide ranges of solubility enhancement, it is not possible to a priori predict a preferred salt form for any drug on the basis of any basic principles. Furthermore, the rate of conversion of a salt form to its unionized state upon being subjected to a change in pH is also not predicted by any known theory or good empirical model. Knowledge of the expected solid form (as predicted by the phase rule) at equilibrium at any given pH is extremely useful in ascertaining whether the formulation as drug product or after administration at the injection site has reached equilibrium or is in the metastable state. Generally, according to the phase rule, the solid form at equilibrium is the unionized form of the drug at all pH values in the K_{sp} controlled region ($pH < pH_{max}$ for bases and $pH > pH_{max}$ for acids).

For monobasic compounds, the relationships are as follows:

$$S_T = S_0\left(1 + \frac{K_a}{[H^+]}\right) \quad \text{when } pH < pH_{max}$$

$$S_T = S_0\left(1 + \frac{[H^+]}{K_a}\right) \quad \text{when } pH > pH_{max}$$

For monoacidic compounds,

$$S_T = S_0\left(1 + \frac{K_a}{[H^+]}\right) \quad \text{when } pH < pH_{max}$$

$$S_T = S_0\left(1 + \frac{[H^+]}{K_a}\right) \quad \text{when } pH > pH_{max}$$

The importance of aqueous solubility of drug has been discussed specifically in the context of biopharmaceutical properties injectable drugs. It is equally important to discuss the fundamental factors that contribute to make drugs insoluble. Considering the general solubility equation (14) provides insights into the physicochemical reasons that make drugs insoluble.

$$\log S_0 = 0.5 - 0.01[T_m(^\circ C) - 25] - \log P$$

According to the above equation, aqueous solubility (S_0) of a drug would be reduced if the melting point (T_m) is high, that is, the solid in the crystalline state has high lattice energy. Alternatively, insolubility may also arise because of high log*P* or lipophilicity of the drug. If both factors, that is, lattice energy and lipophilicity are high then the drug would also be highly insoluble. There are many approaches available for the formulator to enhance the solubility of insoluble compounds. Knowledge of the key factors contributing to insolubility along with its molecular structure can assist in the selection of appropriate solubilization technologies. A few of the many solubility enhancing options available to the formulation scientist developing injectable drug products include pH adjustment, cosolvent solubilization, solubilization by lipids, micellar solubilization, complexation (e.g., with cyclodextrins), amorphous forms of drugs, emulsification, liposomes, etc. Similarly, enhancement of dissolution rates can be achieved by particle size reduction including generation of attrition milled nanoparticles. This discussion has briefly outlined why it is important to consider the various thermodynamic but also the kinetic aspects of equilibrium for all of the physicochemical properties in designing, optimizing and troubleshooting the biopharmaceutical properties of injectable drug products.

DRUG EXPOSURE/PHARMACOKINETICS

Pharmacokinetics (PK) refers to the in vivo time course of blood concentration of an exogenously administered drug. In most cases, this is described by observing the plasma or serum concentration of the drug over time. The various processes that govern the observed concentration-time profile are *a*bsorption, *d*istribution, *m*etabolism, and *e*xcretion. Together, these four processes are described by the acronym ADME. Any changes in the ADME processes, for example, due to disease in the target population, could have changes in the PK profiles and hence the observed safety/efficacy profile of the compound.

Figure 5 illustrates the events that occur after administration of a drug. If the drug is administered in a "depot" [e.g., orally, subcutaneously (SC), etc.], a delay may occur before the drug appears in the blood circulation, possibly because of the time taken for the transport from the site of administration to the circulation. The initial increase in the drug concentration corresponds to the accumulation of the drug in the blood because of absorption from the site of administration. When the absorption rate becomes slower the elimination rate of the drug, the observed plasma concentration profile enters the declining phase. The inflection point at the top of the curve represents the change in this balance of the absorption and elimination rates. A distribution phase occurs where the absorbed drug is deemed to have distributed from the central circulation to other tissues or components of the blood. A rapid decline in the observed concentrations in blood represents distribution from the blood to other tissues such as the liver, kidneys, fat, etc., rather than elimination from the body.

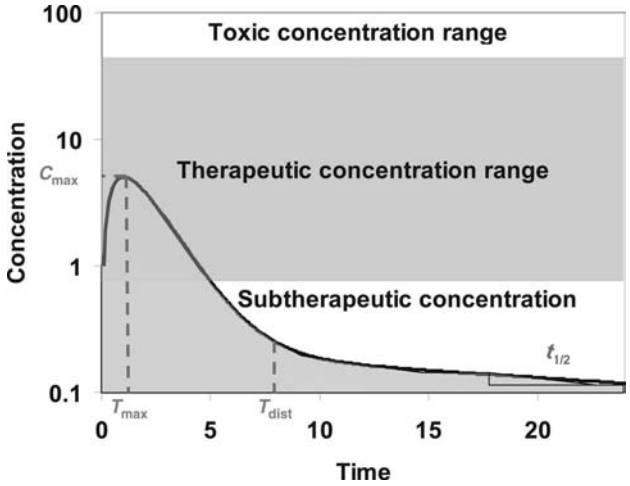

Figure 5 Absorption, distribution, metabolism, and excretion processes and pharmacokinetic summary parameters.

Elimination of the drug occurs as soon as a drug is absorbed and enters the systemic circulation. The last phase of the decline in blood concentrations typically corresponds to the elimination of the drug from the body because absorption is completed and distribution equilibrium is established. The elimination phase is also sometimes referred to as the terminal phase.

The concentration-time profile is often summarized using a set of PK parameters: the maximum concentration (C_{max}), the time to attain maximum concentration (T_{max}), the time taken for the concentration to decline by half (half-life; $t_{1/2}$), and the area under the plasma concentration versus time curve (AUC) (Fig. 5). C_{max} and T_{max}, can be read from the concentration time profiles. The half-life can be read as the slope of the concentration-time curve, where the concentrations are plotted on a log scale. The AUC is typically calculated from the observed concentration-time profile through standard area calculation algorithms.

Taken together, the PK parameters—C_{max}, T_{max}, and AUC—can be used to characterize the rate and extent of absorption of a drug. Thus, they can be used to compare the relative extents to which a particular compound is bioavailable—that is, reaches systemic circulation and is therefore available for therapeutic action—after administration through different routes or from different formulations. Typically, the bioavailability (denoted by the symbol *F*) after an intravenous (IV) administration is assumed to be 100%, and that after other routes of administrations are expressed as fractions of the IV bioavailability.

Most therapeutic compounds exert their pharmacological effect by reversibly interacting with their targets—for example, receptors, enzymes, ion channels, etc. When the systemic drug concentration declines, the extent of modulation of the target also reduces. Thus, the desired therapeutic effect for most compounds, which is the primary objective of the therapeutic dosing regimen, is obtained by maintaining a drug concentration above effective level (therapeutic concentration; Fig. 5). If the concentration is too low, loss of efficacy occurs because of lack of adequate modulation of the receptor. If the concentration is too high, toxicity might occur because of excessive modulation and potential exaggerated pharmacology or because of increasing expression of secondary pharmacological effects such as modulation of other subclasses of receptors. The difference (or the ratio) between the required therapeutic concentration and the toxic concentration is called the therapeutic index of a drug. Drugs with small differences between the therapeutic and toxic concentrations are referred to as narrow therapeutic index drugs and pose challenges in their clinical usage. A successful biopharmaceutical strategy would be effective in maintaining the concentration of the drug within the therapeutic concentration range.

Many biotherapeutics, especially macromolecules, because of their structure and physicochemical properties, possess distinct ADME properties from typical synthetic small molecules. As opposed to small molecules, a detailed understanding of these ADME mechanisms is not yet available for biotherapeutics. However, understanding the ADME processes for biotherapeutics is essential to appropriately design dosing regimens that maximize the therapeutic potential of these compounds.

Absorption

Before a drug can exert a pharmacological effect by modulating its target, it has to be absorbed from the site of administration into the bloodstream. For many synthetic small molecules, the oral route of administration is the preferred route of delivery because of the ease of administration and the related high level of patient compliance. However, biotherapeutics such as peptides, proteins and other macromolecules are, in general, not highly bioavailable after oral administration because of mainly two factors: (*i*) degradation in the gastrointestinal tract and (*ii*) lack of permeability across the GI mucosal barrier. Therefore, biotherapeutics such as monoclonal antibodies are typically administered through injections: IV, SC, and intramuscular (IM) routes being the preferred options. For example, of the 22 approved monoclonal antibodies (15), 4 are administered SC, 17 IV, and one each IM and intravitreally. Each of these sites of administration presents an absorption barrier with a unique set of properties.

For IV administration, there is no absorption barrier since the drug is directly delivered into the bloodstream. For extravascular routes of administration, the rate of absorption can vary widely depending on the site of administration.

SC doses are typically administered in the intradermal SC space in the shoulder, abdomen, thigh, or lower back. Similarly, IM doses are administered in the shoulder and gluteal muscles. After SC and IM injection, it is hypothesized that the drug is absorbed directly into systemic circulation via blood capillaries and through the lymphatic circulation. It has been shown through experiments in sheep that the lymphatic convective transport contributes substantially to the absorption of biotherapeutics after SC and IM administration and that the fraction of the drug absorbed through this process increases as the molecular weight increases (16). Consequently, it is hypothesized that for high molecular weight biotherapeutics such as monoclonal antibodies (approximate molecular weight of 150 kDa) are almost fully absorbed through the lymphatic system. Recent data in rats, where a low—<3%—contribution of the lymphatic route to the overall absorption was observed for erythropoietin, appear to contradict the findings in sheep. Suffice to say that a thorough quantitative understanding of the absorption processes after SC and IM administration of biotherapeutics is not yet available. Typically, absorption is slower for biotherapeutics than for small molecules with T_{max} values in the range of 24 to 72 hours post SC or IM dose.

The rate and extent of absorption from the extravascular site of administration depends on multiple factors and there is loss of drug prior to reaching systemic circulation (bioavailability is less than 100% compared with IV administration). A fraction of the drug administered after extravascular administered dose is subject to presystemic degradation, either at the site of administration, or during lymphatic transport—hence, these routes are clinically relevant only when a limited amount of drug is required to be administered for efficacy.

Other routes of administration such as intravitreal and inhaled routes have also been explored for biotherapeutics. The intravitreal route has been pursued for ranibizumab (Lucentis®), a vascular endothelial growth factor antibody fragment and pegaptinib sodium (Macugen®), a polyethylene glycol conjugated aptamer to promote a local effect. Administration of the drug directly into the site of action typically overcomes systemic PK limitations such as short half-life and minimizes side effects due to interaction with therapeutically inactive targets or targets at organs other than the site of action, thus improving the therapeutic index of the compound. Recently, the inhaled route is being widely explored as an option for biotherapeutics. Exubera® is an inhaled form of insulin for diabetic control. The large surface area of the lungs and the rapid transport of many molecules across the lung epithelial barrier provide attractive options for delivery, especially when the target is present in the airways (17,18). The rate and extent of systemic absorption for biotherapeutics administered at the site of action can vary widely depending on the physiology of the site of action—the density and porosity of the capillary bed, the lymphatic drainage of the site, any existing clearance mechanisms, and the effect of disease (see section "Absorption" under "Physiological Factors That Influence Pharmacokinetics of Injectable Drugs" for more details).

Distribution

Once the drug is absorbed from the site of administration into the blood circulation, it distributes to tissues, including the site of action, to exert its pharmacological effect. Unless the drug is designed to reach only a particular organ or tissue, this distribution of the drug occurs to various extents to all parts of the body. Within the PK field, the term distribution refers to the reversible partitioning of a drug to tissues within the body (19). The rate and extent of overall distribution of a drug from blood circulation to other tissues typically depends on many factors including the ability of the compound to cross tissue membranes, the perfusion rate of the tissues, partitioning into fat, and the tissue composition (20,21). Readers should note that the volume of distribution (V_d) commonly expressed as a PK parameter is a theoretical fluid volume that relates the administered dose and the observed blood concentrations and is not a strictly physiological quantity. For example, drugs that bind extensively to tissue targets have low blood concentrations after dosing, resulting in high estimated V_d, sometimes even higher than body volume (e.g., some basic drugs such as amphetamines)!

Except in the case of active transport, the distribution process for most small-molecule drugs is generally driven by concentration gradients. Therefore, at steady state, the free drug

concentrations in the blood and different tissues are at equilibrium. However, biotherapeutics are typically larger hydrophilic compounds with poor permeability across the tissue membranes. Entry into tissues is thought to be primarily through extracellular pathways (22,23), especially for tissues such as cerebrospinal fluid. Furthermore, return to blood from the tissue is in many cases through the lymphatic drainage (24), which is primarily a convective transport process not dependent on the concentration gradient and the biochemical properties of the compound such as permeability and tissue affinity. Therefore, the concentrations of the drug in blood and other tissues do not reach equilibrium, which is generally the case for small molecules. For example, the serum to cerebrospinal fluid concentration ratio of albumin is approximately 200:1 (22,23). Other investigations have shown that the blood: tissue ratio may also be dependent on the size of the biologic (24). Distribution of a drug to targets is another important factor to consider in the case of biotherapeutics. Many biotherapeutics, because of the very high affinity to their targets, are dosed at stoichiometrically equal molar concentrations to the target. Therefore, binding to the target constitutes a significant distribution pathway. Because the fraction of a drug bound to targets decreases with dose, target binding can lead to nonlinear distribution characteristics—that is, dose-dependant volume of distribution—for some biotherapeutics.

Metabolism

Most drugs begin to be metabolized after they enter the body. The majority of small-molecule drug metabolism is carried out in the liver by *redox* enzymes, termed cytochrome P (CYP)450 enzymes (ubiquitously expressed in the body). As metabolism occurs, a (parent) drug is chemically converted to metabolites. Metabolism eliminates the administered dose of a parent drug. When metabolites are pharmacologically inert, metabolism reduces pharmacological effects in the body as a parent drug is eliminated. Metabolites may also be pharmacologically active, sometimes more so than a parent drug (active metabolites).

The term catabolism is more relevant to describe the process by which biotherapeutics are broken down into smaller molecules such as amino acids. Proteolytic processes through enzymes such as proteases perform this function for biotherapeutics rather than CYP450 types of enzymes. The rate of proteolysis depends on many factors such as the size, carbohydrate content (glycosylation), potential for preproteolytic modification such as desialylation, the primary and tertiary structures (25). The sites of catabolism is also varied with liver, kidneys, and other extravascular sites such as sites of injection, for example, SC space have been implicated in protein catabolism. Many therapeutic proteins such as monoclonal antibodies are glycosylated proteins and are thought to interact with the asialoglycoprotein receptor (ASGPR) expressed on the sinusoidal surface of the parenchymal cells of the liver. ASGPR is believed to mediate the rapid removal and degradation of desialylated circulating proteins containing terminal galactose residues (26). It should be mentioned that characterizing the products of catabolism is substantially more difficult for biotherapeutics because of the wide range of catabolism products arising from an abundance of proteolysis sites and proteolytic enzymes.

An important site of catabolism of biotherapeutics is through the target. Binding of the biologic to the target has been shown to result in target-mediated endocytosis followed by lysosomal degradation for antibodies (27,28) and recombinant proteins (29).

Similar to V_d, clearance (CL) is a theoretical term that is the flow rate at a given concentration that is completely cleared of the drug in unit time and is calculated as the dose divided by the AUC under the assumption of constant clearance during drug elimination. Oxidative metabolism, catabolism, and other elimination processes all combine in achieving clearance of a xenobiotic.

Excretion/Elimination

Drugs and their metabolites are removed from the body via excretion, usually in the urine, in the feces or exhaled in the air. There are three major sites where drug excretion occurs. The kidneys, bile, and lungs. Many hydrophillic small molecules are cleared from the systemic circulation through the kidneys either intact or in the form of their metabolites (glomerular filtration) and excreted in the urine (renal elimination). Macromolecule biotherapeutics, because of their size are typically not cleared intact by filtration through the kidneys. However, small biotherapeutics such as some cytokines, insulin, granulocyte-colony stimulating factor,

interferon α, and erythropoietin have varying degrees of renal elimination, somewhat related to their size. In general, the renal elimination of intact biotherapeutics of molecular weight >30 kDa is expected to be negligible.

Larger molecular weight molecules are excreted into the bile and excrete in feces (biliary excretion). There are species differences in molecular weight cuttoffs for biliary excretion versus renal excretion. In human, the molecular weight cutoff required for biliary excretion is much greater than that for renal excretion. If the molecular weight is lower (e.g., <325–475 Da), the compound may be preferentially excreted in urine. Molecular weight from 325 to 850 Da may be eliminated via both renal and biliary routes. Excretion of molecules larger than 850 Da occurs mainly via biliary excretion. Physicochemical properties of the drug (polarity, lipophilicity, structure) are also critical to the extent of biliary excretion of a drug/metabolite. Biliary excretion has also been reported for biotherapeutics such as insulin (30) and epidermal growth factor.

PHYSIOLOGICAL FACTORS THAT INFLUENCE PHARMACOKINETICS OF INJECTABLE DRUGS

Physiological factors such as age, gender and disease states are known to alter PK of drugs. These factors can affect each component of PK—absorption, distribution, metabolism and elimination—described above.

Absorption

There is no absorption for IV administered drugs, as the drugs will directly circulate into the bloodstream. Therefore, the physiological factors, which influence absorption are minimal for IV dose. SC and IM administered drugs are taken up by the capillaries at the injection site and the permeability of the capillary wall membrane is affected by number of physiological factors.

Proteins larger than 16 to 20 kDa are generally taken up primarily by the lymphatic system and there is a linear correlation between molecular weight (MW 2,500–19,000) and the extent of recovery in the lymph (16). SC administered proteins generally exhibit a slower absorption and elimination compared with IV administration. Absolute bioavailability is generally low possibly because of protein degradation at the site of injection.

The factors affecting lymphatic transport of proteins after SC administration are summarized in the review by Porter and Charman (16). Lymph flow rate increases with exercise or mechanical injury (31). Massage is also known to increase lymph flow (32). Literatures show systemically administered insulin or gonadotropin increases capillary diameter and blood flow rate in rat cremaster muscle (33), although insulin-like growth factor-1 does not increase blood flow in human (34). The site of injection (injected to the abdomen vs. peripheral such as thigh or arm) influences the absorption (35) possibly because of differences in local blood flow and lymph flow.

SC blood flow increases in response to alterations in injection site, skin fold thickness, exercise, orthostatic changes, and ambient temperature (36).

Lymph flow is known to decrease with age (36,37). Membrane fluidity also decreases with age (38). Membrane permeability is also known to be altered with various disease states and with pharmacological agents (39).

Metabolism

Administered small-molecule drugs—either orally or injection—are mainly metabolized in the liver where the major metabolizing enzymes are located. Numerous literature reports suggest age and gender differences in CYP450 enzymes mediated metabolisms (40), however it is difficult to interpret those reports to general terms as those reports use probe drugs and majority of the studies is done in preclinical species.

The liver volume, liver blood flow and biliary function correlate well with body surface area (BSA). The liver size and blood flow decrease with aging, and therefore drug metabolism is reduced with advancing age (41). Renal clearance decreases with age and lower in women than in men at all ages.

Pelletier et al, demonstrated that the gut proteolytic activity is spread over a wide range of pH in younger animals than older ones with a shift from higher pH toward lower pH values with increasing age (42). A review article by Bota and Davies summarizes the regulation of

proteolytic enzymes in human diseases and ageing (43). Several disease states such as muscular dystrophy, cancer, Alzheimer's disease, neurological injury, ischemic injury, atherosclerosis, diabetes and cataract formation are known to alter the regulation of protease activities (44). Similarly, disease severity may also be related to increasing expression of the target and result in increased clearance of some biologics such as herceptin (cleared through the HER-2 receptor pathway) and omalizumab [cleared through immunoglobulin E (IgE)].

Distribution

Intravascular volumes, organ volumes and muscle volumes are generally smaller in elderly than younger people. The impact of reduced volumes is evident when the drug is distributed to those particular organs including muscles.

Drug distribution is also known to change with age because of relative changes in body fat. Lipophilic drugs such as midazolam and diazepam tend to get distributed to fatty tissue resulting in an increased volume of distribution(V_d) in elderly subjects (45,46). Divoll et al. studied PK of diazepam in young and elderly men and women (47). The authors found that the V_d was larger in women than in men but increased with age regardless of gender. Elimination half-life was longer in elderly than in young men partly because of the increased V_d as well as to a reduction in total metabolic clearance. It is noteworthy that the neither age nor gender influenced oral absorption and diazepam was nearly completely absorbed after IM administration (47). The level of α acid glycoprotein increase with age and as a consequence (48) the V_d can decrease for those drugs which bind to this particular protein.

As described above, biotherapeutics are distributed to tissues by blood or lymph, any disease states or aging which alter the blood flow and/or lymph flow can alter the tissue distribution of those large molecules. As mentioned earlier, the expression levels of target tissues (e.g., receptors) can be largely altered by the disease states as well. For example, the level of IgE correlates with the severity of asthma and the distribution of omalizumab, an anti-IgE monoclonal antibody, is related to the level of IgE present in the patient.

EXPOSURE-RESPONSE ANALYSIS

Pharmacokinetic Analysis

The primary aim of PK analysis is to summarize available plasma concentration versus time profiles (PK profiles) for interpretation, comparison, and predictions through the use of a set of parameters. These parameters can be obtained directly from an observation of the PK profile without the assumption of an underlying model quantitatively describing the different ADME processes. This is commonly referred to as a nonparametric, noncompartmental or model-independent analysis. These parameters include maximum concentration, time to reach C_{max}, area under the curve, the clearance [CL derived from the dose and AUC (CL = dose/AUC)]. While this analysis is simple and can represent simple PK characteristics of a compound, it has limited extrapolation ability beyond the studied regimen.

The PK profile can also be described by a set of PK parameters, assuming an underlying mathematical model—typically, a mammillary model with first-order kinetic processes describing the ADME process. This analysis is commonly referred to as compartmental modeling. A simple model is a one-compartment model, which represents central compartment (blood/plasma compartment) (Fig. 6). The rate of drug in (k_a, first-order absorption rate constant) and out (k_{el}, first-order elimination rate constant) of the central compartment is described by first-order kinetics.

Figure 6 Schematic of a simple compartmental pharmacokinetic model.

The versatility of the parametric analysis is founded in the ability of simple mathematical constructs to describe complex ADME phenomenon. By fitting the data to the right model, the model parameters can be estimated and these model parameters can be used to simulate time versus concentration curve with different dose or different routes of administration. Further complexity can be added to the simple one-compartment model to describe more complex PK; standard additions include second (and third) distribution compartments to describe distribution at different rates to different sets of tissues and multiple absorption routes and windows. It should be noted that in this approach, the parameters of the model Ka, CL, V_d, etc., do not have a direct physiological meaning even though they are related to physiological phenomenon.

Physiology-based pharmacokinetic modeling (PBPK) could be considered a special case of compartmental modeling, where the compartments and transfer rates correspond to physiological quantities such as tissues and organ volumes and blood flow rates. PBPK modeling is particularly useful when one wishes to predict the disposition in a particular organ.

Pharmacokinetic-Pharmacodynamic Analysis

The original concept of pharmacokinetic-pharmacodynamic (PK/PD) was described by Gerhard Levy in 1966 (49). PK is a study of a time and drug concentration relationship. Pharmacodynamics is a study of pharmacological responses. PK/PD analysis is a study of the relationship between PK and pharmacodynamics (PD). Understanding the PK/PD relationship is critical to determine the clinical dose and dosing regimen.

There are many different types of pharmacological responses. Mainly they can be categorized as either direct or indirect responses. A direct response is when the observed time course of response is temporally similar to the PK. A simple example of direct response is a receptor binding type response where the relationship between blood drug concentrations and the effect can be described with Hill function (50).

$$E = \frac{E_{\max} \times C^{\gamma}}{\mathrm{EC}_{50} + C^{\gamma}}$$

where $E_{\max}$ is the maximum efficacy (capacity), EC_{50} is the concentration to produce 50% of effect (sensitivity), and γ is Hill factor. Direct PK/PD responses are observed when the drug target is present in blood or when equilibrium is established rapidly between plasma concentration and biophase (Fig. 7). Examples of direct responses are neuromuscular blocking agents, etc., where the response is directly related to the drug concentration and pharmacological effect can be seen immediately.

Those target tissues are often not in the blood and therefore, it is necessary to establish the relationship between plasma concentrations (PK) and the concentrations at the target tissue to understand the PK/PD relationship. The concept of "biophase" (target tissue) was first introduced by Segre in 1968 (51). Indirect PK/PD response is used to describe the case where the time course of PD is time-shifted from that of the PK—that is, the maximum PD response does not occur at the maximum blood concentration (Fig. 8). Such responses occur when the pharmacological effects are results of a cascade of events such as induction, synthesis, secretion or cell trafficking. The very first work in this area was done with anticoagulants by Levy et al. (52,53). The diagram below shows the effect compartment model where the rate of onset and offset of effect is governed by the drug distribution and elimination from the biophase (effect compartment or target tissue).

Basic PK/PD Models

The relationship between PK and PD time courses is usually derived using a PK/PD model. Either observed or model-predicted blood concentrations are used as the forcing function for the PD response and the appropriate PD response parameters—for example, $E_{\max}$, EC_{50}, and γ in the Hill equation—are estimated. PK/PD modeling enables us to quantify pharmacological effects as a function of time in relation to drug concentrations. The direct effect and indirect

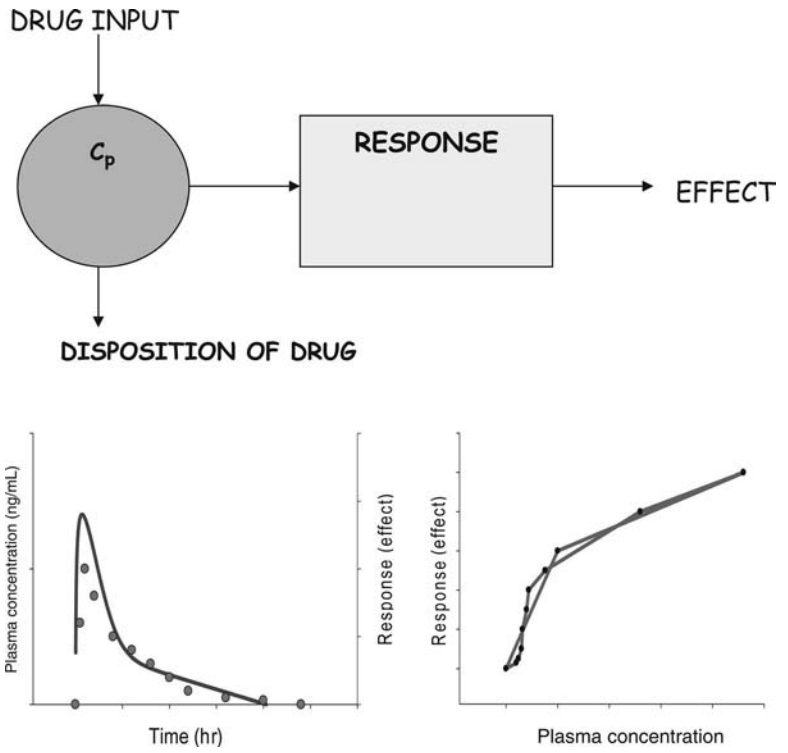

Figure 7 Illustration of a direct pharmacokinetic-pharmacodynamic model. The solid line represents PK profile and the dots represent the PD measures.

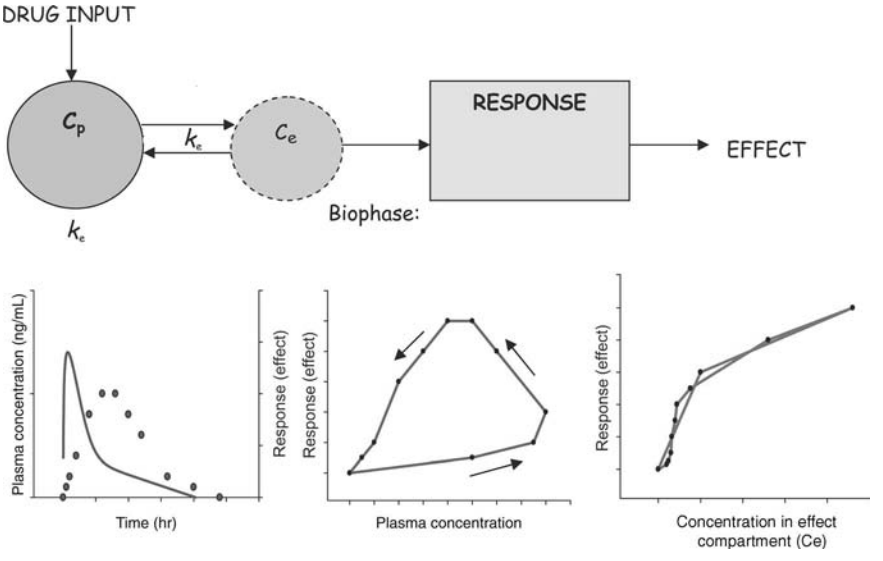

Figure 8 Illustration of an "indirect" or delayed pharmacokinetic-pharmacodynamic effect. In the left panel, the solid lines represent the PK profile and the dots represent PD measures.

effect compartment models shown above are two of the simplest models to describe PK/PD relationships. As stated above, there are many other types of pharmacological responses which cause delayed responses. Because of the diversity of in vivo pharmacological responses, the variety of PK/PD models is quite large and cannot be dealt with in detail here.

BIOTHERAPEUTICS FORMULATION AND IMMUNOGENICITY

All biotherapeutics are potentially immunogenic, and this immunological reaction has the potential to impact the biopharmaceutics of the product. Thus, understanding and mitigating the causes of immunogenicity are critical to the successful application of the biotherapeutic (54).

The causes of immunogencity of biotherapeutics vary widely, and is not necessarily related simply to the amino acid sequence being of foreign origin. General immunological or safety concerns with protein therapeutics include acute infusion or injection site reactions (anaphylactic or anaphylactoid), serum sickness, effects related to the generation of antibodies against the therapeutic, as well as antibodies to therapeutic that may cross-react with endogenous proteins. The latter type of immunological reaction carries the greatest risk because of its potential to impact both safety and efficacy (55,56).

Therapeutic proteins can lead to antibody induction via two pathways: a T cell–independent and a T cell–dependent pathway (57–60). Analysis of antibodies from clinical studies suggests that IgG antibodies make up the majority of the antidrug antibody (ADA) responses, implicating the T cell–dependent pathway as the primary mechanism.

The T cell–dependent pathway requires a cognate T cell–B cell interaction. To initiate the response, the protein must interact with antigen-presenting cells (APCs) such as dendritic cells (DCs), B cells, or macrophages. APCs internalize the antigen (i.e., therapeutic protein), digest it in the endosome, generate peptides that can be loaded into an appropriate MHC class II molecule and present them in a linear conformation on the surface as a complex. These peptides are called T-cell epitopes and may be recognized by T-cell receptors on naïve T (helper) cells in lymph nodes. In parallel naïve B cells also take up the antigen via their specific membrane-bound antigen (B-cell) receptors, process and subsequently present epitopes in MHC class II molecules on their surface. Helper T cells that have been already activated by recognizing the epitope on the APCs, must then proliferate, migrate and encounter B cells with the same epitope on the same MHC class II molecule at the lymphoid follicles. Binding of the T-cell receptor to the peptide:MHC class II complex on the B-cell surface then leads to the expression of costimulatory molecules and secretion of cytokines from T-cell surface that trigger the B cell to differentiate and mature into antibody-secreting cells. A mature but naïve B cell will initially produce an IgM response. Further helper T-cell interactions induce isotype switching to IgG (and other isotype) responses. This T cell–dependent immune response is usually long lasting and of high titer. Once the switch has occurred, some of the activated B cells become long-lived memory cells which react rapidly to rechallenge with the characteristic IgG production. This mechanism requires that B cells (via B-cell receptors) and T cells respond to the same antigen although not necessarily the same epitope. Another important requirement is a costimulatory signal to activate the T cells. These costimulatory molecules can be induced by infection or inflammation—a distress or danger signal in the form of cytokines such as tumor necrosis factor (TNF). In the absence of these distress signals, the peptide:MHC class II complex alone on the APC cannot activate the T cells, thus promoting anergy or tolerance in naïve T cells. On the other hand, the presence of additional molecules that are associated with the therapeutic protein that act like adjuvants (e.g., HCPs or endotoxins), can activate toll-like receptors on the APCs, and may result in reversing tolerance or abrogating T- and B-cell anergy, thus inducing the generation of an immune response.

B cells can also be activated without cognate T-cell help by the so-called T cell–independent pathway. For this purpose, the antigen has to be engulfed by specialized blood-borne peripheral DCs, and presented to B cells. B-cell stimulatory signals are generated when a number of B-cell receptors simultaneously bind to the antigen resulting in their crosslinking and subsequent cell proliferation. A costimulatory signal (e.g., a cytokine) is however required for the activation step. Antibodies produced in this situation are of the IgM type, transient, of low titer and poor specificity. Because of lack of affinity maturation, there is no class switching or generation of memory. This pathway is typically evoked by particulate antigens displaying repetitive epitopes termed pathogen-associated molecular patterns, usually found on bacteria. Again, delivery of a second signal by helper T cells or via pathways mediated by Toll-like receptors would allow for affinity maturation and class switching, creating a more efficient IgG response.

Table 1 Factors That May Impact Immunogenicity of Biotherapeutics

Product-related factors	Patient-related factors	Treatment-related factors
Protein structure (human/nonhuman, posttranslational or chemical modifications)	Disease state being treated	Dose
Product quality parameters (isoforms, chemical and physical degradants)	General immune status of patient	Route
Contaminants and impurities	Genetic background (MHC genotype, HLA phenotypes)	Frequency of dosing
	Concurrent illnesses and concomitant therapy	Length of treatment

Immune response to foreign (exogeneous) proteins also called the "classical" immune response, arises via the T cell–dependent pathway. On the other hand, the human immune system is usually tolerant or anergic to proteins of human origin. In the absence of a neoantigen, an immune response against a human protein though not impossible, is highly unlikely unless the protein is presented to the immune system in a fashion that can reverse tolerance or T- and B-cell anergy by the above T cell–dependent pathway. The likelihood of breakage of tolerance to proteins of human origin or recombinant autologous proteins, is considered a function of the abundance of the endogenous soluble protein. For proteins of low abundance, the immunological tolerance is not complete. T and B cells specific for low-abundance proteins (autoantigens) may not be completely eliminated during early development. Under sufficient provocation (e.g., presence of molecules with adjuvant-like characteristics), these might generate an immune response.

ADAs are broadly classified as binding (BAbs) or neutralizing (NAbs). For biologics of human origin, BAbs and NAbs are of concern because of the possibility of impacting efficacy and PK. BAbs bind to the protein but do not neutralize it. They may mediate infusion reactions or alter the PK/PD profile of the therapeutic. BAbs can enhance clearance or prolong systemic exposure. BAbs can be precursor or triggers for the generation of NAbs through epitope spreading. NAbs bind to the therapeutic molecule and disrupt its ability to bind to the target, that is, neutralize its function. When present at low titers, the impact on efficacy may be minimal but efficacy and biological activity may be impacted at high titers. The most serious type of NAbs response are those that cross-react and neutralize the function of the endogenous analog, especially one that serves a biologically unique function and has no redundancies (61).

There are many factors that can be involved in breaking of tolerance to a protein biotherapeutic and can be broadly classified into three categories as given in Table 1.

Although the factors are categorized above, in practice it is very difficult to deconvolute the impact of specific product attributes from the number of patient and dosing regimen related factors (62–64).

When considered from the perspective of a product development scientist, the causes of immunogencity can be divided into two broad categories.

1. Intrinsic to the molecule and treatment regimen
2. Extrinsic factors related to CMC aspects of the product

Immunogenicity as a Consequence of Molecule and Treatment Aspects of the Biotherapeutic

This category is concerned with the selection and design of the molecule itself and is often the result of a discovery effort intended to realize a certain therapeutic effect. A detailed consideration of this category is therefore outside the scope here but some relevant concepts are covered to provide a background for the subsequent discussion. A nonhuman protein (e.g., streptokinase, botulinum toxin) will induce antibodies by the classical immune response. A similar response can be generated in people who do not have tolerance to a certain protein. For

example, patients with severe hemophilia A involving large deletions or nonsense mutations of the factor VIII gene are more likely to have an antibody response to exogeneous factor VIII than patients with mild or moderate disease since patients with the severe form of the disease do not express functional factor VIII antigen and hence have no immune tolerance (65). In these instances, the generation of ADAs may be considered as a vaccine-like reaction to a foreign protein. As in vaccines, the response is related to a number of factors such as (number, frequency and amount) of dose administered, length of treatment, delivery route, and presence of "adjuvants" (66). More surprising is the observation that "self" proteins can induce an immunological response even in individuals who are not deficient in the protein, but simply produce an insufficient amount for the desired biological effect (67).

Foreign proteins can induce antibodies after a single injection while human proteins may require longer exposure of up to six months (68). Yet, as exemplified by insulin and growth hormone, chronic therapy need not compromise the therapeutic efficacy of the protein. The fact that both types of proteins can induce antibodies implies that the molecular characteristic evoking antibody response is at least more complex than simply being self or nonself to the human system. Nature of the therapeutic (immunostimulatory vs. immunosuppressive) proteins and host immune status also play a role in the observed effect. Cell surface binding therapeutic antibodies generally will have more potential to be immunogenic than those that interact with soluble targets.

The probability of an antibody immune response is considered highest after SC injection, followed by IM, intranasal, and intraveneous routes. SC administration localizes the protein to a small area with a short path to drain into the lymph nodes where B and T cells are present (69). Clinical experience with pulmonary administration of insulin suggests that this route also carries a high risk for generation of immunological reaction (70).

The type of disease plays a role, likely related to the immune status of the patient. Patients with weak or compromised immune systems or those on immune-suppression therapy are less likely to develop ADAs than those with intact immune systems. Acute therapy is less likely to be immunogenic than chronic therapy, although intermittent treatment is more likely to elicit a response than continuous therapy. Also, lower doses are generally more immunogenic than higher, probably related to the fact that the immune system is generally less tolerant of low-abundance proteins.

Porter (71) has prepared a comprehensive review of the literature on immune response to recombinant proteins used in therapy. Among the significant conclusions drawn are that the presence of antibodies has not necessarily been detrimental to the clinical efficacy and that no particular property of a protein has been identified as an obvious predictor of immunogenicity in humans.

Immunogenicity as a Consequence of Chemistry Manufacturing and Control (CMC) Aspects of the Biotherapeutic

The characteristics of a parenteral products are determined by three major factors: process, formulation and package. From the perspective of a product development scientist, the CMC aspects that can play a role in the immunogenicity profile of the product begin with the gene design and cell line selection. Gene sequences are mutated to avoid degradation and aggregation hotspots as well as antigenic epitopes, while maintaining potency (72–74). The choice of host cell line determines the presence (or absence) of glycosylation and the glycosylation pattern. The upstream (bioreactor/fermentation, harvest) process impacts the distribution of glycoforms and other product variants, for example, deamidated variants, disulfide scrambling, and also determines the type of host cell impurities that may ultimately remain in the product. The protein then further undergoes a complex series of processing steps for purification including viral removal. While the overall objective of the post harvest steps is to purify the protein by removing impurities (e.g., host cell proteins, DNA, endotoxins), and product related species (e.g., truncated, hydrolyzed, aggregated, deamidated, oxidized, and improperly glycosylated forms), it is nevertheless impossible to completely eliminate these. The current state of purification processes is such that impurities are routinely reduced to levels well below what is considered a risk in the particular case. Product variants are not as

easily eliminated and a certain minor fraction of some or all of these variants make their way into the final bulk solution. The bulk solution after the purification steps may be stored for a period of time either as a liquid or frozen, before it is finally filtered and pumped into vials or syringes. In some cases, it is subject to the final processing step of lyophilization, before being shipped over an appropriately designed (cold) transport chain to the clinic or pharmacy. Thus, an important objective of the process and formulation development is to stabilize the native state of the molecule and minimize physical and chemical degradation over the shelf-life of the product.

Impact of Process and Formulation

The process that a biotherapeutic undergoes in its product has a significant impact on the product characterisitc. The formulation is intended to stabilize the product during the process and during storage and use. Some aspects of the process and formulation that have the potential to impact immunogenicity are considered below.

Glycosylation. Glycosylation refers to the enzymatic addition of saccharides to the protein as a post-traslational modification. Glycosylation is present in approximately 50% of human proteins and an almost similar proportion of approved biopharmaceuticals. The presence and nature of the glycoform may impact primary functional activity, folding, stability, trafficking and immunogenicity. Although glycosylation is in a way intrinsic to the molecule, it can also be impacted by the production process. For this reason, the choice of the expression system is a critical activity in the development of a biotherapeutic. As mammalian expression systems produce mainly human glycans, these have become the dominant platform for production of therapeutic glycoproteins. However, these platforms require good process control since they display an inherent glycan heterogeneity that is sensitive to culture conditions. Glycosylation can have direct impact on immunogenicity through patterns that are not present in humans. CHO cells produce gycosylation patterns that are close to human, although these cells also express *N*-glycolylneuranimic acid (NGNA), a form of sialic acid not found in humans and reported as immunogenic. Mouse cell lines (e.g., NS0, SP2/0) also produce NGNA in addition to or instead of the *N*-acetylneuraminic acid (NANA) present in human IgGs (75,76). Galactose α(1-3) galactose linkages or terminal α(1-3) galactose can also be added by murine cells (e.g., C127, NS0, SP2/0). This residue has been shown to be recognized by up to 1% of circulating IgG in humans (77). Glycoslation can have an indirect effect on immunogenicty through its impact on folding solubility and (structural) stability. Glycosylation can affect local secondary structure and thereby direct the generation of tertiary structure. Altered or absent glycosylation can therefore alter or eliminate epitopes or expose/generate new ones. Glycosylation can increase solubility by shielding hydrophobic patches and reducing tendency to aggregate, and enhance stability by participating in intrachain stabilizing interactions (78–80).

Purity. Host cell proteins and DNA are contaminants that carry the risk of functioning as adjuvants and thus triggering an immunogenic reaction to the therapeutic given the appropriate antigenic determinants. Lundin et al. (81) summarized that the early pituitary preparations of hGH resulted in about 45% patients developing antibodies. Improvements in processing and purification led to a marked decrease in antibody formation to less than 10% (pituitary source), while it was $<2\%$ for the purest commercial pituitary preparation. Early recombinant preparations, on the other hand, also led to unexpectedly high antibody levels, but were related to *E coli* proteins remaining as impurities in the preparations (82). Bacterial DNA contains unmethylated CpG motifs that are known to activate Toll-Like receptors and are themselves being studied as adjuvants for vaccines. Process improvements resulting in greater purity by reduction of product-related and unrelated species have led to a clear reduction in ADA response. Current purification processes reduce host cell and process contaminants to very low levels.

Product-related impurities and degradation products. Product-related impurities and degradation products for biotherapeutics often overlap and are not readily distinguishable.

For example, charge variants encoded as a consequence of the cell line (e.g., sialylation) and/or generated in the upstream/downstream processes will often overlap with deamidation/isomerization products. Oxidation of susceptible residues can occur at any stage in the production process or subsequent storage and use, as can fragmentation/hydrolysis. Finally, size variants such as truncated, misfolded and aggregated species can also arise at all stages. However, among all the possible chemical and structural changes, the one that causes the most concern is aggregation involving association of multiple protein molecules in partially/wholly unfolded forms, and even in their native state.

Aggregates can form as a result of a variety of interactions between the protein molecules including hydrophobic interactions as well as because of covalent changes caused by chemical modifications such as oxidation. The protein molecules making up the aggregates can be in their native, partially or fully unfolded states. Aggregation is governed by the conformational, that is, thermodynamic stability self-association tendency, that is, colloidal stability of the molecule. A full discussion of the mechanisms of aggregation is outside the scope of this chapter but a number of good reviews are available [Chi et al. (83), Wang (84), Mahler et al. (85)]. Other factors that can impact the level of aggregation in a protein solution includes conditions such as pH, temperature, concentration, ions and ionic strength and stresses such as freeze/thaw, air/liquid and liquid/solid interfacial stress. Chemical modifications such as oxidation can also lead to loss of structural stability and aggregation. Since a protein can undergo aggregation by multiple pathways, all of these factors have to be addressed as part of the formulation development program for the biotherapeutic.

Aggregates are hypothesized to cause immunogenicity through their "repetitive" display of epitopes that are seen by the immune system as resembling the external surfaces of invasive species. As reviewed by Rosenberg (86), it is not the low MW aggregates such as dimers or trimers but the large multimers with molecular weights exceeding 100 kDa that are efficient inducers of immune responses. Native aggregates in which the protein retains a large part of its structure are of greater concern since antibodies could be generated against epitopes that are present on the native monomeric version. Antibodies generated against nonnative aggregates (generated by misfolded species or chemical modifications) could still result in increased clearance as well as raise potential safety concerns. Experiments on animal models have shown that aggregated proteins can lead to an immunological response, but the relevance to human experience is debated (61,87,88).

Aggregation is considered a strong risk factor for generation of immunological reaction and therefore has to be minimized by proper design of process and product. It is a fundamental attribute to assess the quality of a biotherapeutic and control of this parameter is an important aspect of biotherapeutic product development.

Container/Closure System

Container/closure are an integral part of a biological product, be it a vial/stopper, a prefilled syringe or a dual-chamber cartridge. Some component materials which come into contact with the product include the container (glass or plastic, vial or syringe), closure (stopper), administration and infusion components (syringes, bags, infusion lines). The concern for packaging component-dosage form interaction for biologics again arises because of the potential for alteration of the structure of the protein through aggregation or chemical degradation pathways such as oxidation. The impact on the protein can occur directly through the container interface, but also indirectly through any chemical compound that may leach out of the container. Some common leachables from the common container/closures used for biologics include metals, antioxidants, plasticizers, lubricants as well as degradation products of the various components. For example, tungsten residues left behind when preparing staked-needle syringes have been shown to cause oxidation of protein solutions. The FDA also considers the compatibility of container/closure with product as a key requirement in the development of parenteral products. The FDA guidance document on container closure considers inhalation aerosols and solutions, injections and injectable suspensions as products with the greatest level of concern when accounting for route of administration and risk for packaging component-dosage form interaction (89).

Silicone oil coating is commonly used on stoppers and on the inside of syringes or cartridges as a lubricant to enable movement of the plunger. Silicone oil contamination by the syringes used for injecting insulin has been well documented [Chantelau et al. (90), Bernstein (90,91)]. Current processes for siliconization of prefilled syringes or cartridges apply well controlled amounts and involves baking of the silicone emulsion. This tends to reduce the levels of silicone oil extracted into the formulation but the possibility exists. Fibrous aggregates have been shown to form in a a number of model proteins when incubated with silicone oil (92).

Selection of the container/closure system for any product is a critical task. The container/closure must provide adequate protection to the product from the environment and prevent contamination. It must also be compatible with the product and not leach any compunds that could harm the product or pose a safety risk. The experience with vials and stoppers is extensive, but use of devices such as inhalers and injectors increases the complexity of this task.

Safety/Tolerability of Excipients

As stated before, formulation development for a biologic is carried out to identify the optimal composition that will keep the biologic stable for an economically viable length of time. The product format can be a liquid or a lyophilized powder. Excipients are added to accomplish this. A review of the formulation composition for biologics shows that the vast majority comprise a buffer, a tonicity modifier, cryo- or lyoprotectant, and a surfactant. Other additives such as a chelator, antioxidant, and a preservative are occasionally found (93). Most common excipients are generally safe and have long precedence of use, although precautions may be in order in certain cases. Among the common ingredients is the surfactant polysorbate 20 or 80, comprising partial fatty acid esters of sorbital and its anhydrides copolymerized with ethylene oxide. These is known to cause anaphylactic reactions in dogs, and may have an allergenic potential in susceptible individuals. Intravenous immunoglobulin (IVIG) therapy has been connected with numerous episodes of acute renal toxicity and osmotic nephropathy because of a very high sucrose load. The sucrose is added to the product to reduce formation of aggregates as a consequence of the pathogen-removal steps in the process. Sucrose and sorbitol as well as maltitol and fructose can also be contraindicated in patients with hereditary fructose intolerance, the glucose-galactose malabsorption syndrome or sucrose-isomaltase deficiency.

Evaluation and Prediction of Immunogenicity

Animal models have traditionally been used to evaluate the safety of (bio)pharmaceuticals, but their utility in evaluation or prediction of clinical immunogenicity is controversial. Data generated from the animal models must be placed in context of the type of molecule. Bugelski and Treacy (94) group recombinant proteins into classes on the basis of preclinical immunogenicity. For some classes, for example, bacterial proteins, immunogenicity in animals is often predictive for humans. For others, such as fully human proteins, even data from nonhuman primates may have little predictive value. Nonhuman primates with a high level of sequence homology with humans are often seen as most relevant. However, the evidence for success is limited, and mainly governed by the degree of conservation across species. Limited homology means that the animal models are generally over-predictive of human immunogenicity. Transgenic mice that express the appropriate human transgene allow the protein to be tested without generating a xenogenic response. There are many caveats and limitations of this approach (62,94,95), the least of them being that the wild-type strain must be capable of making antibodies to the protein in questions. Limitation in the use of animal models is magnified when trying to decipher the relative impact on immunogenicity of a few percent of product degradants. To be able to detect such changes, the animal models must have a low baseline immune response or a slow development trajectory for immunogenicity, while the studies have to be carefully controlled. In summary, the utility of animal models would primarily lie in assessing the relative immunogenicity risk of CMC related factors.

Computational tools are also being developed for assessment of intrinsic immunogenicity of protein therapeutics including identification and modification or removal of T-cell epitopes (72). Further research is required to develop models with the ability to assess the impact of CMC factors in general and aggregation in particular on immunogenicity.

BIOTHERAPEUTICS BIOEQUIVALENCE/COMPARABILITY

Manufacturers of biotechnological/biological products frequently make changes to manufacturing processes of their products both during development and post-approval. These changes, however minor, could cause undetectable changes in the physicochemical composition of the primary active ingredient of the drug substance or in the profile coproduced compounds such as host cell proteins and other potential impurities. Also, as discussed in previous sections, the dose, frequency, and route of administration all have the potential to change the PK/PD and immunogenicity characteristics. Thus, even minor changes in the drug manufacturing and/or administration process have the potential to affect the overall safety/efficacy profile of the drug product. Demonstration of comparability of the pre- and postchange product is a sequential process, beginning with quality studies (limited or comprehensive) and supported, as necessary, by nonclinical, clinical and/or pharmacovigilance studies. For most changes to the manufacturing process, physicochemical and (quality-related) biological testing can demonstrate that there is no difference in quality of the product that could adversely impact the safety and efficacy of a product. Thus the comparability exercise may be limited to strict process validation of the change or be extended to various quality criteria such as in-process controls, thorough analytical and biological characterization of the product and stability data. However, sometimes an effect on efficacy and/or safety can be expected on the basis of observed difference(s) or cannot be ruled out in spite of the state of the art physicochemical and biological tests. In such cases, additional nonclinical and/or clinical studies will be necessary.

PK studies are a key component of the in vivo comparability testing and are typically performed when analytical characterization is not sufficient to detect differences, or the clinical implication of analytically detected differences is unknown. The study could be performed in animals, if a relevant animal model exists, or in humans. PK studies may not be appropriate for comparability testing when the PK variability of the reference product is in general very high or when the PK variability is of no clinical relevance, when PK is insensitive to clinically relevant changes to the active substance (e.g., in the case of misfolded proteins), drug is active at the site of administration and blood exposure is not a relevant biomarker for safety/efficacy. Despite these limitations, PK testing remains a valuable comparative tool.

Some of the key considerations of a PK for biologics study are: the study population—patients or subjects, dosing regimen—single or multiple doses, parallel or crossover study, the duration of sampling, route and method of administration, doses for evaluation in the study, PK parameters of interest, and the criteria for claiming equivalence. While many of these considerations are also relevant for a small-molecule drug, the PK characteristics of biologics pose a unique challenge in the design and conduct of these studies. The choice of study population depends on the PK and safety profile of the compound of interest and its mode of action. For compounds that are generally well-tolerated and where the PK in healthy subjects is known to be predictive of that in the target patient population, healthy subjects might be appropriate for comparative testing. In other cases, a patient PK study might be considered, especially where relevant PD information can also be gathered. Similarly, many biologics have a long half-life, from days to weeks. Therefore, standard crossover studies can pose limitations due to the duration of treatment and follow-up. Parallel studies could be considered if the duration of the study could become unfeasible. Furthermore, the potential for immunogenic reactions, typically observable after three to four weeks after a single dose, should also be considered in crossover designs. The route of administration should be in accordance with the intended clinical use. If the product is planned to be administered by more than one route (e.g., SC and IV), it may become necessary to test all routes. The selected dose should be in the steep portion of the dose-response curve to detect relevant differences, especially if PD markers are being monitored in the study. Apart from standard PK parameters describing absorption and

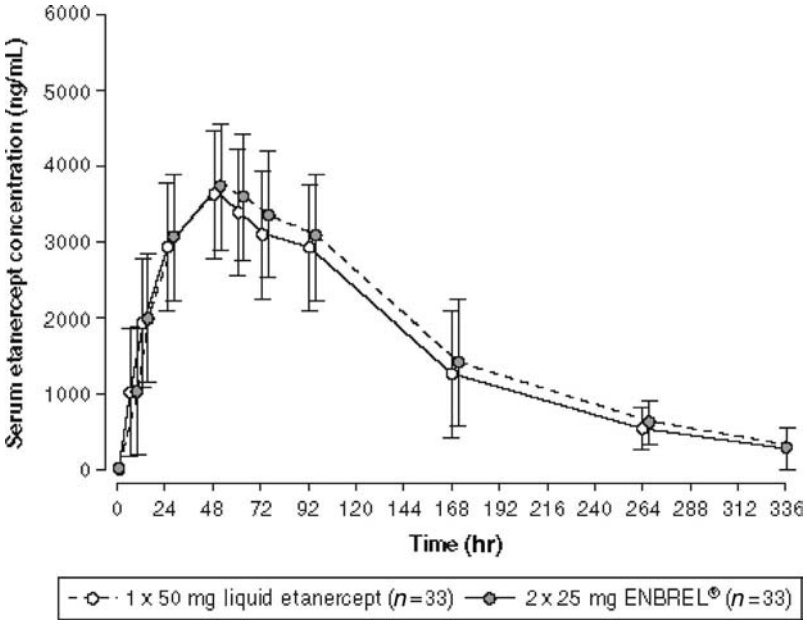

Figure 9 Illustration of pharmacokinetic equivalence for two formulations of etanercept. *Source*: From Ref. 97.

bioavailability (such as C_{max} and AUC), other PK parameters such as elimination half-life and clearance should also be considered for comparability, because of potential changes in the heterogeneity of active substance due to process changes.

The following example by Sullivan et al. (96) illustrate the concept of PK-based comparability assessment for a new formulation of Enbrel® (etanercept). Etanercept is a soluble, fully human, TNF receptor that competitively inhibits the interaction of TNF with cell surface receptors. Etanercept is currently approved for reducing signs and symptoms, inhibiting the progression of structural damage, and improving physical function in patients with rheumatoid arthritis. It is also approved for reducing the signs and symptoms and inhibiting the progression of structural damage in patients with psoriatic arthritis and for reducing the signs and symptoms of active ankylosing spondylitis, juvenile rheumatoid arthritis, and psoriasis. Etanercept was originally introduced commercially in vials containing 25 mg lyophilized powder requiring reconstitution, and to date most patients have received the reconstituted formulation. A 50-mg/mL liquid formulation supplied in a prefilled syringe was approved recently for commercial use. Sullivan et al. (96) present the results of a study in healthy volunteers comparing the PK of the liquid etanercept formulation with that of the reconstituted formulation (Fig. 9). The study was conducted in healthy male and female subjects, where each subject received both formulations (50 mg of etanercept per dose) in a crossover fashion with a minimum of 28 days washout period in between doses. The following PK parameters, obtained from the observed PK profile using noncompartmental analysis, were reported: AUC (to till the final sample collection timepoint and extrapolated to infinity), C_{max}, T_{max}, and terminal $t_{1/2}$. The point estimate of the ratio of geometric means of the PK parameters (AUC and C_{max}) were generated along with their 90% confidence intervals. Equivalence of the two formulations was concluded since the 90% confidence interval of the ratio of PK parameter means lay between 80% and 125%, which is the standard bioequivalence criterion.

Similarly, Paulson et al. (97) performed a PK comparability assessment for adalimumab (Humira) in healthy subjects between two administration routes—as an autoinjector pen and a prefilled syringe. Adalimumab is a murine monoclonal antibody prescribed for the treatment of rheumatoid arthritis, and has a half-life of two to three weeks (PI). Therefore, a parallel group study in 290 subjects was performed in this case to assess the PK equivalence in this

case. The duration of PK assessment was appropriately adjusted to account for the long half-life. The PK and statistical data analysis was similar to that described by Sullivan et al. Comparability was concluded in this case also.

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4 Preformulation

N. Murti Vemuri

INTRODUCTION

Parenteral medication refers to drugs administered by routes other than the oral, typically implying injectable medications. Injectable medications could be presented in various volumes (small volume and large volume), primary packaging (ampoules, vials, cartridges, bags) and specified routes (e.g., intravenous, intramuscular). Many of the preformulation and formulation principles applicable to injectable medications can often be extended to ophthalmic and nasal spray dosage forms as well.

Rational formulation development of parenteral medication should be based on the desired product profile, the physicochemical properties of the drug substance and its interaction with other formulation ingredients, primary packaging components under storage conditions defined by the product profile, as well as the pharmacokinetic properties of the drug substance. Preformulation research comprises pharmaceutical and analytical investigations in acquiring such knowledge base, and these investigations both precede and support formulation development.

On a drug development timescale, preformulation research enables data-driven decisions related to the drug substance and drug product such as salt form selection, polymorph selection, excipient selection, identification of suitable toxicology formulations, and, finally, selection of compositions for clinical and commercial formulations. Additionally, understanding the physical and chemical attributes of the drug substance can often help in troubleshooting formulation, stability, and processing issues that may arise.

Many good reviews and book chapters (1) have been written on the subject of preformulation and physicochemical characterization of drug substances. Although most articles focus on oral formulations, many of the principles carry over to development of parenteral medications. This chapter will attempt to focus more on aspects relevant to development of parenteral dosage forms. Much of the discussion will focus on small molecules and solutions dosage forms, but later sections will touch on specificities related to macromolecules and specialized dosage forms.

CHARACTERIZATION OF THE DRUG SUBSTANCE

Understanding the physicochemical properties of the drug substance is the first step (2) toward building quality into a product using rational formulation design. Drug substances are investigated at various levels of scrutiny to fully understand their behavior—at the molecular/material level, at the particulate level and also at a bulk property level. Table 1 shows a representation of this hierarchy of physicochemical properties. The intended dosage form often dictates where to place the greatest emphasis. For a solid dosage form, it is important to also fully understand the bulk properties, but for parenteral dosage forms, greater emphasis is on understanding the molecular and material properties of the active pharmaceutical ingredient (API).

Molecular Properties

Prior to initiation of preformulation studies, the molecular structure of the drug substance is identified and confirmed by appropriate spectroscopic (NMR, MS) evidence. The material is further identified by its characteristic IR and UV spectrum.

Physicochemical Constants

Two key physicochemical constants of importance are the partition coefficient and the ionization constant. The partition coefficient is an indication of the lipophilicity of a compound and is measured as a ratio of the equilibrium concentrations of the drug in an oily (e.g., octanol) and an aqueous (e.g., water) phase in contact with each other and held at a constant temperature. The

Table 1 Physicochemical Properties of Drug Compounds

Molecular properties *Properties defined by the molecular structure*	**Material properties** *Properties intrinsic to the material or particle*	**Bulk properties** *Properties related to bulk powders*
Molecular weight	Salt form	Powder flow
log *P*/log *D*, pK_a	Crystal form (XRPD)	Bulk density
Chemical stability	Crystal habit	Wettability
Melting point		Powder electrostatics
Solid-state stability		
Solubility		
Spectral characterization (UV, IR, NMR)	Particle size	
		Specific surface area
		Hygroscopicity

Abbreviation: XRPD, X-ray powder diffractometry.

logarithmic value of the ratio of these concentrations is often used and referred as log *P*, or partition coefficient. When an aqueous buffer solution (often pH 7.4) is used instead of water, the value is referred to as log *D*, or distribution coefficient. These coefficients, which are descriptions of the lipophilicity of a compound, are often correlated to the ability of a compound to cross biological membranes as well as their ability to dissolve in formulation vehicles.

The ionization constant (K_a), an intrinsic property of the molecule, describes the ionization behavior of a compound as a function of pH. The negative logarithm of K_a is often used and referred to as pK_a. The pK_a is equal to the pH value when the ratio of the ionized and unionized species is one. The pK_a is thus an important determinant in the pH dependence of ionization and hence solubility as well as salt formation ability of a molecule. These concepts will be further expanded elsewhere in this chapter. If a compound has multiple ionizable groups, each group has a corresponding pK_a value.

The molecular structure of the compound can be utilized for obtaining additional first estimate of properties such as dissociation constants and partition coefficients utilizing prediction software (e.g., from ACD/Labs, Simulations Plus, etc.). Such software packages can also provide a first estimate of the solubility and pH-solubility profiles. These data are especially useful during early development when compound supply is very short and there is a need to provide formulations for discovery pharmacology and early toxicology studies.

Solubility

Solubility is the concentration of drug in solution at equilibrium with excess solid. Typically, when the solid drug is brought in contact with a solvent, it dissolves into the solvent over a period of time and achieves equilibrium asymptotically. Aqueous solubility is of particular relevance to biological activity, bioavailability, and formulation strategy (3).

Solubility is experimentally measured by placing an excess solid in a test tube in contact with a particular solvent with mild agitation and determining the concentration of the drug in a supernatant solution over a period of time using appropriate analytical techniques such as UV spectrophotometry or high-performance liquid chromatography (HPLC). In determining equilibrium solubility, it is important to ascertain that (*i*) an asymptotic value has been achieved (constant over multiple time-points) and (*ii*) the identity of the solid in contact with the solvent is unchanged. The identity of the residual phase can be confirmed by analyzing the residue using techniques such as differential scanning calorimetry (DSC) or X-ray powder diffractometry (XRPD).

During preformulation studies, it is common to determine solubility of the drug compound in aqueous and nonaqueous vehicles used in pharmaceutical formulations. Aqueous systems include buffers, surfactant solutions, and complexant solutions. Nonaqueous

systems include cosolvents (e.g., ethanol, glycerol, polyethylene glycols) and oils (soyabean oil, glycofurol). A more detailed list of excipients is discussed later in this chapter.

pH-solubility profile. Many pharmaceutical compounds contain acidic or basic functional groups and hence show pH dependence in their aqueous solubility. Solubilities can vary significantly in accordance with the pK_a across acceptable pH range. Hence, adjusting pH to achieve requisite solubility can be an important tool in formulating injectable solutions.

The pH dependence of solubility of acids and bases is derived from the ionic equilibria occurring across the pK_a of a compound and is described by the Henderson-Hasselbalch equation (4).

$$\mathrm{pH} = \mathrm{p}K_a + \log\frac{[\mathrm{A}^-]}{[\mathrm{HA}]} \quad (\text{for an acid}) \tag{1}$$

$$\mathrm{pH} = \mathrm{p}K_a + \log\frac{[\mathrm{B}]}{[\mathrm{BH}^+]} \quad (\text{for a base}) \tag{2}$$

Taking the example of a free base, the total solubility of at any given pH is the sum of the solubility of the unionized species (S_0) and the ionized species.

$$S = S_0 + [\mathrm{BH}^+] \tag{3}$$

Figure 1 shows a hypothetical pH-solubility profile for a weak base. At a high pH (pH >> pK_a), the solubility is practically independent of pH and is essentially S_0. As the pH approaches the pK_a, the fraction of ionized species and hence the total solubility increase and are described by

$$S = S_0\left(1 + \frac{[\mathrm{H}^+]}{K_a}\right) \tag{4}$$

The ionized species can associate with a charged counterion to form a salt. This linear increase in solubility ends abruptly when the solubility of the salt form is reached, and at this point the solubility is governed by the solubility product (K_{sp}) of the salt form. For example,

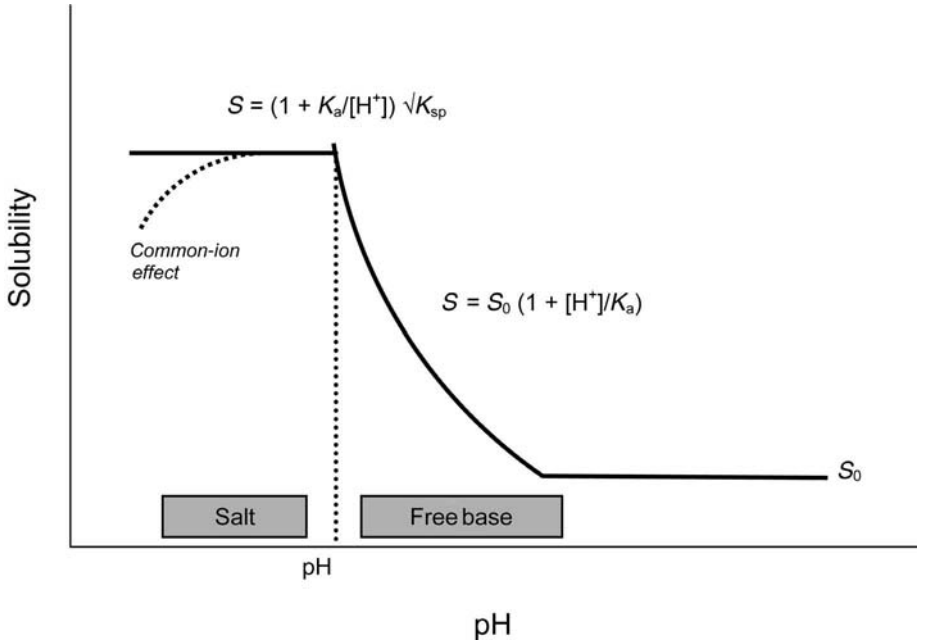


Figure 1 pH-solubility profile of a hypothetical weak base.

Table 2 Properties of Some Commonly Used Solvents

Solvent	Dielectric constant (ε)	log P	Surface tension (γ) (dynes/cm)
Water	81.0	−4.00	72.0
Glycerin	42.5	−2.60	64.9
Propylene glycol	36.7	−1.93	48.8
Ethanol	24.3	−0.31	22.2

assuming that the pH was being changed by titrating with hydrochloric acid, the solubility product is

$$K_{sp} = [BH^+][Cl^-] \tag{5}$$

and the total solubility at this pH_{max} would be

$$S = \left(1 + \frac{K_a}{[H^+]}\right)\sqrt{K_{sp}} \tag{6}$$

Rearranging the equation, the pH_{max} can be determined if the solubility product is known.

$$pH_{max} = pK_a + \log\frac{S_0}{\sqrt{K_{sp}}} \tag{7}$$

Common-ion effect or salting-out effect is also depicted in Figure 1, representing the pH-solubility profile of a weakly basic drug. From the pH of maximum solubility, as one moves toward lower pH values, there is an increase in the concentration of the counterion (e.g., $[Cl^-]$). Depending on the value of the solubility product (a function of the nature of the drug and the counterion), this increase may be compensated by a decrease in the concentration of the ionized drug molecule. This decrease occurs through a precipitation of the drug in its corresponding salt form. This phenomenon is known as "salting-out" or common-ion effect and can be an important consideration in selecting salt forms or buffer systems for formulations.

Solubility in cosolvent systems. Cosolvents such as ethanol, propylene glycol, polyethylene glycols, and glycerol are routinely used in formulating to a higher solubility when aqueous solubility alone is not sufficient to achieve required levels. In case of some drug compounds, the use of appropriate cosolvents can increase the solubility quite significantly. The mechanism behind the increased solubility is frequently related to modifying the polarity or dielectric constant of the solvent system. The principle of "like dissolves like" works—less polar molecules would be better dissolved in a less polar solvent system. Adding a cosolvent with a smaller dielectric constant to water will bring down the overall dielectric constant of the resultant solvent system and make it a better medium for dissolving a less polar or nonpolar molecule. Table 2 shows some physical parameters of common cosolvents (5).

Although cosolvents can be quite effective in achieving solubilization, it should be noted that as excipients these can have toxicological effects (e.g., hemolysis) and potential for local irritation depending on the concentrations used. Additionally, it is very important to consider the potential for the drug to precipitate upon dilution (6). This risk can be assessed both by calculating the degree of precipitation that could occur and by experimentally simulating the dilution that could occur and testing for precipitation potential (7).

Solubility in surfactant systems. Surfactants, a common class of excipients, are amphiphilic molecules (hydrophilic head group and hydrophobic tails), which strongly orient themselves at interfaces. In an aqueous system surfactant molecules would mainly be present at the water-air interface with a small but finite concentration in the bulk of the solution. Surfactants oriented at the water-air interface cause a reduction in the surface tension of water and thereby

improve wettability of drugs being exposed to such a system. With increasing concentration of surfactant in the system, the interface becomes crowded, and at a specific concentration, the surfactant molecules in the bulk orient themselves in micellar structures. Micelles consist of spherical structures with the hydrophobic (lipophilic) tails toward the core and hydrophilic heads forming the external surface. The concentration at which this occurs is called the critical micelle concentration (CMC). Above the CMC, aqueous surfactant systems would contain micellar structures in the bulk.

Lipophilic drugs can be incorporated into the core of micelles, thereby increasing the total solubility of a drug into aqueous systems. The lipophilic cores of micelles present a different environment to the drug molecule providing, in some instances, a stabilizing effect against chemical degradation. Surfactants will preferentially orient toward the surface of nuclei during a precipitation phenomenon and can prevent precipitation occurring due to dilution effects. Thus, surfactants can be a very useful tool in formulating aqueous injectable solutions and suspensions.

Examples of surfactants commonly used in injectable formulations include polyoxyethylene sorbitan monoesters (Tweens), polyoxyethylene-polyoxypropylene copolymers (Pluronics), sodium lauryl sulfate, and lecithins.

Solubility in complexant systems. A complex is an entity formed when two molecules, such as a drug and a solubilizing ligand, are held together by weak, noncovalent forces (dipole-dipole, hydrophobic, or hydrogen bond interactions). Cyclodextrins are a class of such solubilizing ligands that have found a significant application to pharmaceutical compounds. α-, β-, and γ- Cyclodextrins are cyclic oligomers of glucose containing six, seven, or eight glucose residues. Cyclodextrins have gained popularity from a pharmaceutical standpoint because of the ability of these materials to interact with poorly water-soluble drugs and drug candidates resulting in an increase in their apparent water solubility. The mechanism for this solubilization is rooted in the ability of cyclodextrin to form noncovalent dynamic inclusion complexes in solution. As a result of their structure, cyclodextrins present a hydrophilic exterior but a core that is more lipophilic and hence provides a microenvironment for lipophilic drug molecules to engage via hydrophobic interactions. In certain cases, the modified microenvironment of the cyclodextrin core results in improved chemical stability similar to micellar systems. The ability of the cyclodextrin to solubilize a drug compound depends on steric factors (size of the cavity) and thermodynamic factors (decrease in free energy of the system). Additionally, the solubility of the cyclodextrin in water is another key determinant. β-Cyclodextrin has relatively low water solubility (~18.5 mg/mL), but chemical modifications of the basic β-cyclodextrin have imparted improved solubility and lower toxicity. Two of the modified β-cyclodextrins that have gained greater acceptance are hydroxypropyl-β-cyclodextrin (HP-β-CD) and sulfobutylether-β-cyclodextrin (SBE-β-CD). These have water solubilities of about 600 mg/mL and 500 mg/mL, respectively. Both of these modified cyclodextrins have been used in developing injectable formulations that are now FDA-approved products.

During preformulation studies, it is common to assess the solubility of a poorly soluble drug candidate in such cyclodextrins. If solubilization via cyclodextrin complexation is identified as a potential formulation approach, then it is also important to fully characterize the interactions in terms of stoichiometry of the complex as well as the equilibrium constant for the complexation. A number of excellent reviews cover the theoretical and experimental considerations for such determinations in detail (8–10).

Stability and Drug Degradation

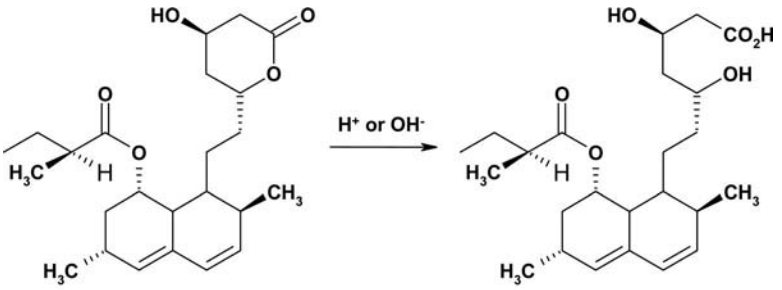
In addition to solubility, stability of the active drug compound is a key determinant in the viability of parenteral drug product. First, it is essential for a drug product to maintain potency relative to label claim over the shelf life to deliver an accurate dose. Second, degradation in the drug product can result in changes in appearance (color, precipitation) or bioavailability. Finally, degradation of the active compound can result in degradation products that may have toxicity that is more significant than that of the active drug substance. Depending on the daily

dose of the active and levels of such degradation products anticipated in a drug product, they may be subject to additional toxicological qualifications as described in ICH Guidance Q3B (R2) (11). When impurities or degradation products are identified as potentially genotoxic, they have to be controlled to very low levels if not completely avoided. This process is detailed in the EMEA Guidance on Limits for Genotoxic Impurities (12). Thus, it is essential to understand the stability and degradation of the active ingredient as a bulk drug substance and in formulation. Understanding the degradation pathways, kinetics, and mechanisms leads to development of a stable drug product (13,14).

During preformulation studies, the goal is to understand the modes of instability of a drug compound, kinetics of degradation, and factors (including formulation factors) influencing the kinetics of such degradation (15). One of the first steps is to develop a stability-indicating method that is capable of resolving and quantifying impurities and degradation products resulting from the drug compound. Typically, HPLC with UV detection is used in preformulation studies, but techniques such as LC-MS and NMR spectroscopy could often aid in the identification of degradation products. HPLC methods are developed to effectively resolve degradation products resulting from forced degradation studies (highly stressed condition of temperature, humidity, or pH).

Modes of degradation. Chemical degradation of small-molecule drugs can occur because of various chemical processes. However, a majority of these fall into three types of reactions.

Hydrolysis This is a very common pathway for drug degradation (16) and is essentially the cleavage of a molecule under the effect of water. Since water, either as a solvent or in the form of moisture in the air, is ubiquitous, the potential for this degradation pathway exists for most drugs. This is of particular relevance to parenteral products, which are mostly formulated in aqueous systems. Chemical bonds that commonly undergo hydrolytic degradation include lactam, ester, amide, and imide bonds. Aspirin is the most common example of a drug undergoing hydrolytic degradation. Lovastatin is a prodrug that undergoes activation through hydrolysis by carboxyesterases in vivo. In vitro it undergoes hydrolysis under acidic and basic conditions by cleavage of the lactone.



lovastatin
(hypolipidemic agent)

Hydrolytic reactions can be significantly influenced by the composition of the medium—pH, buffer concentration, ionic strength, etc. The relationship of the rate of reaction (expressed as rate constant k_{obs}) with pH is quite informative both for understanding the mechanisms involved as well as a guide for formulation. When the reaction is catalyzed by the hydronium (H^+) ion, it results in a slope of negative one on a log k_{obs} versus pH profile, and similarly, when the reaction is catalyzed by the hydroxide ion (OH^-), a slope of one is observed. If no other catalyses are involved, then these two lines meet, forming a V-shaped profile. The pH at which they meet represents pH of maximum stability and is important to know during selection of formulation pH. The shape of curves can be more complicated (U shaped, additional inflections, etc.) depending on the number of ionic species involved (15,17).

In addition to the pH of the medium, concentration of the buffer itself can play a catalytic role in hydrolysis. This can be studied by studying the reaction rate as a function of buffer type and concentration while holding the pH constant. Ester hydrolysis of an experimental compound GW280430A was shown to be catalyzed by citrate, malate, and tartrate buffers but not by a glycine buffer (18). This phenomenon is termed as general acid/base catalysis or buffer catalysis and can often be the cause of deviation from a slope of -1 or $+1$ described in specific acid/base catalysis in the previous paragraph. Additionally, reactions can also be affected by ionic strength, which can be studied by holding the pH and buffer concentration constant and studying the reaction rate as a function of concentration of added ions (e.g., NaCl). Typically, this is not a big effect in pharmaceutical systems.

In summary, hydrolysis is a key degradation pathway for many drug compounds. pH-stability profiles can vary from a simple V shape to more complex profiles depending on the number of ionization states and the different reactivities they present. While some of the pathways can be predicted on the basis of the structure, evaluation of the pH-stability profile and effect of buffer catalysis can be very important in designing the formulation strategy.

Oxidation Oxidation is another common mode of drug degradation. Oxidation can be broadly defined as a loss of electrons in a system; alternately, it could be considered as an increase in oxygen or a decrease in hydrogen atoms. The reaction occurs in concert with reduction of the other reactant, thus forming a redox reaction. If molecular oxygen is involved in the reaction, this is termed as "auto-oxidation." Trace metals and light can catalyze oxidation reactions by initiating free radical chain reactions. Once formed, the radical can be propagated until a termination reaction or a suitable chemical inhibitor intervenes. These reactions can happen in aqueous and nonaqueous media.

Excipients used in formulation can be a source of trace metals and also peroxides, which can have significant effect on oxidative drug degradation. Table 3 shows levels of hydroperoxides measured in some commonly used pharmaceutical excipients (19).

To control oxidation reactions, antioxidants are often included in a formulation. Antioxidants used in a formulation could affect different stages of an oxidation reaction. True antioxidants (e.g., butylated hydroxy toluene, α-tocopherol) react with free radicals, resulting in termination of the chain reaction. Reducing agents (e.g., ascorbic acid) get preferentially oxidized and hence reduce the level of oxygen or the oxidant in the formulation. Chelating agents such as EDTA sequester trace metals which can catalyze oxidation and thereby function as antioxidant synergistic agents. Depending on the reaction involved, a combination of such agents may help control the oxidative degradation (20). Also, during manufacturing and in the primary package, an inert atmosphere generated by nitrogen blanketing can help control oxidative degradation.

Photolysis Photolysis, also referred to as photodegradation, occurs as a result of absorption of light (or radiation energy) (21). When the absorbed energy dissipates through a chemical change in the molecule, photolysis occurs. The changes may result in a color change, precipitate formation or may not be visually detectable. However, there is always loss of potency that is accompanied. Toxicity of the decomposition products is also of concern,

Table 3 Levels of HPO in Some Commonly Used Excipients

Excipient	Number of batches tested	Average HPO (nmol/gm)	Range of HPO (nmol/gm)
Polyvinylpyrrolidone	5	7,300	3,600–11,000
Polyethylene glycol 400	4	2,200	1,000–3,300
Polysorbate 80	8	1,500	180–4,600
Poloxamer[a]	7	30	10–50
Mannitol	5	<10	<10
Sucrose	5	<10	<10–20

[a]Different grades (188, 338, and 407) and batches tested.
Abbreviation: HPO, hydroperoxides.

especially when such products can form by the action of sunlight on the skin or eyes after administration (phototoxicity) (22).

Photodegradation depends on wavelength of the incident light as well as intensity. Primary photochemical reactions usually occur at wavelengths where the drug absorbs light, that is, in regions where the UV/VIS absorption spectrum of the drug overlaps with the spectrum of incident radiation. In some instances it is possible that the energy absorbed by a nondrug molecule (photosensitizer) in the formulation is transferred to the drug molecule, which eventually degrades. Examples of some common drugs that undergo photolytic degradation include methotrexate, furosemide, and tetracyclines. For many drug substances, the kinetics of photodegradation varies significantly with the ionization state of the molecule. Examples would include ciprofloxacin, midazolam, mefloquine, and ameloride (23).

Once a photoinstability is identified, it can be addressed during formulation development through different means. A protective market pack is one of the simplest solutions. Control of pH, ionic strength, trace metals, or even use of complexants (24) can be formulation approaches to also address such instability.

In addition to these major modes of degradation, many other routes are involved in drug degradation such as decarboxylation, racemization/epimerization, acylation, etc. Understanding the causes of drug instability allows for a rational design of a formulation.

Preformulation stability studies. Typically, the drug substance is studied in solid as well as solution states. Stability studies might involve storing the samples under stressed conditions of temperature and humidity such as 40°C/75% RH and 50°C. If the drug is fairly stable, conditions such as 80°C/75% RH and 80°C may be employed to get a first view of drug instability in a reasonable amount of time. These studies are conducted over a short duration such as four to six weeks.

Additionally, the solid drug and an aqueous solution of the drug are exposed to a representative duration and intensity of light in appropriate photostability chambers [as per ICH Q1B (25)]. These studies may be able to indicate not only potential need for protecting the drug product from light but also the need for conducting other stability studies under light-protected conditions. Failure to know this early can produce confounding results.

pH-stability profiles are determined by preparing aqueous solutions of the drug at various pH values ranging from 2 to 12 and studying the kinetics of degradation (loss of active/growth of degradation products) at an appropriate elevated temperature. The solutions are sampled at regular intervals and analyzed using a stability-indicating method. The time course of degradation at a particular pH can typically be expressed as the first-order rate constant k_{obs} (k observed). A log k_{obs} versus pH plot is referred to as the pH-rate profile and can be quite revealing of the mechanisms involved in drug degradation. The pH of maximum stability would be targeted as the pH for the formulation as long as it agrees with the required solubility and local tolerability at that pH.

Form Selection

The solid form of the drug compound can have a significant effect on parenteral drug product processing. During late discovery or early development stages, the solid form of the drug compound needs to be defined and fixed to develop formulations and processes consistent with the expected physical and chemical properties of the API. The solid form is typically described by the salt form used and the crystal polymorph of the chosen salt.

Salt Form

Many drugs are either weak acids or weak bases and can consequently form a range of salts by reacting with various bases and acids, respectively. Salt formation may be employed to alter the physicochemical, biopharmaceutical, and processing properties of a drug substance without modifying the pharmacologically relevant moiety (26).

To form stable salts, the pK_a of the basic center should be greater ($\Delta pK_a \geq 2$) than the pK_a of the conjugate acid to be utilized. Thus, for a basic drug, pK_a of the basic center will determine what salts are feasible.

In the case of parenteral medications, increased solubility is often desired from chosen salts. In general, utilizing counterions with greater acidity, utilizing more hydrophilic counterions (hydroxy acids), and lowering the melting point of the resultant salt (decreased crystal lattice energy) can result in increased solubility. Agharkar et al. (27) demonstrated an increased solubility of an experimental antimalarial drug as a result of decreased crystal lattice energy due to salt formation.

In the case of solution formulations, it is not essential that salt formation is only employed for obtaining a suitable solid form. Salts can be formed in situ in solutions by using the appropriate acid or base to adjust pH of the formulation (28). Sometimes the high aqueous solubility achieved prevents a salt from being easily isolated but can still be utilized as an effective solubilization approach, as previously discussed in the context of pH-solubility profiles.

Polymorph Selection

Polymorphism is defined as the ability of a substance (of constant chemical composition) to exist in two or more crystalline phases that differ in crystal packing arrangement and/or conformation of the molecules in the crystal lattice. The different crystalline forms are then termed as polymorphs.

Crystals are made up of repeating blocks called unit cells. Different polymorphs have distinct unit cells. Polymorphs can differ in various physical, physicochemical, and physicomechanical properties. Differences such as melting point, enthalpy of melt, true density, and powder X-ray diffraction patterns help characterize and differentiate between polymorphs. One can screen for polymorphs by crystallizing a drug from different systems of solvents, evaporation and cooling profiles, and then examining crystals obtained. However, it is not easy to search exhaustively for all possible crystal forms, and often new forms are discovered during development. To reduce the risk, many automated crystallization systems have been developed, which help examine a larger experimental space.

Polymorphism is commonly of concern in the context of solid dosage form bioavailability and processing (29). However, polymorphs also differ in properties that impact a parenteral drug product formulation of which solubility, dissolution rate, and hygroscopicity are of most relevance. Polymorphs differ in their free energy as a result of their packing, and this manifests itself as differences in solubility. The most stable polymorphic form has the lowest solubility. If a metastable polymorph is used in a solution or suspension formulation, there will be a risk of growing crystals of the stable form over a period of time. Solvent maturation studies and temperature cycling of prototype formulations can help identify such problems early.

When a solvent molecule incorporates itself into a crystal lattice associated with a drug compound, it is said to form a solvate. When this solvent is water, it is termed as a hydrate. A hydrate form of the drug is more stable than an anhydrous form and will exhibit lower solubility in an aqueous system. Thus, it is also important to understand and characterize solvate and hydrate forms of the drug compound.

Characterization of Material Properties

Appearance and Microscopy

The solid form of a drug substance is characterized by its appearance in terms of color and subjective description. Additionally, examination under a microscope reveals further details such as crystal morphology and habit.

Crystallinity

Crystalline material can be identified by polarized light optical microscopy where the sample displays birefringence. Crystallinity is also commonly examined by XRPD. An X-ray diffraction pattern is generated because of constructive and destructive interference of X rays reflected off the crystal planes of a powder sample as the angle of incidence is varied. This is described by the Bragg equation.

$$n\lambda = 2d \sin\theta \tag{8}$$

where θ is the incident angle, λ is the wavelength of the X radiation, d is the distance between the crystal planes, and n is an integer representing the order of reflection.

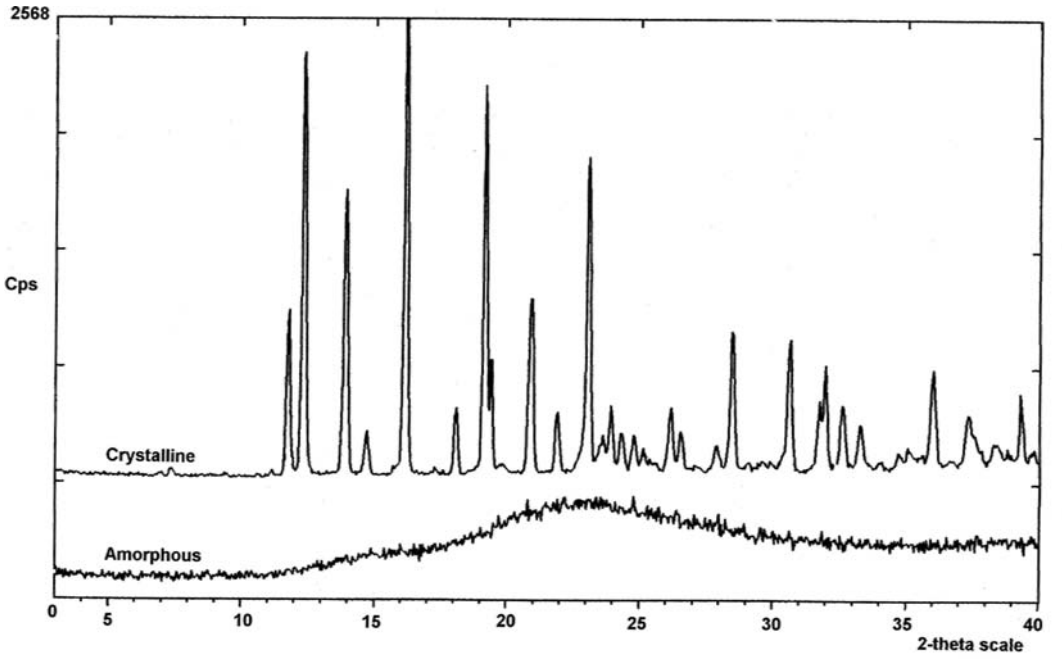


Figure 2 X-ray powder diffraction patterns showing amorphous and crystalline states of an experimental drug compound.

Crystalline forms are characterized by sharp characteristic peaks, while an amorphous material displays a broad halo (Fig. 2) (30). XRPD can be used to distinguish between different polymorphs, solvates, and hydrates. Further, this technique can also be used to quantify mixtures of polymorphs and degree of crystallinity of a crystal form.

Thermal Properties

DSC measures the difference in the amount of heat required to raise the temperature of a sample and a reference as a function of a change in temperature. A typical output shows heat flow into (endothermic event) or out of (exothermic event) the sample as a function of temperature. Melting of a crystalline material is observed as an endothermic event characterized by an onset temperature (melting point) and heat of fusion measured as the area under the endothermic curve. At the glass transition temperature, amorphous materials undergo a transition from a glassy rigid state to a rubbery state of greater mobility (a higher heat capacity), and this is observed on the DSC as a baseline shift characterized by temperature (T_g) and change in heat capacity (ΔC_p). The glass transition is sometimes followed by a small endotherm of enthalpic relaxation related to time-dependent relaxation of this phase. Figure 3 shows the DSC thermogram of an experimental drug compound displaying these transitions along with an overlay of corresponding changes to the X-ray diffraction patterns as observed by variable-temperature XRPD (31).

Modulated DSC (mDSC) is a related technique where an oscillation of temperature is introduced on top of a linear heating rate. This allows deconvolution of the output into reversing (thermodynamic) and nonreversing (kinetic) components, allowing a further understanding of the transitions measured. This can be of particular utility in studying amorphous materials (29).

Thermogravimetric analysis (TGA) measures the weight of the sample as a function of increasing temperature. Loss of water, solvents, or volatile decomposition products can be observed as a weight loss at characteristic temperatures. This analysis is a key technique in characterizing solvates and hydrates. The technique is sometimes further coupled with an IR spectrometer or a mass spectrometer to characterize the evolved volatile components that come off during heating of the sample.

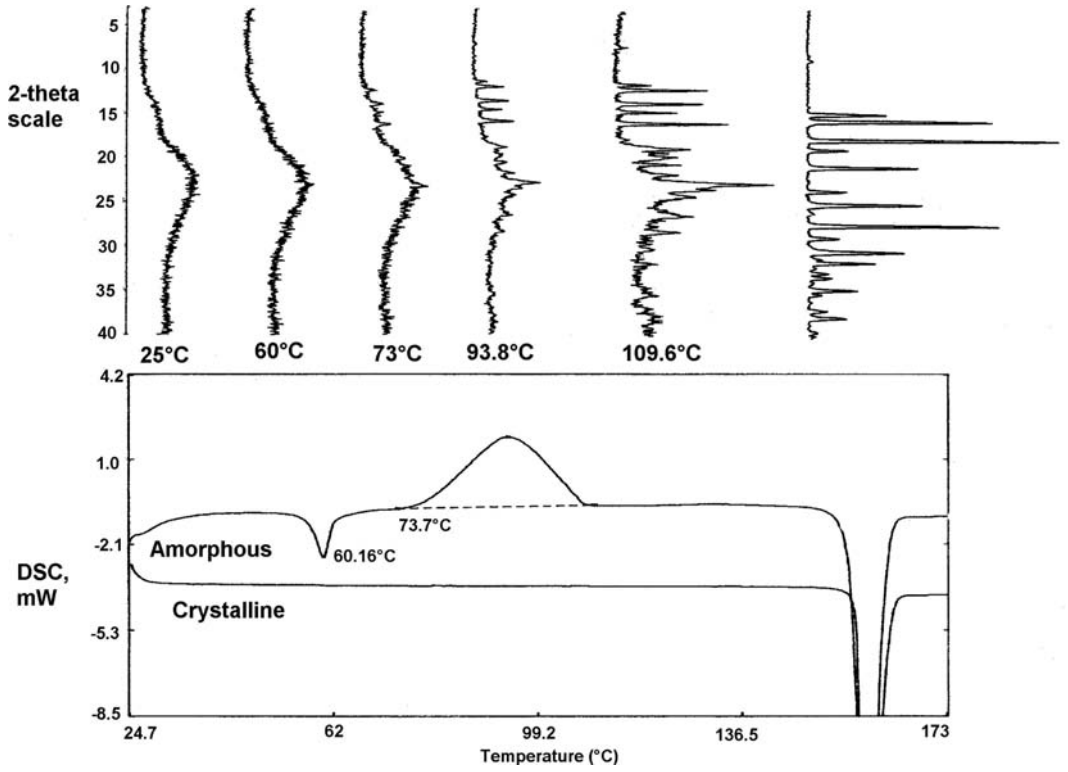


Figure 3 DSC curves of crystalline and amorphous phases of an experimental drug compound overlaid with XRD patterns of the amorphous phase obtained at temperatures corresponding to thermal events in the DSC curve. *Abbreviations*: DSC, differential scanning calorimetry; XRD, X-ray diffraction.

Vapor (moisture) Sorption Analysis

The weight of the sample is monitored as it is exposed to different relative humidities for a period of time approaching equilibrium. The output is a moisture sorption profile, which depicts the sample weight as a function of relative humidity. When a material picks up enough water that causes a change in its physical properties, the material is considered hygroscopic. Crystalline materials typically *adsorb* small amounts of water on the surface unless they pick up water molecules into the crystal lattice to form hydrates. Hydrates are characterized by picking up stoichiometric amounts of water and are physically stable over a range of %RH. Deliquescence occurs when the material adsorbs enough water to dissolve into it thereby turning liquid. This can sometimes happen with salts of hydrophilic molecules and is characterized by a sharp increase in moisture uptake at humidity values greater than a threshold %RH.

Amorphous materials *absorb* water and other solvents into the bulk. The absorbed solvent acts as a plasticizer and reduces the apparent glass transition temperature. When the apparent glass transition temperature drops below storage temperature, the material goes into a mobile rubbery state from which collapse of the structure (liquefaction) with possible recrystallization can occur. This relationship of glass transition temperature as a function of absorbed water is critical to understand when developing a lyophilization process.

INTERACTION BETWEEN THE DRUG SUBSTANCE AND FORMULATION COMPONENTS

Formulation Components

In formulating a parenteral drug product, a number of excipients are employed, and these often form the bulk of a drug product. These excipients are included to dissolve the drug substance, increase the chemical or physical stability of the drug product, give the product

Table 4 Excipients Used in Parenteral Formulations

Solvents and cosolvents
- Glycerin
- Propylene glycol
- Ethanol
- Polyethylene glycol (300, 400)
- *N,N*-dimethylacetamide
- Soyabean oil
- Corn oil
- Ethyl oleate
- Glycofurol

Surfactants (solubilizers, emulsifiers, and suspending agents)
- Polysorbate 80 (Tween 80)
- Polysorbate 20 (Tween 20)
- Polyoxyethylene-polyoxypropylene copolymers (poloxamers)
- Cremophor EL
- Lecithin

Complexants
- Hydroxypropyl-β-cyclodextrin
- Sulfobutylether-β-cyclodextrin (Captisol®)

Buffers
- Citrate
- Phosphate
- Tartrate
- Tromethamine (TRIS)

Chelating agents
- Disodium ethylenediaminetetraacetic acid

Antioxidants
- Ascorbic acid
- Butylated hydroxy anisole
- Butylated hydroxyl toluene
- Sodium bisulfite
- Propyl gallate
- α-Tocopherol

Preservatives
- Benzalkonium chloride
- Benzethonium chloride
- Benzyl alcohol
- Chlorbutanol
- Paraben (methyl, propyl)
- Thimerosal

Tonicity adjusters, bulking agents, lyoprotectants
- Sodium chloride
- Mannitol
- Glycine
- Sucrose
- Trehalose
- Dextran
- Povidone

microbiological protection, or control other product attributes. Since inclusion of new additives could require extensive pharmacological and toxicological evaluation, it is common for formulators to depend on materials already used in marketed parenteral products. Table 4 shows a representation of the classes of excipients that might be used in parenteral formulations and some examples of each of these categories. There is more discussion within this book on the functions and levels of these excipients. Additionally, the reader can refer to some excellent reviews that have been published on this topic (31,32). The FDA also maintains a listing of inactive ingredients used in approved products (33).

Designing Excipient Compatibility Studies

Excipients are often referred to as inactive or inert ingredients to distinguish them from the APIs. However, the lack of pharmacological activity does not necessarily result in a lack of chemical reactivity. Excipients can have significant expected and unexpected effects on the physical and chemical stabilities of a drug product. This is first assessed through well-designed excipient compatibility studies conducted at the preformulation stage (34).

Traditionally, thermal methods such as DSC have been employed as a first screen in determining incompatibilities (35). In these studies, the drug, excipient, and drug-excipient mixture are subjected to a temperature program. If the thermogram of the mixture is not representative (temperature and enthalpy) of the combination of the two single components, then an incompatibility could be suspected. Modifications such as a stepwise isothermal high-sensitivity DSC study have also been tried (36). However, DSC techniques have proved to be of limited predictability.

Isothermal heat conduction calorimetry is a technique that measures heat evolved or absorbed by a sample (relative to a suitable reference) with great sensitivity. Hence, even slow reactions occurring under isothermal (25°C, 45°C/75% RH) can be detected because of the

Table 5 A Plackett–Burman Design

	Variable											Response
Trial	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	Y
1	+	+	−	+	+	+	−	−	−	+	−	
2	+	−	+	+	+	−	−	−	+	−	+	
3	−	+	+	+	−	−	−	+	−	+	+	
4	+	+	+	−	−	−	+	−	+	+	−	
5	+	+	−	−	−	+	−	+	+	−	+	
6	+	−	−	−	+	−	+	+	−	+	+	
7	−	−	−	+	−	+	+	−	+	+	+	
8	−	−	+	−	+	+	−	+	+	+	−	
9	−	+	−	+	+	−	+	+	+	−	−	
10	+	−	+	+	−	+	+	+	−	−	−	
11	−	+	+	−	+	+	+	−	−	−	+	
12	−	−	−	−	−	−	−	−	−	−	−	

sensitivity of the technique (37). This technique has been used to compare the heat signal from a drug-excipient mixture with the sum of the curves generated by the individual components under the same conditions. The magnitude of this interaction curve (difference curve) is an indicator of the extent of the incompatibility (38). However, this technique generally suffers from the fact that it is nonspecific and it is important to carefully design appropriate control experiments to make sure that the recorded heat pertains to a specific chemical incompatibility.

Given some of the challenges described above, the conventional method of chemical analysis of mixtures stored under accelerated storage conditions is still the most commonly employed method. The prerequisite for this methodology is having a stability-indicating method, and most commonly, this is an HPLC method. Since in a parenteral product, the drug and excipients are in very close contact (at a molecular level in the case of solution products) with each other, the stabilizing or destabilizing effect of an excipient is best studied in the presence of all formulation components (prototype formulations) including the targeted primary packaging when possible. High and low levels of each excipient or formulation factor are identified for testing on the basis of conventional levels used in experience or levels approved for use by regulatory authorities.

Different experimental designs can be used for obtaining the required information from a limited number of experimental runs. In such studies excipients constitute factors (at two levels—high and low) in a factorial design of experiments. For such studies screening design is employed at first. A commonly used screening design is a fractional factorial design called a Plackett–Burman design. Table 5 represents a possible design for studying 11 factors by performing 12 trials. This design was employed for a parenteral preformulation study for Naproxen as described by Peswani and Lalla (39). In this study they looked at effects of five excipients, pH, buffer type, autoclaving, and nitrogen blanketing by conducting 12 trials. Although these designs are quite efficient in terms of number of trials, it should be noted that these designs are not capable of identifying interaction terms (e.g., if two factors interact to produce an effect). If such confounding is suspected and needs to be resolved, a full factorial design study could be conducted on a smaller number of identified factors. The reader can get details of the advantages and disadvantages of different experimental designs from other reviews of this specific topic (40).

INTERACTION OF THE DRUG WITH PACKAGING COMPONENTS AND MANUFACTURING SURFACES

Parenteral drug products are in close contact with the primary package of the drug product; so it is useful to carefully consider primary package in the same way other formulation ingredients are evaluated. These packaging materials would include glass vials (or ampoules),

rubber stoppers, infusion bags, etc. Glass vials are most commonly type I (borosilicate glass), but that too can undergo different surface treatments at the manufacturer. Rubber stoppers (commonly butyl or other synthetic rubber and rarely natural rubber because of its sensitizing potential) and bag materials can be quite complex in composition. The formulation scientist works closely with the rubber manufacturer as with the glass manufacturer to choose the appropriate rubber formulation having consistent specifications and characteristics to maintain product stability. It is important during preformulation studies to include an evaluation of likely primary packaging materials to assess potential issues such as adsorption and incompatibilities. Also important to consider are other likely surfaces to be encountered during manufacturing steps, for example, stainless steel, glass, tubing, and filters.

Adsorption

Adsorption occurs when a molecule is attached to another solid surface, most commonly because of Van der Waals forces, hydrogen bonding, or electrostatic interactions. This can often occur with low-solubility hydrophobic compounds as they may prefer another surface as opposed to being in water. When a covalent bond is involved, the adsorption is chemisorption, but this is not commonly observed in the systems being discussed here.

To evaluate adsorption, the formulation (at the most dilute concentration likely) is exposed to the surface and then assayed for loss of drug concentration. For filters and tubing, this might involve passing through the tubing and filters for a fixed duration of time that will exceed the likely duration of a manufacturing run. For stoppers, it might be done by adding a fixed number of stoppers to flasks containing the formulation and storing for a fixed period of time before assaying the concentration. During development of an injectable formulation of Abbott-72517, Gupta et al. observed a 6% of loss of drug (250 mL recirculated for four hours) using a Pall Nylon 66® filter but no loss with a Millipore Durapore disk membrane (41). If adsorption to potential surfaces is identified early on, then it can be used to select appropriate materials for packaging and manufacturing processes.

Compatibility

In addition to adsorption, the degradation of the drug molecule can also be effected by packaging material or manufacturing surfaces. Thus, when feasible, it is useful to conduct excipient compatibility studies using preferred container closure systems. An early readout on any potential incompatibility can lead to an early assessment of alternatives and prevent the loss of time during development. For instance, rubber stoppers can leach out trace quantities of zinc into the formulation and effect oxidation of the drug. If a drug is particularly prone to oxidation, a steel surface may aggravate the issue and a glass-lined tank may be an appropriate measure. Protein drugs could be especially sensitive to silicone that is used on rubber stoppers. A nonsiliconized rubber with a bonded coating may be the answer to the issue.

SPECIALIZED FORMULATIONS

Suspensions and Nanosuspensions

Sterile injectable suspensions comprise of the active compound dispersed in a liquid vehicle either as a ready-to-use formulation or as a dry powder for reconstitution. Such formulations may be engaged either when the drug has solubility that is too low for a solution formulation or for prolonging the release of the drug through depot formulations. Aristocort® is a suspension of triamcinolone diacetate and may be administered by the intramuscular, intra-articular, or intrasynovial routes depending on the situation (42). NPH insulin is a suspension of crystalline zinc insulin combined with the positively charged polypeptide protamine. When injected subcutaneously, it has an intermediate duration of action. Depo-Medrol® is an anti-inflammatory glucocorticoid for intramuscular, intra-articular, soft-tissue, or intralesional injection. One of the challenges of formulating such products involves an evaluation of suspension physical stability with regard to resuspendability and caking.

Another area of specific concern for suspensions is syringeability (drawing a uniform dose) and injectability (pressure applied to expel product through a needle of specified gauge) of the product. The flow properties of the suspension can be characterized using techniques such as rheometry. This technique characterizes the flow of a fluid in response to a range of

applied stresses, resultant strains, and temperatures. Many suspensions and emulsions do not show a linear relationship between applied stress and strain (non-Newtonian behavior) and hence cannot be characterized by a single value for viscosity. A full discussion of this topic is out of the scope of this chapter and is well captured in many reviews on this topic.

For suspension formulations, the solid-state properties are quite relevant. Particle size of the dispersed phase can have a significant impact on the physical stability and syringeability of a suspension. Particle size distributions in suspensions can change over time because of Ostwald ripening—a solution-mediated phenomenon during which larger particles grow at the expense of smaller particles dissolving. An appropriately selected medium and surfactant can minimize the impact of this phenomenon. During screening, subjecting prototype samples to temperature cycling can accelerate the event and help select systems that are the most stabilizing. Crystal growth can also occur because of a more stable polymorph precipitating or a salt being formed. A change in crystal habit can result in significant effects on syringeability and injectability. Hence, there is a greater emphasis to fully understand the solid properties of the drug being formulated as a suspension as opposed to a solution product.

Lately, there has been a growing interest in formulating poorly soluble drugs as nanoparticulate suspensions (43). For compounds that exhibit poor solubility in aqueous and oily vehicles, nanosuspensions could be a preferred formulation option resulting in improved bioavailability. Nanoparticles also form an interesting platform for attaching targeting moieties. Nanoparticles are produced by "top-down" (media milling) techniques (44) or by "bottoms-up" (controlled crystallization) approaches (45). More recently, there have been reports of generating engineered nanoparticles by printing techniques (46).

Well-formulated nanosuspensions are typically nonsettling and hence circumvent some of the concerns mentioned previously with conventional suspension formulations. In such formulations the natural tendency of these small particles to aggregate is overcome by a careful selection of stabilizers, which could include a mix of surfactants and polymers. Compatibility of the drug with a range of possible surfactants and polymers needs to be assessed in parallel to selecting the best options for stabilization. As in conventional suspensions, Ostwald ripening and crystal growth is a concern, and gaining a good understanding of the solid-state properties of the drug is very relevant. Prototype nanosuspensions can be stressed by temperature cycling and freeze-thaw studies to establish their physical stability. It is also useful to assess the physical and chemical stability of the formulated drug to autoclaving conditions to define the strategy for sterilization.

Emulsions

Injectable emulsions have been most commonly used for long-term parenteral nutrition (Intralipid®, Lipofundin®). However, emulsions can also be good carriers of drug substances with good lipid solubility (high log *P*) and poor aqueous solubility (47). Propofol (Diprivan®) and diazepam (Diazemul®) are examples of drugs formulated as emulsions (33), and there are reports on studies conducted with Taxol emulsions (48). With the increased interest in injectable lipid emulsions, there is also a greater awareness of safety issues surrounding such delivery (49).

Typical emulsion formulations consist of oils (long- and medium-chain triglycerides or high-quality food grade oils), emulsifiers (e.g., lecithins, poloxamers, Tweens, and Spans) and an aqueous phase containing appropriate additives to control pH, tonicity, etc. Antioxidants such as α-tocopherol could be included in the oil phase to prevent oxidation of the oils. The emulsions are typically prepared by dissolving the appropriate ingredients in the oil phase and water phase and then homogenizing (e.g., Microfluidizer®, Silverson® homogenizer) the two to obtain the emulsion.

Some attributes to be studied in the specific context of emulsion formulations include assessment of particle (droplet) size and surface charge. Droplet surface charge is measured in terms of the zeta potential. Essentially, zeta potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle. The zeta potential is determined using instruments that measure the elecrophoretic mobility of the particles. The surface change on droplets stabilizes emulsions because of electrostatic repulsion, which prevents coalescence of droplets. A zeta potential of ±30 mV or higher can

help stabilize a colloidal system. Measurement of zeta potential is equally useful while formulating suspensions and nanosuspensions.

SPECIFICITIES RELATED TO BIOLOGICS

Biotherapeutic molecules could range from small oligonucleotides or peptides synthesized using techniques such as solid-phase synthesis to proteins (including interferons, soluble receptors, antibodies, etc.) with tertiary and quaternary structures, which are often produced via genetic engineering technologies. Small oligonucleotides and peptides can often be formulated and analyzed by techniques similar to small molecules. More specialized analytical techniques and formulation considerations are needed for larger proteins. From a preformulation perspective, the goals are the same—to characterize the drug compound and understand the solubility and stability of the drug as well as the interactions with potential excipients that would be used to formulate the drug compound. Early results may determine the formulation strategy of either a ready-to-use solution or a lyophilized product for reconstitution. On the basis of this strategy, additional preformulation studies may be needed to support the formulation choice.

Characterization

In addition to the conventional characterization described earlier in the chapter, additional parameters relevant to protein drugs need to be assessed (50). These include determination of molecular weight, amino acid sequence, and disulfide bonds. Because of a large number of charged groups, proteins are generally soluble in water but can be physically unstable at high concentrations because of their complex interaction with surrounding water. Proteins are zwitterionic in nature as a consequence of the amino and carboxylic groups of individual amino acids. At low pH values, proteins would have a net positive charge, and at higher pH values, due to ionization of the carboxyl groups, they carry a net negative charge. The isoelectric point, pI, is the pH of an aqueous solution of a peptide (or protein) at which the molecules on average have no net charge. In other words, the positively charged groups are exactly balanced by the negatively charged groups. This is an important parameter, which is most commonly determined using an electrophoresis technique called isoelectric focusing.

From a solid-state point of view, protein drugs are frequently amorphous and quite hygroscopic. For large proteins made by genetic engineering technologies, it is also quite common not to routinely isolate the protein as a solid but to hold it in a solution or frozen buffered and stabilized solution.

Stability

The pharmacological activity of proteins and peptides is largely dependent on their intact primary, secondary, tertiary, and quaternary structures. Proteins and peptides are quite fragile and can undergo physical and chemical degradation under a variety of conditions.

Chemical Stability

Chemical degradation can be triggered by changes in temperature, pH, oxygen levels, and trace metals and under the influence of light. Methionine, cysteine, tryptophane, and histidine residues can undergo oxidation under the influence of trace metals and light and higher levels of oxygen. Hydrolysis of the side chains of asparagine and glutamine residues can result in deamidation reaction. Hydrolysis of the amide bond in the protein backbone is another degradation route, which is mainly influenced by the solution pH. β-elimination of cysteine, serine, threonine, and lysine residues is also affected by the solution pH, temperature, and ionic composition.

To characterize the degradation pathways, a multitude of analytical techniques are employed. These include different sequencing (*N*-terminal sequencing), spectroscopic (UV spectral analysis), separation (e.g., ion exchange, reverse phase, gel electrophoresis with protein staining, isoelectric focusing) of the intact proteins or enzymatically digested proteins (peptide map), and mass spectroscopic analysis of proteins to define the chemical modifications occurring. Circular dichroism is used to assess secondary and tertiary structures.

Physical Stability

Native protein structures are not very thermodynamically stable. Proteins easily unfold (denaturation) under the influence of increased temperature and concentration, pH change, buffer species, or chemical and physical stress. Completely or partially unfolded proteins can associate to form irreversible aggregates. Aggregation is not necessarily visible to the eye, but with increasing aggregation, aggregate size increases, and eventually, precipitation can occur, which is clearly visible.

Fluorescence measurements, light scattering techniques (sometimes in combination with reverse-phase or size exclusion chromatographic separation) and field flow fractionation can be used to assess aggregation. Conformational changes leading to aggregation can also be measured by DSC.

Protein unfolding, adsorption to surfaces, and aggregation can be modulated by pH, buffer species, choice of preservatives, and use of appropriate surfactants and stabilizers (sugars) in the formulation. The formulation factors have to be tailored to individual proteins through well-executed studies evaluating formulation, processing, and storage conditions. Other chapters in this book cover protein characterization and formulation aspects in detail.

SUMMARY

The aim of preformulation studies is to gain a thorough understanding of the drug molecule, its physical and chemical properties, as well as its interaction with other formulation ingredients and packaging materials to drive a rational formulation design. This chapter has provided an overview of preformulation studies related to development of parenteral medications.

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5 Formulation development of small and large volume injections

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INTRODUCTION

As described in the U.S. Pharmacopeia, USP-32/NF-27 (1), an injection is a preparation intended for parenteral administration and/or for constituting or diluting a parenteral article prior to administration. It is administered through the skin or other external boundary tissue, rather than through the alimentary canal, so that therapeutic substances, using gravity or force, can gain direct entry to a blood vessel, organ, tissue, or lesion. Parenteral products are required to meet pharmacopeial requirements for sterility, pyrogens, particulate matter, and other contaminants, and, where appropriate, contain inhibitors of the growth of microorganisms.

The USP (1) categorizes sterile preparations for parenteral use according to the physical state of the product as follows:

1. Liquid preparations that are drug substances or solutions thereof, for example, [drug] injection.
2. Dry solids that, upon the addition of suitable vehicles, yield solutions conforming in all respects to the requirements for injections, for example, [drug] for injection.
3. Liquid preparations of drug substances dissolved or dispersed in a suitable emulsion medium, for example, [drug] injectable emulsion.
4. Liquid preparations of solids suspended in a suitable liquid medium, for example, [drug] injectable suspension.
5. Dry solids that, upon the addition of suitable vehicles, yield preparations conforming in all respects to the requirements for injectable suspensions, for example, [drug] for injectable suspension.

Depending on the volume of injection in a package, the USP further designates injection, as either (*i*) small-volume injections or (*ii*) large-volume intravenous (IV) solutions. The term small-volume injection applies to an injection that is packaged in containers labeled as containing 100 mL or less. The large-volume IV solution applies to a single-dose injection that is intended for IV use and is packaged in containers labeled as containing more than 100 mL. Although the term sterile pharmaceutical is applicable to all injections (radiopharmaceuticals included), ophthalmic preparations, and irrigating solutions, this chapter emphasizes the formulation of injectable dosage forms.

FORMULATION OF SMALL-VOLUME INJECTIONS

In terms of number, the small-volume injections constitute the vast majority of all the injectable products in the market - small and large-volume injections combined. Whereas, large volume injections are administered exclusively as IV infusion, the small-volume injectables can be given by IV as well as other routes, although dictated by the volume of injection, as described later.

The goal of formulation development is to have a product that addresses all four requisites of an ideal product from a patient point of view: It should be safe, efficacious, stable, and acceptable/tolerable. From the point of marketing and commercial economics, the product should be easy to manufacture, relatively easy to use or present, and should have optimum shelf life at convenient storage conditions, such as room temperature. Although the preferred goal of the formulation scientist is to develop an injectable formulation that is ready to use (such as an aqueous solution), a number of codependent factors must be carefully evaluated in determining the most appropriate type of formulation. These factors are a) Biopharmaceutical considerations, b) Solubility, and c) Stability.

Biopharmaceutical considerations are aimed at achieving the required drug concentration for pharmacological response and include the intended mode of administration, desired onset of action, and the dose required. The formulation - the drug itself and the excipients used - must be compatible with body tissues, particularly taking care of properties such as hemolysis potential, pain on injection, precipitation of the drug upon administration, etc. Sterility, lack of pyrogenicity, and absence of particulate matter are other important considerations from general safety point of view.

Solubility issues become important when the drug does not have sufficient water solubility to achieve the target concentration in the formulation at physiologically acceptable pH range of 3–10. Various solubilization strategies must be employed to increase the solubility to achieve the required deliverable dose in a minimum possible volume. These techniques include use of buffers, salt formation, use of cosolvent, use of surfactants, etc.

Stability considerations are aimed at developing a formulation that provides sufficient shelf life, which is generally considered to be the time for 10% degradation. The product is optimized in such a way that its intrinsic degradation pathways, for example, the commonly encountered hydrolysis or oxidation, are minimized by appropriate modification of formulation composition, many times by using added substances, such as buffers, chelating agents, etc.

The successful formulation of an injectable small-volume preparation requires knowledge and expertise to effect rational decisions regarding the selection of

1. a suitable vehicle (aqueous, nonaqueous, or cosolvent),
2. added substances (buffers, antioxidants, antimicrobial agents, buffers, chelating agents, tonicity contributors, etc.), and
3. the appropriate container and closure components.

During the course of product development, formulation optimization is an iterative process and evolves as the product moves from the discovery to clinical to commercial stages. Inherent in the above decisions is the obligatory concern for product safety, effectiveness, stability, and reliability. As the injection formulation is finalized, a number of additional supportive studies must be undertaken to establish ruggedness of the formulation.

The majority of parenteral products are aqueous solutions, preferred because of their physiological compatibility and versatility with regard to route of administration. Survey of USP (1) shows that out of >300 pharmacopeial injection entries, nearly 70% are aqueous formulations (a similar trend is expected for nonpharmacopeial products as well). However, cosolvents or nonaqueous substances are often required to affect solution and/or stability of many compounds. Furthermore, for some other compounds, the desired properties must be attained through the use of an alternate dosage form such as suspension, emulsion, or even newer approaches such as liposomes and nanosuspensions.

Although each of these dosage forms have distinctive characteristics and formulation requirements, certain physical-chemical principles are common. Those common principles will be discussed in a general manner and the differences distinctive of each system will be emphasized. It is important to recognize that the pharmaceutical products derived from biotechnology are on the increase and the formulation of these products requires some unique skills and novel approaches. Formulation development aspects of these products are described elsewhere (see chap. 9).

Formulation Principles

Influence of the Route of Administration

Since parenteral preparations are introduced directly into the intra- or extracellular fluid compartments, the lymphatic system, or the blood, the nature of the product and the desired pharmacological action are factors determining the particular route of administration to be employed. The desired route of administration, in turn, places certain requirements and limitations on the formulations as well as the devices used for administering the dosage forms. Consequently, a variety of routes of administration (see chap. 2) are used.

One of the most important considerations in formulating a parenteral product is the appropriate volume into which the drug should be incorporated. The IV route is the only route

in which there are no strict limits of the volumes and as much as fifty milliliters can be administered by the IV route, via hypodermic injection, and several liters can be administered over the course of several hours through an IV administration system. Volumes up to 10 mL can be administered intraspinally, while the intramuscular route is normally limited to 3 mL, subcutaneous to 2 mL and intradermal to 0.2 mL.

The choice of the solvent system or vehicle is directly related to the intended route of administration of the product. IV and intraspinal injections are generally restricted to dilute aqueous solutions, whereas oily solutions, cosolvent solutions, suspensions, and emulsions can be injected intramuscularly and/or subcutaneously.

Isotonicity is another factor that must be taken into consideration. Although isotonic solutions are less irritating, cause less toxicity, and eliminate the possibility of hemolysis, it is not essential that all injections be isotonic. In fact, for subcutaneous and intramuscular injections hypertonic solutions are often used to facilitate absorption of drug because of local effusion of tissue fluids. With IV solutions, isotonicity becomes less important as long as administration is slow enough to permit dilution or adjustment in the blood. However, intraspinal injections must be isotonic because of slow circulation of the cerebrospinal fluid in which abrupt changes of osmotic pressure can give rise to severe side effects.

New routes of administration include intraarticular, directly into the synovial fluid for rheumatoidal diseases and even intradigital, between the fingers, in order to better target the lymphatics. The parenteral routes of administration will influence the design of novel dosage forms and drug delivery systems especially as more potent agents from biotechnology are developed.

This chapter focuses on the physicochemical aspects of formulating a stable product in a suitable container recognizing that safety must be established through evaluation of toxicity, tissue tolerance, pyrogenicity, sterility, and tonicity, and efficacy must be demonstrated through controlled clinical investigations.

Selection of Vehicle

Most parenteral products are aqueous solutions. Chemically, the high dielectric constant (DC) of water makes it possible to dissolve ionizable electrolytes and its hydrogen-bonding potential facilitates the solution of alcohols, aldehydes, ketones, and amines. Water for injection (WFI) is the solvent of choice for making parenterals. When it is not possible to use 100% aqueous solution for physical or chemical reasons, other means of solubilization including the addition of solubilizing agents or cosolvents may be necessary. For instance, nonpolar substances (i.e., alkaloidal bases) possess limited solubility in water and it is necessary to add a cosolvent such as glycerin, ethanol, propylene glycol, or polyethylene glycol. In other cases, to prevent chemical degradation (i.e., hydrolysis, oxidation, decarboxylation, or racemization) water may have to be eliminated partially or totally. Most proteins and peptides require an aqueous environment, and the addition of salt, buffer, or other additives for solubility purposes often leads to conformational changes. Consequently, parenteral product formulators should be aware of not only the nature of the solvent and solute in parenterals but also the solvent-solute interactions and the route of administration. Typically, aqueous solution formulations are prepared by simple solution of the drug and the excipients, by in situ salt formation of the drug in the solution (titrating against an acid or base), or by complexation of the drug with a complexing agent.

Solubility and solubilization. The solubility of a substance at a given temperature is defined quantitatively as the concentration of the dissolved solute in a saturated solution (i.e., the dissolved solute phase). Generally, drugs are present in solution at unsaturated or subsaturated concentrations; otherwise, crystallization of the drug may occur as a result of changes in pH, temperature, by seeding from other ingredients, or particulates in the solution.

The solubilization techniques for injectable formulations include pH adjustment, mixed aqueous/organic cosolvents, oily vehicles, surface-active agents, complexation, as well as formulating the drug in emulsion, suspension, liposomes, nanosuspensions, and combinations of techniques. An excellent review of the solubilizing excipients that could be used in the injectable formulations has been provided by Strickly (2).

Table 1 Expressions for Approximate Solubility

Term	Relative amount of solvent to dissolve
Very soluble	<1
Freely soluble	1–10
Soluble	10–30
Sparingly soluble	30–100
Slightly soluble	100–1,000
Very slightly soluble	1,000–10,000
Practically insoluble or insoluble	>10,000

Table 2 Typical Examples of Drugs Representing the Solubility Terms

Term	Drug	Solubility of drug
Very soluble	Chloral hydrate	>8 g/mL
Freely soluble	Isoniazid	0.330 g/mL
Soluble	Guaifensin	0.050 g/mL
Sparingly soluble	Pyrazinamide	0.015 g/mL
Slightly soluble	Salicylic acid	0.002 g/mL
Very slightly soluble	Griseofulvin	0.000,02 g/mL
Practically insoluble or insoluble	Diclofenec	0.000,002 g/mL

Source: Adapted from Ref. 3.

Solubility expressions. Solubility of a substance can be expressed in a number of ways. Generally, the concentration is expressed as percent (w/v), that is, grams per 100 mL of solution, but molarity and molality have been used. Molarity is defined as the number of moles per 1000 mL of solution. Molality is the number of moles of solute per 1000 g of solvent and, therefore, being a weight relationship, is not influenced by temperature. The USP lists solubility in terms of the number of milliliters of solvent required to dissolve 1 g of substance. If exact solubilities are not known, the USP provides general terms to describe a given range. These descriptive terms are listed in Table 1. Typical examples of drugs representing the solubility terms are listed in Table 2 (3).

Bonding forces. For a substance to dissolve, the forces of attraction that hold the molecules together must be overcome by the solvent. The solubility will be determined by the relative binding forces within the substance (solute-solute interactions) and between the substance and the vehicle (solute-solvent interactions). If an environment similar to that of the crystal structure can be provided by the solvent, then the greater the solubility (i.e., "like dissolves like"). Ionic compounds dissolve more readily in water by virtue of ion-dipole interactions, whereas hydrophobic substances dissolve more easily in organic solvents as a result of dipole or induced dipole interactions.

Often, the solubility of the drug substance is due in large part to the polarity of the solvent, generally expressed in terms of dipole moment, which is related to the DC. Solvents with high DCs dissolve ionic compounds and are water soluble, whereas solvents with low DCs are not water soluble and do not dissolve ionic compounds. The former are classified as polar solvents (e.g., water, glycerin, and ethanol), while the latter are nonpolar (e.g., chloroform, benzene, and the oils). Solvents with intermediate DCs (e.g., acetone and butanol) are classified as semipolar. The DCs of most pharmaceutical solvents are known (4) and values for a number of binary and tertiary blends have been reported (5) and, if not reported, can be readily estimated (6,7). Table 3 is a listing of the DCs of some solvents at 25°C.

The solubility profiles of a number of pharmaceuticals as a function of DC have been reported by Paruta and coworkers (8–10). By determining the solubility of a substance in a system at various DCs, a graph such as that shown in Figure 1 can be constructed to determine the DC that will provide the required solubility for a particular drug substance. As can be seen

Table 3 DCs of Some Solvents at 25°C

Solvent	DC
Water[a]	78.5
Glycerin[a]	40.1
N,*N*-dimethyl acetamide[a]	37.8
Propylene glycol[a]	32.0 (30°C)
Methanol	31.5
Cremophor EL (R) (polyoxyl castor oil 35)[a]	27.0
Ethanol[a]	24.3
N-propanol	20.1
Acetone	19.1
Benzyl alcohol[a]	13.1
Polyethylene glycol 400[a]	12.5
Cottonseed oil[a]	3.0
Benzene	2.3
Dioxane	2.2

[a]Solvents used in parenterals
Abbreviation: DC, dielectric constant.

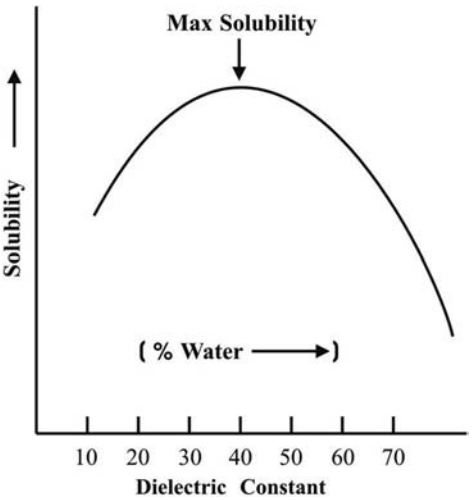


Figure 1 Hypothetical plot of solubility of a substance versus dielectric constant in various mixtures of dioxane and water.

from the plot, to obtain the maximum concentration, a DC of around 40 is required. Not all mixtures will show a maximum, but such a plot illustrates the required DC to obtain the desired concentration. For example, if a DC (DC) of 60 was selected, a mixture of water (DC = 78.5), polyethylene glycol (PEG) 400 (DC = 12.5) and ethanol (DC = 24.3) could be used. Selecting an amount of ethanol necessary to dissolve the drug (e.g., 10%), the percentages of PEG 400 and water can be calculated as follows:

$$(10)(24.3) + (X)(78.5) + (90 - X)(12.5) = (100)(60)$$

where X is the percentage of water required and is calculated to be 73.5%.

Therefore, the vehicle to provide a DC of 60 will have the following composition: Ethanol 10%, PEG 400 16.5%, and Water 73.5%

Since DC is a measure of the polarizability and dipole moment of a compound, several researchers have explored other parameters and polarity indices (11) which include molecular volume, solvent and solute interactions and specific interactions such as hydrogen bonding. In 1952, Hildebrand and Scott (12) introduced solubility parameters to predict solubility of regular solutions. Since pharmaceutical systems deviate from regular or ideal solutions, Martin and coworkers (13) modified the Hildebrand approach to include hydrogen-bonding and

dipolar interactions. The molecular surface area of the solute and interfacial tension between solute and solvent were further used by Amidon (14) and Yalkowsky (15) to predict solubility. Among the many theoretical models available to predict solubility in water, recent reports review the available models and discuss the potential and limitations of these computational approaches (16,17).

Hydrogen bonding, a type of dipole-dipole interaction, is an important determinant of solubility. Because of its small size, the hydrogen atom (proton donor) with its positive center, can approach the negative center (electron donor) of a neighboring dipole more closely than any other atom. As a result of this spatial maneuverability, both intramolecular bonding (i.e., between groups within a single molecule) and the intermolecular type (i.e., among molecules) can occur. The latter is responsible for association in most solvents and dissolution of most drugs. Alcohols dissolve in water by hydrogen bonding, up to an alkyl chain length of five carbon atoms. Phenols dissolve in water and alcohol and, as the number of hydroxyl groups increase, the water solubility is enhanced because of the increased opportunity for hydrogen bonding. Most aromatic carboxylic acids, steroids, and cardiac glycosides are not water soluble but dissolve in alcohol, glycerin, or glycols by hydrogen bonding.

Dipole-ion interaction is another important molecular property that is responsible for the dissolution of ionic crystalline substances in polar solvents (i.e., water or alcohol). Ions in aqueous solution are generally hydrated (surrounded by water molecules) by as many water molecules as can spatially fit around the ion. The attributes of a good solvent for electrolytes include: (*i*) a high-dipole moment; (*ii*) a small molecular size; and (*iii*) a high DC to reduce the force of attraction between the oppositely charged ions in the crystal. Water possesses all of these characteristics and is, therefore, a good solvent for electrolytes. The cation of the electrolyte is attracted to the negative oxygen atom, while the anion attracts the hydrogen atoms of the dipolar water molecules.

Symmetrical molecules, such as benzene and carbon tetrachloride, possess zero dipole moment and are nonpolar. Solubility of such molecules or their existence in a liquid state is due to van der Waals forces. Other intermolecular interactions, such as London forces or Debye interactions are also responsible for solubility of such nonpolar substances.

Effect of temperature. Substances generally dissolve faster if heat is applied to the system and the solubility of most solids is increased by an increase in temperature. This is true if the substance absorbs heat during the course of dissolution. The degree to which temperature can influence solubility is determined by the heat of solution, more specifically the differential heat of solution, ΔH, which represents the rate of change of the heat of solution per mole of solute in a solution of specified concentration. The higher the heat of solution, the greater is the influence of temperature on solubility.

The following equation shows the influence of temperature on solubility:

$$\frac{d\,Ln\,S}{dT} = \frac{\Delta H}{RT^2} \tag{1}$$

where S is the solubility or concentration of a saturated solution, often expressed in terms of molality, molarity, or mole fraction; R is the gas constant; and T is the absolute temperature. Equation (1) can be written as

$$\log S = \frac{\Delta H}{2.303R} \times \frac{1}{T} + \text{constant} \tag{2}$$

By plotting the logarithm of the solubility in moles per liter versus the reciprocal of the absolute temperature as shown in Figure 2, the differential heat of solution can be calculated from the slope of the line, which is equal to

$$-\frac{\Delta H}{(2.303)(1.987)}$$

A positive heat of solution indicates that the process is endothermic (i.e., the solute absorbs heat when dissolving). Therefore, an increase in temperature will increase solubility. A

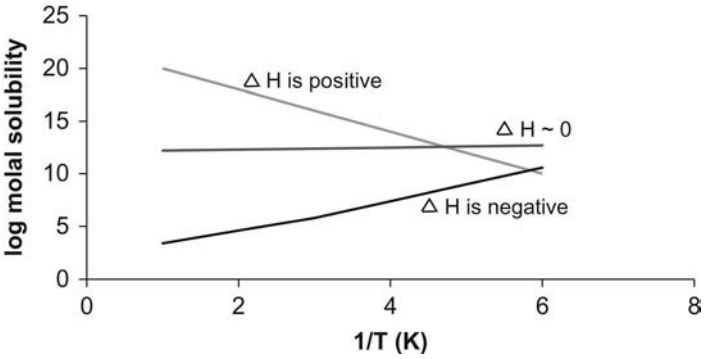


Figure 2 Effect of temperature on solubility of a substance. ΔH represents the differential heat of solution and is calculated from the slope of the line, $-\frac{\Delta H}{(2.303)(1.987)}$.

negative value indicates that the process is exothermic (i.e., the solute evolves heat when dissolving). In this case, increase in temperature results in a decrease in solubility. A differential heat of solution around zero indicates that the solubility is not significantly influenced by temperature.

Measuring solubility. Methods for determining the solubility of drug substances in aqueous solvents have been described (18,19). The standard way to determine the solubility of a compound is to use the "shake-flask" solubility method. This method is inherently low-throughput, labor intensive, and necessitates the addition of drug in a powder form. It involves adding an excess quantity of solid material to a volume of buffer at a fixed pH and the saturated solution is agitated (shake-flask) until equilibrium is reached, generally 12 hours to seven days. Following separation by filtration or centrifugation, the compound in solution is analyzed and quantified by a suitable analytical technique such as UV/Vis spectroscopy or high-performance liquid chromatography (HPLC). The other classical experimental methods used to determine solubility are turbidimetric ranking assays, HPLC-based assays, and potentiometric methods. The newer high-throughput methods which determine both kinetic and thermodynamic (equilibrium) solubilities are based on screening multiple solutes and solvents, in array of compositions, using 96-well format that allows for solubility analysis in a single plate with very low drug amount (19,20).

Solubilization techniques. A variety of approaches to increase the aqueous solubility of an otherwise less soluble or insoluble drug substance to a desired level for optimum injectable product have been reported and reviewed (2,21,22). These include: 1) pH adjustment, 2) salt formation, 3) use of cosolvents, 4) surfactants as solubilizers, 5) use of complexing agents, and others. Metabolizable oils as vehicles have has also been used for certain class of compounds. Beyond these solubilization approaches, it may become necessary in some cases to change the formulation from solution to dispersed system such as emulsion, suspension, and more recently liposomes and nanosuspensions.

pH adjustment Most organic drug substances are weak electrolytes and, therefore, exist in solution in dissociated and undissociated forms. The ratio of these forms is determined by the pH of the solution as per the Henderson-Hasselbach relationship. As a result, properties such as solubility, partition coefficient, and chemical stability, which are markedly different for the undissociated and dissociated forms are influenced by pH.

Many of the organic electrolytes used in parenteral systems contain a basic nitrogen atom in the molecules. These include antihistamines, alkaloids, local anesthetics, and so on, which are practically insoluble in water but dissolve readily in dilute solutions of acids because of salt formation. The addition of alkali to these solutions increases the pH and causes free base to precipitate. Examples are atropine sulfate, ephedrine sulfate, lidocaine hydrochloride, and pyribenzamine hydrochloride.

In compounds that contain an electron withdrawing group, such as oxygen, a positive center is created, which in turn attracts electrons from adjacent nitrogen, and if a hydrogen

atom is attached, the N-H bond is weakened. As a result, in alkaline solution a more soluble anion is formed. The examples are phenobarbital and sulfanilamide.

The addition of acid to the solutions of these compounds will cause the free acid form to precipitate. Even the addition of a salt of a strong acid such as morphine sulfate will result in precipitation.

Most marketed injection products are in the pH range of 4 to 8 for biocompatibility reason, however, some are outside of this range. The pH solubility and pH stability-rate profiles of a drug usually determines the pH at which a product is formulated (23). Additional formulation variables to be considered are the necessity of a buffer, buffer capacity, and drug concentration. These variables are described in details in a further section (see "Added Substances").

Salt formation Salts of acidic and basic drugs usually exhibit higher solubility than their corresponding acid or base forms. Therefore, salt formation is the most preferred and effective method of increasing solubility and dissolution rates of acidic and basic drugs (24,25).

Solubility-pH profiles of weakly acidic or basic organic drugs may be visualized on the basis of classical Henderson-Hasselbach relationship. In the case of monoprotic acid, a saturated solution can be defined by the following equations and corresponding constants (26).

$$\mathrm{HA} \Leftrightarrow \mathrm{H^+} + \mathrm{A^-} \mathrm{K_a} = \frac{[\mathrm{H^+}][\mathrm{A^-}]}{[\mathrm{HA}]} \tag{3}$$

$$\mathrm{HA(Solid)} \Leftrightarrow \mathrm{HA(Solution)} S_0 = [\mathrm{HA}] \tag{4}$$

where [HA] is the concentration of undissociated acid form, $[\mathrm{A^-}]$ is the concentration of corresponding salt form, $[\mathrm{H^+}]$ is the concentration of proton or dissociated hydrogen, and S_0 is the intrinsic solubility of the monoprotic acid. Solubility, S, at a particular pH is defined then as mass balance sum of the concentrations of all of the species dissolved in the aqueous phase.

$$S = [\mathrm{A}]^- + [\mathrm{HA}] \tag{5}$$

Rearranging equations (3), (4), and (5),

$$\begin{aligned} S &= K_a[\mathrm{HA}]/[\mathrm{H^+}] + [\mathrm{HA}] \\ &= S_0(K_a/[\mathrm{H^+}] + 1) \\ &= S_0\left(10^{-\mathrm{p}K_a+\mathrm{pH}} + 1\right), \text{ or} \\ \log S &= \log S_0 + \log\left(10^{-\mathrm{p}K_a+\mathrm{pH}} + 1\right) \end{aligned} \tag{6}$$

For a weakly acidic drug, depending on the pH of the solution, the term, $\log\left(10^{-\mathrm{p}K_a+\mathrm{pH}} + 1\right)$, changes solubility function according to the conditions below.

1. $\mathrm{pH} >> \mathrm{p}K_a$

   The exponent $(-\mathrm{p}K_a + \mathrm{pH})$ remains positive and very large number compared with 1, and hence, 1 is ignored, and

$$\begin{aligned} &\log\left(10^{-\mathrm{p}K_a+\mathrm{pH}} + 1\right) \text{ becomes } \log\left(10^{-\mathrm{p}K_a+\mathrm{pH}}\right) \text{ or} \\ &\left(-\mathrm{p}K_a + \mathrm{pH}\right)\log(10) \text{ or } \left(-\mathrm{p}K_a + \mathrm{pH}\right) \\ &\text{Therefore,} \\ &\log S = \log S_0 - \mathrm{p}K_a + \mathrm{pH} \\ &\text{Since } \mathrm{p}K_a \text{ is a constant.} \\ &\log S = (\log S_0 - \mathrm{p}K_a) + \mathrm{pH} \end{aligned} \tag{7}$$

   Equation (7) is of the form, $Y = c + mX$ or an equation of a straight line with associated intercept and slope, c and m, respectively.

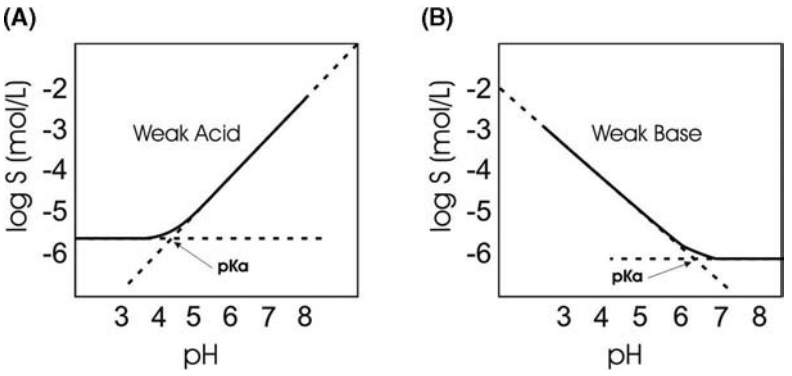


Figure 3 Solubility/pH profile for weak acid ($pK_a = 4.4$) and weak base ($pK_a = 6.1$). *Source*: Adapted from Ref. 26.

Therefore, a plot of log$S$ versus pH, will yield a straight line the slope of which will be equal to +1 and the intercept will be ($\log S_0 - pK_a$). A similar relationship can be made for a weakly basic drug, in which case, the slope will be equal to −1.

Figure 3 (26) shows the solubility-pH profile for a (*i*) weak acid (pK_a 4.4, logS_0 −5.6) and (*ii*) weak base (pK_a 6.1, log S_0 −5.9).

2. $pH = pK_a$, or at the inflection point in the curve.

   The exponent ($-pK_a + pH$) becomes zero and the term

   $$\log\left(10^{-pK_a+pH} + 1\right) \text{ becomes } \log\left(10^0 + 1\right) \text{ or } \log(1+1).$$

   Therefore,

   $$\log S = \log S_0 + 0.3$$

3. $pH << pK_a$, or at the flat line of the curve.

   The exponent ($-pK_a + pH$) remains negative.

   $$\log\left(10^{-pK_a+pH} + 1\right) \text{ becomes } \log\left(0.000\ldots + 1\right) \text{ or close to } 0.$$

   Therefore,

   $$\log S \cong \log S_0$$

Whether certain acidic or basic drugs would form salts and, if salts are formed, dissociation back to the free acid or base forms would depend on several factors, such as pH, pK_a, S_0 (intrinsic solubility), k_{sp} (solubility product) and pH_{max} (pH of maximum solubility). The aqueous solubility of an acidic or basic drug as a function of pH determines if the compound will form suitable salts within the physiologically acceptable pH range. Moreover, the common-ion effect of the salt-forming agents is also important in determining the final solubility. It has been reported that dissolution rates of a hydrochloride salt decrease as the pH of an aqueous medium is lowered when HCl is added or if NaCl is added to the medium. Similarly, the dissolution rate of a sodium salt decreases in the presence of added NaCl in the medium. There are numerous reports in the literature indicating such common-ion effects on salts having relatively low aqueous solubilities (27).

A review by Serajuddin about the principles of salt formation and its utility in formulation has recently been published (28). It surveyed about 120 salts approved by the FDA during the 12-year period from 1995 to 2006 and showed that the hydrochloride salt was the predominant salt form among the basic drugs and the sodium salt was the predominant form for acidic drugs. About 77% of the salts of basic drugs were prepared with relatively stronger counterions (hydrochloride, hydrobromide/bromide, sulfate/bisulfate and nitrate). Similarly, 14 out of 19 salts of acidic drugs were prepared with strong alkalies such as NaOH and KOH.

Use of cosolvents If the pH adjustment or salt formation approach still results in aqueous solubility of a drug well below its therapeutic dose, a mixture of solvents may be used to achieve sufficiently high solubility. A cosolvent is a water-miscible organic solvent that is used to increase the solubility of a poorly water-soluble compound. The addition of cosolvent results in reduction of polarity of water which in effect reduces the surface tension, DC, and solubility parameter of water. The increase in solubility by cosolvents is much more dramatic for nonpolar solutes (can be several orders of magnitude), than for solutes of intermediate polarity. Another advantage of using cosolvents is that a change in solvent property may help considerably in stability for drugs which may exhibit hydrolytic degradation by reducing the concentration of water in the formulation. Cosolvent may also enhance the stability of a drug by providing a less suitable environment for the transition state of the reactants, provided the transition state is more polar than the reactants. It is reported that cosolvents are employed in approximately 10% of the FDA approved injectable products (22).

Cosolvents and solubility J. H. Hildebrand, in a series of papers published begining in 1916, deescribed the basic principles of solutions and solubility and introduced the cosolvency approach (29) and experimental tests of a general equation for solubility (30). Since then, numerous theoretical cosolvency models have been proposed that correlate and/or predict the solubility of drugs in water cosolvent mixtures (31–34) and have been reviewed extensively by Jouban (35). The simplest experimental cosolvency model, that is, the log-linear model of Yalkowsky (36–38), provides an estimate of drug solubility in water-cosolvent mixtures using aqueous solubility of the drug. It is expressed as:

$$\log S_m = f \log S_c + (1 - f) \log S_w \tag{8}$$

Where S_m is the solute's solubility in water-cosolvent mixture, f is the volume fraction of cosolvent, S_c is the solubility of drug in pure cosolvent, and S_w is the solubility of drug in water. S_x values can be expressed in g/L, mole fraction, etc. Equation (8) can be further simplified as

$$\log S_m = \log S_w + f\sigma \tag{9}$$

where

$$\sigma = \log ac_w - \log ac_c \tag{10}$$

And ac_w and ac_c are the activity coefficients for the drug in water and cosolvent, respectively. In a given cosolvent system, σ will be constant. Therefore, if one plots log S_m versus f, the slope will be σ. Comparing slopes of different cosolvent-water systems can easily be done by using σ as a measure of the solubilization potential of the cosolvent. In practice, experimental methods of characterizing the solubility of cosolvent systems can be utilized with the aid of statistical experimental design. Advantage of the experimental approach is that one can use additional excipients, for example, surfactants, buffers, etc., in screening experimental designs.

Cosolvents and stability Cosolvents cannot only increase the solubility of drugs but may also increase the stability of some drugs (31). The addition of cosolvent reduces the collision probability between a water molecule and a drug molecule which is necessary for hydrolysis. As mentioned earlier, the degradation rate of a drug may change with the DC of the medium. Decreasing the polarity of the reaction medium by the addition of cosolvent unfavors the formation of the charged species. It stabilizes a solute against any reaction that produces charged products or proceeds through a charged transition state (39,40). As a general rule, for reactions leading to products that are less polar than the starting material, a less polar medium may accelerate the reaction. On the other hand, reactions leading to products that are more polar than the starting material may proceed rapidly in polar media.

Improvement of stability of a drug in the presence of cosolvent was reported by Ni, et al (41). The authors studied the stability of an anticancer compound, SarCNU (a nitrosourea derivative), in several pharmaceutically acceptable solvents such as water, EtOH, propylene

glycol (PG), propylene glycol monoester of medium chain fatty acids (Capmul PG), dimethylsulfoxide (DMSO), and in different combinations of these cosolvents at four different temperatures. The degradation of the drug was monitored by HPLC and was found to be catalyzed not only by general but also by specific acid and base and followed first-order kinetics. The t_{90} (time for 90% of the drug remaining intact) in pure cosolvent was 25–50 times higher than that in water or semi-aqueous vehicles. Figure 4 shows an Arrhenius plot of the observed rate constants of SarCNU in the solvent mixtures. There was no significant difference in the slopes for the different solvents, suggesting similar degradation mechanism of SarCNU in all solvent mixtures. Furthermore, the order of stabilization by these solvents was Capmul PG> /EtOH> /PE> /PG> /WPE> /water, which was in agreement with decreasing the polarities of the vehicles. The greatest SarCNU stability, as measured by the degradation rate constant derived $t_{90,}$ was observed with Capmul PG as shown in Table 4. Another example where the degradation was significantly reduced in the nonaqueous solvents is described for Eptifibatide, a peptide compound used as an inhibitor of platelet receptor glycoprotein (42). The use of cosolvent to help in solubilization may not, however, lead to favorable stability outcome at all the times. Trivedi, et al, (43) showed that as the fraction of organic solvents was increased, the degradation of zileuton also increased because of the solvolysis of the drug by the cosolvents used.

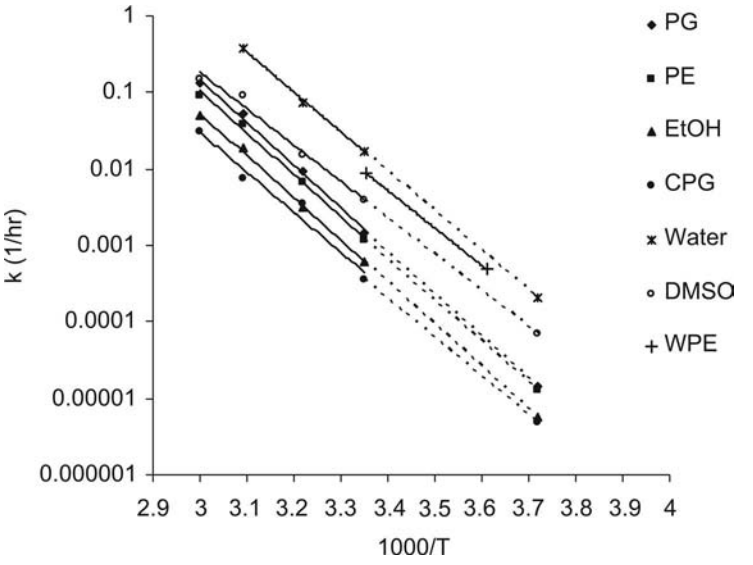


Figure 4 Stability behavior of SarCNU in the presence of various cosolvent.

Table 4 Degradation of SarCNU in the Presence of Various Cosolvent Mixtures

	t_{90} (days)	
Solvent	Room temperature (25°C)	Refrigeration (4°C)
Water	0.25	5.90
Water + propylene glycol + EtOH	0.50	8.96
DMSO	1.14	19.03
Propylene glycol	2.92	77.78
Propylene glycol + EtOH	3.64	89.50
EtOH	7.29	199.52
Capmul PG	12.50	242.57

Source: From Ref. 41.

Table 5 Examples of Marketed Injectable Products Containing Cosolvent Mixtures

Generic name	Trade name	Predominant cosolvent(s) in marketed vehicle
Carmustine	BiCNU	100% ethanol
Diazepam	Valium	Propylene glycol 40% Ethyl alcohol 10%
Digoxin	Lanoxin	Propylene glycol 40% Ethyl alcohol 10%
Melphalan	Alkeran	Propylene glycol 60% Ethyl alcohol 5%
Methocabamol	Robaxin	Polyethylene glycol 50%
Oxytetracycline	Terramycin	Propylene glycol 67–75%
Paricalcitol	Zemplar	Propylene glycol 30% Ethyl alcohol 20%
Phenobarbital Na	Nembutal	Propylene glycol 40% Ethyl alcohol 10%
Phenytoin Na	Dilantin	Propylene glycol 40% Ethyl alcohol 10%
Teniposide	Vumon	*N,N*-dimethylacetamide 6% Cremophor 50% Ethyl alcohol 40%
Docetaxel	Taxotere	Polysorbate 80 100%

Source: From Refs. 44 and 45.

Examples of drugs marketed in water-miscible systems include digoxin, phenytoin, diazepam and others as shown in Table 5 (44,45). These injections are formulated in a water-miscible system containing glycols and alcohol and adjusted to a suitable pH. Other cosolvents used in the past included glycerin in deslanoside, dimethylacetamide in reserpine and dimethylsulfoxide in chemotherapeutic agents undergoing clinical testing. Propylene glycol is used most frequently as a cosolvent, generally in concentrations of 40%. Although such systems are stable in individual vials, care must be exercised on administration. For example, phenytoin is dissolved as the sodium salt in a vehicle containing 40% propylene glycol and 10% ethanol and adjusted to a pH of 12 with sodium hydroxide. However, if this solution is added to a large-volume IV solution and the pH is lowered to a value close to the pK_a of the drug ($pK_a = 8.3$), precipitation of the drug can occur. This is due to the fact that in aqueous systems at pH below 11, the amount of undissociated phenytoin exceeds its solubility.

To be used as solubility/stability enhancer in injectable products, the cosolvent must have certain attributes such as it should be nontoxic, compatibile with blood, nonsensitizing, nonirritating and above all physically and chemically stable and inert. Many cosolvent formulations contain high concentrations of organic solvent and most are diluted prior to injection, however, some may be injected directly and in that case, care must be taken that the rate of injection remains slow.

Surfactants as solubilizers The ability of surfactants to enhance the solubility of otherwise poorly water-soluble compounds in aqueous solution is widely known and used in many injectable formulations. Surfactants are effective solubilizing agents because of their wetting properties and association tendencies as they are able to disperse water-insoluble substances. Surfactants are also used very widely in the biotechnology area for otherwise water-soluble monoclonal antibodies and other proteins and polypeptides, but the primary goal of using surfactant in these products is to minimize hydrophobic interaction related aggregation and not necessarily for the enhancement of solubility. This aspect will be discussed in detail in other chapters.

Surfactants can be either nonionic or ionic (i.e., the ability to lower surface tension rests with the anion or cation in the molecule). In nonionic surfactants, the head groups contain no charged moieties and their hydrophilic properties are due to the presence of hydroxyl groups. Nonionic surfactants are most frequently used in pharmaceutical systems because of their compatibility with other surfactants, stability, and relatively low toxicity. Some examples of

water-soluble nonionic surfactants include long-chain fatty acid analogs such as fatty alcohols, glyceryl esters, and fatty acid esters. Among the most widely used water-soluble nonionic surfactants in injectable products are polyethylene oxide (PEO) sorbitan fatty acid esters, or Polysorbates.

In anionic surfactants, the head groups are negatively charged. The most widely used anionic surfactants are those containing carboxylate groups, such as soaps, sulfonates, and sulfate ions. In cationic surfactants, the head groups are positively charged. Some examples include amine and quaternary ammonium salts. Cationic surfactants are not used in pharmaceutical systems because of their toxicity since they adsorb readily to cell membrane structures in a nonspecific manner, leading to cell lysis (46).

As shown in Figure 5, surfactants typically orient themselves at polar/nonpolar interfaces because of the presence of discrete hydrophobic and hydrophilic regions. As the bulk concentration of surfactant in solution is increased, the surfactant molecules begin to associate into small aggregates called micelles, whereby their hydrophobic regions are shielded from aqueous contact by their hydrophilic regions. All surfactant molecules in excess of that concentration associate into micelles, while the concentration of nonassociated surfactant molecules remains nearly constant. The concentration at which such association occurs is called critical micelle concentration (CMC). Using soap as a micelle forming substance, Lawrence proposed in 1937 that poorly soluble hydrophobic molecules locate in the hydrocarbon core of the micelle, while polar molecules would associate with the polar end (47). Molecules that contain polar and nonpolar groups align themselves between the chains of the micelle with the nonpolar part directed into the central region and the polar end extending out into the hydrophilic chains (Fig. 6).

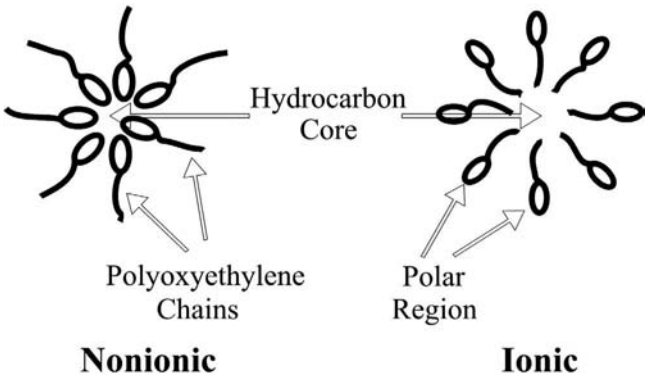


Figure 5 Illustration of spherical orientations of nonionic and ionic micelles.

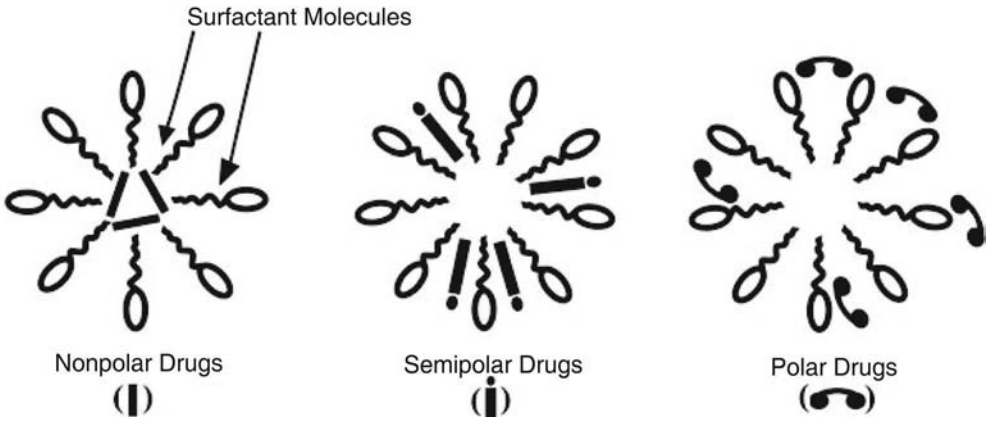


Figure 6 Schematic representation of mechanisms of miceller solubilization.

Table 6 Effect of Surfactants on the Solubility of Furosemide

	Distilled water		0.1 N hydrochloric acid	
Surfactant % (w/v)	Total solubility (μg/mL)	Miceller solubility (μg/mL)	Total solubility (μg/mL)	Miceller solubility (μg/mL)
0	41.2	–	15.0	–
Polysorbate 20 (C12)				
0.005	31.2	–	40.0	–
0.05	45.0	3.7	41.1	26.1
0.5	57.0	15.7	50.0	35.0
1.0	167.0	125.7	145.0	130.0
5.0	705.0	663.7	670.0	655.0
Polysorbate 40 (C16)				
0.005	32.5	–	25.0	–
0.05	45.0	3.7	22.5	7.5
0.5	112.5	71.2	72.5	57.5
1.0	143.7	102.4	137.5	122.5
5.0	792.5	751.2	887.0	872.0
Polysorbate 80 (C18)				
0.005	43.7	2.4	15.9	0.9
0.05	43.7	2.4	18.7	3.7
0.5	141.2	100.0	74.0	59.0
1.0	205.0	163.7	160.0	145.0
5.0	980.0	938.7	808.0	793.0

Source: From Ref. 49.

Generally, the solubilization capacity of a same amount of surfactant is high for those with lower CMC value. The solubilizing ability of nonionic surfactant toward water-insoluble drugs has been extensively studied (48). Akbuga and Gursoy (49) showed how the solubility of furosemide, a very insoluble compound commonly used as diuretic, was dramatically affected by the surfactant concentration and alkyl chain length (Table 6).

The CMC can be measured by a variety of techniques, for example, surface tension, light scattering, osmometry, all of which show a characteristic break point in the plot of the operative property as a function of concentration. Figure 7, a plot of surface tension against concentration of surfactant shows a break in the linearity of the curve, indicating the CMC (50). Many factors such as temperature, pH of the solution, electrolytes, and other ingredients affect micellization and hence solubilization (51,52). For nonionic surfactants, the CMC value decreases with increasing temperature whereas for ionic surfactants, it increases as the temperature increases (53). Since the pH can affect the equilibrium between ionized and nonionized solute species, it can have an effect on the capacity of micellar solubility as shown by Castro et al, for atenolol, nadolol, midazolam and nitrazepam (54). For ionic surfactant micelles, electrolyte addition causes a decrease in the CMC resulting in an increase in the micellar solubilization capacity (55), whereas in the case of nonionic surfactant, polysorbate 80, the solubility of furosemide increases in the presence of sodium chloride because of increased micellar packing and micelle volume (56). Other ingredients present in the formulation can also have a profound effect on the solubilizing capacity of surfactants. Surfactants may precipitate in the presence of some organic additives or micellization may be abolished if high enough concentrations of, for example, alcohols are present. Excipients such as phospholipids also affect the CMC. Many water-soluble drugs themselves are remarkably surface active: they lower the surface and interfacial tension of water, promote foaming, and associate into micelles, such as antibacterial (hydrochlorides of acridines, benzalkonium chloride, cetylpyridinium chloride) tranquilizers (hydrochlorides of reserpine and phenothiazine derivatives), local anesthetics (hydrochlorides of procaine, tetracaine, dibucaine, and lidocaine), nonnarcotic analgesic (propoxyphene hydrochloride) and narcotic analgesic (morphine sulfate and meperidine hydrochloride), antimuscarinic drugs (propantheline bromide, methantheline bromide, methixene hydrochloride), cholinergic agents (pilocarpine hydrochloride, and other

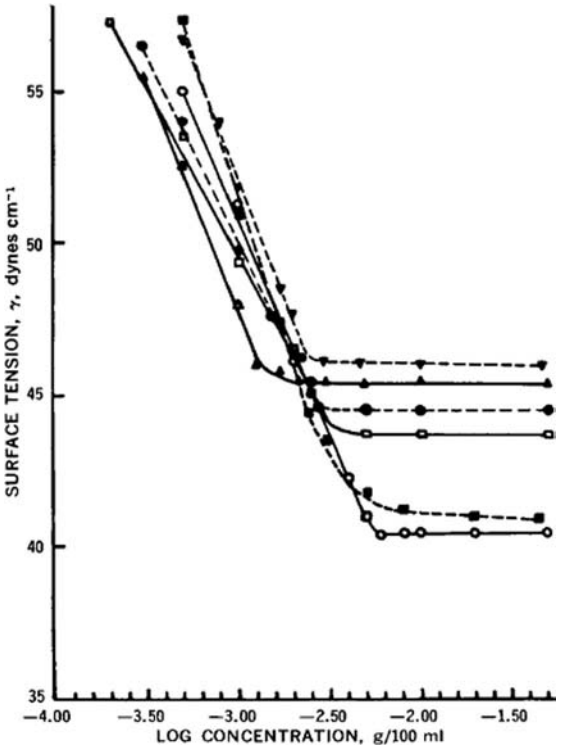


Figure 7 Surface tension versus concentration of surfactant. Break in the curve denotes CMC. *Source*: From Ref. 50.

alkaloidal salts), antihistamines (pyrilamine maleate, tripelennamine hydrochloride, chlorcyclizine hydrochloride, diphenhydramine hydrochloride), anthelmintics (lucanthone hydrochloride), and antibiotics (sodium fusidate, some penicillins, and cephalosporins) (46).

Selection of surfactant in the injectable products should be based on its safety and toxicology profile (LD50, tissue tolerance, hemolysis, etc.), solubility of the drug in the in surfactant, and drug-surfactant compatibility. Since surfactants act as nonspecific solubilizers, stabilizers, emulsifiers and wetting agents, they can also cause toxicity and disrupt normal membrane structure. As mentioned earlier, only nonionic surfactants are generally used in parenterals because of their relative less destruction to biological membranes. Table 7 lists some commonly used surfactants, their properties, and examples of marketed injection products that contain surfactants for the purpose of solubility enhancement. Polysorbate 80 is

Table 7 List of Some Surfactants in Injectable Products and Their Properties

Surfactant	Chemical name	HLB[a] value	CMC (% w/w)	Injection product (chemical/ brand/% surfactant)
Cremophor	Polyoxyethylated castor oil	12–14	0.02	Paclitaxel/taxol/52.7 Tenoposide/vumon/55 Cyclosporine/sandimmune/65
Solutol HS	Polyethylene glycol 660 hydroxystearate	14–16	0.03	Vitamin K /Aqua-mephyton/25
Pluronic-F68	Polaxomer	>24	0.1	Recombinant Growth hormone/accretropin/0.2
Polysorbates	Tween-80	15	0.0014	Amiodorone/cordarone/10 docetaxel/taxotere/100 Vitamin A palmitate/aquasol-A/12
–	Sodium desoxycholate	16	0.08	Amphotericin/fungizone/0.4
–	Sodium dodecyl sulfate	40	0.03	Aldesleukin/proleukin/0.018

[a]Hydrophilic Lipophilic Balance

the most commonly used surfactant and is used in the range from fraction of percent in many products to 100% in the case of taxotere injection.

Cyclodextrins as solubilizers Cyclodextrins are oligomers of glucose produced by enzymatic degradation of starch. The number of α-1,4-linked glucose units determine the classification into α, β, or γ cyclodextrins having six, seven, or eight glucose units, respectively (57–59). The cyclodextrins exert their solubilizing effect by forming soluble inclusion complexes in aqueous solutions. The cyclodextrins are amphipathic (i.e., the exterior is hydrophilic due to the hydroxy groups oriented on the exterior while the interior is hydrophobic) and can form soluble, reversible inclusion complexes with water-insoluble compounds. The unsubstituted cyclodextrins are too toxic for parental use but the chemically modified cyclodextrins appear to be well tolerated when administered parenterally and have been shown to effectively enhance the solubility of several drugs including steroids and proteins (60,61). The solubility of alfaxalone, an insoluble anesthetic, was increased by 5000 times to 19 mg/mL in 20% hydroxypropyl-β-cyclodextrin (62). Some other examples of injectables that are currently in the market which contain chemically modified cyclodextrin for the purpose of enhancement of solubility are: Aripiprazole (Abilify®) (63), ziprasidone (Geodon®) (64) and voriconazole (Vfend®) (65) containing sulfobutylether β cyclodextrin (SBECD), itraconazole (Sporanox®) (66) containing hydroxypropyl-β-cyclodextrin, and others.

Having reviewed the factors that govern solubility and solubilization during the formulation development of injectable products, the next considerations are the elements of formulations.

Types of vehicles

Aqueous The vast majority of injectable products are administered as aqueous solutions because of the physiological compatibility of water with body tissues. Additionally, the high DC of water makes it possible to dissolve ionizable electrolytes, and its hydrogen-bonding potential facilitates the solution of alcohols, aldehydes, ketones, and amines. The current USP (1) has monographs for purified water, sterile purified water, WFI, sterile WFI, bacteriostatic WFI, sterile water for inhalation, and sterile water for irrigation.

WFI is the solvent of choice for making parenterals. It must be prepared fresh and be pyrogen-free. It must meet all the chemical requirements for sterile purified water and in addition the requirements for bacterial endotoxins. The tests required for WFI are generally the same among the various pharmacopeias but differences do exist with regards to limits. WFI may be prepared by either distillation or reverse osmosis but the distillation method is by far the most common and accepted method. Because of the excellent solvent properties of water, it is both difficult to purify and maintain purity. Microorganisms, dissolved gases, organic and inorganic substances, and foreign particulate matter are the most common contaminants of water.

Prior to distillation, the water used as the source for WFI is usually subjected to chlorination, carbon treatment, deionization, and, sometimes, reverse osmosis treatment (forced passage through membrane materials). After distillation, it is filtered and then stored in a chemically resistant tank (stainless steel, glass, or blocked tin) at a cold temperature around 5°C or at an elevated temperature between 65°C and 85°C to inhibit microbial growth and prevent pyrogen formation. Generally, the hot water is continually circulated in the manufacturing areas during storage and usually filtered again prior to use. Sterile WFI and Bacteriostatic WFI are permitted to contain higher levels of solids than WFI because of the possible leaching of glass container constituents into the water during sterilization and storage. Bacteriostatic WFI, which generally contain 0.9% (9 mg/mL) of benzyl alcohol as a bacteriostatic preservative, should not be sold in containers larger than 30 mL to prevent injection of unacceptably large amounts of bacteriostatic agents (such as phenol and thimerosal).

Other water-miscible cosolvents These have been discussed earlier.

Nonaqueous vehicles Drugs that are insoluble in aqueous systems are often incorporated in metabolizable oils. Steroids, hormones, and vitamins are incorporated in vegetable oils such as peanut, sesame, corn, olive, and cottonseed. Oil injections are only administered intramuscularly. There are strict specifications for the vegetable oils used in manufacturing

Table 8 Official Injections Containing Oils as Vehicles

USP (1)	Oil commonly used
Desoxycorticosterone acetate	Sesame
Diethylstilbestrol	Sesame, cottonseed
Dimercaprol (suspension)	Peanut
Estradiol cypionate	Cottonseed
Estradiol valerate	Sesame
Estrone	Sesame
Ethiodized iodine	Poppyseed
Fluphenazine decanoate	Sesame
Fluphenazine enanthate	Sesame
Hydroxyprogesterone caproate	Sesame
Menadione	Sesame
Nandrolone decanoate	Sesame
Nandrolone phenpropionate	Sesame
Penicillin G procaine (combinations)	Vegetable
Propyliodone (suspension)	Peanut
Testosterone cypionate	Cottonseed
Testosterone enanthate	Sesame
Testosterone propionate	Sesame

intramuscular injections. Storage of these preparations is important if stability is to be maintained. For example, they should not be subjected to conditions above room temperature for extended periods of time. Although the oils used for injections are of vegetable origin, federal regulations require that the specific oil be listed on the label of a product, because some patients have exhibited allergic responses to certain vegetable oils.

Sesame oil is the preferred oil for most of the compendial injections formulated with oil. It is the most stable of the vegetable oils (except to light), because it contains natural antioxidants. Sesame oil has also been used to obtain slow release of fluphenazine esters given intramuscularly (67). Excessive unsaturation of oil can produce tissue irritation. In recent years, the use of injections in oil has diminished somewhat in preference to aqueous suspensions, which generally have less irritating and sensitizing properties. Benzyl benzoate may be used to enhance steroid solubility in oils if desired. Table 8 lists the oil injections official in the current USP (1).

Added Substances

Added substances such as buffers, antioxidants, antimicrobial preservatives, tonicity adjusting agents, bulking agents, chelating agents, solubilizing agents, and surfactants must frequently be incorporated into parenteral formulas in order to provide safe, efficacious, and elegant parenteral dosage forms. However, any such additive may also produce negative effects such as loss of drug solubility, activity, and/or stability. Any additive to a formulation must be justified by a clear purpose and function. No coloring agent may be added, solely for the purpose of coloring the finished preparation, intended for parenteral administration (1). The reader is encouraged to refer to a number of publications that provide comprehensive listing of formulation components used in all marketed injectable products (1,68–74). Hospital pharmacists who are involved in IV additive programs should be aware of the types of additives present in products that are being combined. Commonly used parenteral additives and their usual concentrations are listed in Table 9.

Pharmacopeias often specify the type and amount of additive substances that may be included in injectable products. These requirements often vary from compendia to compendia, so it is important to refer to the specific pharmacopeia that applies to the product in question. USP (1) specifies following maximum limits in preparations for injection that are administered in a volume exceeding 5 mL: for agents containing mercury and the cationic surface-active compounds, 0.01%; for chlorobutanol, cresol, phenol, and similar types of substances, 0.5%; and for sulfur dioxide, or an equivalent amount of the sulfite, bisulfite, or metabisulfite of

Table 9 Commonly Used Parenteral Additives and Their Usual Concentration

Added substance	Usual concentrations (%)
Antibacterial preservatives	
Benzalkonium chloride	0.01
Benzethonium chloride	0.01
Benzyl alcohol	1–2
Chlorobutanol	0.25–0.5
Chlorocresol	0.1–0.3
Metacresol	0.1–0.3
Phenol	0.5
Phenylmercuric nitrate and acetate	0.002
Methyl p-hydroxybenzoate	0.18
Propyl p-hydroxybenzoate	0.02
Butyl p-hydroxybenzoate	0.015
Thimerosal	0.01
Antioxidants	
Acetone sodium bisulfite	0.2
Ascorbic acid	0.01
Ascorbic acid esters	0.015
Butylhydroxyanisole (BHA)	0.02
Butylhydroxytoluene (BHT)	0.02
Cysteine	0.5
Nordihydroguaiaretic acid (NDGA)	0.01
Monothioglycerol	0.5
Sodium bisulfite	0.15
Sodium metabisulfite	0.2
Tocopherols	0.5
Glutathione	0.1
Chelating agent	
Ethylenediaminetetraacetic acid salts	0.01–0.075
DTPA	0.01–0.075
Buffers	
Acetic acid and a salt, pH 3.5–5.7	1–2
Citric acid and a salt, pH 2.5–6	1–5
Glutamic acid, pH 8.2–10.2	1–2
Phosphoric acid salts, pH 6–8.2	0.8–2
Tonicity adjustment	
Dextrose	4–5.5
Sodium chloride	0.5–0.9
Mannitol	4–5

potassium or sodium, 0.2%. Ethylenediaminetetraacetic acid derivatives and salts are sometimes used to complex and thereby inactivate trace metals that may catalyze oxidative degradation of drugs. The properties and function of these added substances will be reviewed next, except solubilizing agents and surfactant, which have been reviewed earlier.

Buffers. Maintenance of appropriate pH of the formulation is essential for proper solubility and stability. Changes in the pH of a formulation may occur during storage because of degradation reactions within the product, interaction with container components (i.e., glass or rubber), and absorption or evolution of gases and vapors. Buffers are added to many products to resist a change in pH. Excellent reviews on pH control within pharmaceutical systems by Flynn (75) and Nema et al (76) are recommended to the reader. A suitable buffer system should have an adequate buffer capacity to maintain the pH of the product at a stable value during storage, while permitting the body fluids to adjust the pH easily to that of the blood following administration. Therefore, the ideal pH to select would be 7.4, the pH of the blood. Extreme deviation from this pH can cause complications. Tissue necrosis often occurs above pH 9, while extreme pain and phlebitis are experienced below pH 3. The acceptable range for IV injections is 3 to 9 because blood itself is an excellent buffer and can very quickly neutralize the

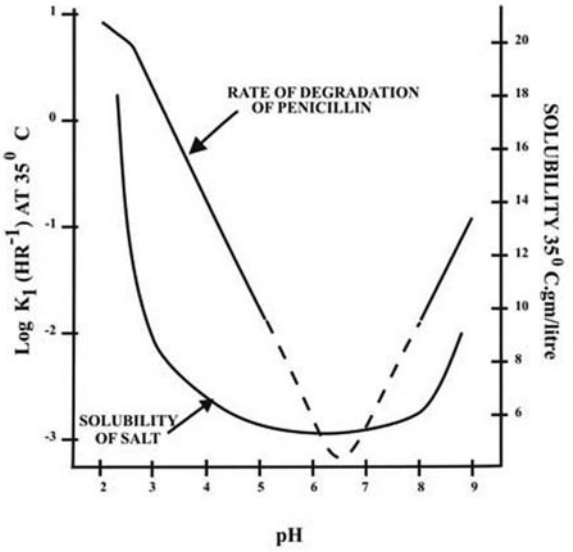


Figure 8 Solubility/stability pH profile of procaine penicillin. *Source*: From Ref. 77.

pH outside of 7.4. Parenterals administered by other routes are generally adjusted to a pH between 4 and 8.

A suitable buffer system can be selected from knowledge of a solubility/stability pH profile of the drug in solution. A typical pH profile of both solubility and stability is shown in Figure 8 for procaine penicillin G (77). By following the degradation over a given pH range and plotting the rate constants versus pH, the pH of maximum stability (pH 6.6) can be determined. In the case of procaine penicillin G, the solubility is lowest between the pH 6 and 7, which is desirable since the product is formulated as a suspension. Once the desired pH is determined, a buffer system that provides sufficient buffer capacity can be selected. The buffer capacity, β, is an indication of the resistance to change in pH upon the addition of either basic or acid substances and can be represented by the following expression:

$$\beta = \frac{dB}{d\text{pH}} = 2.303C\frac{K_a H^+}{(K_a + H^+)} \tag{11}$$

where

dB = change in concentration of base or acid,
dpH = change in pH,
C = molar concentration of buffer system, and
K_a = dissociation constant of the buffer.

A hypothetical plot of β versus pH-pK_a is illustrated in Figure 9 for a monobasic acid. A maximum value at zero indicates that the greatest buffer capacity occurs at a pH equal to the pK_a of the buffer system and further suggests that a buffer system with a pK_a within ±1.0 unit of the desired pH should be selected.

Buffer systems for parenterals generally consist of either a weak base and the salt of a weak base or a weak acid and the salt of a weak acid. Figure 10 shows the effective range of typical pharmaceutical buffers. The distance indicated by the arrows represents the effective buffer range for each system and the dashed lines represent the pK_a for the system. Commonly used buffers are phosphates, citrate, acetate, and glutamates.

The Henderson-Hasselbach relationship is used to calculate the quantities of buffer species required to provide a desired pH.

$$\text{pH} = \text{p}K_a + \log\frac{C_{salt}}{C_{acid}} \tag{12}$$

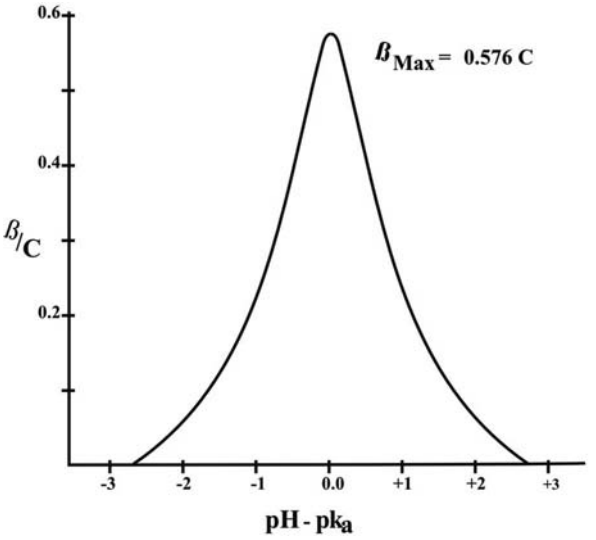


Figure 9 Theoretical buffer capacity curves of a monobasic acid.

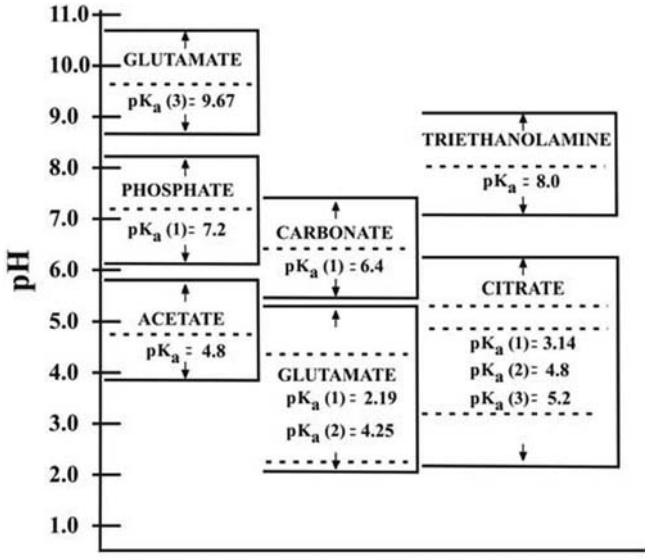


Figure 10 Effective range of pharmaceutical buffers, indicated by the arrows. The dashed line represents the pK_a value.

Where C_{salt} and C_{acid} are the molar concentrations of the salt form and the acid form, respectively. As shown from the following calculation, an acetate buffer system (pK_a = 4.8) consisting of 0.1 M acetic acid and 0.05 M sodium acetate would result in a pH of 4.5.

$$\text{pH} = 4.8 + \log\frac{0.05}{0.1} = 4.8 - 0.3 = 4.5$$

Although buffers assure the stability of pH of solution, the buffer system itself can affect other properties such as reaction kinetics and solubility aspects. Buffers can act as general acid or general base catalysts and cause degradation of some drug substances. Such a mechanism occurs with a number of amine and amine derivative drugs in systems containing polycarboxylic acids (e.g., citric, tartaric, and succinic). In one such case, as shown in Figure 11, the degradation of vitamin B_1 increases with increase in citrate buffer concentration (78).

The ionic strength contributions of the buffer system can also affect both isotonicity and stability. For example, if adjustment of pH is made with sodium hydroxide, say of a solution

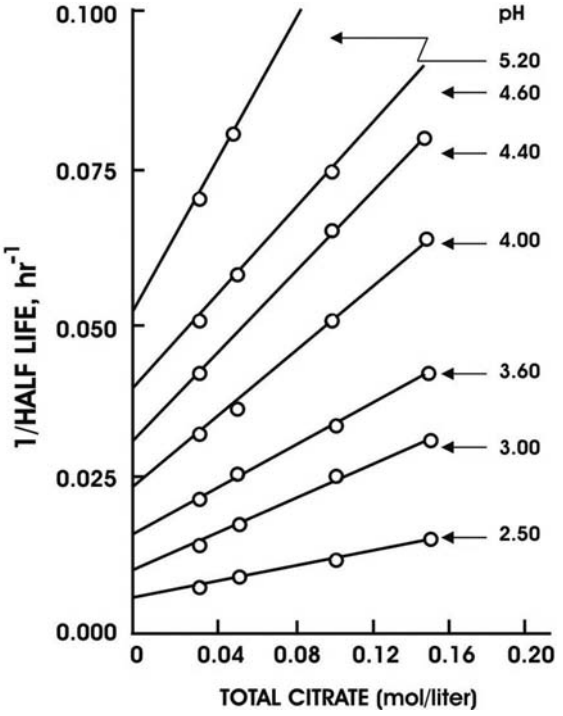


Figure 11 Effect of citrate buffer concentration on thiamine hydrolysis (vitamin B1) at 96.4°C at constant ionic strength and at different pH values. *Source*: From Ref. 78.

containing monosodium phosphate, the effect of the generation of disodium salt on isotonicity and the effect of HPO_4^{-2} must be taken into account (79,80).

Antioxidants. Many drugs in solution are subject to oxidative degradation. Such reactions are mediated either by free radicals or by molecular oxygen and often involve the addition of oxygen or the removal of hydrogen. For products in which oxygen is directly involved in the degradation, protection can be afforded by displacing oxygen (air) from the system. This is accomplished by bubbling nitrogen, argon, or carbon dioxide through the solution prior to filling and sealing in the final container. Oxidative decomposition is catalyzed by metal, hydrogen, and hydroxyl ions. Drugs possessing a favorable oxidation potential will be especially vulnerable to oxidation. For example, a great number of drugs are formulated in the reduced form (e.g., epinephrine, morphine, ascorbic acid, menadione, etc.) and are easily oxidized. Oxidation can be minimized by increasing the oxidation potential of the drug. As illustrated in Figure 12 (81), lowering the pH of the solution will increase the oxidation potential. This occurs because according to a simplified version of the Nernst equation:

$$E = E^0 + \frac{RT}{2} \log \frac{[H^+] \cdot [Ox]}{[Rd]} \tag{13}$$

an increase in hydrogen ion concentration causes an increase in the actual oxidation potential, E. In this equation E^0 is the standard oxidation potential, R the gas constant, T the absolute temperature, and constant 2 represents the number of electrons taking part in the oxidation-reduction reaction.

Agents that have a lower oxidation potential than the drug in question, and thus can be preferentially oxidized, are called antioxidants. Such agents are added to parenteral solutions either alone or in combination with a chelating agent or other antioxidant and function in at least two ways: (*i*) by being preferentially oxidized and thereby gradually consumed or (*ii*) by blocking an oxidative chain reaction in which they are not usually consumed.

Morphine in aqueous solution undergoes a pH-dependent oxidative degradation. The rate is slow and constant between pH 2 and 5, where morphine exists in the protonated form as

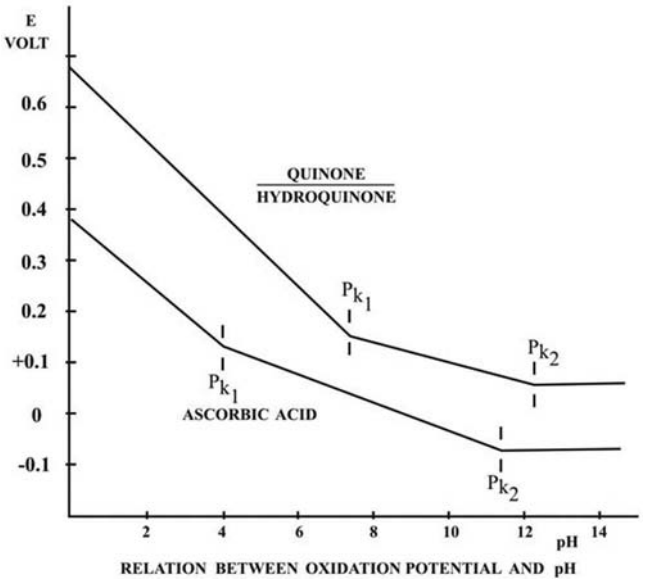


Figure 12 Relationship between oxidation potential and pH. *Source*: From Ref. 81.

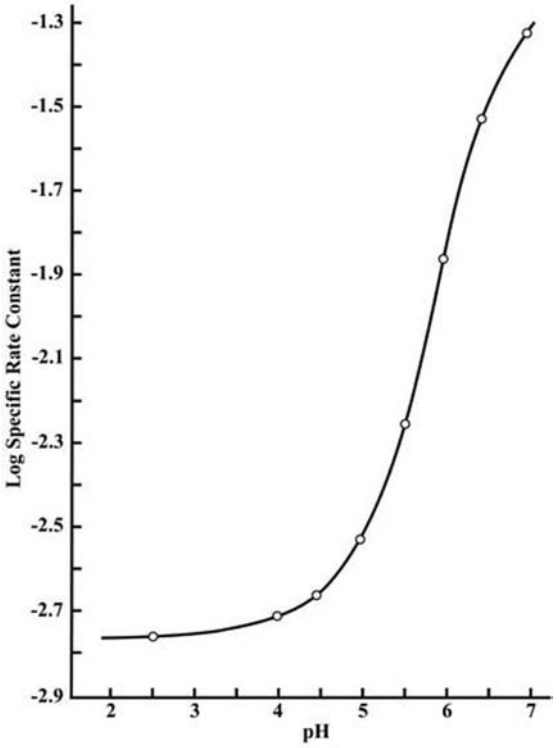


Figure 13 Reaction rate constant for the first-order oxidative degradation of morphine at 95°C as a function of pH. *Source*: From Ref. 82.

shown in Figure 13. However, above pH 5, the oxidation increases with increase in pH (82). Therefore, morphine can be stabilized by lowering the pH or by adding an antioxidant such as ascorbic acid which will be preferentially and reversibly oxidized between pH 5 and 7. Ascorbic acid, in turn, can act as an antioxidant for hydroquinone because it has a lower oxidation potential and will be preferentially oxidized. Table 10 lists some standard oxidation

Table 10 Some Commonly Used Antioxidants and Their Oxidation Potentials

Substance	E^a (V)	pH	Temperature (°C)
Riboflavin	+0.208	7.0	30
Dithiothreitol	+0.053	7.0	30
Sodium thiosulfate	+0.050	7.0	30
Thiourea	+0.029	7.0	30
Ascorbic acid[a]	+0.003	7.0	25
	−0.115	5.2	30
	−0.136	4.58	30
Methylene blue	−0.011	7.0	30
Sodium metabisulfite[a]	−0.114	7.0	25
Sodium bisulfite[a]	−0.117	7.0	25
Propyl gallate[a]	−0.199	7.0	25
Acetylcysteine[a]	−0.293	7.0	25
Vitamin K	−0.363	–	20
Epinephrine	−0.380	7.0	30
Hydroquinone	−0.673	–	–
Resorcinol	−1.043	–	–
Phenol[a]	−1.098	–	–

[a]Common in parenteral products

potentials. Salts of sulfur dioxide, including bisulfite, metabisulfite, and sulfite are the most common antioxidants in aqueous solutions. Irrespective of which salt is added to the solution, the antioxidant moiety depends on the final concentration of the compound and the final pH of the formulation (83). The metabisulfite is used at low pH values (84). Some drugs can be inactivated by bisulfites. For example, in the presence of bisulfite, epinephrine forms addition product as epinephrine sulfonate, which is inactive (85). Ortho or para-hydroxybenzyl alcohol derivatives such as parabens react in a similar manner.

While undergoing oxidation reactions, the sulfites are converted to sulfates. Since small amounts (picograms) of barium or calcium can be extracted even from type I glass, an insoluble sulfate can form in the solution (86). Therefore, additional care must be exercised to visibly inspect preparations containing sulfite antioxidants or sulfate drugs for the presence of fine particles which will appear, upon gently shaking, as a swirl originating from the bottom of the container. Sulfite levels are determined by the reactivity of the drug, the type of container (glass seal vs. rubber stopper), single or multiple-dose use, container headspace, and the expiration dating period to be employed.

Another antioxidant, Glutathione, an electron donor, stabilized the photooxidation of menadione, a synthetic analogue of Vitamin K by a charge transfer complex formation (87), thereby blocking the light-catalyzed oxidative chain reaction.

Often a single antioxidant may not be sufficient to completely protect the product. Certain compounds have been found to act as synergists, increasing the effectiveness of antioxidants, particularly those that block oxidative reactions, e.g., ascorbic acid and citric acid. Frequently, chelating agents such as ethylenediaminetetraacetic acid (EDTA) salts are used because these salts form complexes with trace amounts of heavy metals which otherwise would catalyze oxidative reactions. While incorporating such antioxidants, the formulator must be aware of their potential side effects. Although, very widely used, sulfites are associated with several effects upon parenteral administration, including flushing, pruritus, urticaria, dyspenia, and bronchospasm (88).

In practice, several approaches can be utilized by the formulator to protect the product from oxidative instability, such as purging the solution and headspace with inert gas to exclude oxygen, lowering the pH, and addition of an antioxidant. One must ensure use of high purity excipients since trace impurities, namely peroxides and metals, carried into a formulation through ingoing components, may also have a catalyzing effect on the auto-oxidation pathway. Well-protected, properly sealed packages that provide an acceptable headspace-to-product ratio can also provide some robustness to the product, thus making it

Table 11 List of Commonly Used Antibacterial Preservatives and Their MICs

Agent	MIC[a] range	Amount most often used (%)
Benzalkonium chloride	0.005–0.03	0.01
Benzethonium chloride	0.005–0.03	0.01
Benzyl alcohol	1.0–10.0	1.0
Chlorobutanol	0.2–0.8	0.5
Chlorocresol	0.1–0.3	0.1–0.25
Cresol	0.1–0.6	0.3
Parabens (methyl, ethyl,	0.05–0.25 methyl	0.18
propyl, butyl esters)	0.005–0.03 others	0.02
Phenol	0.1–0.8	0.5
Phenylmercuric nitrate	0.001–0.05	0.002
Thimerosol	0.005–0.03	0.01

[a]Affected by product pH, ionic strength, storage temperature, packaging materials, etc.
Abbreviation: MIC, minimum inhibitory concentration.

less sensitive to oxidation (89). Process control is required for assurance that every container is deareated adequately and uniformly.

Antimicrobial preservatives. Agents with antimicrobial activity must be added to preparations packaged in multiple-dose containers unless prohibited by compendial monograph or unless the drug itself is bacteriostatic, for example, methohexital sodium for injection and most of the cytotoxic anticancer products. A partial list of antimicrobial preservatives used in pharmaceutical systems along with their minimum inhibitory concentrations (MICs), is presented in Table 11.

An excellent review is published by Meyer et al (90) that provides a comprehensive summary of antimicrobial preservatives that are commonly used in licensed parenteral products to date. It was noted that the most commonly used eight antimicrobial preservatives in all parenteral products at the present are: phenol, benzyl alcohol, chlorobutanol, m-cresol, methylparaben, phenoxyethanol, propylparaben, and thimerosal with the three most commonly used preservatives in small molecule injection products are phenol, benzyl alcohol, and parabens.

Phenol is a bacteriostatic when present in 1% w/v solution and has activity against mycobacteria, fungi, and viruses (91). The solubility of phenol in water is 1 in 15 (w/w) at 20 °C. Aqueous solutions of phenol are stable, can be sterilized by dry heat or autoclaving, and should be maintained in containers that are protected from light. Phenol is incompatible with albumin and gelatin, which will result in precipitates possibly due to phenol-induced denaturation of these molecules. There is a low likelihood of adverse reactions from phenol in parenteral products due to the low concentrations used in these products.

Benzyl alcohol is an aromatic primary alcohol and is effective against most Gram-positive bacteria, yeast, and mold, but is less effective against gram-negative bacteria. Its solubility in water is 1 in 25 (w/w) at 25°C. The optimum antimicrobial activity occurs at pH less than 5 and is less active above pH 8.3. It may be stored in glass or metal containers or in polypropylene containers coated with Teflon or other inert fluorinated polymers (92).

Parabens are benzoic acid esters and have a broad spectrum of antimicrobial activity at a pH range of 4–8, but are more effective against yeasts and molds when compared with bacteria. Antimicrobial activity is normally enhanced when combinations of parabens are used with excipients such as propylene glycol, phenylethyl alcohol, and edetic acid (93). Aqueous solutions of parabens are stable at a pH range of 3 to 6, but degrade by hydrolysis at pH greater than 8. The solubility of methylparaben and propylparaben in water is 1 in 400 (w/w) at 25 °C, and 1 in 2500 at 20°C, respectively (92). Because of inherent low solubilities, sodium salts are frequently utilized in the final dosage forms.

Antimicrobial agents are specifically excluded in the large-volume injections that are used to provide fluids, nutrients, or electrolytes, such as dextrose and sodium chloride injection, dextrose injection, ringer's injection, lactated ringer's injection, and sodium chloride

injection. Bacteriostatic agents may be added to dextrose and sodium chloride injection when it is labeled for use as a sclerosing agent, because the amount of injection used for such purposes is small, and the quantity of antibacterial present would not be harmful to the patient.

The two main considerations while selecting an antimicrobial preservative in the injection products are their compatibility and effectiveness.

Many papers have been published describing the incompatibilities or binding of preservatives with surfactants, pharmaceuticals, and rubber closures (94–99).

Antimicrobial activity of preservative parabens, which was due to the concentration of the free form, was shown to be significantly reduced in the presence of polysorbate because of binding (96). Rubber closures and rubber extractives have also been found to influence significantly preservative loss from solution and antimicrobial activity, respectively. Lachman and coworkers (98,99) studied the interaction of preservatives with various types of rubber and found significant losses of a number of preservatives (i.e., chlorobutanol, chlorophenylethyl alcohol, methylparaben, and benzyl alcohol) with natural and neoprene rubber whereas the loss was minimal in the presence of butyl rubber.

The effectiveness of antimicrobial agents can be determined using a test described in compendia as "antimicrobial effectiveness testing." The test typically consists of inoculating 10^5–10^6 CFU/mL microorganisms (e.g., bacteria and fungi) per container at time zero, and evaluating the log reduction over time. The criterion used for passing this test is as follows:

> Bacteria: Not less than 1.0 log reduction from the initial calculated count at 7 days, not less than 3.0 log reduction from the initial calculated count at 14 days, and no increase from the 14 days' count at 28 days.
> Yeasts and molds: No increase from the initial calculated count at 7, 14, and 28 days.

It is recommended that this test should be performed with the formulation throughout and near the end of the expiration date to ensure that adequate levels of preservative are still available.

While the need for an antimicrobial is clearly obvious, there have been recent concerns and evidence of irritation from these agents. Therefore, it is essential to keep the concentration as low as possible, recognizing that these agents act by killing living cells and do not differentiate the good cells from the bad ones.

Tonicity. To minimize tissue damage and irritation, reduce hemolysis of blood cells, and prevent electrolyte imbalance upon administration of small-volume parenterals, the product should be isotonic, or nearly so. Isotonic solutions exert the same osmotic pressure as blood plasma. Solutions may also exert less (hypotonic) or more (hypertonic) osmotic pressure than plasma. Red blood cells (RBCs; erythrocytes) when introduced into hypotonic solution will swell and often burst (hemolysis) because of diffusion of water into the cell. If the cells are placed into hypertonic solutions, they may lose water and shrink (crenation). In isotonic solutions (e.g., 0.9% sodium chloride) the cells maintain their "tone" and the solution is isotonic with human erythrocytes. Isotonicity of formulation is not always feasible as a result of the high concentrations of drug utilized, the low volumes required for some injections, the wide variety of dose regimens and methods of administration, and product stability considerations. Historically, there has been concern over the osmolarity or tonicity of IV infusion fluids because of the large amounts of solution administered to hospitalized patients, but in the last few years there has also been interest in the osmolarity of other parental dosage forms.

Sodium or potassium chloride and dextrose are commonly added to adjust hypotonic solutions. There are several methods available to calculate tonicity (100). The sodium chloride equivalent method is the most convenient. The sodium chloride equivalent of a substance can be determined from its ability to lower the freezing point of water. A 1% sodium chloride solution has a freezing point of -0.58°C and is assigned a sodium chloride equivalent, E, of 1.00. The freezing point of blood (serum) is -0.58°C, the same as a 0.9% w/v solution of sodium chloride. If a 1% solution of a substance has a freezing point of -0.058°C, the E value will be 0.1. Therefore, 1.0 g of the substance will be equivalent to 0.1 g of NaCl.

Table 12 Sodium Chloride Equivalents and Freezing Point Depression for 1% Solutions

Agent	Sodium chloride equivalent	Freezing point depression (°C)
Atropine sulfate	0.13	0.075
Barbital sodium	0.30	0.171
Benzyl alcohol	0.17	0.09
Boric acid	0.50	0.288
Calcium chloride	0.51	0.298
Calcium disodium edetate	0.21	0.120
Calcium gluconate	0.16	0.191
Chlorobutanol	0.24	0.14
Citric acid	0.18	0.10
Codeine phosphate	0.14	0.080
Dextrose	0.16	0.091
Dimethyl sulfoxide	0.42	0.245
Edetate disodium	0.23	0.132
Ephedrine HCl	0.30	0.165
Isoproterenol sulfate	0.14	0.078
Mannitol	0.18	0.1
Penicillin G potassium	0.18	0.102
Phenol	0.35	0.20
Pilocarpine nitrate	0.23	0.132
Polyethylene glycol 300	0.12	0.069
Polyethylene glycol 400	0.08	0.047
Sodium bisulfite	0.61	0.35
Sodium cephalothin	0.17	0.095
Sodium chloride	1.00	0.576
Sodium citrate	0.31	0.178
Sodium phosphate, dibasic	0.42	0.24
Sodium sulfate, anhyd	0.58	0.34
Sucrose	0.08	0.047
Urea	0.59	0.34

To make 100 mL of a 1% solution of the substance isotonic, 0.8 g of sodium chloride must be added. A partial list of sodium chloride equivalents of variety of parenteral additives is shown in Table 12.

In the absence of a sodium chloride equivalent the L_{iso} method can be used as shown by Goyan, et al, in 1944 (101). The L_{iso} is the value at which a specific compound type will be isotonic with blood. It is related to sodium chloride equivalent in the following manner:

$$E = 17\frac{L_{iso}}{M} \tag{14}$$

where M is the molecular weight of the substance. Table 13 shows some L_{iso} values for various types of compounds. The calculation of tonicity is illustrated by the following example.

It is desired to make a 2 g/100 mL solution of sodium cephalothin isotonic using sodium chloride. Sodium cephalothin has a molecular weight of 238.

Table 13 L_{iso} Values for Various Types of Additives in Parenteral Formulations

Compound type	L_{iso}	Example
Nonelectrolyte	1.9	Sucrose
Weak electrolyte	2.0	Phenobarbital
Divalent electrolyte	2.0	Zinc sulfate
Univalent electrolyte	3.4	Sodium chloride
Unidivalent electrolyte	4.3	Sodium sulfate
Diunivalent electrolyte	4.8	Calcium chloride
Unitrivalent electrolyte	5.2	Sodium phosphate
Triunivalent electrolyte	6.0	Aluminum chloride

Table 14 Comparison of Measured Osmolality Values with Those Calculated from Sodium Chloride Equivalents

Solution (g/100 mL)	Measured osmolality mean mOsm ± SD	Sodium chloride equivalent method	
		Osmolality	Percent of measure
Dextrose			
5.0	262 ± 5.9	249	95.0
10.0	547 ± 6.2	499	91.2
20.0	1176 ± 14.9	998	84.9
Alanine glycine			
1.0	246 ± 0.5	256	104
2.0	480 ± 1.7	512	107
5.0	1245 ± 10.8	1281	103
0.2 NaCl in 5% dextrose	311 ± 5.85	312	100
0.45% NaCl in 5% dextrose	385 ± 5.48	390	98.7
Ringer's solution, USP	294 ± 4.98	281	95.6
Lactated ringer's, USP	264 ± 3.23	248	93.9
Travasol 5.5%	554 ± 11.4	596	107.6
67% travasol (5.5%) 33% dextrose (50%)	1330 ± 29.6	1323	91.9

As shown in Table 13 the L_{iso} for univalent electrolytes has a calculated value of 3.4. Therefore,

$$E = 17x\frac{3.4}{238} = \frac{57.8}{238} = 0.24g - \text{eq.}$$

Since 2 g of drug is used in the 100 mL of fluid, $2 \times 0.24 = 0.48$ g – eq. is contributed by sodium cephalothin toward the 0.90 g of sodium chloride needed for isotonicity.

Hence 0.90 g − 0.48 g = 0.42 g of sodium chloride must be added to 2 g of sodium cephalothin in 100 mL to achieve isotonicity of the resulting solution. The sodium chloride equivalent method was used for determining the osmolarity of a number of infusion solutions and compared with measured values. As shown in Table 14, there is good agreement between measured and calculated values until the concentrations become very high.

Isoosmosity, determined by physical methods, should be distinguished from isotonicity, determined by biological methods (i.e., the hematocrit method with human erythrocytes). This distinction is necessary because of the variable diffusibility of different medicinal substances across the cell membrane, which does not always behave as a truly semi-permeable membrane. Solutions that are theoretically isoosmotic with the cells may cause hemolysis because solutes diffuse through the cell membrane. For example, a 1.8% solution of urea has the same osmotic pressure as 0.9% sodium chloride, but the urea solution produces hemolysis, because urea permeates the cell membrane. If a solution is hypertonic, not much can be done with the formulation unless it can be diluted with water prior to administration. Administration of a hypertonic solution should be done slowly to permit dilution by the blood. In some cases, where injection of such solutions produces pain, as in an intramuscular injection, a local anesthetic may be added. The effect of isotonicity on reducing pain on injection is somewhat vague, although it may at least reduce tissue irritation.

Special Types of Parenterals

Suspensions. A parenteral suspension is a dispersed, multiphased, heterogeneous system of insoluble solid particles intended principally for intramuscular and subcutaneous injection.

Suspension formulation is desired when the drug is too insoluble or unstable to be formulated as a solution, as well as when there is a need to retard or control the release of drug from a suspension. The desirable parenteral suspension is sterile, stable, resuspendable, syringeable, injectable, and isotonic/nonirritating. Because a delicate balance of variables is required to formulate a suitable product, a suspension is one of the most difficult parenteral

forms to prepare. Such a product must not cake during shipping and storage, and should be easy to suspend and inject through 18- to 21-gauge needle throughout its shelf life.

To achieve these goals, it is necessary to control the crystallization, particle size reduction (micronization), and sterilization of the drug substance, as well as the processes involved in wetting of the drug with surfactants, aseptic dispersion and milling, and final filling into containers. Uniform distribution of the drug is required to ensure that an adequate dose is administered to the patient. Parenteral suspensions exhibit instability in ways not applicable to solutions and dry solids. This is due to the problem of crystal growth, caking, and product-package interactions.

Injectable suspensions may be made with either vegetable oils or aqueous vehicles. Many contain low concentrations of solids (5% or less) but a few, such as procaine penicillin G, may contain up to 58% w/v solids. Therefore, properties such as resuspendibility, zeta potential, rheology, and particle size distribution become important, and often need to be monitored as a part of a stability program for these products. When particles interact to form clumps or aggregates, the process is termed flocculation or agglomeration. The process of dispersing these aggregates into individual particles is called deflocculation. The size of individual particles may also change because of temperature fluctuation during storage and/or polymorphic changes. For example, if the solubility of a drug is very temperature dependent, individual crystals can dissolve or grow in size depending on the circumstances encountered. If the rate of absorption or injectability of the drug depends on the particle size distribution of the dispersed insoluble drug, the intended performance of the product may be altered.

The requirements for, limitations of, and difference between the design of injectable suspensions and other suspensions have been summarized by several authors (102,103). The requirements and limitations relate to (*i*) microbiological purity, (*ii*) ingredients allowed, and (*iii*) mechanical flow properties. The microbiological purity requirements, like all parenterals, involve sterility and freedom from pyrogens.

There are 38 official parenteral suspensions in the current USP (1). The wide variety of injectable suspensions can be illustrated with the following examples. Sterile Ampicillin for suspension, USP, represents a powder to which an aqueous diluent is added to make an injectable suspension. Sterile aurothioglucose suspension, USP, is an example of a ready-to-use suspension in vegetable oil. Aqueous ready-to-use suspensions include betamethasone acetate suspension, USP, and insulin zinc suspension, USP.

As shown in Table 15, a formula for an injectable suspension might consist of the active ingredient suspended in an aqueous vehicle containing an antimicrobial agent, a surfactant for wetting and preventing crystal growth (by reducing free surface energy), a dispersing or suspending agent, antioxidant, and perhaps a buffer or salt, etc. Table 16 lists materials commonly used to formulate parenteral suspensions.

Two basic methods are used to prepare parenteral suspensions: (*i*) sterile vehicle and powder are combined aseptically or (*ii*) sterile solutions are combined and the crystals are formed in situ. In the first method, an aqueous vehicle containing the water-soluble components are heat sterilized, when possible; or filtered through a 0.22 μm sterilizing membrane filter into a presterilized mixing/filling tank. The sterile drug powder is gradually added to the sterile solution, aseptically, while mixing. The sterile drug powder, in turn, is obtained by aseptically filtering a solution of the drug through a sterilizing membrane into a

Table 15 Examples of Injectable Suspension Formulations in the Market

Active/Brand/Conc.	Dexamethazone/ Decadron® (8 mg/mL)	Medroxyprogesterone Acetate/Depo-Provera® (100 and 400 mg/mL)	Triamcinolone Acetonide/Kenalog® (10 and 40 mg/mL)
Surfactant	Polysorbate 80	Polysorbate 80	Polysorbate 80
Suspending agent	Sodium CMC	PEG 3350	Sodium CMC
Antimicrobial agent	Benzyl alcohol	Parabens	Benzyl alcohol
Antioxidant	Sodium bisulfite	–	–
Others	Disodium edetate, sodium chloride, creatinine	Sodium chloride	Sodium chloride

Table 16 Partial List of Ingredients Used in Aqueous Parenteral Suspensions

Suspending agents
Aluminum monstearate
Gelatin (nonantigenic)
Mannitol
Povidone
Sodium carboxymethylcellulose
Sorbitol
Surfactants
Lecithin (soybean)
Polyoxyethylene-polyoxypropylene ethers
Polyoxyethylene sorbitan monolaurate
Polysorbate 80
Silicone antifoam
Sorbitan trioleate
Solubilizing agents
Polyethylene glycol 300
Propylene glycol
pH adjustment
Citric acid
Sodium citrate

sterile vessel into which a presterilized solution of antisolvent is introduced causing the drug to crystallize. The crystals or powder are separated aseptically by filtration or centrifugation, washed, dried, and sized through milling. After all tests have been completed on the bulk material, it is aseptically filled.

In the second method, the vehicle is prepared and sterilized by filtration. The drug is dissolved separately in a nonaqueous solvent and sterilized by filtration. The sterile drug solution is aseptically added to the sterile vehicle, causing the drug to crystallize. The resulting suspension is then diluted with sterile vehicle, mixed, the crystals are allowed to settle, and the supernatant solution siphoned off. The suspension is then brought to volume and filled in the normal manner. In few cases, the filled vials may be subjected to terminal sterilization if chemical properties and particle size characteristics remain unchanged post sterilization.

Rheologically, an injectable suspension can present some formidable challenges. While a suspension can usually be formulated so that it can be filled, shipped, and injected, it is frequently difficult to formulate a product in which these three qualities remain relatively unchanged throughout its shelf life. Rheological evaluation should be done with a recording viscometer that continuously measures the shear throughout the hysteresis loop.

The critical nature of the flow properties of parenteral suspensions becomes apparent when one remembers that those products are frequently administered through 1- to 1.5-in or longer needles, having internal diameters in the range of only 300 to 600 μm. In addition, microscopic examination shows a very rough interior needle surface, further hindering flow. The flow properties of parenteral suspensions are usually characterized on the basis of syringeability or injectability. Syringeability refers to the handling characteristics of a suspension while drawing it into and manipulating it in a syringe, clogging and foaming tendencies, and accuracy of dose measurement. The term injectability refers to the properties of the suspension during injection. It includes such factors as pressure or force required for injection, evenness of flow, aspiration qualities, and freedom from clogging. The syringeability and injectability characteristics of a suspension are closely related to viscosity and to particle characteristics.

Emulsions. An emulsion is a heterogeneous dispersion of one immiscible liquid in another.

This inherently unstable system is made possible through the use of an emulsifying agent, which prevents coalescence of the dispersed droplets (104). Parenteral emulsions are rare because it is necessary (and difficult) to achieve stable average droplets of less than 1 μm to prevent emboli in the blood vessels. In addition, they are also thermodynamically unstable

by nature, that is, on standing they will eventually separate into two phases. However, proper choice of emulsifier (generally 1–5%) and optimum preparation conditions can delay the separation of phases and thus lead to more desirable nominal shelf lives of >2 years. An emulsion can be characterized as oil-in-water (o/w), containing up to 40% oil or water in oil (w/o), depending on the identity of the dispersed and continuous phases (105).

Preparation of an emulsion requires mixing the two immiscible phases with the surfactant(s) and applying energy (generally mechanical) in order to create shear forces to deform the interface and form droplets, using sufficient force and/or time to achieve the required droplet size. This can be done in either batch or continuous modes of operation. Typically, the surfactant or mixture of surfactants is dispersed in the aqueous phase along with any water-soluble components by stirring and heating as necessary until a homogenous mixture is formed. The oil phase is then added with stirring or shaking to form a "premix" with large (>10 μm) droplets, which is then subjected to a high-energy mechanical homogenization. The final droplet size depends on the formulation composition as well as the operating conditions (e.g., temperature, homogenization pressure, and duration of homogenization) (106). The preferred method for sterilization of parenteral emulsion is terminal autoclaving. If the components of a particular drug-emulsion formulation preclude autoclaving because of stability problems, sterile filtration of the product may be a viable alternative, requiring that the emulsion droplets pass through a 0.22 μm sterilizing membrane filters. Apart from the requirements of sterility and absence of pyrogens, parenteral emulsion product must show acceptable physical stability properties such as particle (droplet) size distribution, viscosity, osmolarity, and zeta potential, as well as good chemical stability.

Parenteral emulsions are used for several purposes, including

1. water-in-oil emulsions of allergenic extracts (given subcutaneously),
2. oil-in-water sustained-release depot preparations (given intramuscularly), and
3. oil-in-water nutrient emulsions (given intravenously).

IV oil-in-water nutrient emulsions provide the source of calories and essential fatty acids for patients requiring parenteral nutrition for extended periods of time (usually for longer than five days). IV fat emulsions are prepared from either soybean (5–30%) or safflower oil (5–10%) and provide a mixture of neutral triglycerides, predominantly unsaturated fatty acids. The major component of fatty acids are linoleic, oleic, palmitic, stearic and linolenic acids. In addition, these products contain 1.2% egg yolk phospholipids as an emulsifier and glycerol to adjust tonicity. The emulsified fat particles are approximately 0.4 to 0.5 μm in diameter, similar to naturally occurring chylomicrons. The prime destabilizers of emulsions are excessive acidity (low pH) and inappropriate electrolyte content. Careful consideration must be given to additions of divalent cations (calcium and magnesium) which cause emulsion instability (107). Amino acid solutions, on the other hand, exert a buffering effect protecting the emulsion (108).

For IV oil-in-water nutrient emulsions, the current USP (1) specifies special requirement for the globule size: The volume-weighted, large-diameter fat globule limits of the dispersed phase, expressed as the percentage of fat residing in globules larger than 5 μm (PFAT5) for a given lipid injectable emulsion, must be less than 0.05%.

Liposomes. Liposomes are small, spherical vesicles which consist of amphiphilic lipids enclosing an aqueous core. The lipids are predominantly phospholipids which form bilayers similar to those found in biomembranes. Depending on the processing conditions and the chemical composition, liposomes are formed with one or several concentric bilayers.

Liposomes are often distinguished according to their number of lamellae and size. For example, small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs), and large multilamellar vesicles (MLVs) or multivesicular vesicles (MVVs). SUVs show a diameter of 20 to approximately 100 nm. LUVs, MLVs, and MVVs range in size from a few hundred nanometers to several microns. The thickness of the membrane (phospholipid bilayer) measures approximately 5 to 6 nm (109).

Liposomes are unique as drug carriers in that they can encapsulate drugs with widely varying polarities. Liposomal formulation can significantly increase the apparent aqueous solubility of a lipophilic drug, making possible delivery of a dose much higher than its water solubility, Therefore, a stable formulation with a water-insoluble drug is often achievable with no precipitation upon dilution. Drugs formulated in liposomes distribute differently in the body than conventional pharmaceuticals, since liposomes have distinct pharmacokinetic pathways of distribution and elimination (110). Encapsulation of drugs in liposomes thus results in an increase of drug levels at the targeted sites, such as inflammation, infection, or neoplasm, compared with the conventional formulations. This site-specific action reduces the toxicity of drugs without loss of their efficacies (111,112).

Phospholipids are the principal raw material of forming liposomes. These are susceptible to hydrolysis and oxidative degradation, latter due to unsaturated acyl chains. Large liposomes form spontaneously when phospholipids are dispersed in water above their phase transition temperature. The preparation of SUVs starts usually with MLVs, which then are transformed into small vesicles using an appropriate manufacturing technique.

Mechanical dispersion method is the most frequently used in the production of the large-scale liposomes. Usually it is two-step process: the film preparation and hydration step, and the particle size reduction step. The hand-shaken method and proliposome method are the two commonly used methods in the first step. For particle size reduction, sonication or microfluidization techniques are used. The liposomal preparations are then aseptically filtered through 0.22 μm membrane filter to render them sterile for IV use since both lipids and the structure of liposomes are unstable at high temperatures and hence conventional terminal steam sterilization is not suitable.

Currently, there are two liposomal formulations approved for the U.S. market by the FDA: AmBisome®, a liposomal formulation of amphotericin B, and DOXIL®, a liposomal formulation of doxorubicin.

Nanosuspensions. Nanosuspension can be defined as colloidal dispersion of nano-sized drug particles that are produced by a suitable method and stabilized by a suitable stabilizer. Nanosuspensions are used to formulate drugs that are poorly water soluble as well as poorly lipid or organic solvent soluble. A number of reports have been published on the nanosuspenion development in general (113–116), nanosuspension based injectable products (117–121), and their preclinical and clinical aspects (122). Major advantages of nano-suspensions for IV use are (*i*) avoidance of organic cosolvents, (*ii*) capability of packing higher mass-per-volume per dose, and (*iii*) potential stability improvement due to presence of unsolubilized solid-phase drug.

Nanosuspensions of drugs are typically produced either by controlled crystallization or by a high-energy particle size reduction process. Examples of the latter include wet milling and high-pressure homogenization (115,116). A third approach was reported recently, wherein crystallization and particle size reduction were combined to produce injectable nano-suspensions (117). Some of the important considerations in development of injectable nanosuspensions include: a) Nanoparticles should be stable and not susceptible to phenomena such as aggregation or Ostwald ripening, b) The nanosuspension should be free of contamination from any media used during processing, c) The nanoparticle manufacturing should be possible by aseptic processing, if terminal sterilization by heat or membrane filtration is not feasible, and d) Surfactants and excipients used should be acceptable for injectable applications.

Particle size distribution and its stability is an important element in the formulation in nanosuspension and requires careful optimization of surfactants to be used in the formulation. Adsorption kinetics and affinity of the surfactant to the newly formed crystal surface play a determining factor on the final particle size and stability of the nanosuspension. A number of surfactants have been explored for the stabilization of nano-crystals including polysorbates, phospholipids, phosphatidylcholine, etc.

Recently, a nanosuspension product containing Paclitaxel (a very water-insoluble anticancer agent), Abraxane®, has been approved by FDA for IV administration. Abraxane

contains lyophilized particles with 10% (w/w) paclitaxel and 90% (w/w) albumin. The particle size of the suspension is about 130 nm (123). Another example of IV nanosuspenion is sterile powder of busulfan, encapsulated in a mixture of phospholipids - dimyritoylphosphatidylcholine and dilauroylphosphatidylcholine - in a buffer containing mannitol (124).

Dried forms. Sterile solids are drugs or drug products packaged in a dry form which must be reconstituted or suspended in sterile vehicle prior to administration. Many drugs, particularly the cephalosporins and penicillins, are not sufficiently stable in aqueous solution to permit packaging them "ready to use." The dry solids which are intended to be reconstituted by the addition of suitable solvents to yield solutions, conforming in all respects to the requirements for injections (solutions for injection), are described by a title in the form "for injection or sterile." Examples are thiopental sodium for injection (USP), in which the preparation contains added substances in addition to the drug, and sterile nafcillin sodium (USP), in which there are no additional ingredients other than the drug. In any such labeling, the product is intended to be appropriately reconstituted as a solution. Some reconstituted products must be further diluted prior to use, an example being methohexital sodium for injection (1).

Dry products which are to be reconstituted as suspensions by the addition of a suitable vehicle to yield a product meeting all requirements for sterile suspensions are labeled as "sterile—for suspension." An example is sterile ampicillin trihydrate for suspension. Such preparations are manufactured and packaged as dry sterile solids by sterile filtration and freeze-drying or bulk sterilization and aseptic powder filling. The sterile bulk powder in the latter process can be achieved by either aseptic crystallization or spray-drying.

The powder filling procedure is briefly described below.

Powder filling. This method involves filling sterile powder into individual containers (vials) under aseptic conditions in which a measured quantity, either on a weight or volume basis, is delivered. If the material is free flowing, a machine method is used whereby the solid material is fed from a hopper to the container by means of an auger in the stem of the hopper or an adjustable cavity in the rim of a filling wheel.

Particle size and shape are important factors in powder filling since electrostatic charge, hygroscopicity, and flow are generally influenced by these properties. Additionally, the dissolution rate can be influenced by particle size. The humidity of the filling room should be carefully controlled. If the room is too dry, the powder will become electrostatically charged and will not flow. If the humidity is too high, compaction will occur because of moisture in the powder.

For parenteral products, the powder is generally prepared under aseptic conditions by crystallization or spray-drying, which provides greater assurance of sterility within the material. In the crystallization technique, the drug is dissolved in an appropriate solvent and sterilized by 0.2 μm membrane. Next, under controlled conditions, another sterile solvent in which the drug is not soluble is added to the solution to induce crystallization of the drug. The sterile crystals are removed, washed and dried, then usually tested for particle size distribution, dissolution rate, and correct crystalline form prior to filling.

In order to obtain a uniform product from lot to lot, strict adherence to the procedures developed for a particular crystallization must be followed, including control of pH, rates of addition, solvent concentrations, purity, temperature, and mixing rates. Each crystallization procedure has to be designed to ensure sterility and minimize particulate contamination. Subtle changes, such as using absolute ethyl alcohol instead of 95% ethanol during the washing step of crystallization procedure, can destroy the crystalline structure if the material being crystallized is a hydrate structure.

If the drug powder is to be prepared by spray-drying, as shown in Figure 14, a sterile solution of the drug is prepared in a similar manner as for aseptic crystallization but instead of crystallizing the drug by adding another solvent, the sterile solution or a resultant slurry is sprayed through an atomizer with a fine orifice into a drying chamber, generally conical in shape.

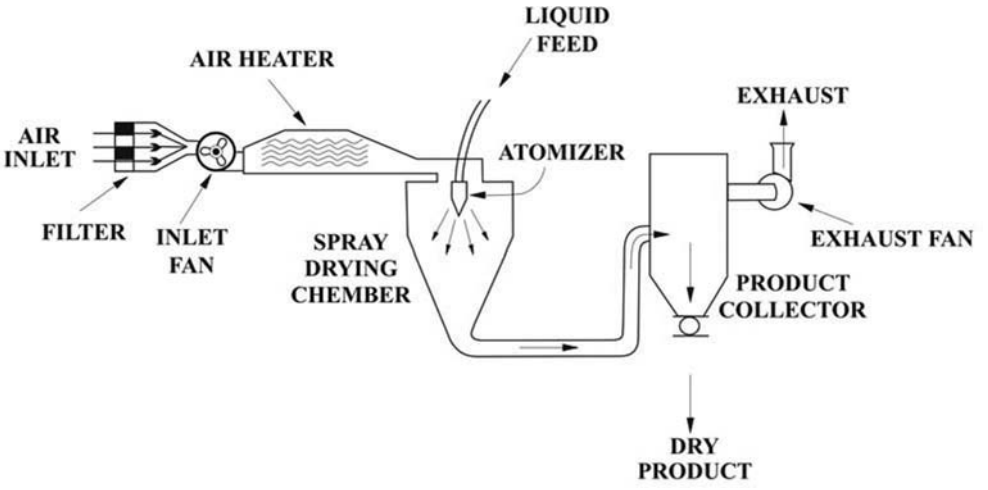


Figure 14 Schematic representation of spray-drying process.

Upon contact with a stream of hot sterile gas, the solvent rapidly evaporates and the resulting powder is collected in a sterile chamber. The type of atomizer and method of spraying, the concentration of the solution to be sprayed, the pressure at which it is atomized and the temperature and pressure of the gas in the chamber are factors that influence the particle size and porosity of the resultant powder. The drug powder, present as hollow spheres, is then filled into vials as a dry powder.

Freeze-drying or lyophilization. The freeze-drying or lyophilization of injectable products is described in sufficient detail elsewhere (chap. 17, volume 2), so only a brief discussion will be included here.

Freeze-drying, also known as lyophilization, is widely used for pharmaceuticals to improve the stability and long-term storage stability of labile drugs (125–127). Freeze-dried formulations not only have the advantage of better stability, but also provide easy handling (shipping and storage). There are currently more than 125 small molecule lyophilized injection products in the market and the number of lyophilized proteins and vaccines exceeds 50 (128). Most of these are formulated as lyophilized products because of their instability in aqueous solutions, however, as in the case of acyclovir sodium, lyophilization is necessary to minimize interaction of the alkaline formulation with glass material. Table 17 shows the examples of products of whose aqueous stability was only for few hours, but once they were converted into dry product by lyophilization, the resulting products had acceptable market shelf life.

Table 17 Comparison of Stability of Lyophilized and Solution Forms

Product	Bulking agent	Lyophilized product	Reconstituted product
Actreonam/Azactam	Arginine	3 yr	2 days (RT)
Amphotericin/Fungizone	–	2 yr	1 day (RT)
Cyclophosphamide/Cytoxan	Mannitol	3 yr	1 wk (13% loss)
Carboplatin/Paraplatin	Mannitol	2 yr	Particulates
Fosaprepitant/Emend	Lactose	>2 yr	24 hr at RT
Gemcitabine/Gemzar	Mannitol	>2 yr	24 hr at RT
Lansoprazole/Prevacid	Mannitol	>2 yr	1 hr at RT
Ixabepilone/Ixempra	None	>2 yr	1 hr at RT

Although there are those who would consider freeze-drying only as the last resort, there are others who view it as a panacea - a way to get into clinical trials quickly or a way to exclude contaminants and inert particles, especially in comparison with powder filling. Certainly, freeze-drying does offer the advantage over powder filling of accuracy of dosage, since the drug is filled into the final container as a solution. Microgram quantities can be filled precisely. The desired characteristics of a freeze-dried pharmaceutical dosage form include

1. an intact cake occupying the same shape and size as the original frozen mass,
2. sufficient strength to prevent cracking, powdering, or collapse,
3. uniform color and consistency,
4. sufficient dryness to maintain stability, and
5. sufficient porosity and surface area to permit rapid reconstitution.

Of course, as with any injectable dosage form, freedom from contamination (i.e., micro-organisms, pyrogens, and particulates) is an essential attribute. The desired characteristics can be achieved by proper formulation of the product and by employing optimum freeze-drying cycles.

A freeze-drying cycle essentially consists of three distinct phases: a) Freezing of the solution, b) primary drying or sublimation, and c) secondary drying. Loading of the filled vials in the chamber, maintenance of vacuum throughout the drying phases, supply of refrigeration during freezing and heat during the drying phases, and completion of the drying cycle by stoppering the dried vials and unloading them out of chamber are some other required actions. For a systematic approach to the development of a suitable freeze-dried product, knowledge of the various stages of the process is necessary. Comprehensive reviews of principles and practice of freeze-drying in pharmaceutical are widely reported in pharmaceutical literature (129–132).

The initial freezing process is of critical importance since it will influence the pattern of the sublimation phase. During freezing, pH change may arise from crystallization of buffer salts as well as large increase in ionic strength that may result into stability problems. The pH shift during freezing can be minimized by optimal choice of buffer salts or by reducing buffer concentrations. Upon freezing, the entire formulation must be in a completely frozen state otherwise collapse or meltback may happen during drying. The temperature above which the freeze-dried product loses macroscopic structure and collapses during freeze-drying is termed as collapse temperature or T_c and is usually about 2°C higher than T_g', which is often associated with the glass transition temperature in the frozen state (133). T_c equals the eutectic temperature (T_{eu}) if solutes are crystallized in the frozen solution. Well designed cooling cycle (ramp and hold times) must be used in order to obtain an appropriate structure of the frozen mass, which is a function of the rate of freezing and the final freezing temperature. The rate of freezing affects the size of ice crystals. Slower rate of freezing results in larger ice crystals and vice versa. If the frozen system exhibits metastable or amorphous-glassy structures, these structures may need to be ruptured by appropriate thermal treatment or annealing process (a succession of cooling and rewarming periods), thereby inducing crystallization of the amorphous material for efficient sublimation.

Most freeze-dried drug products are organic electrolytes which exhibit eutectic points or glass transition temperatures and super-cooling tendencies. Several methods have been used for determining eutectic temperatures: (*i*) thermal analysis, (*ii*) differential thermal analysis, and (*iii*) electric resistivity (131).

Knowledge of the eutectic temperature of the additive is essential since the addition of a salt such as sodium chloride to a drug with a eutectic significantly above that of sodium chloride would only succeed in lengthening the cycle because lower temperatures would have to be maintained. In addition, some additives, such as the phosphates, tend to form crusty-appearing cakes. This occurs during freezing and drying, probably because of the phenomenon of recrystallization. Volatile substances are generally considered to be of little value to the finished cake but can be used if they accelerate the drying cycle, for example, t-butanol (134,135). The next step in the freeze-drying process is primary or sublimation drying which is

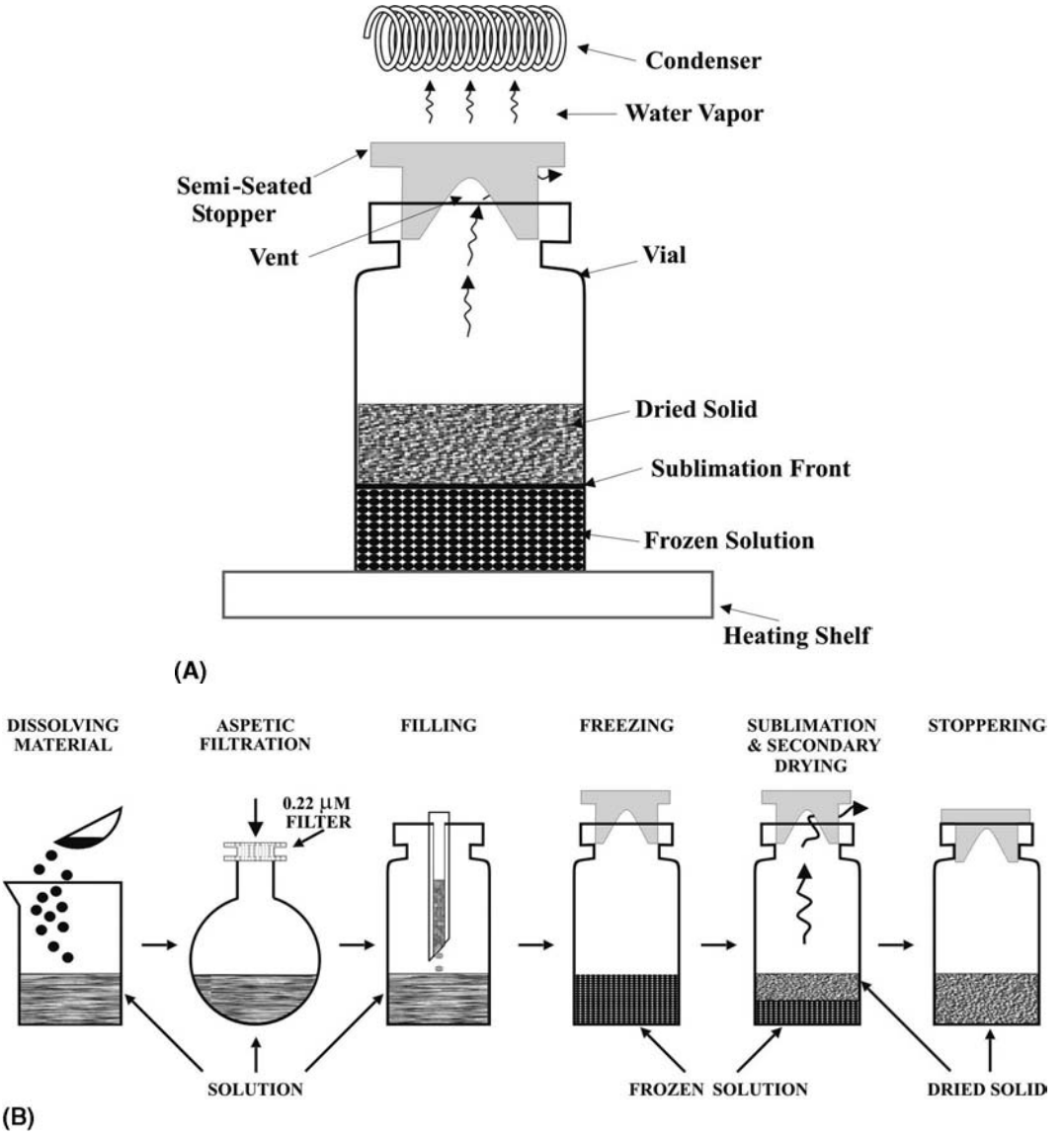


Figure 15 Schematic representation of freeze-drying process.

conducted under low chamber pressure conditions, for example, 200 mTorr or lower, under which sublimation of ice, as dictated by the ice/water-vapor equilibrium line of the phase diagram of water, takes place and the water vapor from the frozen matrix is transferred out of the vial, traveling into the headspace of the vial, through the vents of the closure, into the chamber, and eventually onto the cold condenser, where it is condensed again as ice (Figure 15A). Thus, frozen water from the vial is vaporized by sublimation and collected on the cold plates of condenser. The sublimation is a phase change, requiring energy, which must be supplied as heat from the carefully controlled heated shelf. The sublimation drying phase is a combined heat-mass transfer process in which both the transfer phenomena must be carefully balanced so that sustained drying rate (mass transfer) prevails without collapsing or melting of the frozen mass because of accumulation of heat from the heated shelf (heat transfer). During

the entire sublimation phase, the product temperature should always be several degrees below Tc in order to obtain a dry product with acceptable appearance. Factors influencing the rate of vaporization have been discussed extensively (136–139). The faster heat can he applied, the faster the drying proceeds, provided that, a) The temperature of the product remains below its liquefying point, and b) sufficiently low pressure is maintained in the system by efficient vacuum pumps. If a sufficiently low pressure is not maintained, the temperature of the product will rise resulting in the partial softening or puffing of the product.

In developing a formulation for freeze-drying, the optimal formula will permit the overall cycle to be carried out in the least amount of time, while providing a stable and efficacious product which contains a low moisture content, undergoes rapid reconstitution, and possesses the desired appearance. The potency of many lyophilized products is so high that relatively small amounts are required for the lyophilized injectable dosage form. Therefore, the need for suitable filler or bulking agent is often indicated. The percentage of solids in the frozen plug will vary depending on the dosage and nature of the active ingredient; generally, it should be above 5% and not exceed 30%, with a 10 to 15% content being optimum. Materials to choose from to add to the solution to improve the physical characteristics of the finished cake are limited but include mannitol, lactose, sucrose, dextran, amino acids, sorbitol, gelatin, mono- and dibasic sodium phosphate, albumin, sodium chloride, etc. It should be kept in mind when adding bulking agents that drying will be accelerated if the solute concentration is kept low. However, solutions with too low concentration ($\leq$ 1% w/w) may result in very brittle cake and there is a likelihood that some of the powder may fly off the cake into the chamber resulting in low drug content in the vial.

If degradation is a risk during freezing due to concentration effects or pH changes, stabilizers or buffers may have to be added. The problem of collapse has been discussed earlier (140) and if the substance is vulnerable to collapse, a rigidizer such as glycine or mannitol may need to be added. If damage during freezing is a problem, a cryo-protective agent such as sucrose or albumin may be added. If the ingredients that are added are found to adhere to the glass surface, such as albumin, then the containers with thin walls, such as ampuls and tubular vials, may need to be coated with silicone to minimize sticking. The depth of fill in a container is critical. While this depends on the volume of the container, a rule of thumb has been 1 to 2 cm in depth but never exceed one-half the capacity of the container otherwise breakage of vials may be seen.

Freeze-dried products are generally packaged in ampuls or vials. Ampuls would only be used for single-dose administration, and provide even drying because the tubing is thin and bottoms are reasonably flat. However, they must be sealed after removal from the chamber and reconstitution is sometimes cumbersome if shaking is required. Additionally, the generation of glass particles is a problem. Vials are used for both single- and multiple-dose application. If molded glass is used, there is greater incidence of variation of thickness and uneven bottoms. The containers must be sealed with a closure that can be accomplished inside the chamber, lessening the risk of contamination and providing an opportunity to seal under an inert gas or under vacuum.

The next stage in freeze-drying cycle is secondary drying. When sublimation drying phase is completed, the temperature of the product progressively rises (following the temperature of the shelves). The goal of desorption is to remove traces of moisture in the product (the majority of the water in the form of ice already been removed during the sublimation phase). The secondary drying process consists in removing the molecules by having the product under the highest possible shelf temperature (e.g., 20–35°C) compatible with its stability and the chamber pressure at its lowest value.

Typical process of freeze-drying is illustrated in Figure 15B. It involves: (1) dissolving the drug and excipients in a suitable solvent, generally water; (2) sterilizing the bulk solution by passing it through a bacteria-retentive filter; (3) filling into individual sterile containers with semi-stoppered closures; (4) freezing the solution by placing the open vials on cooled shelves in a freeze-drying chamber, (5) applying a vacuum to the chamber and heating the shelves in order to sublime the water from the frozen state, and (6) breaking the vacuum at

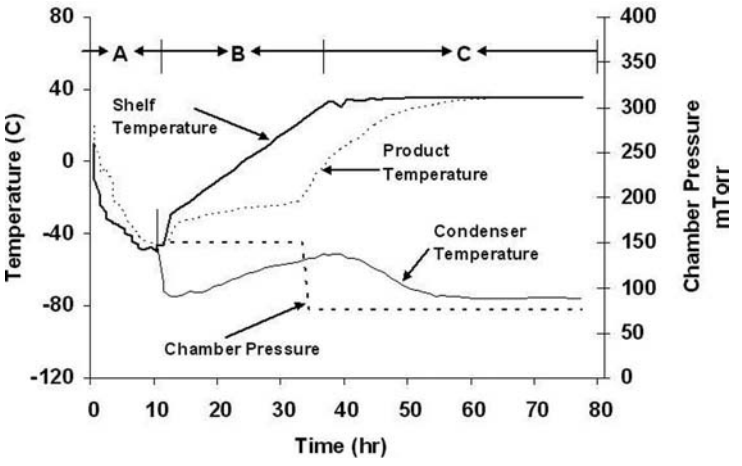


Figure 16 Typical product temperature/chamber pressure curve during freeze-drying.

the end of drying using sterile air or nitrogen, fully stoppering the containers, and unloading of the vials.

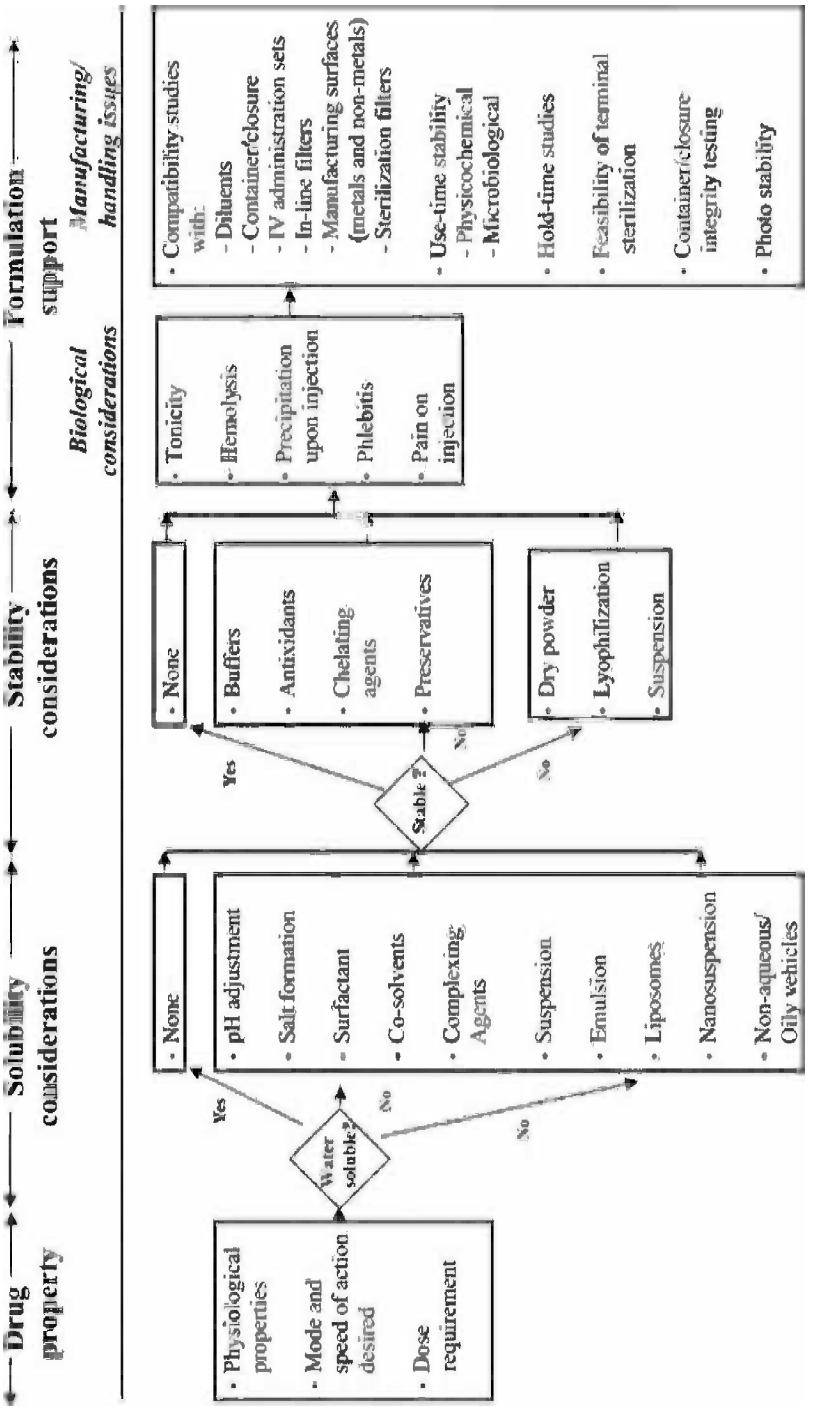
Temperature and pressure curves for a typical cycle are illustrated in Figure 16 for Mannitol solution (5% w/w) filled into 10 mL glass vial (5 mL/Vial). Freezing stage is denoted by "A," primary drying by "B," and secondary drying by "C." During freezing as the shelf temperature is lowered the product cools down and freezes and eventually reaches its target temperature of $<-40^{\circ}C$. At this time, the condensers are chilled to below very low temperature ($<-70^{\circ}C$) and the vacuum is initiated in the chamber. Once the vacuum has reached its target value, say 150 mTorr in this case, then the primary drying begins wherein the shelf is heated slowly to provide heat to sustain sublimation. Around 34 hours, at the end of phase "B, " the product temperature starts rising swiftly indicating that the ice is removed and the heat is consumed not just for the phase change in sublimation, but results in increase in the temperature of the product. The phase denoted by "C" is secondary drying where the continuously heated shelves provide heat to remove residual moisture by desorption process, aided by lower chamber pressure than before. At the end of secondary drying, the vials are fully stoppered, vacuum is broken to return the chamber to the atmosphere and the vials are unloaded.

Formulation Development Process

From preceding sections, it is clear that successful formulation of an injectable small-volume preparation requires a broad knowledge of physical, chemical, and biological principles as well as expertise in the application of these principles. Moreover, formulation is a highly specialized task requiring not only specific knowledge but also years of experience. During the course of development, formulation design and optimization is an iterative process and evolves as the product moves from the discovery to clinical to commercial stages. Although, most of the times, the development is an empirical approach based on principles mentioned earlier, there are number of strategies or decision trees that one can adopt to proceed with the product design. There are even published reports that the suggest use of "expert systems," comprising of databases and decision making processes, to aid parenteral development (141).

Table 18 summarizes one such approach that can be considered as a template for parenteral formulation development process which considers many of the essential factors necessary for the formulation design and lists various formulation-supporting studies that are needed from patient use, manufacturing, and marketability point of view. These

Table 18 Suggested Template for Small-Volume Injectable Formulation Development Process



studies are not mutually independent, though. Not only the formulator must arrive at an optimum formula from stability/solubility point of view alone, but he/she must ensure that the product is acceptable from patient's acceptability/tolerance point of view and it poses minimal difficulty or constraints from the manufacturing and/or marketing point of view.

Formulation-supporting studies. In finalizing the formulation, a number of supporting studies are needed to address the biological or patient-related issues, support the manufacturing process, and define the boundaries under which the product's qualities will be maintained throughout the shelf life of the product.

1. Biological considerations:
   a. Evaluation of impact of formulation toward hemolysis, precipitation, phlebitis, and pain on injection
   b. Tonicity
2. Manufacturing and handling support studies:
   a. Compatibility with commonly used diluents and IV administration sets, etc.
   b. Compatibility with manufacturing equipment
   c. Compatibility with membrane filters, if aseptic processing is used during the manufacture
   d. "In-use" stability studies
   e. Feasibility of terminal sterilization
   f. Photostability

Biological considerations.

Hemolysis, precipitation, phlebitis, and pain on injection Some injection products are prone to formulation-related problems such as hemolysis of the RBCs; precipitation of the drug and ensuing phlebitis; and pain at the site of injection.

Hemolysis results from disintegration of RBC membrane and release of the cellular contents into the plasma, particularly that of hemoglobin. Once outside of the RBC, hemoglobin molecule quickly dissociates into its component polypeptide chains which can pose many serious physiological problems, mainly the renal failure. Hemolysis usually results from hypotonicity or from the effect of drug or the formulation components on cell membranes (142,143).

Precipitation of the drug at the site of administration can happen once the solubilizing principles are diluted away or removed from the vicinity of the drug.

Phlebitis occurs because of inflammation of a vein with symptoms such as tenderness, edema, erythema, and a local temperature rise. In severe cases, it can lead to thrombus and even more severe complications. Although a number of factors have been implicated as causes of phlebitis; particulate matter, precipitation of drug, and local pH effects are the most likely causes (144–148).

Injectable formulations are often painful and irritating following injection as a result of cell damage such as phlebitis. Sometimes the pain/irritation response is associated with the active drug (s) present in the formulation, for example, macrolide antibiotic (149) and excipients (150). Pain on injection may occur during and immediately following the injection or it may be a delayed or prolonged type of pain which increases in severity after subsequent injections. The actual cause of the pain is often unknown and will vary significantly among patients according to the product. In some cases pain may be reduced by minor formulation changes such as adjusting tonicity and pH or adding an anesthetic agent such as benzyl alcohol or lidocaine hydrochloride. In other cases pain is more inherent to the drug and the problem is more difficult or impossible to resolve. Pain, soreness, and tissue inflammation are often encountered in parenteral suspensions, especially those containing a high amount of solids. A number of in vivo (animal studies) and in vitro studies to evaluate hemolysis, precipitation, phlebitis and pain upon injection have been published (151–154). It is important that the

formulator evaluate the potential of the formulation to causes of the above mentioned problems using these or other suitable techniques.

Tonicity Tonicity has been previously discussed under "Added Substances."

Manufacturing and handling support studies.

Compatibility with commonly used diluents and IV administration sets Many IV parenteral products are often administered via large-volume parenteral (LVP) solutions. In such cases, the solubilized portion of the product, either withdrawn directly from the ready-to-use solution or from the reconstituted dry product, is directly added to the diluent bag or added through the Y-site of the IV administration set. Obviously, the potential for drug stability and compatibility problem is great because of the long duration of contact time and exposure to ambient conditions of temperature and light (155). The potential physical and chemical incompatibilities associated with such dilutions are compiled in a treatise by Trissel (74) and is often the primary reference book on this subject in the practice of pharmacy.

Typically, compatibility of the drug product with the reconstitution diluents (precipitation and stability), at the recommended storage temperature and at the likely extreme concentrations of administration, is demonstrated with most commonly used diluents and IV fluids, such as normal saline, dextrose solutions, ringer's solution, etc., and combinations thereof (156). It is also important that compatibility information is generated for the drug in contact with potential delivery devices such as the IV administration sets, in-line filters, syringes, etc.

Compatibility studies with manufacturing equipment contact surfaces Various contact surfaces are encountered during the manufacture and storage of injection products. Compatibility studies of the drug product with such surfaces must be evaluated to ensure that there are no adverse interactions and the quality of the product is unaffected. Typical product contact surfaces during the manufacture are transfer tubing, manufacturing equipment, filtration surfaces and devices, filling machine parts (pumps, filling needles) surfaces, etc. These are comprised of variety of materials such as rubber, plastic, ceramics, and metals. Typically, the component under investigation is placed in contact with the drug product solution for 24 to 96 hours at room temperature, at which point the samples are analyzed for various physicochemical attributes such as pH, appearance, UV/FT-IR spectroscopy, and potency.

Compatibility with packaging components During the storage of the product in the final container, the product comes in contact with the rubber-based or polymeric stoppers, glass in the case of vials, or other plastic materials in the case of syringes and plastic bags. Compatibility studies of the drug product with such packaging components is performed similarly by contacting the packaging components with the drug product and analyzing for physicochemical attributes of both the solution and the components.

Compatibility with membrane filters Bulk solutions of many aseptically produced injection products are sterilized by membrane filtration using 0.22 μm filters. It is important that the compatibility of the drug product with that of material of the sterilization membrane filter (and prefilter, if used) as well as the filter assembly is evaluated to ensure that the product quality is not affected as well as no undesired components are added to the drug product. Some of the techniques used in practice for this purpose include the following:

1. Microbial membrane retention testing to demonstrate that the formulation of the product does not adversely affect the effectiveness of removal of any microbial contamination from the bulk solution. This is typically done by filtering a challenge solution containing large number of bacteria in the drug product solution (or its equivalent placebo) and testing for the filtrate for any microbial presence.
2. Membrane compatibility study to ensure that the prolonged exposure of the product does not affect the key membrane characteristics. This is typically done by soaking the membrane disks in the drug bulk solution for 24 to 48 hours and then evaluating

the filters for key parameters such as water permeability (flow rate), product bubble point, weight change, and appearance.
3. Filter extractability testing to assess the effect of formulation on the extractables from the filter. This is typically performed by subjecting the filter device to worst-case sterilization conditions (time, temperature, and repeated cycles) followed by extended exposure to organic solvents such as 100% denatured ethanol and then analyzing the extract for volatile and nonvolatile organic compounds.
4. Product specific bubble point measurement as a tool to monitor the integrity of the filter during routine manufacturing.

"In-use" stability Use-time stability studies are performed to establish the following:

1. How long the drug product solution is stable at ambient (use) conditions, if normally the drug product is supplied in dried form.
2. How long the drug product is stable at ambient (use) conditions, if normally kept at refrigerated storage.
3. In what diluent and how long the diluted drug solution is stable, from both physicochemical and microbiological perspectives.

The above information is then included in the package insert that is provided with the final drug product and forms the basis for the proper use of the drug and instructions for suitable use of diluents and delivery devices.

Feasibility of terminal sterilization Injection products are rendered microbiologically sterile by terminal sterilization by using steam or dry heat. Steam sterilization, which offers the greatest assurance of sterility, can be expected to cause some changes in the product, however subtle. Drugs are reactive substances and autoclave temperature (121°C) for 15 to 30 minutes could give rise to degradation processes and interactions with the container. Additionally, materials could leach form the rubber closure. In addition to loss of drug, antimicrobial agents and antioxidants can be absorbed or consumed during sterilization. Lately, it is becoming a well-accepted principle that sterile drugs should be manufactured by aseptic processing only when terminal sterilization is not feasible because of excessive thermal degradation of the product. There are many categories of the products that may qualify for not subjecting to terminal sterilization (157); however, regulatory agencies may require a written justification to address why a product is not being terminally sterilized. With such restrictions, the formulator of an injection product must assess the effect of terminal sterilization conditions on the stability of the product, the acceptable level of degradants, and offer alternate sterilization techniques such as aseptic processing or adjunct processing step(s) in addition to aseptic processing, for example, addition of heat exposure condition which may provide increased level of sterility confidence (158).

Photostability Exposure to irradiation such as light can influence the stability of the formulation, leading to changes in the physicochemical properties of some products. The most obvious result of drug photodecomposition is a loss of potency of the product. In few cases, trace amounts of photodecomposition products formed during storage and administration may lead to adverse effects (159). The excipients used may also often contribute to the photoreaction (160–163) and hence stability evaluation in the presence of excipients is important. The selection of a protective packaging must be based on knowledge about the wavelength causing the instability. A review by Tonnesen (164) has focused on practical problems related to formulation and stability testing of photolabile drugs. An ICH guideline, "Guidelines for the photostability testing of new drug substances and products," describes photostability methodology, including the decision flow-cart, choice of light source, sample preparations, and interpretation of results (165).

In the case of injection products, transparent glass or plastic vial offers little protection toward radiation (166). The stabilizing effect of amber glass as the only means of

photoprotection is not satisfactory for highly photolabile drugs like molsidomine (167). Even brown glass can offer inadequate protection as demonstrated for drugs like epinephrine, isoprenaline and levarterenol (168). In practice, a secondary container, such as a cardboard box or carton is often necessary to prevent photodegradation. Similarly, for extremely high light-sensitive drugs, the manufacturing operations (compounding, filling, and packaging) may also need to be carried out by minimizing light exposure or by using yellow lights in the process areas.

At the conclusion of the formulation development process, the formulator must be in a position to compile all the knowledge generated in the process for regulatory scrutiny. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has published guidance for industry, "Q8 Pharmaceutical Development," which provides necessary elements of the development process that includes the concepts of quality by design (QbD), use of quality risk management, and use of knowledge management (156). This guidance recommends summarizing the development of the formulation including identification of those attributes that are critical to the quality of the drug product. As per the guidance, the summary should highlight the evolution of the formulation design from initial concept up to the final design. This summary should also take into consideration the choice of drug product components (e.g., the properties of the drug substance, excipients, container closure system, any relevant dosing device), the manufacturing process, and, if appropriate, knowledge gained from the development of similar drug product(s). The guideline further describes the use of principles of quality by design (QbD) during the development of a drug product. The QbD identifies characteristics that are critical to quality from the perspective of the patients, translates them into attributes that the drug product should possess, and establishes how the critical process parameters can be varied to consistently produce a drug product with the desired characteristics. Reader is encouraged to study and practice the quality principles laid down by this guidance.

Container Effects on Formulation

Containers for parenteral products serve several purposes; facilitate manufacturing; maintain product protection including sterility and freedom from pyrogen; allow inspection of the contents; permit shipping and storage; and provide convenient clinical use. The container components must be considered as integral parts of the product because they can dramatically affect product stability, potency, toxicity, and safety, and therefore must be evaluated carefully with a variety of tests. For details on this topic, reader is directed to chapters 11 and 12 of this book.

Stability Evaluation

Throughout the world, there has been phenomenal increase in laws, regulations and guidelines for reporting and evaluating data on safety, quality and efficacy of new medicinal products. Although different regulatory systems have the same fundamental concepts to evaluate the quality, safety and efficacy, the process of evaluation has diverged over time to such an extent that the industry has found it necessary to duplicate many time-consuming and expensive test procedures, in order to market new products, internationally. To address this, initiation of ICH was pioneered by the European Community, in the 1980s, and later joined by the representatives of the regulatory agencies and industry associations of the United States and Japan. The key goals of the ICH have been the development of the "ICH process" for developing harmonized guidance on technical issues and under this process many guidance have been published. For details on the topic of stability studies regarding the stability procedures, sampling requirements, storage conditions, testing schedules, and evaluation of data, reader is directed to chapter 10, volume 3 of this book.

Process Effects

The processing of parenteral products has been covered elsewhere in this textbook, but some specific cautions associated with the effects on formulation will be highlighted. There comes a

point in the development process of a product to characterize the production process and assess its effect on the formulation. This requires scale-up procedures to identify the process and equipment variables and with knowledge of the formulation and package variables assess how product quality and manufacturing productivity will be affected. In the manufacture of a sterile product, the assurance that the finished product possesses the desired quality control characteristics depends on a number of independent but interrelated events commencing with the initial design of the dosage form and carrying forth through the process design and validation and culminating with the establishment of standard procedures for manufacturing.

To provide for the assurance that all quality attributes will be achieved on a repetitive basis, the following are essential: (1) the dosage form is designed with knowledge of the desired functional and quality control characteristics of the finished product; (2) the qualification procedures are adequate to ensure reliability of the equipment, effectiveness of the process, and the integrity of the processing environment; (3) personnel are trained in contamination control techniques; and (4) there is adequate documentation of all procedures and tests. Such a development sequence combined with validation requirements suggests a formalized program culminating in a product that can be reliably processed. The process characterization is a principal step in assuring that the process can be translated to manufacturing on a routine production basis. Although this chapter is not intended to cover processing in the broad sense, those responsible for developing formulations should have an understanding of the following:

1. Scale-up procedures
2. Preliminary technical documentation
3. Design of processing and validation protocols
4. Use of process analytical technologies (PAT) for monitoring and control purposes
5. Qualification/validation runs
6. Final technical documentation and authorizations

The overall approach must be organized, scientific, and thorough. Moreover, the issues in shipment of the product, especially if refrigeration or some other storage temperature restrictions apply must be addressed. Lastly, addressing the usual unplanned deviations in the manufacturing processes and the provision of rework or rescue procedures must also be considered.

FORMULATION OF LARGE-VOLUME INJECTIONS

Introduction

LVPs or injections are primarily used for IV nutritional therapy which is required when normal enteral feeding is not possible or is inadequate for nutritional requirements. Specific nutritional requirements and administration mode depends on the nutritional status of the patient and the duration of the parenteral therapy (45). To meet IV nutritional requirements, one or more of the following nutrients may be required:

- Protein substrates: These include various amino acids formulation used for general replacement purpose, for hepatic failure, for encephalopathy, and for metabolic stress conditions.
- Energy substrates: These include dextrose and IV fat emulsion.
- Electrolytes: Saline, ringer's solution, etc.
- Vitamins and trace metal supplements.

Besides providing the water, electrolytes, and simple carbohydrates needed by the body, LVPs also a) act as the vehicle for infusion of drugs that are compatible in the solution, b) provide solutions to correct acid-base balance in the body, c) act as plasma expanders, d) promote diuresis when the body is retaining fluids, d) act as dialyzing agents in patients

with impaired kidney function, and e) act as x-ray contrast agents to improve diagnostic abilities. It is now almost a standard practice to begin infusing a patient with a LVP, often dextrose and electrolytes, shortly after admission to the hospital. One of the reasons for this is to provide a readily accessible link to the central compartment if additional medications are required, while at the same time providing fluids and electrolytes to achieve an optimum balance for further treatment. IV administration, however, bypasses protective mechanisms of the body, and the onset of adverse reactions, including the cases of nosocomial bacteremias in hospitals (169), which may come about from many causes, can be as rapid as the beneficial effects. The National Intravenous Therapy Association (NITA) as well as many technical books have developed recommendations for procedures to be followed during IV therapy (170,171). The procedures are designed to minimize undesired reactions.

Formulation Principles

Physiological Parameters

The physiological parameters of a LVP formulation are limits on those characteristics of the solution that impart some effect on the biochemistry of the body.

Some constituents that are basic to the sustenance of life in the human organism can be influenced by IV therapy. These are water, electrolytes, carbohydrates, amino acids, lipids, and micronutrients such as vitamins, minerals, and trace elements.

The living cell, the body's basic unit, is bathed in tissue fluid kept constant in composition by the interaction of many processes, some of which are outside the scope of this chapter. Alteration in the amount or composition of tissue fluids can cause significant physiological derangements. Such imbalances may occur as a major or minor feature of illness, trauma, or surgical procedures. Under such circumstances it is necessary to anticipate and correct deficits and imbalances by administration of suitable fluids. The body fluids, named for the compartments in which they are found, are intravascular (within the blood vessels), intracellular (within the cells), and interstitial (within the space between cells). Extracellular fluid is the total of intravascular and interstitial fluids. The fluids consist of water containing a mix of electrolytes, neutral solutes in a wide range of high and low molecular weights, and undissolved substances. The composition of each fluid differs, yet a chemical balance is maintained in each fluid. Approximate figures for the electrolytic composition of body fluids are shown in Table 19.

Table 19 Electrolyte Composition of Body Fluid Compartments

	Intravascular	Interstitial	Intercellular
Electrolytes	(mEq/L)	(mEq/L)	(mEq/L)
Cations			
Sodium (Na^+)	142	145	10
Potassium (K^+)	4	4	160
Calcium (Ca^{2+})	5	5	2
Magnesium (Mg^{2+})	2	2	26
Total	154	156	198
Anions			
Chloride (Cl^-)	102	115	2
Bicarbonate (HCO_3^-)	27	30	8
Phosphate (HPO_4^{2-})	2	2	120
Sulfate (SO_4^{2-})	1	1	20
Organic acids	6	7	–
Protein	16	1	48
Total	154	156	198

Extracellular fluid is characterized by high concentrations of sodium and chloride ions. The intravascular fluid contains a much higher concentration of protein than is found in interstitial fluid because the large plasma protein molecules are not diffusible. The retention of protein anions on one side of, the semi-permeable membrane causes a redistribution of the anions that are permeable, in order to maintain chemical balance (172). As a result, the concentration of other anions is lower in intravascular fluid than in interstitial. Intracellular fluid is characterized by very high concentrations of potassium, phosphate, and protein.

An LVP formulation must be developed to ensure that desired levels of the solution are administered in a therapeutically active and available form. In order to obtain the desired response, the physiological intent of the formulation must be considered and the physiological, chemical, and physical properties of the formulation defined. The formulator must understand the biochemistry of the body and the chemistry of the in vivo parenteral because it is through their interaction that the result is achieved. These factors are discussed in the sections to follow.

Formulation Parameters

Physiological. Body fluids rapidly exchange both water and electrolytes between the cells and extracellular compartments, maintaining equilibrium within and between the compartments. The movement of solvent and solute through the semi-permeable membranes that separate the compartments is called osmosis. If the concentration of solutes in adjoining compartments differs, water moves very rapidly into the compartment with the higher concentration in the effort to establish equilibrium. Simultaneously, disassociated solutes diffuse at a slower rate to the compartment with the lower concentration. Because some components of the fluid cannot move through the semi-permeable membrane, the fluid in the compartment must make adjustments to maintain its own ionic equilibrium (mentioned previously with respect to the difference in the ions contained in extracellular and interstitial fluids).

The resistance to unrestricted movement between compartments is defined as osmotic pressure and is expressed as osmoles per kilogram (osm/kg) or, more conveniently, milliosmoles per kilogram (mOsm/kg). Osmolarity values of dilute solutions can be calculated and their levels expressed as milliosmoles per liter (mOsm/L) by using the formula:

$$\text{mOsmol/L} = \frac{\text{g/L of solute}}{\text{molecular weight of solute}} \times 1000 \times \text{number of ions}$$

Sodium chloride, for example, has a molecular weight of 58.5 and forms two ions, Na^+ and Cl^-, in solution. The osmolarity of 0.9% sodium chloride injection would be calculated as $\text{mOsm/L} = 9/58.5 \times 1000 \times 2 = 307.7$, rounded to 308.

An immediate concern of introducing large amounts of fluid into the body system is that of maintaining the "tone" of the living body cells, RBCs circulate in blood, which has an osmolarity of 306. Using osmolarity as a measure of tonicity, one would expect no physical change in the RBC if 0.9% sodium chloride injection, with an osmolarity of 308, were infused into the vein. This is the case, as can be demonstrated by putting red cells into the 0.9% Sodium Chloride Injection and microscopically examining the cells for physical change. No changes result, and the solution is termed isotonic. If RBC are placed in a hypertonic solution, for example, 20% dextrose (1010 mOsm/L), the water in the cell will diffuse out, causing the cell to shrivel. Conversely, RBC placed in a hypotonic solution, such as 0.45% sodium chloride (154 mOsm/L), will swell because of the flow of water into the cell and, if the effect is great enough, may rupture. For this reason, WFI, USP, which has no dissolved solids, despite its name is never injected alone. Table 20 shows the relationship between osmolarity and tonicity.

Tonicity, as defined by numerical calculation, is only one consideration that must be taken into account and it must be used with judgment. For example, a solution of 1.85% urea is isotonic but quite unsuitable for administration at the rate isotonic solutions are normally infused; it can cause hemolysis as well as upset the body's nitrogen balance. A solution of amino acids, which is hypertonic at about 850 mOsm/L, may be life sustaining and the

Table 20 Relationship Between Osmolarity and Tonicity

Osmolarity (mOsm/L)	Tonicity
>350	Hypertonic
329–350	Slightly hypertonic
270–328	Isotonic
250–269	Slightly hypotonic
0–249	Hypotonic

problems of tonicity can be overcome if it is introduced slowly into a large vein where there is ample blood volume to assure dilution. Hypertonic and hypotonic solutions can be used if administered slowly. The rates of shift of water into or out of the vascular system are determined by the rate of administration, rate of diffusion of the solute, and tonicity of the solution. Calculation of tonicity has been described in the earlier section.

Physicochemical

Solubility Compared with the solubility challenges in compounds used in small-volume parenteral as described earlier, most of the solutes used in LVP solutions are extremely soluble relative to their therapeutic concentrations. This means that solubility is rarely a consideration during formulation and, once in solution, the ingredients remain dissolved under normal storage and handling conditions. There are occasional reports of crystallization in highly concentrated solutions, such as 15% mannitol; this is caused by a reduction in solubility when the bottle is cold and the crystals go back into solution readily when the bottle is warmed. The solubility of mannitol is 13 g/100 mL water at 14°C; the package inserts for mannitol solutions caution the user that concentrations over 15% may show a tendency to crystallize.

In some cases, as with amino acid or high-concentration dextrose solutions, the temperature of the WFI is elevated during mixing. Although the ingredients are soluble at lower temperatures, minimizing the preparation time reduces the time the solution is exposed to ambient microorganisms. The order in which ingredients are added to the mix tank may have an effect on how rapidly the mix is completed or whether it can be completed. For example, when one is preparing amino acid solutions the pH changes after the addition of each amino acid and some amino acids are soluble only at specific, narrow pH ranges. Consequently, the order of adding the various amino acids can be critical unless preblended powdered amino acids are used. In general, solubility only becomes a consideration when the LVP is used as a carrier for other drugs.

pH The pH of a formulation must be considered from the following standpoints: the effect on the body when the solution is administered; the effect on stability of the product; the effect on the container closure system, and the possible degradation of drugs that are added. The pH of blood is normally between 7.35 and 7.45, and the immediate effect of intravenously introducing fluids outside this range depends on the buffer capacity of the solution, determined by the amount of weak acids or bases that are part of the formulation. The solution is rapidly diluted in the bloodstream, and the body's buffering system can maintain the proper pH level when high or low pH LVPs are administered, although it does so less easily if the solutions are highly buffered.

Because of its lower cost, type II glass, a flint glass with a surface treatment, is used for many LVPs that are packaged in glass. Solutions with pH values approaching or over 7.0 accelerate glass attack and must be packaged in the more expensive type I borosilicate glass. Since this glass is resistant to attack by alkaline solutions, it is used to prevent the pH from rising even higher. Other problems associated with degradation of the glass surface, such as the formation of glass flakes in the product, can be avoided by the use of type I glass. Chapter 11 provides a thorough discussion of glass containers that are used for packaging parenterals.

Vehicles WFI is the vehicle used for all LVPs. All ingredients are dissolved, and the resulting aqueous solution is clear and generally colorless. The IV fat emulsion, an LVP that may be administered alone or in combination with amino acid and dextrose solutions for total parenteral nutrition (TPN) therapy, is the exception. Triglycerides, egg phospholipids, glycerin, and WFI are homogenized to produce a stable emulsion with fat particles approximately 0.3 μm in size.

Physical Parameters

The sensitivity of a solution when exposed to light and extremes in temperature must be evaluated during the development of a formulation. Certain vitamin solutions require protection from light, for example, in the form of an amber bottle or an opaque unit carton. A light protective cover must be put over containers of solutions to which photodegradable drugs have been added. Solutions with high concentrations of dextrose or combinations with dextrose that have a tendency to develop slight discoloration with age will do so more rapidly if stored at high temperatures. The physical parameters that are defined for a solution are stated on the labeling and packaging inserts.

Packaging Parameters

The chapters on containers and closures in this textbook provide detailed information about the characteristics of materials available for packaging parenteral medications.

Stabilization of LVPs

Added substances. Buffering agents, chelating agents, antimicrobial preservatives, and antioxidants, commonly added to parenteral medications, are rarely used in LVPs. Buffering agents generally are not added as such, although acids and bases, which are used to adjust pH, can raise or lower the buffering capacity of the solution. By their nature and use, LVPs introduce large amounts of fluid and chemicals into the body. The active ingredients are present for a therapeutic effect, and although present in only very low percentages, added substances might, in total, have an effect on the patient who receives many bottles of solution during the course of treatment.

Very minute quantities of metals such as iron, copper, or calcium may be introduced into LVPs because of ingredients used and hence the quality of the incoming raw materials must be ensured. When drugs are administered orally, the gastrointestinal tract prevents aluminum from being absorbed into patient tissues; however, when the drugs are administered parenterally the aluminum can be deposited in tissues, potentially at toxic amounts. Therefore, according to the latest FDA guideline, the aluminum content of LVP drug products used in TPN therapy must not exceed 25 μg/L (173).

Antioxidants such as sodium bisulfite or sodium metabisulfite are part of some LVP formulations. They are added to protect the active ingredients from the action of oxygen in the solution or headspace of the container. The presence of oxygen, even very small amounts, can accelerate color formation or degradation of such products as 5% Dextrose in lactated ringer's or amino acid solutions. In lieu of the addition of an antioxidant, which might be added in concentrations of up to 0.1%, processing to displace the oxygen with an inert gas, usually nitrogen, may be done during mixing and filling operations. If both nitrogen and an antioxidant are used, the use of nitrogen will reduce the amount of bisulfite needed to protect the product during its shelf life.

Electrolytes, Carbohydrates, and Nutritionals

Typical examples of LVP formulations are shown in Tables 21 to 23. They are only a few of the many formula variations that represent the basic theme of each grouping.

Electrolyte solutions. The multiple electrolyte injection is an example of a solution that must be packaged in type I glass or plastic because its high pH, 7.3, can chemically attack type II

Table 21 Typical Examples of Electrolyte Solutions

Electrolyte	Plasma-Lyte R®	Isolyte S pH 7.4®	Normosol R®
Na^+	140	141	140
K^+	10	5	5
Ca^{++}	5	–	–
Mg^{++}	3	3	3
Cl^-	103	98	98
Lactate	8	–	–
Acetate	47	27	27
Gluconate	–	23	23
Phosphate	–	1	–
Osmolarity (mOsm/L)	312	295	294
pH	5.5	7.4	6.6

Source: From Ref. 45.

Table 22 Examples of Carbohydrate Solutions

Dextrose concentration		Caloric content	Osmolarity
%	g/L	(cal/L)	(mOsm/L)
2.5	25	85	126
5	50	170	253
10	100	340	505
20	200	680	1010
25	250	850	1330
30	300	1020	1515
40	400	1360	2020
50	500	1700	2525
60	600	2040	3030
70	700	2380	3535

Source: From Ref. 45.

Table 23 Examples of Nutritional Solutions

Solution	Amino acids with electrolytes	Intralipid® 10%
mOsm/L	357–1300	260
cal/L	–	1100
Total nitrogen (g/100 mL)	0.55–2.3	–
Formulation	May contain up to 8 essential	10% soybean oil
Electrolytes	and 11 nonessential amino acids and electrolytes (Na^+, K^+, Cl^- acetate, and phosphate)	1.2% egg yolk phospsolipids 2.25% glycerin –
Antioxidant	May be present	No
Buffering capacity	Moderate	Low
Light Protection	Yes	Yes
Container	Glass, plastic	Glass, plastic

Source: From Ref. 45.

glass surfaces. Each 100 mL of lactated ringer's injection contains 0.60 g sodium chloride, 0.03 g potassium chloride, 0.02 g calcium chloride, and 0.31 g sodium lactate (anhydrous) in WFI. The lactate ion in this solution is metabolized in the liver to glycogen, which becomes carbon dioxide and water, requiring the consumption of hydrogen ions; the result is an alkalinizing

effect. Again, the addition of dextrose, 5.0 g/100 mL, is for the caloric value and results in lower pH and higher osmolarity.

Electrolyte solutions make it possible to maintain or, in the case of specific clinical disorders, bring about the balanced levels of water and electrolytes required for proper body functioning.

Carbohydrate solutions. A standard solution that provides a source of water for hydration and carbohydrate calories contains Dextrose as a energy substance (Table 22). The dextrose is metabolized rapidly, and the water moves into other body compartments. If it is necessary to replace large losses of body water the injection can be administered, the patient's condition permitting, at a rate as high as 8–10 mL/min. Higher concentrations of the dextrose injection provide more calories without overloading the body with water.

Nutritional solutions. For proper nutrition an individual must have an intake of carbohydrates, amino acids, and fatty acids, along with trace minerals and vitamins. Carbohydrate and amino acid solutions have been available as injections for a number of years and can supply part of the patient's nutritional needs. Problems of toxicity, stability of the emulsion, particle size, and formation of free fatty acids had to be overcome before fat emulsions became viable products. Successful commercial production of fat emulsions that could be administered intravenously made it possible to provide the additional calories and essential fatty acids needed to implement TPN for the patient unable to take food enterally.

Fat emulsions typically contain a metabolizable vegetable oil, emulsifying agent, tonicity agent, and WFI. Table 23 shows a formula of fat emulsion in which each 100 mL contains 10 g soybean oil, 1.2 g egg yolk phospholipids as an emulsifying agent, 2.25 g glycerin as tonicity agent, and WFI. Sodium hydroxide is used to adjust the pH to approximately 8.0. In the soybean oil, the major fatty acids are linoleic (50%) and oleic (26%), with palmitic, linolenic, stearic, myristic, arachidic, and behenic acids making up the remainder. Size of the fat particles is controlled to about 0.3 μm. The emulsion is opaque, so the visible signs of incompatibility with additives might be concealed, although breaking of the emulsion results in visible free oil floating on the surface.

Complete amino acid solutions which contain L-amino acids provide the eight essential and as many as ten nonessential amino acids. Studies of blood serum levels of amino acids in normal individuals have established the ranges of each that are present and provide the basis for formulation. Each manufacturer of these solutions has particular combinations of amino acids that have been shown to be effective. There are over 70 amino acid injection formulations now being marketed including specialized amino acid injections (e.g., Aminess®, Aminosyn RF®, HepatAmine®, NephrAmine®, RenAmin®) for patients (e.g., those with renal or hepatic disease) who may have specialized requirements for amino acids or who may not tolerate amino acids contained in conventional solutions (45).

An essential amino acid cannot be converted to another amino acid and must be used by the body to fill a need for that particular one or be converted into uric acid. A nonessential acid may be used if needed, metabolized to another nonessential acid that is needed or converted to uric acid. When amino acids are administered parenterally, adequate calories must be provided concurrently to bring about synthesis of proteins; high-concentration dextrose injection or fat emulsion provides the source of calories. Concentrations of amino acid solutions vary from 3.5% to 15% depending on the indication for use. With some amino acids, however, there are limitations on the amount that will go into solution because the presence of other amino acids has an effect on solubility; the formulation of amino acid solutions is difficult because of this interaction and changing behavior.

Parenteral Nutrition

It has been estimated that approximately 40–55% of hospitalized patients are malnourished to some degree (174). Nutritional assessment and introduction of parenteral nutrition therapy based on the particular needs of the patient can reverse the nutritional status, minimize the harmful effects of poor nutrition, and accelerate the healing process.

Standard IV therapy usually provides dextrose, water, and electrolytes. Dextrose solutions are available in concentrations of 2.5% to 70%; a 5% solution supplies 170 cal/L and has an osmolality of 280 mOsm/L. These solutions are nutritionally incomplete, cannot supply enough calories without overhydrating the patient, and are suitable only for a few days as a source of nutrition. Electrolytes and vitamins may be added to correct imbalances and ensure normal body functions, including utilization of nutrients.

Amino acid therapy prevents nitrogen loss, is used for treatment of negative nitrogen balance, and provides the building blocks for the protein that is necessary for the return to proper health. These solutions may be given concurrently with oral feeding and, as with any IV solution, provide a route for other medications. They are, like the dextrose solutions, when used alone, nutritionally incomplete and should be given only in the short term to help preserve body protein in a stable patient.

TPN via the central venous route is used for patients with a need for calories and nutrients over a long period of time. High-concentration solutions of dextrose and amino acid solution, for example, 50% dextrose and 8.5% amino acid solution, are admixed in the hospital pharmacy. Trace elements, vitamins, or electrolytes are added to the mixture as needed. This solution will be quite hypertonic, with an osmolarity of around 2000 mOsm/L, and must be administered at a carefully controlled rate into a large vein with a high rate of blood flow to achieve proper dilution and minimize irritation of the vein. Infusion is accomplished by inserting a catheter with the tip extending into the superior vena cava and then, via an administration set, connecting the catheter to the bag that contains the admixture. The catheter may remain in place for as long as 30 days with proper care and precautions to avoid sepsis. The 10 or 20% fat emulsion may be administered intermittently through the central vein, through a peripheral vein, or be combined with the dextrose and amino acid solutions in the "mixing bag" prepared in the hospital pharmacy.

Stress Testing

Stress testing, testing after exposure to exaggerated conditions, is done throughout the developmental process and is designed to establish "safety factors." The data obtained from chemical, microbiological, biological, and physical tests, when compared with the results of tests on samples prepared under normal conditions, provide additional assurance that a safe and effective product will reach the market. Stress testing may take many forms.

Materials that will be in contact with the solution are subjected to extractions that far exceed the normal surface-volume ratios and the extracts are used for chemical, physical, biological, and toxicity testing. Tests for plastic and rubber are listed in various the pharmacopoeias. In addition, the LVP manufacturer may prepare concentrated extracts for tissue culture tests, a screening test for direct cell effects, and tests in rodents and other animals for indications of toxicity. The identity of the material extracted can be established chemically, quantified, and, with the results of the biological tests, related to its effect on humans.

During development of the sterilization cycles, temperature distribution and penetration studies are performed to ensure that the lethality is imparted to the entire sterilizer load. These studies are followed with evaluation lethality of biological indicators in the load. Often, the filled containers are subjected to two or three sterilization cycles and then checked for physical or chemical change.

Product filled containers are tested for drop tests, thermal shock tests, internal pressure tests, and impact resistance. The procedures for these tests are given in manuals that are available from the American Society for Testing Materials (ASTM). Alternating cycles of low and high temperatures provide information about how the solution and container react to adverse storage conditions. Such an evaluation may become part of the initial stability evaluation or the subject of a special stability study.

Stability Evaluation

Stability evaluation studies are aimed to support expiration dating of the product and also to provide labeling information about shipping and storage conditions, maximum and minimum

temperatures, or the necessity to prevent exposure to light. These studies encompass many aspects: physical (change of color or formation of a precipitate), chemical (change in pH or assay), microbiological (there are no antimicrobial agents in LVPs), or the packaging, which must be nonreactive and protect the solution during the shelf life. For details on the topic of stability studies regarding the stability procedures, sampling requirements, storage conditions, testing schedules, and evaluation of data, reader is directed to chapter 10, volume 3 of this book.

Processing Conditions Affecting Formulation of LVP

Some aspects of water quality, filtration, and sterilization are described below as they relate to LVP formulation (these have been described in detail in separate chapters elsewhere in this textbook).

WFI is the main ingredient of an LVP formula. Produced in large amounts by distillation or by reverse osmosis, the water must be tested frequently to assure that it is of the quality specified in the compendia. For particulate matter, pharmacopoeias require that each LVP unit must contain no more than 25 particles/mL that are equal to or larger than 10 μm and no more than 3 particles/mL that are equal to or larger than 25 μm in effective linear measurement. Particle generation from any source to which the solution will be exposed must be identified and controlled. Likely sources are air, processing liquids and gases, or components. Each source may contribute only a few particles but in combination can have a significant effect on the quality of the solution. Emphasis should be placed on reducing the generation of particles as well as effective filtration of liquids and gases at the point of use in the process.

LVPs are terminally sterilized, that is, sterilized after the product is filled and sealed in its final container. The sterilization methods generally used is steam under pressure. The type of container, size of container and solution has an effect on the cycle. Plastic containers, for example, are flexible and permeable. Air overpressure inside the sterilizer must be adjusted during the cycle to counteract the internal pressure in the container in order to avoid distortion. The air that prevents distortion also can enrich the oxygen content of the solution and airspace in the container; the result is that 5% dextrose in lactated ringer's develops more color in plastic than in glass. Amino acids are particularly susceptible to oxygen and all but a few are currently packaged in glass. Glass containers are rigid and impermeable but are subject to breakage because of thermal shock if the temperature differentials between the content of the bottle and sterilizer are excessive. The rate of heat up or cooling must be carefully controlled to avoid thermal shock. During sterilization of product in glass containers, the air overpressure in the sterilizer prevents lifting of the closure, which may be brought about by the internal pressure of the bottle. Cycle adjustments must be made for container size; smaller sizes have more surface area available per unit volume than larger sizes and may be used as worst-case samples for studying the effects of heat history.

Admixture Considerations

Of all LVPs infused, 60% to 80% are estimated to be admixed with one or more drugs (175,176). The number of new drugs and possible combinations is increasing steadily. Appropriate compatibility and stability studies must be performed to ensure that the drugs introduced into LVPs are compatible. The phenomenon of incompatibility occurs when the LVP and drugs produce, by physicochemical means, a product that is unsuitable for administration to the patient. Physical incompatibility may be detected by a change in the appearance of the solution, such as the formation of a precipitate, a haze, a change of color, or the breaking of an emulsion. Subtle incompatibilities, such as a change in pH or drug concentration, may not result in a visual change or may not become evident until a later time.

Instability occurs when an LVP product or admixture is modified because of sorption or such storage conditions as time, light, or temperature. The modified product may not be suitable for administration, and unless the combination has been studied in the laboratory, the only clue to a stability problem may, be failure to get the expected clinical result.

The parameters of tonicity, pH, solubility, and added substances, which were considerations in the design of the LVP formulation, also must be considered in a different context when drugs are added to the solution. The drug product may contain solvents, preservatives, stabilizers, buffers, antioxidants, and other ingredients that, when added to the LVP, can result in instability and incompatibility problems. Sodium benzoate, a preservative in some drugs, precipitates as benzoic acid when added to an LVP with an acidic pH. Copper, a trace metal needed by the body, can cause precipitation in amino acid solutions. Stability of the combination must be maintained after mixing and during infusion if the desired result is to be achieved. Stability problems may be caused by pH, solubility, sensitivity to light or temperature, absorption, or chemical incompatibility. Stability may also be related to time, and this is one reason that it is recommended that admixtures not be stored for prolonged periods.

One example of the role of pH would be that of ampicillin B in dextrose solutions. Unless the pH of the dextrose solution is greater than 5.0, the combination is incompatible. The monograph for Dextrose for Injection allows a pH range of 3.5 to 6.5. When the pH of 5% dextrose in lactated ringer's injection is below 5, some nerve-blocking agents, such as succinylcholine, will precipitate from solution.

Chemotherapeutic drugs and vitamin preparations generally should be protected from light. Sodium bisulfite, an ingredient added to some LVPs to reduce degradation caused by oxidation, may be present in only the quantity needed for protection of the solution during sterilization and shelf life. It may not be present in sufficient quantity to provide protection from the air that may be introduced to the container during admixing or storage in plastic containers.

The order of introduction of drugs to the LVP may either highlight or mask visible incompatibilities. If a drug is incompatible at a given pH and the pH of the LVP must be adjusted, the pH should be adjusted before the drug is added. A fat emulsion, white and opaque, masks reactions that might be visible in a clear solution, and the package insert cautions not to add electrolytes directly to the emulsion.

The potential physical and chemical incompatibilities associated with such dilutions are compiled by Trissel (74) and is often the primary reference book on this subject in the practice of pharmacy.

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6 Drug solubility and solubilization

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SOLUBILITY AND PARENTERAL PRODUCTS

This chapter provides a practical description of the physical phenomena leading to molecular level solubilization or dispersion of solutes (drugs) in a way that should enable the formulator to make informed decisions regarding formulation strategies for parenteral delivery. Solubility is discussed from the perspective of a thermodynamically defined equilibrium requiring several energetic steps in going from solute in a condensed phase to a solute in solution. Discussions will include the nonequilibrium state of supersaturation while focusing on the fit-for-purpose definition of solubility targeting parenteral drug delivery. The definition of solubility can relate to the solubility of any physical state of matter in another, or even in a similar state (miscibility), but this chapter will focus on solubility of a solid state in a liquid media, resulting in a solution mixture, which is of primary pharmaceutical importance for parenteral drug delivery (1).

Thermodynamic solubility can be described as the condition where the chemical potential of solute (μ_{solute}) in solution is in equilibrium with, and equal to, the chemical potential of the solute in its respective solid phase (μ_{solid}) under consideration (2). At a constant temperature and pressure, this equilibrium defines the saturated solution with respect to the designated solid phase and respective media. Any perturbation in the solute phase or solvent phase can result in a temporary metastable state of either supersaturation ($\mu_{solute} > \mu_{solid}$) or subsaturation ($\mu_{solute} < \mu_{solid}$), where the chemical potentials differ and the system will spontaneously attempt to reestablish equilibrium. Any effort to intentionally alter solubility will require a modification in the chemical potentials of either the solute solid state or the solute in solution.

To better understand strategies to modify solubility, three key energetic drivers for the solubilization process should be considered (2). The first step is the necessary energy input to overcome the intermolecular interactions of the solute in its respective condensed state (Fig. 1). The second step is the energy input necessary to overcome solvent-solvent interactions and create a cavity in the solvent which accommodates the solute. The unfavorable energy input to this point is then countered with the energy release occurring upon collapse of the solvent cavity around the solute and ensuing intermolecular interactions between solute and solvent.

Alterations in the solvent can influence both solvent-solvent interactions and subsequent solvent-solute interactions. This is the basis for many of the cosolvent strategies used for solubilization, wherein the μ_{solute} is decreased shifting the equilibrium toward increased amounts of drug in solution. Solubilization through changes in the solid form of a drug (amphorous, polymorphs, etc.) leads to increases in the μ_{solid}, which also shifts the equilibrium, but also runs the risk of conversion to a more thermodynamically stable and less soluble solid form with time. Solubilization obtained through alterations in the solute's molecular structure has the potential to significantly alter solubility by impacting specific solvent—solute interactions or solute—solute interactions. This is probably the preferred strategy for enhancing solubility, but such molecular modifications are difficult to introduce once the drug development process on an entity has been initiated. Hence, molecular design modifications are best instituted through interactions with medicinal chemists in the discovery organization prior to drug candidate selection.

One of the most commonly used strategies to provide apparent increases in solubility, or total drug in solution, is to create alternative equilibria for the drug or solute to reside in. While these equilibria enhance the total amount of drug in solution, the μ_{solute} remains equivalent to that of the solid phase, that is, the intrinsic solubility is not altered but instead the μ_{solute} residing in some additional equilibrium is reduced through specific interations or altered solvation. Creation of alternative equilibria to "sequester" drug provides the basis for solubilization strategies, such as micellar partitioning, chemical ionization, complexation, and partitioning into emulsions.

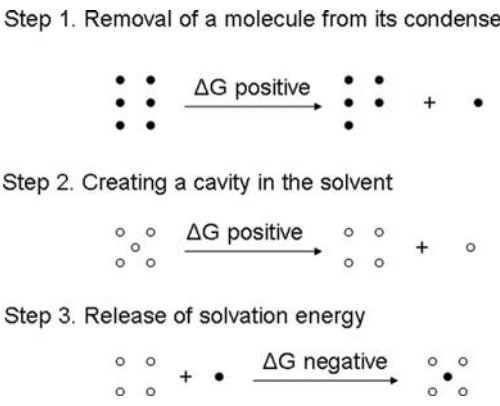

Figure 1 An illustration of the three steps needed for drug solubility.

In the simplest of terms, the solubility of a solute in a given solvent system, as defined by amount of drug dissolved, seems easily determined, but reliable, reproducible and meaningful numbers can be difficult to obtain. The more common methods are best described as "fit for use," wherein the solid phase of interest is incubated in solvent and the total amount of solute present in solution is measured. The method of solid-phase separation is critical and really defines the utility of the apparent solubility obtained. Typically, either filtration or centrifugation is used with subsequent assay of filtrate (filtration) or supernatant (centrifugation). Details of separation can be particularly important when colloid scale dispersions exist. Furthermore, as solubilities begin to drop below 1 μg/mL, issues of nonspecific adsorption to surfaces (filter, container), coupled with analytical detection limitations can result in highly variable values across labs.

Factors such as temperature, energy input and the nature of both the solid phase and the solvent can significantly impact how rapidly equilibrium is obtained. Approaching equilibrium from both a state of supersaturation and subsaturation taking measurements as a function of time is probably the best approach. At equilibrium both should approach similar values. When solubilities are >1 μg/mL, 24-hour incubation will generally approach 90% to 95% of equilibrium value, assuming particle sizes are small (3).

IMPLICATIONS OF SOLUBILITY FOR PARENTERALS

A common challenge in development of drugs intended for parenteral administration is the solubilization of a poorly soluble active ingredient (4). For intravenous (intravascular) injection, solubility of the active ingredient in the plasma needs to be below saturation upon dilution to prevent precipitation or formation of phlebitis. Injection of a drug into an extravascular site may establish a depot depending on the type of formulation administered. Drug absorption from a depot by passive diffusion and partitioning is dependent on drug solubility. Only the fraction of drug in solution is available for absorption. A critical difference between the pH of the administered drug solution and the physiological pH at the injection site (and/or solubility of the drug in a cosolvent vehicle and in physiological tissue fluid) can cause an unpredicted decrease in absorption due to precipitation of the drug at the injection site. Phenytoin is formulated as a sodium salt in a pH 12 solution of 40% propylene glycol, 10% alcohol and water for injection. When injected into muscle tissue, the large difference in pH and simultaneous dilution of propylene glycol with tissue fluids cause conversion of the sodium salt to less soluble free acid and precipitation at the injection site. Amphotericin B has a low aqueous solubility of 0.1 mg/mL at pH 2 or pH 11. However, Amphotericin B is highly soluble in liposomal intercalation and becomes an integral part of the lipid-bilayer membrane. These liposomal products permit administration by IV infusion. Another commonly studied low solubility drug is paclitaxel with an aqueous solubility of 0.1 μg/mL. Wheelar et al. manufactured an emulsion and liposome blend using corn oil, cholesterol and egg phosphotidylcholine containing 5 mg/mL of paclitaxel, a 50,000-fold increase in solubility (5).

PROPERTIES OF THE SOLVENT

A popular aphorism used for predicting solubility is "*like dissolves like*" (6). This statement indicates that a solute will dissolve best in a solvent that has a similar polarity to itself. This view is rather simplistic, since it ignores many solvent-solute interactions, but it is a useful rule of thumb. Strongly polar compounds like sugars or ionic compounds like inorganic salts dissolve only in very polar solvents like water, while strongly nonpolar compounds like oils or waxes dissolve only in very nonpolar organic solvents like hexane. The dielectric constant, solubility parameter and interfacial/surface tension are among the most common polarity indices used for solvent blending to improve solubility.

Generally, the dielectric constant of the solvent provides a rough measure of a solvent's polarity. It is the electric permittivity ratio of solvent to vacuum. It measures the solvent's ability to reduce the strength of the electric field surrounding a charged particle immersed in it. This reduction is then compared with the field strength of the charged particle in a vacuum. In general, polar solvents have higher dielectric constant values than nonpolar molecules. Solvents with a dielectric constant of less than 15 are generally considered nonpolar (7). The dielectric constants of some commonly used solvents and cosolvents in parenteral products are listed in (Table 1).

Gorman and Hall (10) studied the solubility of methyl salicylate in isopropanol-water mixtures, and obtained a linear relationship between log mole fraction of the methyl salicylate and the dielectric constant of the mixed solvent.

For a solution to occur, both solute and solvent molecules must overcome their own intermolecular attraction forces, so called van der Waals forces, and find their way between and around each other. This is accomplished best when the attractions between the molecules of both components are similar. The solubility parameters are defined to express the cohesion between like molecules. It is a numerical value that indicates the relative solvency behavior of a specific solvent and can be calculated from heats of vaporization, internal pressures, surface tensions, and other properties, as described by Hildebrand and Scott (11). The heat of vaporization in conjunction with the molar volume of the species, when available at the desired temperature, probably affords the best means for calculating the solubility parameter. It can be expressed as equation (1).

$$\delta = \left(\frac{\Delta H_v - RT}{V_l}\right)^{1/2} \tag{1}$$

where ΔH_v is the heat of vaporization and V_l is the molar volume of the liquid compound at the desired temperature, R is the gas constant, and T is the desired absolute temperature. Hildebrand and Scott include solubility parameters for a number of compounds in their book. A table of solubility parameters has also been compiled by Hansen and Beerbower (12), wherein the authors introduced partial solubility parameters δ_D, δ_p, and δ_H. The parameter δ_D accounts for nonpolar effects, δ_p for polar effects, and δ_H to express the hydrogen bonding

Table 1 Dielectric Constant, Solubility Parameter, and Surface Tension of Common Solvents and Cosolvents

Solvent	Dielectric constant	Solubility parameter $(cal/cm^3)^{1/2}$	Surface tension 20°C (dyne/cm)
Water	78.5	23.4	72.8
Ethanol	24.3	12.7	22.4
Propylene glycol	32	14.8	38.0
Glycerin	43	16.5	64.3
PEG 300 or 400	35	9.9	43.5 (PEG 200)
Benzyl alcohol	13	12.1	40.7
Dimethyl sulphoxide (DMSO)	47	12.0	43.5
N,N-dimethylacetamide (DMA)	38	10.8	36.7
N,N-dimethylformamide (DMF)	37	12.1	39.1
N-methyl-2-pyrrolidone (NMP)	32	23.0	40.8

Source: From Refs. 8 and 9.

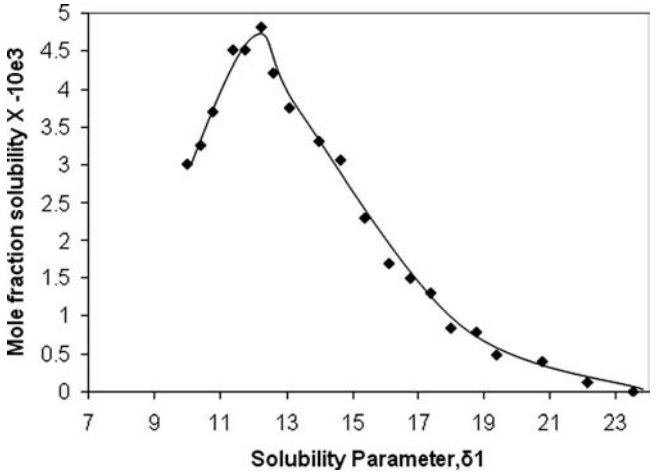

Figure 2 Solubility of trimethoprim in dioxane-water mixture of varying solubility parameter. *Source*: From Ref. 14.

nature of the solvent molecules. The sum of the squares of the partial parameters gives the total cohesive energy density $\delta^2_{(\text{total})}$ [eq. (2)]. Kesselring et al. have determined both total and partial solubility parameters using gas-liquid chromatography (13).

$$\delta^2_{(\text{total})} = \delta^2_{\text{D}} + \delta^2_{\text{P}} + \delta^2_{\text{H}} \tag{2}$$

The more alike are the δ values of two components, the greater is the mutual solubility, miscibility, of the pair. For example, the δ value of phenanthrene is 9.8; it would be expected to be more soluble in carbon disulfide with a δ value of 10 than in normal hexane with a δ value of 7.3. Conversely, δ of a drug can be estimated from measured solubility as a function of solvent solubility parameter (14) (Fig. 2).

Interfacial/surface tension is another solvent property caused by the attraction between the liquid's molecules by various intermolecular forces. It is a measure of the work required to create a cavity of unit area of surface from molecules in the bulk, hence relating to cavity formation for solutes. Polar solvent generally has higher surface tension than nonpolar solvent. Some surface tension and interfacial tension (against water) at 20°C are listed in Table 1 (15).

PROPERTIES OF THE SOLUTE

Drug molecules contain different structures and functional groups. The collective contributions from each functional group make the macroscopic physicochemical properties of the drug, which are a reflection of inter- or intramolecular interactions. For example, the stronger the attractions between molecules or ions, the more difficult it is to separate the molecules, therefore, the higher the melting point and poorer the solubility. The intra- or intermolecular forces are dictated by intrinsic molecular properties, such as polarizability, electronic factors, topology and steric factors, lipophilicity, hydrogen bonding, surface areas, volumes and connectivity, etc.

Molecular Properties

Polarizability and Electronic Factors

Polarizability is a characteristic property of the particular molecule. It is defined as the ease with which an ion or molecule can be polarized by any external forces. From electromagnetic theory, there is a relationship between polarizability α_{p} and dielectric constant ε of a molecule, where n is the number of molecules per unit volume [eq. (3)].

$$\frac{\varepsilon - 1}{\varepsilon + 2} = \frac{4}{3}\pi \cdot n \cdot \alpha_{\text{p}} \tag{3}$$

When a molecule cannot be represented by a single Lewis structure, that is, using an integral number of covalent bonds between two atoms, but rather has properties in some sense

intermediate to these, resonance structures are then employed to approximate the true electronic structure. Because of confusion with the physical meaning of the word resonance, as no elements actually appear to be resonating, it has been suggested that the term resonance be abandoned in favor of delocalization and delocalization energies (16).

An electric dipole is a separation of positive and negative charges. It can be characterized by dipole moment, μ, which is equal to the product of charge on the atoms and the distance between the two atoms bounded with each other. Many molecules have such dipole moments because of nonuniform distributions of positive and negative charges on the various atoms. Such is the case with polar compounds like hydroxide (OH^-), where electron density is shared unequally between atoms. Dipole moment is the polarity measurement of a polar covalent bond. The higher the polarity of a molecule the greater the dipole moment and the value can be calculated through the comparison of dielectric constant and the refractive index of the solutions.

Some drugs are known to form a charge-transfer complex with certain solvents. A charge-transfer complex (or CT complex, electron-donor-acceptor-complex) is a chemical association of two or more molecules, or of different parts of one very large molecule, in which the attraction between the molecules (or parts) is created by an electronic transition into an excited electronic state, such that a fraction of electronic charge is transferred between the molecules. The resulting electrostatic attraction provides a stabilizing force for the molecular complex. The association does not constitute a strong covalent bond and is subject to significant temperature, concentration, and host (e.g., solvent) dependencies and occurs in a chemical equilibrium with the independent donor (D) and acceptor (A) molecules.

The great majority of drugs contain ionizable groups; most are basic, some are acidic. The ionization constant (K_a) indicates a compound's propensity to ionize. It is a function of the acidity or basicity of group(s) in the molecule. Because of the many orders of magnitude spanned by K_a values, a logarithmic measure of the constant is more commonly used in practice, wherein the pK_a is equal to $-\log_{10} K_a$. The equilbria for acids [eqs. (4) and (5)] and for bases [eqs. (6) and (7)] are described as follows:

$$HA = H^+ + A^- \tag{4}$$

$$pK_a = -\log([H^+] \cdot [A^-]/[HA]) \tag{5}$$

$$HB^+ = H^+ + B \tag{6}$$

$$pK_a = -\log([H^+] \cdot [B]/[HB^+]) \tag{7}$$

Rearranging the pK_a equations give the well-known Henderson-Hasselbalch equations for both weak acid (HA) and weak base (B) and the ability to calculate the percentage of ionized species at any particular pH [eqs. (8) and (9)].

$$pH = pK_a + \log[A^-]/[HA] \tag{8}$$

$$pH = pK_a + \log([B]/[BH^+]) \tag{9}$$

When the pH is two units either side of the pK_a, then the drug will be almost completely ionized (BH^+, A^-) or unionized (B, HA). The solution pH and the pK_a are important because the charged form of a drug is more soluble than the neutral form. To have any realistic chance of significant pH-solubility manipulation for a parenteral, the pK_a for a base must be greater than 3 and for an acid less than 11.

Lipophilicity

Lipophilicty is the tendency of a compound to partition into a nonpolar lipid matrix versus an aqueous matrix. Lipophilicity is readily calculated, thanks to the work of Hansch and Leo (17).

It is a rapid and effective tool for initial compound property assessment. One traditional approach for assessing lipophilicity is to partition the compound between immiscible nonpolar and polar liquid phases. Traditionally, octanol is the nonpolar phase and aqueous buffer as the polar phase with the partition value, log*P* defined below [eq. (10)]. Log*P* is measured at a pH of the buffer where all of the compound molecules are in the neutral form.

$$\text{Log}\, P = \log \frac{[C_{\text{nonpolar}}]}{[C_{\text{polar}}]} \tag{10}$$

Hydrogen Bonding

The assumption that the solubility of a solute in a given solvent is related simply to the bulk properties of the pure components, that is, "like dissolves like," was originally intended strictly for systems involving only London dispersion forces. For quite polar solution components, the specific intermolecular interactions, such as hydrogen bonding, when they occur, are often the dominant factors in determining solubility (18).

A hydrogen bond is a special type of attractive interaction that exists between an electronegative atom and a hydrogen atom covalently bonded to another electronegative atom. Usually the electronegative atom is oxygen, nitrogen, or fluorine, which has a partial negative charge and is the hydrogen bond acceptor. The hydrogen then has the partial positive charge and is the hydrogen bond donor. The typical hydrogen bond is stronger than van der Waals forces, but weaker than covalent or ionic bonds and can occur intermolecularly, or intramolecularly. When hydrogen bonding between solute and solvent is possible, solubility is greater than expected for compounds of similar polarity that cannot form hydrogen bonds. Hansen and Beerbower (12) have introduced hydrogen bond partial solubility parameter, δ_H, to account for the nonideality effect from hydrogen bonding on total solubility (see above).

Topology and Steric Factors

It is believed that the variations in the magnitude of solubility of different solutes in water are caused by their dissimilar chemical structures and much attention has been paid to quantitative structure activity relationship (QSAR) studies of modeling the relationship between chemical structure and solubility of organic compounds. Molecular topology as one of the structure indices has been used widely to study the solubility of compound in different models (18,19).

Molecular topology is the mathematical description of molecular structure allowing a unique and easy characterization of molecules by means of invariants, called topological indices, which are the molecular descriptors to correlate with the experimental properties. Different from the conventional physicochemical descriptors, topological indices (TIs) allow the use of the QSAR relations to design new compounds from scratch. This is possible because, contrary to the physical parameters, the algebraic descriptors are not indirectly related to structure but they are a mathematical depiction of the structure itself.

Besides the chemical structure of the molecules, the spatial arrangement of their functional groups can play a significant role in compound solubility when it influences the degree of interaction between solute and solvent. For example, two isomers can exhibit very different solubilities in the same solvent (20). The influence of the location of the functional groups is referred to here as the steric effect. For strongly interactive solvents like water, the steric effect is particularly severe and sometimes dominating when it hinders or promotes hydrogen bonding interaction. On the other hand, structural alterations that are not in the vicinity of an interacting functional group and do not alter the functionality of the group, have little influence on solubility.

Surface Areas, Volumes, Connectivity

Theoretically, the dissolution process of a crystalline solid can be carried out in four hypothetical steps: (1) melting of the crystalline solute, (2) separation of a solute molecule from the molten bulk, (3) creation of a cavity in the solvent for accommodation of a solute, and (4) placement of the solute molecule into the cavity created. The energy required for these

processes can be characterized using the enthalpy of melting, the cohesive energy of the solute and solvents, and the adhesive energy at the interface, which are directly proportional to the interfacial area. Hence, solubility can be related to the molecular surface area of a solute.

The solubility in water of aliphatic compounds has been successively related to molecular surface area by Amidon and associates (21,22). They investigated the aqueous solubility of hydrocarbons, alcohols, esters, ketones, esters, and carboxylic acids. Excluding olefins, a linear relationship was found between log (solubility) and total surface area with 158 compounds that they investigated. Similarly, molar volume of the solute is another property impacting solubility. It is related to molecular weight and affects the size of the cavity that must be formed in the solvent to solubilize the molecule.

Molecular connectivity is a measure of extent of molecular branching and normally used as a connectivity index. The connectivity index, easily computed, based on the degree of connectedness at each vertex in the molecular skeleton, is shown to give highly significant correlations with water solubility of branched, cyclic, and straight-chain alcohols and hydrocarbons as well as boiling points of alcohols (23). These correlations are superior to those based on well-founded theory relating to solvent cavity surface area.

Macroscopic Properties

The melting point or freezing point of a pure crystalline solid is strictly defined as the temperature at which the pure liquid and solid exist in equilibrium. The heat absorbed when a gram of a solid melts, or the heat liberated when it freezes, is known as the latent heat of fusion. The heat added during the melting process does not bring about a change in temperature until the entire solid has disappeared, since this heat is converted into the potential energy of the molecules that have escaped from the solid into the liquid state.

The heat of fusion may be considered as the heat required to increase the interatomic or intermolecular distances in crystals, thus allowing melting to occur. Heat of fusion is dictated by crystal packing. A crystal that is packed by weak forces generally has a low heat of fusion and a low melting point, whereas one packed together with strong forces has a high heat of fusion and a high melting point.

Solubility, as discussed earlier, is strongly influenced by intermolecular forces, similar to melting point. This similarity was demonstrated by Guttman and Higuchi, who studied the melting points and solubilities of xanthines. When the side chain at 7 position changed from H (theophylline) to propyl (7-propyltheophylline), the melting point decreased from 270 to 100°C, while solubility in water at 30°C increased from 0.045 to 1.04 mol/L. An empirical equation was derived by Yalkowsky and Banerjee (24) to estimate solubility on the basis of the lipophilicity and melting point [eq. (11)].

$$\mathrm{Log}S = 0.8 - \log P_{\mathrm{ow}} - 0.01(\mathrm{MP} - 25) \tag{11}$$

Here S is solubility, $\log P_{\mathrm{ow}}$ is the octanol/water partition coefficient (a measure of lipophilicity), and MP is the melting point (a measure of crystal packing).

Polymorphs exist when two crystals have the same chemical composition but different unit cell dimensions and crystal packing. Compounds that crystallize as polymorphs generally have different physical and chemical properties, including different melting points, x-ray diffraction patterns, and solubilities. Generally, the most stable polymorph has the highest melting point and lowest solubility; other polymorphs are metastable and convert. A consideration of the data in the literature indicates that improvements in solubility of metastable crystal forms can be expected to be as high as twofold (25).

When the crystal lattice contains solvents that induce polymorphic changes, they are called solvates. If the solvent is water, these pseudo-polymorphs are called hydrates. These hydrates and solvates are easily confused with true polymorphism and lead to the term pseudo-polymorphism. The solvates may be discriminated by DSC/TGA, where an additional endotherm due to the solvent will be apparent in DSC provided the heating rate is slow, and weight loss at similar temperature is observed in TGA.

Hydrate formation generally leads to a lower solubility since the preexistence of water in the crystal lattice reduces the energy available for solvation. For example, glutethimide

anhydrate has melting point 83°C and solubility 0.42mg/mL, but its hydrate has melting point 68°C but solubility only 0.26mg/mL. However, solvates tend to have higher solubility than the neat form because of the weakening of the crystal lattice by the organic solvent. For example, succinylsulphathiazole neat has a solubility of 0.39mg/mL, and its pentanol solvate has solubility of 0.80mg/mL (26).

Amorphous solids may be considered as supercooled liquids in which the molecules are arranged in a random manner somewhat as in the liquid state and do not have melting points. Amorphous solids are in a high energy state relative to their respective crystalline solids, therefore, leading to differences in dissolution rate, chemical reaction rate and mechanical properties. Amorphous solids also have a higher solubility than their crystal form. The solubility advantage compared with the most stable crystalline counterpart was predicted to be from 10 to 1600 fold, as shown by Hancock and Parks (25). However, the experimental solubility advantage was usually considerably less than this, because determining solubility for amorphous materials under true equilibrium conditions is difficult because of the tendency for such materials to crystallize upon exposure to small quantities of solvents.

When particles are in the submicron range, a small increase in the saturation solubility is expected as described by the Freundlich–Ostwald equation [eq. (12)] (27,28).

$$\frac{RT}{V_m}\ln\frac{S}{S_0}=\frac{2\gamma}{r} \tag{12}$$

where S is the saturation solubility of nanosized particle, S_0 is saturation solubility of an infinitely large crystal, γ is the crystal-medium interfacial tension, r is the particle radius, V_m is the molar volume, R is a gas constant, and T is the temperature. Assuming a molecular weight of 500, density of 1 gm/mL, and a value of 60 to 70 mN/m for the crystal-water interfacial tension, the above equation would predict a 62% to 76% increase in solubility at a particle size of 100 nm.

IONIZATION AND THE SOLUBILITY PROFILE

The total solubility of a compound at a particular pH is the sum of the "intrinsic solubility" of the neutral species in solution plus the solubility of the charged species. For a weak base, when the aqueous medium at a given pH is saturated with free base, the total solubility at that pH may be expressed as described [eq. (13)]. The typical solubility profile of a weak base when $pH > pH_{max}$ is shown in (Fig. 3).

$$S_{base}(pH > pH_{max}) = [B]_s + [BH^+] = [B]_s\left(1+\frac{[H_3O^+]}{K_a}\right) \tag{13}$$

When there are counterions present in the solution, at low enough pH, the entire free base will be converted into salt form, and the salt is the solid form. In this case, the equilibrium solubility at a particular pH may be expressed by equation (14).

$$S_{base}(pH < pH_{max}) = [B] + [BH^+]_s = [BH^+]_s\left(1+\frac{K_a}{[H_3O^+]}\right) \tag{14}$$

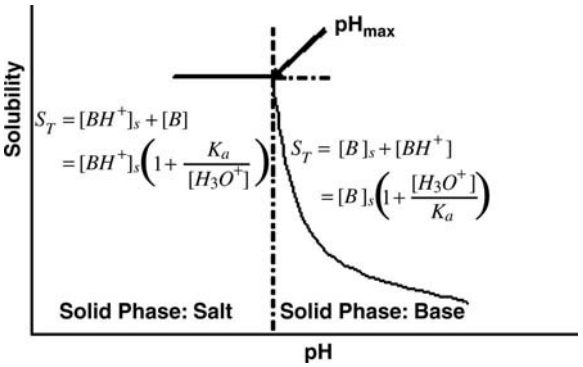

Figure 3 Schematic representation of the pH-solubility profile of a weakly basic compound.

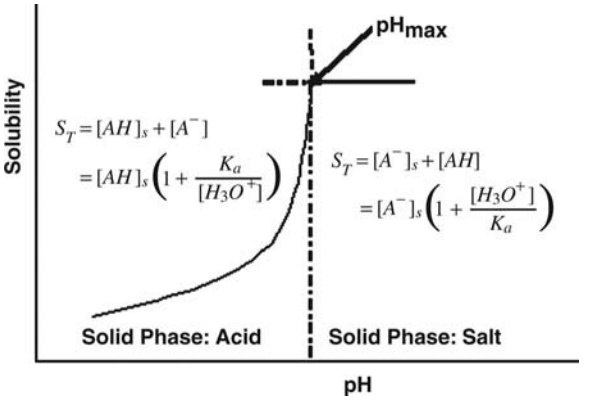

Figure 4 Schematic representation of the pH-solubility profile of a weakly acidic compound.

When these two independent curves in solubility pH profile intersect, the point is called pH_{max} as shown in the Figure 3. Similarly, the pH-solubility profile for a weak acid is also shown (Fig. 4).

Zwitterions refer to compounds with oppositely charged groups, but carry a total net charge of 0 and is thus electrically neutral. Solubility of zwitterions at certain pH is the combination of the contributions from all the charge groups. For compounds with two ionizable groups, solubility can be expressed by the following equation [eq. (15)].

$$S = S_0(1 + 10^{pK_{a1}-pH} + 10^{pH-pK_{a2}}) \tag{15}$$

It depends on its ionization constants, pH and intrinsic solubility, S_0, which is defined as the solubility of the neutral form of the compound. The solubility profile is U-shape characteristic for zwitterionic compounds.

For weak electrolyte drugs, salt formation is a common approach to improve solubility. Acids form salts with basic drugs and bases form salts with acidic drugs (29). For the salt of a basic drug, the dissolution equilibrium can be described as equation (16).

$$(BH^+X^-)_{solid} \leftrightarrow [BH^+]_s + [X^-] \tag{16}$$

Where $[BH^+]_s$ is the salt solubility and $[X^-]$ is the counterion concentration. The apparent solubility product K_{sp} can be derived as equation (17).

$$K_{sp} = [BH^+]_s[X^-] \tag{17}$$

In the absence of excess counterion, $[BH^+]_s = [X^-]$, solubility is the square root of K_{sp}. Under such conditions, drug solubility does not change with pH, as indicated in the figures above. On the other hand, if a significant amount of counterions exit in the formulation, decrease in solubility may be observed according to equation (18).

$$[BH^+]_s = K_{sp}/[X^-] \tag{18}$$

SOLUBILITY PREDICTION

A number of approaches to solubility prediction have been developed over the years and continue to be used (30). Recently many successful attempts were made for predicting aqueous solubility of compounds, but it is still a challenge to identify a single method that is best at predicting aqueous solubility (31). The first hurdle in the prediction of aqueous solubility is the estimation of melting point or enthalpy of sublimation (32). In addition, it is difficult to predict the solubility of a complex drug candidate on the basis of the presence or absence of certain functional groups. Conformational effects in solution may play a role in solubility and cannot be accounted for by a simple summation of contributing groups.

Because of the complexity involved in developing the prediction models, most models were completed using nonelectrolytes.

The prediction of aqueous solubility tends to use three approaches: methods correlating experimentally determined melting points and log$P$, correlations based on group contributions, and correlations with physicochemical and quantum chemical descriptors calculated from the molecular structure [quantitative structure property relationship (QSPR) approaches] (1).

Methods using melting point and log$P$ are best exemplified by the general solubility equation (GSE) model (33). The GSE model is based on the fact that the aqueous solubility of a nonelectrolyte solute depends on its crystallinity and its polarity, wherein the melting point and the octanol-water partition coefficient act as good surrogate measures, respectfully. For compounds with melting points $< 25^{\circ}$C, the melting point is taken to be 25°C. Ran, Yalkowsky and coworkers (34) revised equation 11 to equation (19).

$$\text{Log}S = 0.5 - \log P_{\text{ow}} - 0.01(\text{MP} - 25) \tag{19}$$

The theoretical treatment of this solubility prediction method is presented in more details elsewhere (1). With this prediction model, the absolute average error ranged from 0.5 to 1 log molar solubility unit for drug-like compounds (35).

The aqueous functional group activity coefficients (AQUAFAC) model is based on group contribution values, which are based on experimental aqueous solubilities (36). In this model, the molar aqueous solubility can be calculated using equation (20).

$$\text{Log}S = 1.74 - \log \gamma_{\text{w}} - \frac{\Delta S_{\text{m}}(T_{\text{m}} - T)}{2.303RT} \tag{20}$$

Where, γ_{w} is the aqueous activity coefficient of a compound, which is obtained from the AQUAFAC model. ΔS_{m} is the entropy of melting, T_{m} is the melting point, and T is the ambient temperature, both in Kelvin, R is the gas constant.

Using QSPR models, aqueous solubility is controlled predominantly by solute molecular size and shape, by its polar nature and hydrogen bonding capabilities. In addition, hydrophobicity, flexibility, electron distribution and charge have been found to play important roles in prediction (37). Many molecular property desciptors are now available computationally. Aqueous solubility has been modeled by correlating measured solubilities with one or more physicochemical and/or structure properties. Most methods use linear methods such as multiple linear regression (PLS) or nonlinear methods such as artificial neural networks (ANN). In general, nonlinear methods appear to provide better predictions (38). The root mean squared errors for models based on QSPR tends to range from approximately 0.7 log units to 1 log units. Recently, the effect of crystal packing on solubility has been added into the computational model (39).

Jain et al. applied two methods to compare aqueous solubility estimation of 1642 organic nonelctrolyte compounds ranging from 10^{-13} to 10^{0} in experimental molar solubility (33). The average absolute errors in the solubility prediction are 0.543 log units for AQUAFAC and 0.576 log units for the GSE. About 88.0% of the AQUAFAC solubilities and 83.0% of the GSE molar solubilities are predicted within one log unit of the observed values. The marginally better accuracy of AQUAFAC is assumed to be due to the fact that it utilizes fitted-parameters for many structural fragments and is based on experimental solubility data. The AQUAFAC also includes reasonable estimate of the role of crystallinity in determining solubility. The GSE on the other hand is a simpler, nonregression based equation, which uses two parameters (MP and logK_{ow}) for solubility prediction. The major assumption in the GSE is that octanol is an ideal solvent for all the solutes. This may not be true for strongly hydrogen bonding compounds, and consequently might result in larger error for such compounds.

With some computational packages it is now possible to make predictions on aqueous solubility that are as good as experimental measurements (± 0.5 log unit) for many compounds. However, all of the commercial programs were trained on selected organic chemicals and the predictive ability for drug-like compounds is still a challenge. When the commercial software programs do not yield good results for internal compounds, it may be necessary to evaluate various QSAR models and develop an in-house model (30).

SOLUBILIZATION AND "ENHANCED SOLUBILITY"

Modifications to the Solid State

Salt formation is probably the most common way to increase both the solubility and dissolution rate of ionizable drugs (29). The solid form, clearly distinct from the free acid or base solid form, provides significant enhancement in solubility through the provision of alternative equilibria, thus driving the total solubility (intrinsic + ionized) up significantly. This alternative equilibria results in a more readily solvated ionized form in hydrolytic solvents. As discussed earlier, the saturation solubility of the salt will be defined in conjunction with the Ksp, resulting pH and relative pK_a of the drug. As shown earlier (Figs. 3 and 4), changes in the pH or media composition can alter the solubility through common ion effects, or if the pH deviates well away from the pK_a, can actually result in precipitation of the free acid or base solid.

Selection of the counterion can actually be used to control the solubility by varying the Ksp. As pointed out by Anderson and Conradi (40), the impact of hydrogen bonding within the conjugate species can play a role in the Ksp and ends up also being translated into effects on the melting point of the salt. Common ion effects are manifested through the relationship defined by the Ksp. The solubility of the hydrochloride salt of the zwitterionic molecule lomefloxacin is a good example where excess chloride ion, as in admixtures with normal saline, can impact the solubility of the salt (41) (Fig. 5).

It is important to recognize that with any salt, the resulting pH of the media will be paramount in avoiding precipitation of the free base or acid. The strong acid conjugate salt of a weakly basic drug will end up driving the pH of the solution acidic, and conversely for strong base conjugate of weakly acidic drug. Care must be taken when such salts are dissolved into buffered systems where supersaturated solutions of the free base or acid may occur and have the propensity to precipitate with time. In such cases, a full understanding of the solubility versus pH curve is critical when using salts to provide improved solubility.

Cocrystals, similar to salts, provide a means to generate a crystalline form of the drug. While these solid phases can provide increased dissolution rates there has been minimal use of cocrystals to facilitate parenteral drug delivery. The properties and description of cocrystals has been discussed at length in a recent review (42).

The use of high energy amorphous solids can often result in temporary increases in solubility, but with a propensity to generate more stable crystalline forms. In parenteral

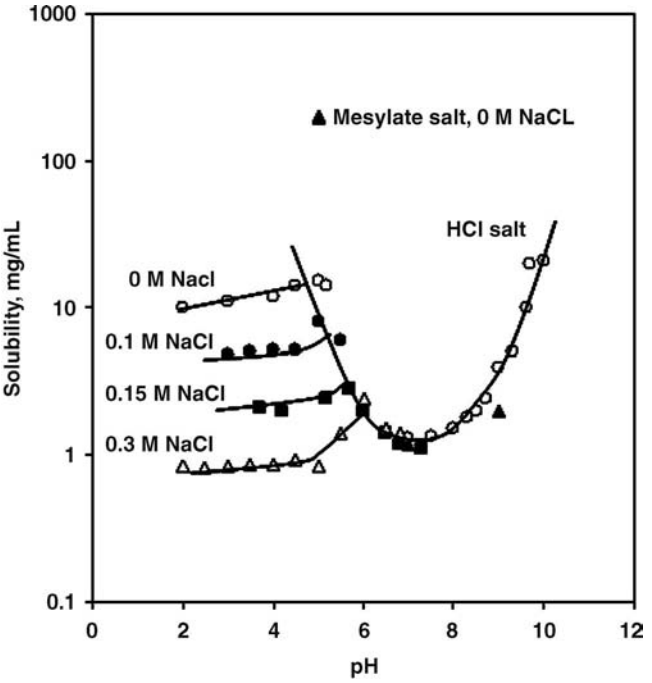

Figure 5 Effect of pH and NaCl concentration on the solubility of the zwitterionic quinolone lomefloxacin. *Source*: From Ref. 41.

products, the importance of metastable solids can many times play a role with lyophilized products upon reconstitution. The process of lyophilization often results in higher energy polymorphs or amorphous solids which allow for a very rapid dissolution and reconstitution back to the solution state. A thorough understanding of the dynamic nature of the lyophilized solid forms and the more stable crystalline forms which may exist is critical, whether they are hydrates, solvates, or polymorphs. The intentional use of such high energy states to increase solubility is limited because of its unpredictable behavior.

The best way to adjust solid form and impact solubility is via molecular modification, either as an analog or through formation of a prodrug. While these must be considered new chemical entities, they can provide a broad range of possible properties. Analog strategies are often focused on attempts to either decrease the lipophilicity and/or introduce hydrogen bonding groups which can enhance solvation in more hydrophilic media. In either case, especially with introduction of hydrogen bonding groups, increased interactions in the solid phase and its melt can actually increase as well, thus offsetting any gains afforded by increases in solvation. When possible, the introduction of ionizable groups can provide great solubility advantages (43).

In those cases where the perservation of the pharmacophore or desired biopharmaceutical properties does not permit molecular modifications leading to a more soluble molecule, a prodrug strategy can be invoked, overcoming immediate solubility limitations, yet when appropriately triggered, can release the active parent of interest (44).

Modifications to the Solution Phase

The use of cosolvents as was discussed earlier, has the ability to alter the dielectric constant of the solvent, influence the energy required to overcome hydrogen bonding forces in aqueous media and reduce the amount of energy necessary to create a cavity sufficient to accommodate the solute. Furthermore, these changes in solvent can greatly alter the degree of solvation of the solute once molecularly dispersed in the solvent. Soubility enhancement by addition of cosolvent is very typically log linear with respect to the cosolvent (Fig. 6). The degree of solubilization is dependent on both the lipophilicty, or log*P*, of the drug and type of cosolvent (45) (Fig. 6). Cosolvency and solubilization have been discussed by Rubino (46).

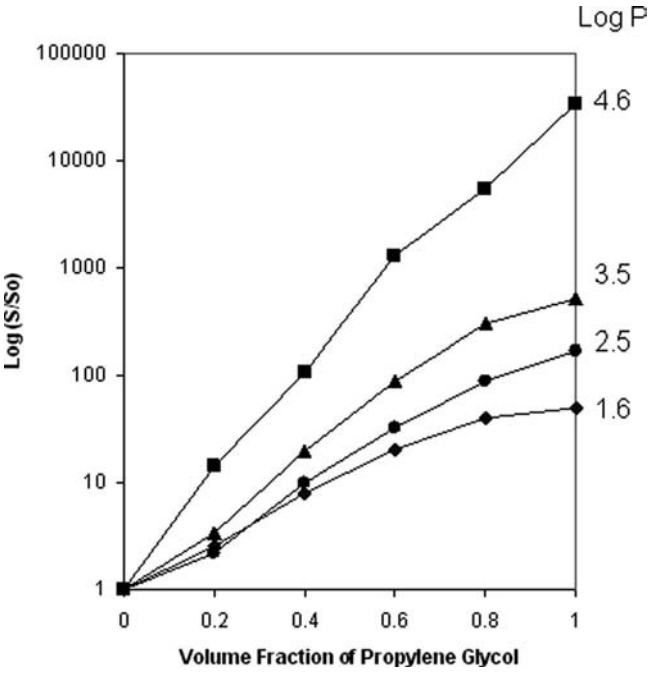

Figure 6 Propylene glycol solubilization of hydrocortisone esters. *Source*: From Ref. 45.

Modification due to Alternative Equilibria for Solute

An excellent overview of various methods to provide alternative equilibria for solubilization was presented by Yalkowsky (1). The rational selection of a solubilizing agent should be based on the structure of the drug to be solubilized and on the degree of solubilization needed to obtain the desired dose. The generation of alternative equilibria for the drug to exist in is one of the most commonly used methods to provide enhancements in the overall "apparent solubility" of the drug in solution. This strategy includes the use of ionization equilibria (discussed above in conjunction with salts), complexation equilibria, partitioning into surfactant micelles, partitioning into emulsion systems, and liposomal type systems.

Complexation and Association

Strategies of complexation include the use of chelating agents, organic molecular associations and inclusion complexes. The most common formulation strategies using complexation are centered around the use of cyclodextrins, with more emphasis generally placed on derivatized cyclodextrins because of their greater solubility and improved in vivo safety margin. Typically only those drugs with an aromatic ring or a nonpolar side chain are solubilized by cyclodextrin complexation (4). If complexation alone is insufficient, then a combination of complexation and pH modification or/and cosolvent may be used (47).

Complexation is an equilibrium process and the binding constant (or stability constant) for the formation of a 1:1 complex is given by equation (21).

$$\kappa_{1:1} = \frac{[\text{Drug}]_{\text{complex}}}{[\text{Drug}]_{\text{free}}[\text{Ligand}]_{\text{free}}} \tag{21}$$

$[\text{Drug}]_{\text{free}}$, $[\text{Ligand}]_{\text{free}}$ and $[\text{Drug}]_{\text{complex}}$ (m molecules of drug, n molecules of ligand) are the equilibrium molar concentrations of the free drug, the ligand and the drug in the complex form, respectively. Often, it is impossible to separate the individual binding constants and the apparent binding constant (κ_{app}) is used [eq. (22)].

$$\kappa_{\text{appm:n}} = \frac{[\text{Drug}_{\text{m}}\text{Ligand}_{\text{n}}]_{\text{complex}}}{[\text{Drug}]^{\text{m}}[\text{Ligand}]^{\text{n}}} \tag{22}$$

The total solubility of the drug in the presence of ligand is the sum of the intrinsic solubility of the drug in the absence of the ligand and the solubility of the drug in the ligand(s) [eqs. (23) and (24)].

$$[\text{Drug}]_{\text{total}} = [\text{Drug}]_{\text{intrinsic}} + \tau[\text{Ligand}]_{\text{total}} \tag{23}$$

$$\tau = \frac{\text{m}\kappa_{\text{appm:n}}[\text{Drug}]^{\text{m}}_{\text{intrinsic}}}{1 + \kappa_{\text{appm:n}}[\text{Drug}]^{\text{m}}_{\text{intrinsic}}} \tag{24}$$

A plot of $[\text{Drug}]_{\text{total}}$ versus $[\text{Ligand}]_{\text{total}}$ gives an intercept of $[\text{Drug}]_{\text{intrinsic}}$ and a slope τ. According to the above equation, the total solubility of a drug undergoing complexation is a linear function of the ligand concentration. The intercept of this line is equal to the solubility of the free drug and its slope is given by τ. Rearrangement of the equation allowed the calculation of the apparent binding constant, $\kappa_{\text{appm:n}}$ [eq. (25)].

$$\kappa_{\text{appm:n}} = \frac{\tau}{\text{m}[\text{Drug}]^{\text{m}}_{\text{intrinsic}} - \tau[\text{Drug}]^{\text{m}}_{\text{intrinsic}}} \tag{25}$$

The value of κ is a measure of the strength of the drug-ligand interactions and is dependent on the properties of the drug and the ligand molecules. For a particular ligand, the size, shape, aromaticity and the nonpolarity of the drug molecule play important roles in determining this strength. The properties of the solubilizing medium, such as temperature and polarity also influence the strength of these interactions (48–50).

Complexation of lomefloxacin with five metal ions (Al^{3+}, Ca^{2+}, Mg^{2+}, Bi^{3+}, and Fe^{3+}) was found to increase solubility of lomefloxacin (50). The stoichiometrics of the various complexes were different. In the presence of 0.25 M Ca^{2+} ion, solubility of lomefloxacin was raised by two to threefold at pH 5, while 0.25 M Al^{3+} increased the solubility by nearly 30 fold. The stability constants were determined from the solubility, which ranged from 11.2 for L:Ca^{2+} complexes to 2.34×10^{10} for L:Al^{3+} complexes. The authors concluded that the higher order of stability for lomefloxacin-Al ion complex was related to the higher charge density of the metal ion.

Hydrotropic agents (hydrotropes) have been used to increase the water solubility of poorly soluble drugs, and in many cases, the water solubility has increased by orders of magnitude (51). Several hydrotropic agents such as urea, caffeine and other xanthine derivatives, tryptophan, sodium benzoate, PABA-HCl, Procaine-HCl and nicotinamides have been identified. Solubilization diagram for riboflavine exhibits a positive deviation from linearity, which implies a greater solubilizing power at higher concentrations of PABA-HCl and is characteristics of hydrotropic solubilization (52). In the study to increase the solubility of paclitaxel, 5.95 M of *N*,*N*-doethylnicotinamide was found to raise the solubility by 1700 fold (from 0.30 μg/mL to 512 mg/mL or 0.6 M). The authors indicated that an effective hydrotropic agent should be highly water soluble while maintaining a hydrophobic segment (51). Almost all highly effective hydrotropic agents have a pyridine or a benzene ring in their structure.

Complexation of a drug molecule with a ligand molecule reduces the exposure of former's hydrophobic region to water resulting in an increase in its solubility. The practical and phenomenological implications of phase-solubility analysis were developed by Higuchi and Connors in their pioneering work published in 1965 (53). On the basis of the shape of the generated phase-solubility relationships, several types of behaviors can be identified (Fig. 7). The two major types are A and B. Only A-type of profile will be discussed in this Chapter.

In an A-type system, the apparent solubility of the substrate increases as a function of CD concentration. In A_L subtype, the solubility is increased linearly as a function of solubilizing concentration. A_P system indicates an isotherm wherein the curve deviates in a positive direction from linearity and the A_N system indicates a negative deviation from linearity. The equations related to complexation with cyclodextrin were presented in the previous section except that the ligand is replaced with cyclodextrin.

The use of CDs to enhance solubilization of a poorly soluble drug is often preferred to organic solvents (54). As a solution is administered, both the drug and CD concentration are reduced in a linear manner making precipitation is less likely. Drug release from parenteral administration of CD complexes is thought to be associated with complete and almost instantaneous dissociation via the dilution of the complex (49). For strongly bound drugs, or for those cases where dilution is minimal, contributions from competitive displacement by endogenous materials, drug binding to plasma and tissue components, uptake of the drug by tissue not available to the complex or CD, and CD elimination may also be important (55). In ophthalmic applications where the possibility for dilution is more limited, factors associated with partitioning and secondary equilibria may be the main mechanisms for drug release.

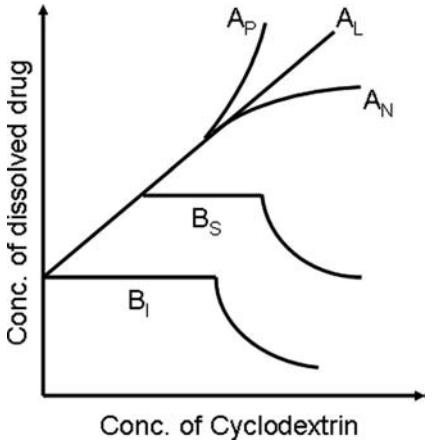

Figure 7 Graphical representation of A- and B-type phase-solubility profiles with applicable subtypes (A_P, A_L, A_N, and B_S, B_I). *Source*: From Ref. 53.

Inclusion complexation is restricted to drugs that have a hydrophobic region that can be inserted into a cavity that has the fixed dimensions. For α-, β-, and γ-cyclodextrins, the cross section of the solute protrusion must be less than 6, 8, and 10 Å, respectively. The αCD can preferentially accommodate aliphatic chains, and the βCD accommodates aromatic rings most efficiently. Fused ring or branched compounds can often best accommodate in the larger γCD cavity. Modified cyclodextrins are very water soluble and form moderately nonviscous solutions (1). Because of the large molecular weight and relatively high cost of cyclodextrins, their use is generally limited to solutes for which a low molar solubility is desired.

Cyclodextrins are cyclic oligosacchrides derived from starch containing six (αCD), seven (βCD), eight (γCD), nine (δCD), ten (εCD) or more (α-1,4)-linked α-D-glucopyranose units (54). In addition to increase the aqueous solubility of poorly water-soluble drugs and stability, CDs can be used to reduce or prevent irritation and prevent drug-drug interactions (56). The central cavity of the CD molecule carries lipophilic characteristic (57). In aqueous solution, the hydroxy groups form hydrogen bonds with the surrounding water molecules resulting in a hydration shell around the dissolved CD molecule (54). In general, the natural cyclodextrins exhibited less than 10-fold improvement in the solubility of compound.

The rates of formation and dissociation of drug:CD complexes are very close to diffusion rate-controlled with drug: CD complexes continuously being formed and broken apart (55). The equilibrium constants were reported to have a mean value of 130, 490 and 350 M^{-1} for αCD, βCD and γCD (58). A marketed parenteral solution, Caverject Dual® (alprostadil IV solution), contains αCD in which αCD is mainly excreted unchanged in the urine after IV injection and it has a higher solubility of 145 mg/mL at 25°C in water (59). βCD is limited in its parenteral application by its low aqueous solubility of 18.5 mg/mL at 25°C and adverse nephrotoxicity.

The natural CDs and their complexes are of limited aqueous solubility. Substitution of the hydrogen bond-forming hydroxyl groups results in improvement in their aqueous solubility. Modified CD include the hydroxypropyl derivatives of βCD (HPβCD) and γCD (HPγCD), the randomly methylated βCD (RMβCD) and sulfobutylether βCD (SBEβCD) (54). The modified cyclodextrin has been reported to increase solubility of progesterone by 3600 fold in with 300 mM of HPβCD (60). HPβCD and SBEβCD are considered nontoxic at low to moderate i.v. doses (54). HPβCD and SBEβCD are much more water soluble than natural βCD and have been used in several parenteral products, including Itraconazole (Sporanox) and Voriconazole (Vfend®, containing 16%w/v SBEβCD). After i.v. injection, HPβCD is almost exclusively eliminated through the kidneys. HPγCD has been incorporated in an eye drop solution and a parenteral diagnostic product.

Cyclodextrins can be used in combination with pH adjustment for synergistic drug solubility enhancement, according to the following equation [eq. (26)].

$$[\text{Drug}]_{\text{total}} = [\text{Drug}_u] + [\text{Drug}_i] + [\text{Drug}_u\text{CD}] + [\text{Drug}_i\text{CD}] \quad (26)$$

Where $[\text{Drug}_u\text{CD}]$ is unionized drug-cyclodextrin complex, and $[\text{Drug}_i\text{CD}]$ is ionized drug-cyclodextrin complex. The synergistic effect is generated because of the ionized drug-ligand complex $[\text{Drug}_i\text{CD}]$, which is absent in situations where pH adjustment or cyclodextrin is used alone (61). The interactions of charged and uncharged drugs with neutral (HPβCD) and anionically charged (SBEβCD) modified β-cyclodextrins have been studied (62). The authors found the binding constants for the neutral forms of the drugs to be greater with SBEβCD than with HPβCD. For the anionic drugs, the binding constants between SBEβCD and HPβCD were similar, while the binding constants for the cationic agents with SBEβCD were superior to those of HPβCD. Therefore, a clear charge effect on complexation, attraction in the case of cationic drugs and perhaps inhibition in the case of anionic drugs, was seen with the SBEβCD.

Micellar

If a drug is not solubilized by aqueous pH-modification, cosolvents, complexation, or combinations of these, surfactants are often used. The formulations are usually concentrated drug solutions in water-miscible organic solvent(s) that are diluted prior to intravenous administration (4). Water-miscible surfactant molecules contain both hydrophilic and

hydrophobic portions which self-associate to form micelles once the surfactant monomer concentration reaches the critical micelle concentration (CMC). Surfactants in parenterals can increase drug solubility through micellization, improve drug wetting, prevent drug precipitation upon injection, improve the stability of a drug in solution, modulate drug release or to prevent aggregation due to liquid/air or liquid/solid interfacial interactions (63).

A simple equation illustrates the principle of surfactant induced micellization and its impact on drug dissolution is as follows [eq. (27)].

$$[\text{Drug}]_{\text{total}} = [\text{Drug}]_{\text{aqueous}}(1 + \kappa[\text{Surfactant}]_{\text{m}}) \tag{27}$$

Where $[\text{Drug}]_{\text{total}}$ is the total solubility, $[\text{Drug}]_{\text{aqueous}}$ is the drug aqueous solubility, κ is a distribution coefficient, $[\text{Surfactant}]_{\text{m}}$ is the difference between the surfactant concentration and the CMC. The total drug in solution increases linearly with the linear increase in surfactant concentration once the surfactant concentration exceeds the CMC. While the linear response limits the degree of solubilization, it minimizes the potential for supersaturation or precipitation upon dilution.

The surfactants commonly used for intravenous infusion formulation include cremophor EL, cremophor RH60, and polysorbate 80. The solubilizing solvent is typically a mixture of surfactant and solvent(s) such as cremophor EL/ethanol/propylene glycol. The upper limit of surfactant administered in vivo is 10% for the cremophor EL and up to 25% polysorbate 80 for IV infusion. Cremophor EL is known to have significant side effects such as hypersensitivity reactions and liver damage (64).

Polysorbate 80 is a nonionic surfactant commonly used in parenteral formulations. Chlordiazepoxide (Librium™) comprises 4% of polysorbate 80 along with 20% propylene glycol and is injected undiluted intramuscularly. Quite often the surfactant containing formulation is diluted prior to intravenous administration to reduce toxicity. For example, amidarone hydrochloride has a water solubility of 0.7 mg/mL, is solubilized to 50 mg/mL in Cordarone™ by a combination of 10% polysorbate 80 and pH adjustment to 4.1. It is administered by intravenous infusion after a 25-fold dilution with dextrose 5%. Solutol HS-15 is a newer nonionic surfactant for parenteral formulation. Solutol HS-15 is used up to 50% to solubilize Propanidid, 7% to solubilize Vitamin K_1. Solutol HS-15 has also been used in preclinical formulations to prepare supersaturated injectable formulations of water-insoluble molecules (65).

Emulsions

Highly lipophilic, low melting point drugs can be quite soluble in oils and formulated for intravenous administration by employing an oil-in-water emulsion stabilized by surfactants in interfacial phases. A recent review by Strickley provides an excellent summary of excipients used in commercially available lipid-based formulations (4). Emulsions typically contain 10% to 20% oil and 2% glycerol for isotonicity, 1% phospholipid surfactant (e.g., lecithin), at pH 7 to 8 and an oil-soluble drug partitioned into the oil phase. The surfactant is applied to provide an energy barrier to agglomeration of the emulsion droplets. Lipid-based systems can exist in a wide variety of microstructures depending on the components used and their concentration, such as w/o or o/w emulsion and microemulsions, micelles, reverse micelles, bicontinuous phases, or mesomorphous pases (66). The solubilization capacity and drug release rate of the active molecules are related to the microstructure. Understanding solubility in lipid mixture is complicated by the fact that these systems are strongly affected by their interfacial nature, the nature of the oil, surfactant, cosurfactant, the size of the droplet and the preferred location of the drug within the system (67). The unique structural organization of the microemulsion results in additional domains which may increase their solubilization capacity as compared with nonstructured solutions containing the same fraction of components.

A marketed emulsion in the United States, Diprivan®, in which propofol, a water-insoluble compound is solubilized to 10 mg/mL in an emulsion composed of 10% soybean oil, is administered by IV bolus or IN infusion (4). There are other commercial emulsions in Europe and Japan, including diazepam, PGE1, dexamethasone palmitate and flurbiprofen.

Emulsions are being prepared with an energy input, such as ultrasonication, homogenization, or high-speed stirring and are thermodynamically unstable because of high

interfacial energy. Stabilization hinges on the ability to reduce interfacial tension, forming an interfacial film barrier to kinetically impede coalescence of droplets. There are four types of stabilizing agents: inorganic electrolytes, surfactants, macromolecules and solid particles. Detailed discussion is available elsewhere (68).

Microemulsions are a thermodynamically stable isotropically clear dispersion composed of a polar solvent, an oil, a surfactant, and a cosurfactant. The potential to form self-emulsifying drug delivery systems was evaluated by Pouton in 1985 (69). Recently, development of injectable microemlsuions has received considerable attention for IV delivery of drugs because of its potential to increase solubility (e.g., solubility of felodipine was increased by 10,000 fold in the microemulsion), reduce toxicity and hypersensitivity, reduce pain upon injection, as a long circulating formulation for drug targeting, and as a depot for IM delivery of drugs (70–72).

Microemulsions offer many advantages compared with macroemulsions: smaller particles (often <100 nm), require less energy to process and have higher physical stability (73). Microemulsions generally have very low interfacial tension at the water-oil interface, and form a highly fluid interfacial surfactant film. Because of the numerous small droplets, the surface area to volume ratio of microemulsions are very high and it forms easily because of the low surface tension, typically due to high levels of surface active species.

Most drugs that can be formulated in emulsions are generally liquids or low melting solids that have high octanol-water partition coefficients (74). In the Diprivan emulsion, Propofol has a high solubility in vegetable oil (>0 mg/mL), a low melting point of 18°C, and a large octanol-water partition coefficient (log*P* 3.83 in pH 6–8.5). Drugs with moderate to high melting point often cannot be formulated as emulsions because of the high lattice energy and low solubility in oil. High melting drugs possess some degree of polarity (i.e., presence of permanent dipoles and ability to form hydrogen bond), and these strong intermolecular forces cannot be readily overcome by the weak dispersion forces operating between solute and oil. Malcolmson studied the effect of oil on the solubility of testosterone propionate in nonionic o/w microemulsions and reported that larger molecular volume oils such as triglycerides miglyol 812 significantly increased the solubility of the compound over the corresponding micellar solution (75).

Predicting the solubility in lipid emulsions may be quite complicated because of the interfacial nature of the systems and the distribution of the drug in the continuous or dispersed phase and sometimes preferred location at the surfactant interface (67). If the drug preferentially resides at the interface in microemulsions, the creation of a larger interfacial area upon mixing the components may result in higher solubility. Testard studied the solubilization of a lipophilic molecule, lindane, in a microemulsion with a nonioinc surfactant. They found the solubility of lindane increased in the microemulsion region compared with the bulk oil; it was attributed to the incorporation of lindane in the surfactant interface (76). Addition of an amphiphilic block copolymer to medium chain surfactants has been shown to favorably alter the interfacial structure and significantly boost the solubilization capacity of microemulsions (77).

Surfactants are added to emulsion systems to reduce interfacial tension, reduce initial droplet size and size distribution, draw a liquid fill between droplets in areas where film thinning may have occurred, impart steric stabilization and in the case of charged surfactants give rise to charge distribution. The presence of surfactant and cosurfactant could make microemulsion supersolvents for drugs relatively insoluble in both aqueous and hydrophobic solvents (78). Using mixed oils and/or mixed surfactants in microemulsion may offer significant advantages over using pure single component materials (79). Prediction of absolute solubility in lipid vehicles is difficult since it requires similar knowledge as needed for aqueous solubility prediction, but also knowledge of the drug's specific interactions between the solute and formulation components, including an understanding of the lipid microstructure (67).

Liposome

Liposome formulations can be used as a means to solubilize some drugs for intravenous administration, to improve pharmacokinetics, enhance efficacy, and reduce toxicity (4). Liposomes are closed spherical vesicles composed of one or more bilayers of amphipathic lipid molecules enclosing one or more aqueous core compartments (80). Moderately hydrophobic

drugs can be solubilized by liposomes if the drug becomes encapsulated or intercalated within the liposome. Hydrophobic drugs can also be solubilized by liposomes as an integral part of the lipid bilayer. Water-soluble drugs reside within the aqueous inner core and are released as the liposome erodes in vivo or by leakage. A typical liposome formulation contains water with phopholipid at ~5 to 20 mg/mL, an isotonicifier, a pH 5 to 8 buffer, and potentially cholesterol.

Liposomes are injectd either by IV infusion or intrathecally. Upon IV administration, most conventional liposomes are easily taken up by the reticuloendothelial system (RES, in the body. There are several liposome formulations on the market. Amphotericin B, a compound with low aqueous solubility of ~0.1 mg/mL at pH 2 (anion) or pH 11 (cation), is solubilized to 5 mg/mL by liposomal intercaltion and becomes an integral part of the lipid bilayer (81). The amphotericin B liposomal products are being administered by IV infusion and have a longer in vivo half-life. Upon formulation in liposomes, paclitaxel, a low solubility drug (< 2 μg/mL), has been reported to achieve a solubility of 3.39 mg/mL in a liposomal formulation of polyethylene glycol 400, soybean phosphatidylcholine (PC) and cholesterol (82). Liposomes can be classified on the basis of liposome size or lamellarity as multilamellar large vesicles (MLVs), small unilamellar vesicles (SUVs), and large unilamellar vesicles (LUVs).

The lipids normally used are the unsaturated PC, phosphatidic acid (PA), phosphatidylglycerol (PG), and the saturated lipids L-a-dimyristoylphosphatidylcholine (DMPC), dipalmitoyl phosphatidylcholine (DPPC), dipalmitoyl phosphatidic acid (DPPA), and L-a-dimyristoylphosphatidylglycerol (DMPG). ABELCET® is an example of MLV consists of amphotericin B complexed with DMPC and DMPG in a 1/0.7/03 molar ratio. The complex assumes a flattened, ribbon-like mutilamellar structure with a particle size ranging from 1600 to 11,000 nm. Upon administration, ABELCET exhibits large volume of distribution, high clearance from blood and long terminal elimination half-life.

Large unilamellar liposomes (LUV) refer to vesicles > 100 nm in diameter bounded to single bilayer membrane. LUV provides higher encapsulation of water-soluble drugs, economy of lipids and reproducible drug release rates; however, these LUV liposomes are difficult to produce. Small unilamellar liposomes (SUV) are formed by dispersing multilamellar vesicles into water using sonication, extrusion through filters of various pore sizes, or homogenization to form optically clear suspensions. AmBisome® is an example of closed fluid-filled unilamellar bilayer liposomes made of a single phospholipid bilayer with amphotericin B intercalated within the membrane at drug:lipid molecular ratio 1:9, and particle size 45 to 80 nm. Upon injection, AmBisome exhibits smaller volume of distribution than the multilamellar ABELCET. Several excellent reviews on liposome technology and its application have been published (83,84).

Combined Solubilization Strategies

Various methods have been reported to enhance solubility of poorly soluble compounds by utilizing a combination of more than one of the solubilization techniques (54,85,86).

Combined use of pH with surfactants was reported to significantly increase drug solubility. The total solubility of a weak electrolyte undergoing ionization and micellization can be accounting for the free unionized drug D_u, free ionized drug D_i, micellized unionized drug D_uM, and micellized ionized drug D_iM as equation (28).

$$[\text{Drug}]_{\text{total}} = [\text{Drug}_u] + [\text{Drug}_i] + K_u[\text{Drug}_u][M] + K_i[\text{Drug}_i][M] \tag{28}$$

where K_u and K_i are the micellar equilibrium constants for the unionized and ionized drug, respectively. This equation is valid for surfactants that are either neutral or completely ionized in the pH range studied. Li discussed this approach using polysorbate 20 on flavopiridol, a weakly basic compound with an apparent pK_a of 5.69 and a low intrinsic solubility of 0.025 mg/mL for its zwitterionic form (87). The solubility of flavopiridol in 10% polysorbate 20 solution at pH 4.3 (27.3 mM) is much higher than that could be expected by increasing the total solubility through appropriate pH adjustment from pH 8.4 and solubilization of the unionized drug in the micelles (3.3 mM). The authors pointed out that high solubility of the ionized drug in the micelles is the source of synergism for solubility enhancement in the

pH-surfactant solutions. Furthermore, this formulation does not precipitate upon dilution with isotonic Sorensen's phosphate buffer.

Combination usage of pH control and cosolvent has been reported to increase solubility of flavopiridol (87). Since solubility of the unionized form is pH independent, the authors concluded the higher total solubility at low pH is attributed to the solubilization of the ionized species by the cosolvent. The pH related solubilization produced by cosolvent can be described by equation (29).

$$[\text{Drug}]_{\text{total}} = [\text{Drug}_u]10^{\sigma_u f} + [\text{Drug}_u]10^{(pK_a - pH)}10^{\sigma_i f} \quad (29)$$

Where f is the volume fraction of cosolvent, σ_u and σ_i are the solubilizing powers of the cosolvent for the unionized and the ionized species, respectively.

Redenti reported that hydroxylcarboxylic acids (such as citric acid, lactic acid, malic acid, tartaric acid), or bases (such as tromethamine, diethanolamine, triethanolamine) can be used in drug-cyclodextrin solutions to enhance drug solubility by several orders of magnitude through formation of a "multicomponent complex" while that of cyclodextrin can be enahanced more than10 fold (54). The synergistic effect was rationalized due to the specific interaction of the hydroxyl acid groups with the hydrogen bond system of the host and/or the modification of the hydrogen bond network of the surrounding water molecules. Astemizole, upon βCD multicomponent complexation with tartaric acid, achieved 27,600-fold enhancement of solubility. The resulting amorphous complex dissolved rapidly and generated supersaturation that remains stable for several days.

Loftsson reported that addition of small percentage of hydrophilic polymers in cyclodextrin-based formulation can further enhance drug solubility (88). With the addition of 0.25% polyvinylpyrrolidone, the solubility of a number of compounds was increased from 12% to 129% in a 10% (w/v) HPβCD vehicle. The authors suggested that the polymer increased the stability constants of the drug-cyclodextrin complexes because of increased negative enthalpy change together with an increased negative entropy change.

Pitha reported that gradual addition of ethanol decreased and eventually abolished the formation of inclusion complexes of testosterone with HPβCD in aqueous solutions (89) (Fig. 8). Initially, at ethanol concentration $<30\%$, the solvent acted as a competing for the cavity of HPβCD and reduced the solubility of testosterone; at higher ethanol concentrations

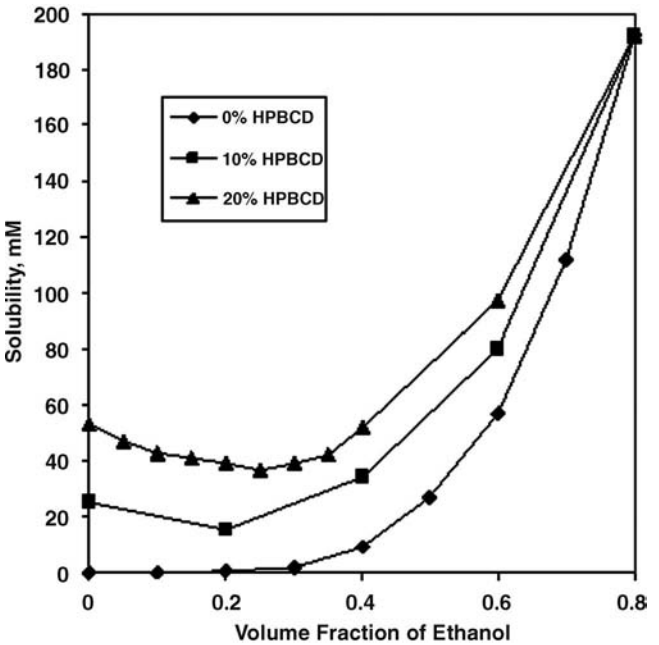

Figure 8 Effect of ethanol on solubilization of testosterone into aqueous solution containing hydroxyl-β-cyclodextrin. *Source*: From Ref. 89.

the solubility of testosterone started to rise, in which the dissolution primarily occurred through nonspecific solvent effects.

The effect of pH variation on complexation and solubilization of naproxen (a weak acid with pK_a 4.2) with natural βCD and various neutral, cationic and anionic βCD derivatives, and hydrophilic polymers has been investigated (86). The authors found the presence of 0.1% PVP increased the solubility of naproxen in the presence of 25 mM HPβCD complex by approximately 30%, at pH 1.1 and 6.5. This integrated strategy of pH control and polymer addition to the CD complexing medium allows a smaller quantity of CD be used to solubilize a given amount of drug.

Propylene glycol, PEG, ethanol, cremophor EL, cremophor RH60, and polysorbate 80 are water-miscible solvents and surfactants in commercially available injectable formulations. These solvents and surfactants are used in combination with each other, usually as a concentrate for dilution just prior to IV injection (4). In general, the cosolvent increases the CMC of the surfactant and increases solubility of the drug. Paclitaxel, a water-insoluble compound (aqueous solubility of 0.1 μg/mL), is solubilized in Taxol® to 6 mg/mL (i.e., 60,000-fold aqueous solubility) with 51% cremophor EL and 49% ethanol, and is diluted 5 to 20 fold with dextrose 5% or lactated Ringer's prior to administration. The final dosing formulation of Taxol is a micellar dispersion (90). The combination of cremophor EL and ethanol has also been used to solubilize teniposide, valrubicin, tacrolimus and cyclosporin.

Trace amount of polymer may decrease the precipitation rate (91), stabilize micelles and other type of aggregates in aqueous solutions and increase the solubility of the compounds by about twofold (92). Water-soluble polymers not only solubilize βCD and its complexes, but they are also able to enhance formation of complexes between drugs and βCD (54). Quarternary complexs of drug, cyclodextrin, polymer and tartaric acid have been reported to further enhance drug solubility (93). However, contrary results have been reported that formation of polymer/cyclodextrin complexes reduced the ability of the cyclodextrin to solubilize drug through complexation (54).

SUMMARY

The decisions regarding solubilization strategy often reside in the intrinsic solubility of the drug, solubilization capacity of the particular strategy, dose of drug to be delivered, infusion time, and potential safety concerns with the excipients, all coupled with the therapeutic area and unmet need. Technologies such as cosolvency and pH modification (indirectly salts) are often favored because of their very high capacity for solubilization. They typically result in exponential increases in solubility and can be very valuable for very low intrinsic solubility drugs (i.e., less than 10 mcg/mL), leading to apparent solubilities in excess of 50 mg/mL. However, given the exponential nature of solubilization and linear nature of subsequent dilution on administration, they are much more prone to precipitation upon dilution. Other approaches (micellar, complexation, emulsions, liposomes) often have lower capacity, but tend to solubilize in a more linear proportionality to concentration of solubilizer, thus being much less prone to precipitation upon dilution. These more linear alternative equilibrium type approaches are not likely to provide solubilization in excess of 20 mg/mL, often much less.

The risk in any sort of solubilization strategy is the propensity for precipitation upon administration and dilution into biological media. The presence of proteins and lipoproteins upon dilution can often facilitate supersaturation and allow for the time necessary to get further dilution and distribution in vivo. In essence, they often provide alternative equilibria for drug solubilization in vivo. The use of in vitro methods (94) and in vivo methods (95) to explore propensity for precipitation can often be very useful.

Solubility, coupled with dose and therapeutic indication, often define the ability to adequately deliver a drug parenterally. While the thermodynamic solubility ultimately dictates the actual chemical potential of the drug in solution under specified conditions, the total "solubilized drug" probably becomes the more relevant descriptor for drug delivery in parenteral systems. Efforts to solubilize drugs are highly dependent on altering either the conditions of the solvent system, creating alternative equilibria for the drug to reside in, changing the macroscopic solid form of the solute, or actually changing the solute at the molecular level (i.e., creating a new chemical entity). These alterations can increase the

escaping tendency from the solid state, facilitate the cavity formation in the solvent necessary for solute insertion, enhance the level of interactions between the solute and solvent, or simply provide an alternative state in which the molecule can reside. As will be discussed elsewhere in this book, the ultimate success of these strategies resides in the ability to deliver the molecule of interest to the in vivo milieu without deleterious results of precipitation upon administration.

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7 Formulation of depot delivery systems

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INTRODUCTION AND DRIVERS FOR DEPOT DELIVERY

Depot delivery systems, also known as sustained-release systems, are parenteral formulations containing multiple doses of drug that, when introduced into the body, are designed to release the drug over a specified, often prolonged, period of time. Depot formulations come in many forms, designed for several different administration routes, and have been in use for over half a century. In addition to the many depot pharmaceuticals approved for use today, the development of novel systems remains an active area of research because of the ability of depot systems to overcome several well-recognized challenges often associated with conventional delivery. These include variations in drug plasma levels between doses that can lead to adverse effects or compromised efficacy, poor patient compliance due to frequent dosing, and difficulty localizing exposures to the target organ or tissue.

Advantages of Depot Delivery Systems

As earlier chapters in this book have highlighted, parenteral drug delivery can overcome many of the challenges associated with oral delivery of bioactive molecules, including degradation in the gut, low permeation through intestinal mucosa, and high first pass metabolism. It is generally recognized that, for certain therapeutic molecules, such as proteins, parenteral dosing is indeed often the only viable way to deliver pharmacologically relevant doses. At the same time, relative to other routes of administration, injection is invasive and is generally less preferred as a means of administering therapeutics. Depot delivery systems provide one way to mitigate this issue by decreasing the frequency of administration. For example, BYDUREON™, pending FDA approval for the treatment of type II diabetes, promises to reduce the frequency of dosing from twice daily with the current product, BYETTA™, to once weekly with the depot formulation (1).

Certain classes of drugs have relatively narrow therapeutic windows, defined as the concentration in vivo above which a compound is therapeutically effective, but below that at which toxic effects are observed (2). For these drugs, it can be challenging to maintain plasma concentrations within the therapeutic window (Fig. 1). In some cases, such as when the molecule has a very short half life, and is not well absorbed along the length of the GI tract, oral dosing may simply not be feasible. The gold standard for maintaining precise control over plasma drug levels is continuous infusion, typically via the intravenous route (3). Clearly, despite the degree of control it offers, continuous IV infusion is often not practical because of heightened risk and the need for close medical supervision during treatment. Depot delivery systems can avoid the peaks and troughs in plasma concentrations common with conventional dosing, and maintain the plasma concentration within the therapeutic window, by providing an infusion-like profile without the drawbacks of IV delivery.

In some cases, such as cancer treatment, it may be desirable to limit drug exposure to the site of action, and minimize systemic exposure altogether. The GLIADEL® wafer, a depot formulation of carmustine, which is implanted at the surgical site after brain tumor resection, is one example of this approach (4). Intra-articular injection of corticosteroid depots is another example where local effects at the site of action can be maximized relative to systemic effects (5).

Poor compliance is increasingly recognized as a significant factor in the failure of therapy in certain patients and there is an inverse relationship between dose frequency and compliance (6). Schizophrenia is one such example, where compliance rates are estimated at about 50% (7). Depot formulations of antipsychotics were first introduced in the early 1960s, initially for patients with suicidal or violent tendencies, but later became well-accepted as maintenance therapies (7,8). Depot antipsychotics are also reported to reduce the frequency of side effects

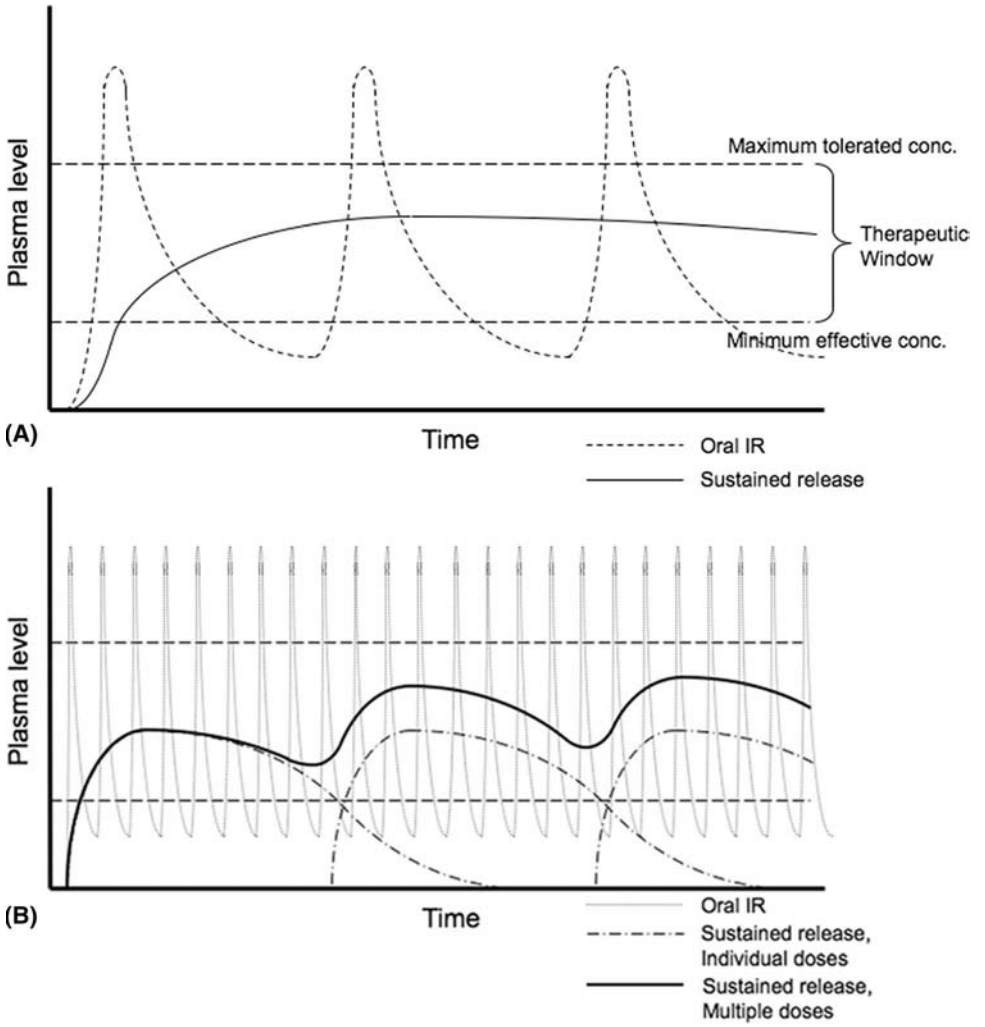

Figure 1 (**A**) An idealized representation of plasma concentration versus time obtained following oral dosing and administration of a sustained-release formulation. Note that oral dosing can result in large variations in plasma concentrations between doses, and that plasma concentrations may not be maintained within the therapeutic window. In contrast, sustained-release formulations are capable of maintaining relatively constant plasma profiles over time. (**B**) Plasma concentration versus time profiles for oral and multiple sustained-release doses over a longer period of time.

(7). RISPERDAL® CONSTA® was the first atypical antipsychotic approved as an injectable depot. As evidence of its impact, sales have grown to $870 million (IMS, 2006).

Despite their many advantages, there are some drawbacks to depot delivery, including difficulty in removing the dose once administered, lack of dosing flexibility, the need for injection or implantation, and potential local adverse tissue reactions (9). These limitations can, in many cases, be managed or overcome. In cases where it is necessary to maintain the ability to cease dosing, nondegradable implant systems can be utilized. Several products, such as Lupron Depot®, include formulations that release for varying periods of time to improve dosing flexibility. Most modern sustained-release formulations can be delivered through conventional needles (although admittedly large-bore by current standards), and the excipients used in the formulations are generally nonirritating. The properties of an ideal depot delivery system include extent and duration of release matched to the needs of the

indication, tolerability and lack of toxicity, and biodegradability (in most cases). Zero order release is often desired, although this can be difficult to achieve in practice, and many products have been commercialized without meeting this criterion. The ability to alter the release rate during administration, while not currently possible in commercialized systems, would also be a desirable option, and is an active area of research.

History and Types of Depot Formulations

Depot formulations have been in use for well over half a century; implantation of testosterone pellets was employed in the 1930s (10). The first widely marketed depot formulations, launched in the 1950s, were injectable intramuscular (IM) suspensions of drugs in aqueous and oily vehicles. A number of additional suspension and oily-vehicle depot formulations, based on poorly soluble alkane ester prodrugs, were developed and launched during the 1960s and 1970s. Use of the biodegradable polyester, poly(lactic-co-glycolic acid) (PLGA), for drug delivery began in the 1970s (11), culminating in the U.S. launch of the PLGA microsphere product, Lupron Depot®, in 1989. In the years following, a number of additional PLGA depot products were launched, including a microsphere formulation of a protein, and extruded PLGA rods. The 1990s saw the introduction of new polymers and lipid-based strategies for sustained-release delivery, as well as the development of implantable device-based depot systems. These strategies have enabled a number of product launches that have continued into recent years.

Requirements for Pharmaceutical Actives Suitable for Depot Delivery

Given the practical constraints and technical challenges associated with developing parenteral sustained-release formulations, pharmaceutical actives must meet certain requirements to be suitable for depot delivery. These requirements vary according to the specific depot strategy selected, but several criteria are general. Most importantly, actives should be potent to allow incorporation of the entire quantity of active needed for dosing over the lifetime of the depot, at a reasonable drug loading within the system. The required potency should be estimated by considering the desired duration of release, and injection volume and drug loading constraints. Note that, when oral PK/PD data exist, it is important to consider the impact that parenteral dosing may have on exposures; this often works to the advantage of the formulator in terms of reduced doses because of absorption limitations and first pass metabolism via oral dosing. Stability is the second criterion, as it is necessary to ensure that the active remains stable not only during the manufacturing process and over the shelf life of the product, but also after administration, within the environment of the body. Stability at body temperature, in an aqueous environment, and in the presence of proteins and enzymes, may become important considerations. Solubility, in aqueous media, solvents that may be used in the manufacturing process, and within the formulation itself, is the third important criterion. Specific solubility requirements will vary according to the formulation approach, and may indeed dictate the formulation strategy. Additional criteria include PK/PD profile (therapeutic window), lack of irritation of the active to local tissues, and the absorption profile of the active. In determining the suitability of an active for depot delivery, it is also important to consider the requirements of the therapeutic area. Therapeutic areas that require extended periods of dosing, high compliance rates, and localized delivery lend themselves to depot formulations. Specific examples of relevant therapeutic areas include hormone therapy (testosterone, estrogen, GnRH antagonists, etc.), corticosteroid treatment, basal insulin delivery, antipsychotics, and contraception.

SUSPENSION AND OILY-VEHICLE DEPOT SYSTEMS

Formulations based on suspensions of drug substance in aqueous or oily vehicles were amongst the first long-acting injectable delivery systems developed (Table 1). These systems rely in large part on the dissolution properties of the suspension particles to govern the release rate from the depot. When the solubility of the drug substance in an oily vehicle allows, an alternate approach is to formulate an oil solution of the drug; in this case the formulator relies

Table 1 A Partial List of Injectable Suspension and Oily-Vehicle Sustained-Release Products Approved in the United States

Trade name	Active	U.S. approval	Excipients (reference number)
Aristocort	Triamcinolone diacetate, 40 mg/mL	1961, Fujisawa	PEG 3350, 3% Tween 80, 0.2% Sodium chloride, 8.5 mg/mL Benzyl alcohol, 9 mg/mL pH ~ 6 (12)
Aristospan	Triamcinolone hexacetonide, 5, 20 mg/mL	1969, Fujisawa	Sorbitol, 50% Polysorbate 80, 0.2–0.4% Benzyl alcohol, 0.9% pH 4.5–6.5
Bicillin C-R	Penicillin G Benzathine, 300,000 U/mL, Penicillin G Procaine, 300,000 U/mL	1953, Wyeth-Ayerst	CMC, 0.55% Lecithin, 0.5% Povidone, 0.1% Methylparaben, 0.1% Propylparaben, 0.01% Sodium citrate pH 6–8.5 (12)
Bicillin L-A	Penicillin G Benzathine, 600,000, 300,000 U/mL	1958, Wyeth-Ayerst	Lecithin, 0.5% CMC, 0.6% Povidone, 0.6% Methylparaben, 0.1% Propylparaben, 0.01% Sodium citrate buffer
Celestone Soluspan	β-methasone sodium phosphate / acetate, 3 mg/mL	1965, Schering	Sodium phosphate dibasic, 7.1 mg/mL Sodium phosphate monobasic, 3.4 mg/mL EDTA, 0.1 mg/mL Benzalkonium chloride, 0.2 mg/mL pH 6.8–7.2 (12)
Cortone	Cortisone acetate, multiple strengths	1950	Sodium CMC, 5 mg/mL Tween 80, 4 mg/mL Sodium chloride, 9 mg/mL Benzyl alcohol, 9 mg/mL (12)
Decadron-LA	Dexamethasone acetate, 8 mg/mL	1973	Sodium CMC, 5 mg/mL Tween 80, 0.75 mg/mL Sodium chloride, 6.7 mg/mL Creatinine, 5 mg/mL EDTA, 0.5 mg/mL Benzyl alcohol, 9 mg/mL Sodium bisulfite, 1 mg/mL pH 5.0–7.5 (12)
Deca durabolin	Nandrolone decanoate, 25, 50 mg	1962, Organon	Arachis oil Benzyl alcohol,10%
Delalutin	Hydroxyprogesterone caproate, 125, 250 mg/mL		Castor oil Benzyl benzoate Benzyl alcohol (13)
Delatestryl	Testosterone enanthate, 200 mg/mL	1953, Squibb	Sesame oil Chlorobutanol, 5 mg/mL (12)
Delestrogen	Estradiol valerate, 10, 20, 40 mg/mL	1954, Squibb	Castor oil Benzyl benzoate Benzyl alcohol Ethanol (13)
Depinar	Cyanocobalamin-Zn-tannate	1980, Armour	Sesame oil Aluminum monostearate, 2% (14)
Depo-Estradiol	Estradiol cypionate, 5 mg/mL	1979, Upjohn	Cottonseed oil Chlorobutanol anhydrous, 5.4 mg/mL
Depo-Medrol	Methylprednisone acetate, 20, 40, 80 mg/mL	1959, Upjohn	PEG 3350, 3% Tween 80, 2 mg/mL Sodium phosphates, 2 mg/mL Benzyl alcohol 9 mg/mL Sodium chloride (isotonic) pH 3.5–7.0 (12)

(*continued*)

Table 1 A Partial List of Injectable Suspension and Oily-Vehicle Sustained-Release Products Approved in the United States (*continued*)

Trade name	Active	U.S. approval	Excipients (reference number)
Depo-Provera	Medroxyprednisolone (progesterone), acetate, 100, 400 mg/mL	1960, Upjohn	PEG 3350, 20–29 mg/mL Tween 80, 2.4 mg/mL Sodium chloride, 8.7 mg/mL Methylparaben, 1.4 mg/mL Propylparaben, 0.15 mg/mL (12)
Depo Sub Q Provera 104	Medroxyprogesterone acetate, 104 mg	2004, Pharmacia and Upjohn	PEG Polysorbate 80 Povidone Monobasic sodium phosphate Dibasic sodium phosphate Methionine Sodium chloride Parabens
Depo-testadiol	Estradiol cypionate, 2 mg/mL, testosterone cypionate, 50 mg/mL	1980, Upjohn	Cottonseed oil Chlorobutanol anhydrous, 5.4 mg/mL
Depo-testosterone	Testosterone cypionate, 200 mg	1979, Upjohn	Cottonseed oil (15)
Ditate-DS	Testosterone enanthate, 180 mg/mL, estradiol valerate, 8 mg/mL	1982, Savage	Ethyl oleate BP (15)
Haldol	Haloperidol decanoate, 50, 100 mg	1986	Sesame oil Benzyl alcohol, 1.2% (12)
HP Acthar	ACTH-Zn-tannate	1952, Armour	Gelatin,16% Phenol, 0.5% (16)
Hydeltra-TBA	Prednisolone Tebutate, 20 mg/mL	1956	Sorbitol Polysorbate 80 Sodium citrate Benzyl alcohol (17)
Hydro-cortone	Hydrocortisone acetate, 50 mg/mL	1951	Sodium CMC, 5 mg/mL Tween 80, 4 mg/mL Sodium chloride, 9 mg/mL Benzyl alcohol, 9 mg/mL (12)
Kenalog-10, 40	Triamcinolone acetonide, 10, 40 mg/mL	1960	Sodium CMC Polysorbate 80 Sodium chloride Benzyl alcohol (17)
Lantus	Insulin glargine, 100 U/mL	2000, Sanofi-Aventis	Glycerol 85% M-cresol Polysorbate 20 Zinc
Lunelle	Medroxyprogesterone acetate, 25 mg Estradiol cypionate, 5 mg	2000, Pharmacia and Upjohn	PEG, 28.56 mg/mL Polysorbate 80, 1.9 mg/mL Methylparaben, 1.8 mg/mL Propylparaben, 0.2 mg/mL Sodium chloride, 8.56 mg/mL
Percorten	Desoxycortisone pivalate, 25 mg/mL	Ciba	Methylcellulose Sodium CMC Polsorbate 80 Sodium chloride Thimerosal (17)
Plenaxis	Abarelix, 100 mg	2003, Praecis	CMC Reconstituted in sodium chloride
Prolixin Decanoate, 25 mg/mL	Fluphenazine decanoate	1972, Squibb	Sesame oil Benzyl alcohol, 1.2%
Prolixin Enanthate, 25 mg/mL	Fluphenazine enanthate	1967, Squibb	Sesame oil Benzyl alcohol

Table 1 (*continued*)

Trade name	Active	U.S. approval	Excipients (reference number)
Sus-phrine	Epinephrine HCl, 5 mg/mL	1951	Glycerin, 325 mg/mL Thioglycolic acid, 6.6 mg/mL Ascorbic acid, 10 mg/mL Phenol, 5 mg/mL (12)

Note: Note that some of these products have been discontinued. Approval dates were referenced from Drugs@FDA (http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm?fuseaction=Search.Search_Drug_Name).
Abbreviation: CMC, carboxymethylcellulose.

chiefly on the oil/water partition coefficient and dispersion of the vehicle to govern release. We will discuss both approaches in more detail in this section. A third approach is adsorption of the active component to a solid adsorbent (3); this approach is commonly used in vaccine formulations, but will not be discussed in more detail here. Suspension and oily-vehicle formulations are generally suitable only for compounds with low aqueous solubility. If the aqueous solubility of the drug substance is too high to enable formulation by these approaches, solubility can be reduced by formation of a poorly soluble prodrug. One common approach is esterification with alkanes (e.g., to form enanthates, decanoates, or cypionates), an approach used extensively for hormones such as testosterone (3). Alternatively, poorly soluble complexes or salts can be formed, such as zinc-insulin and carboxymethylcellulose (CMC) complexes (3). Suspension and oily-vehicle depot formulations are most often administered by the IM route, although they can also be administered via the subcutaneous (SC), intra-articular, and intradermal routes.

Physical Stability of Suspensions

Injectable suspensions are dispersions of solid drug in an aqueous or oily liquid vehicle. The most common are coarse suspensions, which typically have a mean particle size of less than about 50 μm to ensure that they can be administered through a suitably sized hypodermic needle, and to slow the rate of settling (18). The rate of settling of particles in a dispersion is governed by Stokes' law.

$$\nu = \frac{\Delta\rho g d^2}{18\eta}$$

where ν is the velocity of sedimentation, $\triangle\rho$ is the density difference between the phases, g is the gravitational acceleration, d is the particle radius, and η is the viscosity of the continuous phase (18). Given the goal of slowing the sedimentation rate, Stokes' law instructs the formulator to decrease the particle size and density difference between the phases, and increase the viscosity of the continuous phase. We will later examine how excipients can be used to accomplish these goals.

Stokes' law assumes uniform and noninteracting particles. In reality, interactions between suspended particles are significant and include attractive van der Waals forces, and repulsive electrical double layer and solvation/hydration forces (19). One of the primary failure modes in the formulation of suspensions is caking, which results from the settling of particles and the formation of a densely-packed layer of solids (17). The distance between particles is sufficiently decreased within the cake so that attractive van der Waals forces dominate and cause irreversible aggregation of the particles, preventing their redispersion. One technique used to prevent caking is to formulate the suspension to flocculate. Flocculated particles interact to form a loosely aggregated structure, where interparticle distances are sufficiently large that the system is easily resuspended (e.g., by brief shaking). Formulation at the secondary minimum of the potential energy function can maximize the stability of the flocculated system (18).

A second failure mode, particularly in nanoparticulate systems, is particle growth over time through Ostwald ripening. This phenomenon is described by the Ostwald-Freundlich equation.

$$\ln \frac{C_1}{C_2} = \frac{2M\gamma}{\rho RT}\left(\frac{1}{r_1} - \frac{1}{r_2}\right)$$

where C_1 and C_2 are the saturation solubility at the surface of particles of radius r_1 and r_2, respectively. M is molecular weight, γ is the surface energy of the solid in contact with the solution, ρ is the density of the solid, R is the gas constant, and T is absolute temperature (17). The phenomenon is driven by the higher saturation solubility at the surface of small particles relative to larger ones, as a result of curvature effects. Drug therefore dissolves from the surface of small particles, diffuses to the vicinity of larger particles where saturation solubility is exceeded, and deposits on to the surface of the larger particles, causing a net upward shift in particle size of the system. It must always be appreciated that micro- and nanosuspension systems are thermodynamically unfavorable, and that one must rely on slowing the kinetics to ensure physical stability of these formulations.

Formulation of Parenteral Suspensions

The ideal parenteral suspension is easily resuspended with mild shaking and does not cake upon storage, does not settle rapidly and remains homogenous long enough to allow reproducible dosing, maintains stability and elegance over its shelf life, maintains sterility during storage and use, and is easily administered through a 20- to 25-gauge needle (17). Like all formulations, the formulation of injectable suspensions should begin with a thorough preformulation characterization including solubility in water over a range of pH and in the presence of stabilizing surfactants and polymers, chemical stability in both solid state and solution, and full characterization of drug forms including polymorphs, hydrates and solvates. Drug form can significantly influence the rate of absorption from the injection site (20). After IM administration, aqueous suspensions tend to form a loose agglomerate within the fibrous or membranous tissues between muscle fibers, while the vehicle is rapidly absorbed (21).

Particle Size

Drug particle size can have a significant impact on formulation physical stability, syringeability, and release rate, and therefore should be well characterized and controlled through approaches such as controlled crystallization or milling (17). It is important that the process used provide a narrow particle size distribution to minimize Ostwald ripening, and that the potential for form change, for example, to the amorphous form, be well-understood given the potential adverse impact on physical stability (22). Particle size reduction techniques include jet milling, spray drying, and supercritical fluid processing (18). Wet media milling can be used to generate nanocrystalline dispersions (23): the Elan NanoCrystal® technology is used in Janssen's product INVEGA SUSTENNA™. Compared with coarse suspensions, reduction of particle size to the submicron range enhances physical stability (reduced settling rate), homogeneity, syringeability (reduced viscosity), and options for sterilization (23).

Particle size has a significant effect on syringeability, and it is critical to evaluate suspension systems for syringeability and injectability. A typical recommendation to prevent particle "bridging" that could lead to clogging, is to limit the size of the largest particles to no larger than one-quarter to one-third the inner diameter of the needle (17). The viscosity of the formulation should be optimized to ensure a balance between physical stability of the suspension and syringeability. Thixotropy and shear-thinning behavior can be leveraged to accomplish both goals, as in the case of penicillin G procaine suspensions (24).

The impact of particle size on release rate and pharmacokinetics has been the subject of a number of published studies. Procaine penicillin G aqueous IM suspensions demonstrated faster release as particle size was reduced from 60 to 100 mesh to micronized size (25). The trend was similar for oil suspensions, unless the system was gelled by addition of aluminum monostearate, in which case the trend was reversed. In a separate study, in which aqueous

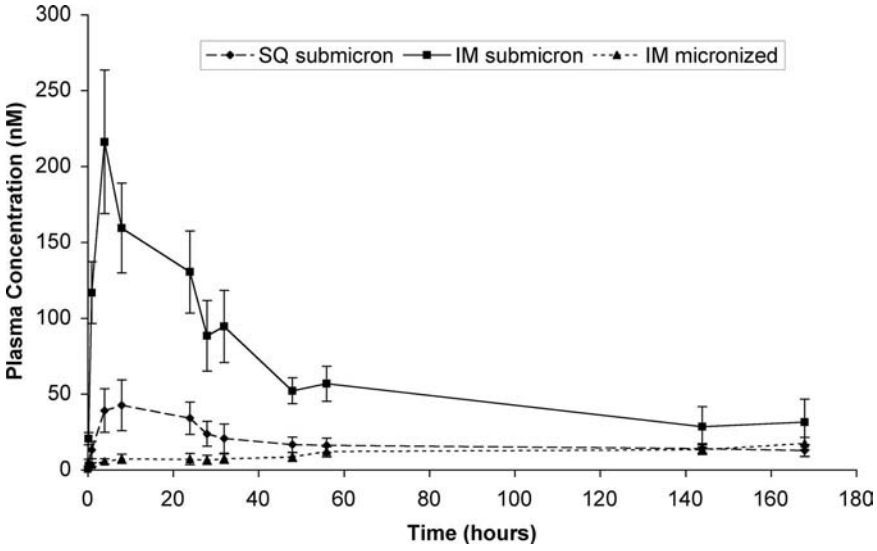

Figure 2 Mean plasma concentration versus time profiles for a poorly-soluble Merck compound after subcutaneous or intramuscular administration to rats of coarse or submicron drug suspension formulations. The composition of the suspension vehicle was the same for both suspensions, as was the dose (20 mg/kg). Data are mean ± SE, $n = 3$–4).

phenobarbital suspensions were administered intramuscularly to dogs, the area under the blood level curve was found to decrease as particle size increased from 6.63 to 29.96 μm (26). Studies of IM aqueous suspensions of model compounds in rats also demonstrated that the absorption rate constant increased with decreasing particle size (21). This effect was particularly apparent as particle size was decreased to 2 to 3 μm or smaller, possibly because of the ability of smaller particles to migrate more easily through the fibrous tissues at the injection site, enabling the depot to spread further following injection. In studies performed in our laboratories on aqueous suspension formulations of a poorly soluble drug, we similarly observed that reduction of particle size led to much faster absorption from a submicron suspension as compared with a coarse suspension (Fig. 2), by IM dosing. We also observed much faster absorption of the submicron suspension when administered by the IM route, compared with the SC route, highlighting the importance of administration route.

Theoretically, the release rate of drug from the depot under sink conditions is given by the following equation:

$$\left(\frac{Q}{t}\right)_{\mathrm{d}} = \frac{S_{\mathrm{a}} D_{\mathrm{s}} C_{\mathrm{s}}}{\delta_{\mathrm{s}}}$$

where Q is the amount of drug released in time t. S_{a} is the surface area of drug in contact with the surrounding fluid, D_{s} is the diffusion coefficient of drug molecules in the fluid, C_{s} is the saturation solubility of the drug, and δ_{s} is the thickness of the hydrodynamic diffusion layer surrounding the solid (3). The faster dissolution of smaller particles is explained by their higher surface area, but this relationship is only relevant if the particles remain at least partially dispersed after administration, as demonstrated by the results of the gelled oil system referenced previously.

Excipients

Earlier in this section we discussed the use of excipients to aid in the stabilization of suspensions. Nonionic surfactants, such as polysorbate 80, are commonly used to wet and sterically stabilize the drug particle surface (18). Povidone and lecithin have been used less

commonly for this purpose (17). Polymers such as CMC and high molecular weight polyethylene glycol (e.g., PEG 3350) are commonly used to increase the viscosity of the continuous phase. Additional excipients may include buffers, antimicrobial preservatives, and electrolytes such as sodium chloride. The latter may be used both to ensure tonicity, and to adjust ionic strength to impact flocculation (18). The total solids content in parenteral suspensions is often limited by the syringeability and injectability of the system, and may impose an upper limit on drug concentration.

Manufacture and Control of Parenteral Suspensions

Suspension formulations can be very challenging to develop and manufacture. They can be prepared either as ready to use suspensions, or as powders for reconstitution. It is typically not possible to sterilize suspension systems by sterile filtration, so they must either be manufactured under aseptic conditions, or terminally sterilized by heat or ionizing radiation. Two processes used to prepare parenteral suspensions are aseptic combination of sterile powder and vehicle, and in situ crystallization from sterile solutions (17). For the latter, sterile powder can be prepared by aseptic antisolvent crystallization, lyophilization, or spray drying (17). Particle size reduction is often required and can be accomplished by milling, and the vehicle is typically sterilized by either filtration or heat sterilization (17). Additional manufacturing considerations include entrapped air and foam, and particulate matter control (17).

Oily-Vehicle Solution Depot Systems

Compounds with low solubility, poor stability, or the potential for causing irritation in aqueous vehicles can be formulated as injectable solutions in oily vehicles. Advantages of this approach relative to suspension formulations include greater ease of manufacture, fewer physical stability concerns, and the ability to sterilize by filtration. Clearly, for this approach to be viable, the active must be sufficiently soluble and stable in the selected vehicle. As for suspensions, hydrophilic compounds can be converted to lipophilic prodrugs for formulation as a depot.

Ideally, oils for use in depot formulations should be chemically stable and inert to reactions with the drug, relatively low in viscosity, physically stable across a wide range of temperature, nonirritating, and free of antigenic properties (27). Oils acceptable for injection include fixed oils such as olive oil, corn oil, sesame oil, arachis oil, almond oil, peanut oil, poppyseed oil, soybean oil, cottonseed oil, and castor oil (28). Vegetable oils, as natural products, contain a variety of triglyceride components, including olein, linolein, stearin, palmitin, and myristin (29). Sesame oil is generally preferred because of its enhanced stability, imparted by natural antioxidants, however, it is light-sensitive (28). Isopropyl myristate, ethyl oleate, benzyl benzoate, polyoxyethylene oleic triglycerides (Labrafils), thin vegetable oil (fractionated coconut oil, Viscoleo) and PEGs are synthetic alternatives (28). Ethyl oleate is sometimes preferred because of lower viscosity. The fixed oils are generally well-tolerated, however, some patients may have allergic reactions to vegetable oils (28). Oily depots are typically administered intramuscularly, as SC injection has resulted in pain and irritation at the injection site (28).

Many oily vehicles are eliminated from the injection site slowly, by dissolution in body fluids or conversion to soluble species, or via the shedding and transport of oil microdroplets from the depot surface (29). Visual observation after IM administration has indicated that oil depots do not spread as extensively as aqueous systems and take on a flattened, pod-like shape (29). This is important because the surface area of the depot is expected to be a key determinant of release rate. The absorption of drugs from oil solutions has been shown to obey first-order kinetics in cases when the absorption of the vehicle is slow relative to the active. In this case, diffusion of the active through the aqueous phase surrounding the depot is rate limiting, and the rate constant is controlled by both the oil/water partition coefficient and the vehicle injection volume. By contrast, the absorption of drugs from oily suspensions can obey zero order kinetics, since the solubility of the drug in the vehicle is maintained at the saturation solubility until the suspension particles have completely dissolved (29).

DEGRADABLE POLYMERIC DEPOT DELIVERY SYSTEMS

Poly(Lactide-co-Glycolide) Systems

Polyesters of poly(lactic acid) (PLA) and copolymers of lactic and glycolic acids, referred to as PLGA, are the most commonly used polymers in biodegradable depot dosage forms. These biocompatible polymers undergo random, mostly nonenzymatic, ester linkage hydrolysis to form lactic acid and glycolic acid, which are normal metabolic compounds in the body. Resorbable sutures, clips and implants were the earliest applications of these polymers (30). The application of PLA and PLGA as biodegradable and biocompatible polymers for drug delivery was initiated in the 1970s (11,31,32). Southern Research Institute developed the first synthetic, resorbable suture (Dexon®) in 1970, and the first patent describing the use of PLGA polymers in a sustained-release dosage form appeared in 1973 (33).

PLGA is synthesized by means of a random ring-opening copolymerization of two different monomers, the cyclic dimers of glycolic acid and lactic acid. Thus, the polymers or copolymers may be produced by the polycondensation of the lactic acid and/or glycolic acid in the presence of an inorganic acid (34). Today, PLGA polymers are commercially available from multiple suppliers, including Boehringer-Ingelheim (Resomer®), PURAC (Purasorb®), Absorbable Polymers International (Lactel®), and Alkermes (Medisorb®). PLGA polymers are available commercially as end capped or acid terminated and with inherent viscosities ranging from 0.15 to 6.5 dL/g (35).

Polymer Selection and Degradation

Understanding the physicochemical and biological properties of a polymer is important prior to selection of a polymer for depot drug delivery. PLGA polymer can generally be characterized by molecular weight (inherent viscosity), polydispersity, lactide to glycolide ratio, and chemistry (end capped vs. acid terminated). The selection of the polymer for depot delivery would depend on the target release profile of the drug, with the drug release mainly governed by the degradation of the polymer. A vast amount of literature is available on the characterization of PLGA, its biodegradation, and drug release properties. The polymer PLA exists in an optically active (L-PLA; semicrystalline) and an optically inactive (DL-PLA; amorphous) form. The amorphous form is preferred, as it enables a more homogenous dispersion of the drug in the polymer matrix (36). The glass transition temperature of the DL-PLA and PLGA is about 30°C to 60°C and is represented by the following equation:

$$T_g = T^0{}_g - \frac{K}{M_n}$$

where $T^0{}_g$ (60.1°C for PLA) is a limiting T_g of a material of infinite MW, M_n is a number average MW, and K (37.1×10^4 °C for PLA) is a constant for the polymer (37,38).

Lactic acid is more hydrophobic than glycolic acid and hence, PLGA polymers rich in lactic acid are more hydrophobic, absorb less water, and degrade at a slower rate (31,39–41). Generally, a bulk erosion mechanism (a homogenous chain cleavage reaction throughout the matrix) has been considered as the main degradation pathway for PLA and PLGA (42,43). However, recent studies on the degradation of various PLGA copolymers have demonstrated a heterogeneous degradation mechanism. The degradation products generated in the interior autocatalytically accelerate the degradation process, because of an increased amount of carboxylic acid end groups and thus, a decrease in the microclimate pH (43–45). Enzyme catalyzed degradation has been hypothesized, but these studies are not convincing (46). The factors that can influence the hydrolytic degradation of lactide/glycolide homopolymer and copolymer include: water permeability and solubility (hydrophilicity/hydrophobicity), chemical composition, mechanism of hydrolysis (noncatalytic, autocatalytic, enzymatic), additives (acidic, basic, monomers, solvents, drugs), morphology (crystalline, amorphous), device dimensions (size, shape, surface to volume ratio), porosity, glass transition temperature (glassy, rubbery), molecular weight and molecular weight distribution, physicochemical factors (ion exchange, ionic strength, pH), sterilization, and site of implantation (47). The kinetics of biodegradation of PLGA microspheres were studied in rats using steroid microspheres prepared with radiolabeled PLGA of varying composition (48–50). The

degradation of PLGA ranged from 10 weeks (50:50 lactide to glycolide) to approximately 30 weeks (87:13 lactide to glycolide), and finally to 45 weeks with 100% lactide (49). PLGA has found application in multiple depot products in the market. Some of the marketed PLGA based depots are summarized in Table 2.

PLGA Microspheres

PLGA microspheres are by far the most commonly used polymer-based injectable depot drug delivery systems, and are advantageous for several reasons. PLGA microspheres are biocompatible, can be easily administered through a syringe, can provide sustained release for prolonged periods of time, and can encapsulate active molecules with wide-ranging physicochemical properties, including small molecules, peptides, proteins and nucleic acids (51).

A number of techniques have been developed for the microencapsulation of drugs, such as solvent evaporation and solvent extraction [oil-in-water (o/w) emulsion, water-in-oil-in-water (w/o/w) emulsion, and solid-in-oil-in-water (s/o/w) emulsion], phase separation or coacervation, spray drying, extrusion, and supercritical fluid based encapsulation. Although each process is associated with certain advantages and disadvantages, in general, the selection of the microencapsulation process is dependent on the nature of the polymer, the drug, the intended use, and duration of therapy (36,41,45–54). The microencapsulation method selected should (41,51,52,55)

- ensure stability or biological activity of the drug;
- yield microspheres in a desired size range (microparticles of size less than 250 μm, ideally less than 125 μm have been determined to be suitable for depot delivery);
- be reproducible with regards to the quality and drug release profile from the microspheres;
- be scalable to support clinical development and commercialization; and
- not exhibit microsphere aggregation or adherence.

A number of proprietary technologies, based on minor variations in the basic encapsulating techniques discussed above, have been developed for preparing microspheres. A brief listing of those technologies is provided in Table 3. We will be discussing the various encapsulation techniques briefly in the following section.

Solvent evaporation and solvent extraction.

Oil-in-water emulsion The o/w single emulsion/solvent evaporation technique is the most favorable technique to encapsulate hydrophobic drugs with poor aqueous solubility but good solubility in water-immiscible organic solvents, such as methylene chloride and ethyl acetate. In this process, the drug and the polymer are dissolved in the organic solvent, followed by emulsification of the organic (oil) phase in water to form the o/w emulsion (Fig. 3A). The water phase generally contains an emulsifier, such as polyvinyl alcohol (PVA) and polysorbate 80 (PS 80). It is desirable that the drug has low solubility in the planned aqueous phase to enhance encapsulation efficiency and yield. The volatile solvent is generally removed by either evaporation to a gas phase (56), which involves prior dissolution into the continuous phase (57), or is extracted into the continuous phase (58,59). The rate of solvent removal from both the evaporation and extraction processes is dependent on the temperature and solubility characteristics of the solvent, polymer and dispersion medium, and in the case of extraction process, on the ratio of the emulsion volume to the quench medium volume (60). Solvent removal by the extraction process is faster than that by the evaporation process, and hence the microspheres made by the extraction process are generally more porous than the ones made by the evaporation process under similar conditions (60).

The o/w method has been used for a large number of drug candidates, such as neuroleptics (thioridazine, chlorpromazine, bromperidol), local anesthetics, diazepam, L-methadone, anticancer compounds (aclarubicin, lomustine, and paclitaxel) and steroids (36,61). It should be noted that for high drug loading formulations, precipitation of the drug

Table 2 Marketed PLGA Depot Products

Product name	Active ingredient	Product owner	Partner	Drug release duration	Comments
Atridox®	Doxycycline hyclate	Tolmar, Inc.	–	1 wk	PLA-based in situ gelling formulation delivered to the gum for the treatment of chronic adult periodontitis. The system consists of 450 mg of the ATRIGEL® delivery system, which is a bioabsorbable, flowable polymeric formulation composed of 36.7% (PLA) dissolved in 63.3% *N*-methyl-2-pyrrolidone. Doxycycline hyclate equivalent to 42.5 mg doxycycline is mixed prior to administration.
Atrisorb FreeFlow-D	Doxycycline Hyclate	Tolmar, Inc.	–	–	PLA-based system for guided tissue regeneration
Decapeptyl SR	Triptorelin acetate	Debiopharm Group	Ipsen (ex–United States)	1, 3 mo	PLGA microspheres containing water-insoluble salts of triptorelin for prostate cancer. Other excipients include mannitol, carmellose sodium, and polysorbate 80.
Eligard	Leuprolide acetate	QLT, Inc.	Astellas Pharma, Inc. (Europe) Sanofi-Aventis (United States, Canada) Sosei (Japan) MediGene AG (Europe) Tecnofarma (Latin America), Luxembourg Pharmaceuticals (Israel), Han All (Korea), Tecnofarma International (Central and South America)	1, 3, 4, 6 mo	PLGA/PLA in situ forming gel administered SC for the treatment of prostate cancer.
Lectrum depot	Leuprolide acetate	Eriochem S.A. (Latin America and Asia)	–	1, 3 mo	Lyophilized PLGA/PLA-based microspheres for uterine leiomynomas, endometriosis, prostate cancer. or precocious puberty.
Leuprolide 14 day	Leuprolide acetate	Oakwood Laboratories, LLC	–	2 wk	PLGA microsphere of GnRH for the treatment of prostate cancer.
Leuprorelin acetate injection	Leuprolide acetate	Dongkook Pharmaceutical Co. (Korea)	–	1, 3 mo	PLGA microspheres for SC administration to treat breast and prostate cancer
Lupride 1 month	Leuprolide acetate	Sun Pharmaceutical Industries (India)	–	1 mo	PLGA microspheres for IM administration to treat prostate cancer and endometriosis.

(*continued*)

Table 2 Marketed PLGA Depot Products (*continued*)

Product name	Active ingredient	Product owner	Partner	Drug release duration	Comments
Lupron depot	Leuprolide acetate	Takeda Pharmaceutical Co. Ltd.	Abbott Laboratories, Inc. (United States, Canada); Orion Corp (Northern Europe)	1, 3, 4 mo	PLGA/PLA microspheres with diluent presented in prefilled syringe for IM administration to treat endometriosis and prostate cancer. The front chamber prefilled dual-chamber syringe contains leuprolide acetate, PLGA/PLA and D-mannitol. The second chamber of diluent contains CMC sodium, D-mannitol, polysorbate 80, water for injection, USP, and glacial acetic acid, USP to control pH.
Lupron depot—PED	Leuprolide acetate	Abbott Laboratories, Inc.	–	1 mo	PLGA microspheres for IM administration to treat central precocious puberty. Presentation similar to Lupron Depot.
Luprorelin/ luposhere 1 month	Leuprolide acetate	Peptron, Inc.	Daewoong Pharmaceutical Co. Ltd. (Korea)	1 mo	PLGA microspheres for SC administration for the treatment of breast cancer, prostate cancer, endometriosis, and precocious puberty.
Nutropin depot	Somatropin (growth hormone)	Genentech (Roche), Inc.	–	2 wk, 1 mo	PLGA microspheres for SC administration to treat growth failure. Supplied in vials containing somatotropin, zinc acetate, zinc carbonate, and PLGA. The diluent is composed of 3% CMC sodium salt, 0.1% polysorbate 20 in normal saline.
Risperdal Consta	Risperidone	Janssen Pharmaceutica Products LP	Janssen-Cilag N.V. (Europe, United Kingdom); Janssen-Cilag N.V. (Japan)	2 wk	PLGA microspheres with 38% drug loading administered IM for the treatment of schizophrenia, bipolar disorder. The microspheres are reconstituted in 2 mL diluent (polysorbate 20, sodium carboxymethyl cellulose, disodium hydrogen phosphate dihydrate, citric acid anhydrous, sodium chloride, sodium hydroxide, and water for injection) prior to administration.
Salvacy/Moapar 3 months	Triptorelin pamoate	Debiopharm Group	European Pharmaceutical Partner	3 mo	Lyophilized PLGA microspheres for SC administration for reversible reduction of testosterone to castrate levels. The diluent consists of 8.5% mannitol USP, 3% CMC sodium USP, 0.2% polysorbate 80 NF.

Sandostatin LAR	Octreotide acetate	Novartis AG	–	1 mo	PLGA microspheres administered IM after reconstitution for the treatment of acromegaly.
Sinofuan	Fluorouracil	Simcere Pharmaceutical Group (China)	–	–	Sustained-release implant for multiple cancer indications.
Somatuline LA	Lanreotide acetate	Ipsen (Europe)	Teijin Pharma Ltd. (Japan)	2 wk	PLGA microspheres for IM administration for the treatment of Acromegaly and for the relief of symptoms associated with neuroendocrine tumors.
Suprefact depot	Buserelin acetate	Sanofi-Aventis	–	2, 3 mo	PLGA-based rods for SC implantation for treatment of advanced prostate cancer.
Trelstar depot Trelstar LA	Triptorelin pamoate	Debiopharm Group	Watson Pharmaceuticals, Inc. (United States), Paladin Labs, Inc. (Canada), Ferring Pharmaceuticals (Asia, Europe), West Pharmaceutical services, Inc.	1 mo (depot) 3 mo (LA)	Lyophilized PLGA microspheres for IM administration for treating prostate cancer. The diluent consists of 8.5% mannitol USP, 3% CMC sodium USP, 0.2% polysorbate 80 NF.
Vivitrol injectable suspension	Naltrexone	Alkermes, Inc.	Janssen Pharmaceutica Products, LP (Russia)	1 mo	PLGA microspheres for reconstitution prior to IM administration with Naltrexone loading of ca. 33% for the treatment of alcohol abuse and opioid dependence. Delivery diluent contains CMC sodium salt, polysorbate 20, sodium chloride and water for injection.
Zoladex implant	Goserelin acetate	AstraZeneca PLC	–	1, 3 mo	Cylindrical 1mm–diameter PLGA-based implant administered SC using a 16G hypodermic needle for the treatment of advanced prostate cancer, breast cancer and endometriosis.

Abbreviations: PLGA, poly(lactide-co-glycolide); PLA, poly(DL-lactide); CMC, carboxymethylcellulose.

Table 3 Proprietary Encapsulation Technologies and Related Patents

Encapsulation technology	Company	Encapsulation process	Reference
PolyShell	Akina, Inc.	Solvent exchange—double emulsion	WO03053325 (A3), EP1404516 (A3)
Injectable depot technology by coacervation	Alkermes, Inc.	Coacervation	US2004228833
Medisorb	Alkermes, Inc.	Solvent evaporation/extraction—emulsion method	US2003113380, US6110921, US5650173
ProLease®	Alkermes, Inc.	Solvent evaporation/ extraction—emulsion method	US6051259
West Pharma injectable depot technology	Archimedes Pharma	Solvent extraction—emulsion method	US5869103
Microcoat	Biotek, Inc.	Solvent evaporation	US4623588
SRI/Brookwood injectable microspheres	Brookwood Pharmaceuticals (now SurModics)	Solvent extraction—emulsion method	US4897268, US5407609
Depocore	CeNeS Pharmaceuticals	Coacervation	US2003180368
Debio® PLGA-2	Debio	O/W emulsion method/phase separation	US5445832
Mimplant microgranules	Debio	Solventfree extrusion process	US6319512
Extruded injectable beads	Debio	Solventfree extrusion process	US5134122
SynBioSys	InnoCore	Solvent evaporation/extraction—emulsion method	WO2005068533, EP1555278
Oligosphere®	MacroMed, Inc.	Solvent evaporation/extraction—emulsion method	US5100669, US5665428
ChroniJect	Oakwood Laboratories	Solvent evaporation/extraction—emulsion method	US5945126
SmartDepot	Peptron	Spray drying	WO2004112752, WO2005023224
TheraPhase ProPhase CoPhase	PR Pharmaceuticals	Solvent evaporation/extraction—emulsion method	US6706289 and family
CriticalMix™	Critical Pharmaceuticals	Supercritical CO_2 without solvents or high temperature	US6414050, US6670407

out of the polymer phase is very likely and thus, understanding the phase behavior of the drug polymer system and kinetics of precipitation, including particle size and polymorphism of the drug, become critical factors influencing drug release from the matrix.

Solid-in-oil-in-water emulsion The s/o/w emulsion technique is applicable when a specific drug is not soluble in the carrier solvent or solvent mixture, or when extensive drug loss to the continuous phase cannot be avoided when employing a cosolvent system. A lot of early research on hydrophobic drug encapsulation (such as norethisterone) as a contraceptive utilized this technique (49). Recently, the s/o/w technique has been used for the encapsulation of hydrophobic drugs such as levonorgestrel (62), β-estradiol (63), haloperidol (64), and camptothecin and its derivatives (65). Since drug particles are encapsulated directly, it is important that the particle size of the drug is small and well controlled. Generally, particle sizes of less than 10 μm, preferably in the 1 to 2 μm range, are desirable to improve drug loading and the uniformity of drug distribution within and amongst microspheres. Besides small particle size of the drug, careful control of drug sedimentation (in the suspension medium) or floatation (due to adhesion of bubbles to hydrophobic surfaces) during the encapsulation process must be achieved. Drug particles adsorbed on the surface of prepared microspheres (especially if the drug particle size is large) could lead to a burst release (63). This

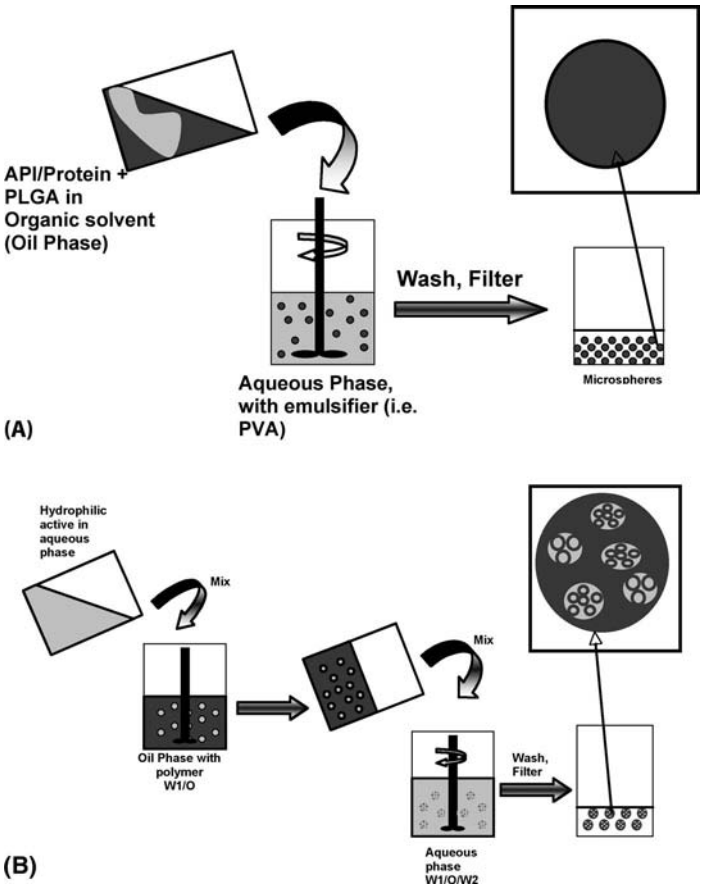

Figure 3 Schematic representations of the (**A**) single oil-in-water emulsion and (**B**) double water-in-oil-in-water emulsion processes for making microspheres.

issue could potentially be alleviated by addition of an extra polymer coating step for s/o/w microspheres, as has been suggested in the literature (66). The s/o/w microspheres tend to form large voids and channels as the drug particles dissolve, leading to better access of the dissolution medium into the microspheres, and resulting in a faster release profile as compared with monolithic microspheres prepared by the o/w emulsion technique.

Water-in-oil-in-water emulsion The $w_1/o/w_2$ encapsulation method is a commonly used method for hydrophilic compounds with high aqueous solubility, such as peptides, proteins, and vaccines (40,41,53). One of the first challenges with this technique was low encapsulation efficiency of hydrophilic molecules, as described by Okada et al. (U.S. patent 4652441), which was overcome by performing w_1 phase solidification. Briefly, the process comprises dissolving the active molecule in a suitable buffer, and then adding this to an organic phase (e.g., dichloromethane) containing dissolved PLGA, under controlled stirring to form the first w_1/o emulsion (Fig. 3B). This emulsion is then introduced with stirring into the second water phase, containing an emulsifier (e.g., PVA) to form the $w_1/o/w_2$ emulsion. The organic solvent is either removed by evaporation (reduced pressure or stirring) or extraction (dilution into a large quantity of water with or without surfactant). The microspheres are then washed, separated (e.g., by filtration, sieving, or centrifugation), and then dried or lyophilized to give the final product. During the development of this technique, various formulation and process variables were evaluated to optimize drug loading, encapsulation efficiency and release profiles (67–71).

Phase separation technique. Unlike the o/w emulsification technique, the phase separation, or coacervation, technique is suited for both water-soluble and water-insoluble drugs. However,

the coacervation process is mainly used for hydrophilic molecules, such as peptides and proteins. The process consists of precipitating (or phase separating) the polymer from the organic solution by the addition of a nonsolvent to yield drug-containing microspheres. In brief, the drug is either dissolved in water and then added to the polymer-containing organic phase (o/w emulsion), or directly added to form a solution in the organic phase. To this, an organic nonsolvent is added with stirring, which extracts the polymer solvent. This leads to phase separation of the polymer to form coacervate droplets, which entrap the drug. The microspheres thus formed are hardened by transferring to a larger quantity of organic nonsolvent, washed, filtered, sieved and dried (54,72). The various factors that influence the final product include addition rate of the first nonsolvent, concentration of the polymer, stirring rate, temperature, or addition of an additive (to alleviate stickiness of the coacervate droplets). Since the process does not utilize addition of an emulsion stabilizer, agglomeration might become a frequent problem.

Melting and spray-drying techniques. Melting and spray drying have been utilized to prepare microspheres in cases where conventional processing methods, such as o/w and w/o/w emulsion, do not provide the required throughput and product stability. Spray drying is rapid, convenient, easy to scale-up, utilizes mild conditions, and is less dependent on the solubility parameter of the drug and the polymer (41,73,74). Compared with the conventional emulsion methods, the spray drying method requires larger batch sizes (limitation if small amounts of bulk available), results in larger losses due to adhesion of microparticles to the apparatus, and is reported to cause agglomeration of the microparticles (74). Modifications to the spray drying technique have been incorporated, such as a double nozzle technique to reduce agglomeration. Spray dried formulations for a range of compounds, such as theophylline, progesterone, and piroxicam, have been reviewed in the literature (36).

Melting is a technique that avoids the use of organic solvents, but requires the dispersion or melting of the drug in a polymer melt. To generate microspheres from this hot melt, a water-soluble polymer that is not miscible with the drug/polymer melt can be employed. The resulting emulsion can be solidified by cooling, and the microspheres can be collected by dissolving the water-soluble polymer matrix in a large volume of water (European Patent EP 934 353). An alternative method is to grind/jet-mill the drug/polymer matrix after cooling (33,61,75). The improvements in this technology have focused on generating more uniform particles by introducing an extrusion step in the process, and getting spherical and smaller particles by emulsification in a hot solution containing an emulsifier (61). Microspheres produced by the melt technique generally lead to nonporous polymer matrices, which subsequently lead to slower release rates from the depot, especially for hydrophobic drugs.

PLGA Gel/Rod Systems

Although microspheres (Fig. 4A) have been predominant, other PLGA-based depot systems have also been developed, including in situ forming gels, and rods for implantation. In this section we will discuss some of the PLGA-based gel and rod systems briefly, and highlight the advantages and disadvantages of such systems.

The in situ gelling systems are presented as liquids or semi-solids with a wide range of viscosity, containing a biodegradable polymer and drug dispersed or dissolved in the liquid phase of the delivery system (solvent/cosolvent system). Upon SC or IM administration, a depot is formed at the site of injection (Fig. 4B). Such systems are usually manufactured through aseptic processing, however, γ-irradiation for terminal sterilization of the product has been evaluated as well. The in situ forming depots have been classified into different categories, depending on the depot-forming mechanism (76,77).

The in situ precipitating system consists of PLGA dissolved in a water-immiscible or partially miscible organic solvent, which also dissolves/disperses the drug to form a solution/suspension. Once administered, the organic solvent escapes, allowing water ingress and precipitation or phase separation of the drug/polymer system, leading to the formation of a depot. Depending on the solubility of the drug in the organic phase, these systems are generally associated with high initial burst. The initial burst is also dependent on the

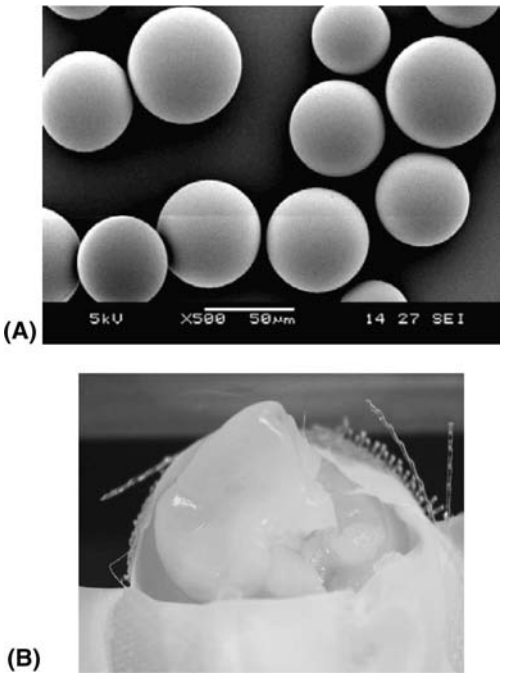

Figure 4 (**A**) Scanning electron micrograph of PLGA microspheres and (**B**) photograph of an in situ forming PLGA gel depot explanted from a rat. *Abbreviation*: PLGA, poly(lactide-co-glycolide).

hydrophobicity and concentration of PLGA, water miscibility of the organic solvent, and the aqueous solubility and loading of the drug. Subsequent drug release from the depot is dependent on the degradation/erosion of PLGA. Eligard®, which uses the Atrigel® technology from QLT, (78) has received regulatory approval. The Atrigel® technology involves the dissolution of polymer and drug in *N*-methyl-2-pyrrolidone, but has also utilized other organic solvents such as propylene glycol, dimethyl sulfoxide, tetrahydrofuran, triacetin and ethyl benzoate to control initial burst. The biocompatibility and systemic toxicity of these organic solvents, when administered intramuscularly or subcutaneously, have been of concern. Alzamer® technology, developed by Alza, also utilizes PLGA as a carrier for in situ depot formation, however, this technology utilizes more lipophilic solvents, such as benzyl alcohol, to reduce irritation and initial drug burst.

Thermally induced gelling systems are exemplified by the water-soluble ReGel® triblock copolymer, composed of the hydrophobic PLGA (A) and hydrophilic PEG (B) blocks in the ABA configuration, which is a solution under ambient conditions, but turns into a gel at body temperature. OncoGel® is a six-week sustained-release depot of paclitaxel that utilizes this system. The release from the ReGel polymer system is controlled by controlling the hydrophilicity/hydrophobicity, molecular weight, concentration and polydispersity of the copolymer (79).

Implantable PLGA-based biodegradable systems have also been explored. Zoladex® is a one- and three-month PLGA depot of goserelin acetate for the treatment of prostrate cancer. Durect is developing the PLGA-based Durin® implant, containing Leuprolide for Alzheimer's disease. Durin is a reservoir-type implant where the drug release is controlled by the drug loading, polymer molecular weight and composition, geometry of the device, and permeability of the membrane (WO03000156 from Southern Biosystems Inc).

Delivery of Proteins and Peptides

Peptides and proteins have become a vital class of therapeutics, however, many issues exist in the delivery of biologically active macromolecules to target tissues. Upon injection, peptides and proteins are rapidly cleared because of proteolytic degradation, efficient renal clearance, neutralization by antibodies, and rapid distribution to tissues outside the blood stream. The

rapid clearance results in the need to dose peptides and proteins on a very frequent basis, which is a painful and inconvenient dosing regimen and often results in poor patient compliance. Several strategies have evolved to overcome the challenge of short half-life, including increasing the molecular size of the protein via conjugation of high molecular weight biopolymers (80) and site-directed mutagenesis to remove proteolytic cleavage sites. Over the last 25 years, much pharmaceutical research has gone into developing improved delivery systems aimed at delivering real patient value by providing another means of overcoming these challenges. Formulation strategies include injection of crystalline or amorphous peptide or protein particles (81), implantable osmotic pump devices, and sustained-release polymeric depot systems.

The development of peptide and protein depot systems can involve significant challenges beyond those typically encountered with small molecules. Polypeptides are inherently unstable because of their physicochemical and biochemical properties, which stem in part from their large molecular size. Quite simply, more can go wrong with larger molecules. Proteins have secondary, tertiary and often quaternary structure that all contribute to the three dimensional orientation necessary for proper protein function. The processes outlined earlier for manufacturing depot systems, which can include high-shear mixing, pumping, organic solvent/aqueous interfaces, surfactants, contact with hydrophobic surfaces, sudden pressure differentials, heat, and drying, are all detrimental to the delicate structure of a protein. The more successful formulation strategies have sought to minimize protein unfolding and aggregation by reducing process stress and carefully considering the additives/solvents used. Additives and solvents can cause protein denaturation by perturbing their physicochemical stability, and the use of solvents is therefore an important consideration for polypeptide depot development (82). In addition to their inherent physicochemical instability, proteins are also sensitive to chemical degradation (83). In particular, asparagine deamidation and hydrolytic cleavage are accelerated as a result of the acidic environment created when PLGA breaks down via ester bond hydrolysis (84,85).

Despite the aforementioned challenges, several peptides are commercially available as sustained-release depots, including leuprolide, triptorelin, histrelin, goserelin and octreotide. Images include biodegradable microspheres and rods, as well as nonbiodegradable polymer rods and titanium-based implantable osmotic pump devices. Once-monthly Lupron Depot® (Leuprorelin acetate suspension for SC injection) was the first sustained-release peptide approved in the United States, in 1989 (38). Since this approval, longer-acting images have been produced and today three-, four-, and six-month and one-year delivery options are available.

The only protein depot to receive FDA approval was Nutropin Depot™. Nutropin Depot™, approved in 1999 as a treatment for growth hormone deficiency in pediatric patients, is a sustained-release form of Genentech's human growth hormone [somatropin (rDNA origin)] using Alkermes' PLGA-based ProLease® technology. The once or twice-monthly injection (based on the patient weight) offered an alternative to multiple weekly injections. Unfortunately, the product had a short lifetime and was pulled from the market in June of 2004, citing the high cost of production and commercialization. Although the drug was discontinued, the successful development and approval of this complex dosage form signified major success for those working on sustained-release dosage forms for biologics. There was a large leap in complexity in producing Nutropin Depot™ compared with the smaller octa-, nona- and decapeptides mentioned previously. These peptides do not possess the secondary structure of most proteins (alpha-helix or beta-sheet) and are quite stable, having properties more like small molecules. In contrast, human growth hormone contains 191 amino acids and both secondary and tertiary structure.

The Nutropin Depot™ approval took years of commitment and was the result of a well-designed manufacturing strategy, which focused specifically on stabilizing the protein structure (86–88). The manufacturing process, based on the work of Gombotz (89), was different from other, more conventional s/o/w microsphere manufacturing processes, as it utilized low temperature processing, excipient-based protein stabilization, and release-controlling agents. On the basis of this work, and the work of many others, many of the technical challenges inherent to developing PLGA-based sustained-release biologics have been defined, opening the way for rational design of molecules (especially peptides) for sustained-release delivery. Synthetic peptides can be designed and/or screened to be less sensitive to the low pH environment of a degrading microsphere. Reactive amino acids like lysine, with its

nucleophilic primary nitrogen, can be removed or capped to avoid amide formation that can result in covalent peptide-PLGA conjugates. If the desire is a PLGA-based protein delivery system, early forced degradation screening utilizing conditions which mimic PLGA degradation, as well as a screen of manufacturing stress conditions, should be conducted to select the protein with the highest stability. Having very early insight into the desired final product image will better allow for the rational design of the proper characteristics, which will, in turn, ensure manufacturability later in development.

Other Degradable Depot Delivery Systems

Natural and Synthetic Polymers

A number of natural and synthetic biodegradable polymers have been investigated for depot delivery, although only few of them have demonstrated biocompatibility. Natural biodegradable carriers like bovine serum albumin (BSA), human serum albumin (HSA), collagen, gelatin, and hemoglobin have been studied for drug delivery (41), but their use is limited by their high costs and questions over purity. Thus, in the last two decades, synthetic biodegradable polymers have been widely used. In this section we will summarize such biodegradable depot systems and highlight the various depot delivery technologies utilizing those polymers.

Polycaprolactones. Poly-ε-caprolactone (PCL) is a biodegradable polyester with a melting point around 60°C and a glass transition temperature of approximately −60°C (90). It is semicrystalline and is known to degrade slower than polylactide under physiological conditions and thus, is suitable for release extending to a period of greater than one year. A variety of drugs including antigens, antihypertensives, chemotherapeutic agents, and antibiotics have been evaluated with regards to encapsulation in PCL microspheres (91).

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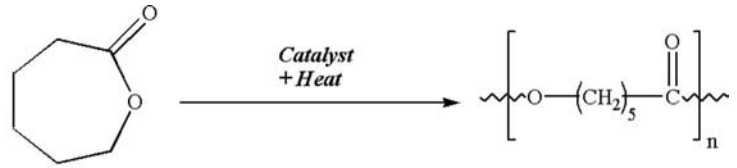

PCL can be an attractive polymer for encapsulating proteins since the degradation of PCL will not result in an acidic environment that is detrimental to protein stability (9). This has been exemplified with PCL microspheres of insulin (92). Block copolymers of caprolactone with PLA, PLGA, PEG, or PEO have also been evaluated for drug delivery (93–95). Capronor is a biodegradable polymer system for the sustained subdermal delivery of contraceptive steroids. Capronor utilizes PCL as the polymer and was evaluated in phase II clinical trials as a contraceptive however, the product did not make it to market.

Polyphosphoesters. Polyphosphoesters (PPE) are a group of structurally versatile biodegradable polymers (degrade via hydrolysis and possibly enzymatic digestion at the phosphoester linkages) that have found application in drug delivery because of their biocompatibility and similarity to bio-macromolecules such as nucleic acids (96).

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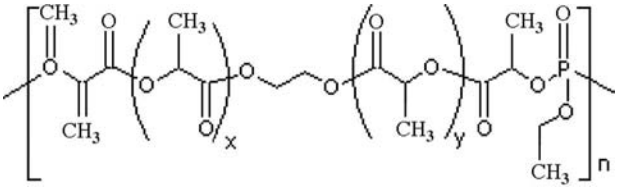

PPE has been used as a carrier for sustained delivery of low molecular weight drugs (97), proteins (98), and DNA (99). Guilford Pharmaceuticals (now MGI Pharma) had a product

candidate, Paclimer®, a poly (lactide-co-ethylphosphate) microsphere formulation of paclitaxel, designed to deliver paclitaxel over eight weeks for the treatment of ovarian cancer.

Polyanhydrides. Polyanhydrides (PA), as the name suggests, are biodegradable copolymers with a hydrophobic backbone of anhydride linkages formed by the condensation reaction of two fatty acids. Their applications in parenteral drug delivery have been reviewed for a variety of therapeutic agents such as growth hormone, anticancer agents, antibiotics, local anesthetics, anticoagulants, anti-inflammatory, and neuroactive drugs (100,101). Polyanhydride microspheres can be prepared by spray drying, hot-melt encapsulation or emulsion methods. Because of release mediated by surface erosion, they are believed to better protect unreleased drug from the release medium (9,101). Various types of homo- and hetero-PAs consisting of aliphatic, aromatic, heterocyclic and other monomers have been studied in detail and extensive work on PA carriers resulted in clinically used implants like Gliadel® (MGI Pharma, Inc.) and Septacin™ (Abbott Laboratories). Gliadel is a polyanhydride polymer matrix of poly[bis(p-carboxyphenoxy) propane] with sebacic acid P(CPP:SA) (20:80 molar ratio) containing BCNU for the treatment of brain tumor. BCNU is a nitrosourea with short half-life but is considered a "gold standard" for treating glioblastomas. Gliadel wafer is a sterile, off-white to pale yellow wafer with a diameter of 1.45 cm and 1 mm thickness. Each wafer contains 7.7 mg BCNU and 192.3 mg PA copolymer. Septacin™ is a PA implant consisting of P(FAD:SA) (1:1 weight ratio) polymer and gentamicin for the treatment of osteomyelitis. Each implant has five beads in a strand with each bead being 12 mm long and 4 mm in diameter weighing 150 mg (contains 20 mg gentamicin as gentamicin sulfate) (102).

Polyortho esters. Polyortho esters (POE) are generally synthesized by condensation of diols and a diketene acetal, and often involve copolymerization with a latent acid such as glycolic acid and lactic acid (a class of POE called Biochronomer®, which have been developed by AP Pharma) to allow control over the hydrolytic degradation of the ortho ester linkages (9,103). POEs are thermoplastic polymers that have been demonstrated to be stable to 24 kGy γ-irradiation and can be easily formulated as microspheres using extrusion followed by cryogenic milling (104,105). Various processes have been employed to prepare POE microspheres including spray congealing (106), emulsion-solvent evaporation (low encapsulation efficiency with water-soluble drugs) (107,108), and extrusion of block copolymers of PEG and POE to enhance encapsulation efficiency with water-soluble compounds (109).

Block copolymers of polybutylene terephthalate. Multiblock copolymers of hydrophilic PEG and hydrophobic polybutylene terephthalate (PBT), known as PolyActive™, have been developed by OctoPlus. The degradation of these biodegradable and biocompatible polyether ester copolymers occurs by hydrolysis of the ester bonds and oxidation of the ether linkages (110,111). OctoPlus is currently developing Locteron™, a microsphere formulation of interferon α, using this technology.

Cross-linked dextran. Cross-linked dextran is a biodegradable and biocompatible (112,113) hydrogel system for drug delivery, specifically protein delivery, which has been developed by OctoPlus. A modified dextran derivatized with hydroxyethyl methacrylate (dex-HEMA), referred to as OctoDEX®, has been reported to be able to tailor the release of proteins from microspheres from days to months (114–116).

Polyamino acid polymers. Polyamino acid polymers, as the name suggests, are composed of naturally occurring amino acids. The release duration can be tailored, in principle, by modifying the hydrophobicity of the participating amino acids in the block copolymer. Flamel Technologies has developed these polymer systems for protein delivery. An amphiphilic block copolymer, composed of L-leucine and L-glutamate, is referred to as Medusa I® (117). These are self-assembling systems, which are noncovalently associated with proteins. Insulin (Basulin®) is one of the proteins that is being investigated with this technology for type I diabetes, with a target release duration of two days. Flamel has also developed Medusa II®,

which is hydrophobically modified L-glutamate, for release over two weeks. Interferon α2b and Interleukin-2 are also being developed using this technology (118).

Cellulosic polymers. Water-soluble anionic polymers, such as CMC, have been utilized to form water-insoluble complexes with soluble cationic peptides. Such insoluble complexes, formed by ionic interactions, have been developed (Rel-Ease®) for sustained drug delivery by Praecis. Plenaxis® is an abarelix-CMC complex that utilized Rel-Ease® technology and was approved in 2003 for the treatment of advanced prostate cancer; however, it was withdrawn in 2005 because of financial considerations (119–121).

Cross-linked albumin. Use of cross-linked albumin for sustained-release applications is exemplified by the ProMaxx® drug delivery technology, which was developed by Epic Therapeutics, Inc, a wholly-owned subsidiary of Baxter Healthcare Corporation. ProMaxx is a protein matrix-based technology developed for protein, peptide, and small molecule delivery. The microspheres, in the particle size range of 0.5 to 40 um, are produced in an aqueous medium by mixing a carrier protein (e.g., HSA), a water-soluble polymer (e.g., hetastarch), a polyanionic polysaccharide (e.g., dextran sulfate, heparan sulfate, and polyglutamic or polyaspartic acid), and a divalent metal cation (e.g., Ca^{2+} and Mg^{2+}). The release from the microspheres can be controlled by varying the concentration of hetastarch, temperature, pH, albumin, or length of heat exposure of microspheres. Baxter is developing LeuProMaxx® (one- and three-month release of leuprolide acetate) using the ProMaxx technology, for the treatment of prostate cancer (9,122).

Other gel-forming polymer systems. The SABER® system, from Durect Corporation, consists of a hydrophobic polysaccharide, sucrose acetate isobutyrate (SAIB), as the drug release-controlling matrix. SAIB, along with the drug, is dissolved/dispersed in ethanol, benzyl alcohol, or other water-miscible solvents. Since this system has a relatively low viscosity, administration with a smaller gauge needle is easier compared with PLGA-based gel systems. Sustained-release formulations of bupivacaine (123) and rhGH (124) are being considered for feasibility assessment or development.

A cross-linked PEG-based copolymer (containing multiple thio (-SH) groups along the polymer backbone) which forms a hydrogel when mixed with α, omega-divinylsulfone-PEG (2 kDa) dissolved in a neutral phosphate buffer has been reported (125). The system has been proposed to achieve a release over two to four weeks, with application mostly suited toward large molecules. Mild adverse tissue reactions have been reported in biocompatibility studies in rabbits and rats.

GelSite® polymer, from DelSite biotechnologies, is a natural acidic polysaccharide extracted and purified from the aloe plant. The polymer forms a gel in the presence of calcium (in situ cross-linking) when injected subcutaneously or intramuscularly, and thus entraps a water-soluble drug (e.g., a protein) providing sustained release (U.S. patent 5929051). The polymer has also been shown to specifically bind to, and stabilize, heparin binding proteins, thus providing additional control over drug release without affecting the biological function (U.S. patent 6313103).

Chitosan is a pH-dependent cationic polymer (amino polysaccharide) that has been demonstrated to be biocompatible and biodegradable. Chitosan can form an in situ thermosensitive gelling system when combined with an anionic polyphosphate salt, glycerophosphate (GP) (126,127). A chitosan-GP gelling system has been evaluated for camptothecin delivery, providing zero-order release over four weeks (128).

Poloxamer® 407 is a triblock copolymer of polyoxyethylene and polyoxypropylene units in the ABA configuration. Mostly utilized as a nonionic surfactant, this water-soluble polymer demonstrates reverse gelling properties. A 20% or higher polymer solution is a liquid at low temperatures, but gels at body temperature (129). Although this approach potentially provides an exciting system for sustained release of large molecules, because of the lack of organic solvents, its application has been limited by a lack of biodegradability, cytotoxicity concerns, and reports of increased levels of plasma cholesterol in rats administered with poloxamer intraperitoneally (130).

Lipid-Based Systems

Conventional lipid-based depot systems, such as oil solutions or suspensions, have been discussed earlier in this chapter. Conventional lipid systems rely on the partition of drug from the oil phase into the aqueous phase at the injection site to control release. Advanced lipid-based dispersed systems, with particles in the submicron size range, have been developed for water-soluble and water-insoluble drugs for parenteral administration. Natural and synthetic phospholipids, with or without further chemical modifications, have not only been used in stabilizing triglyceride-based lipid formulations, but also are the major structural components of lipid vesicles. Though lipid-based systems including emulsions provide an opportunity for sustained release, the duration of release is seldom over one week. In this section we will briefly discuss a few such lipid-based systems.

Liposomes. Liposomes are vesicles composed of an inner aqueous core surrounded by a phospholipid bilayer. Liposomes are primarily categorized into three types—multilamellar vesicles (MLV), small unilamellar vesicles (SUV) and large unilamellar vesicles (LUV). Optimization of the bilayer composition, charge, and size of liposomes, as well as the internal aqueous composition, allows efficient incorporation of a wide variety of drugs (131). Liposomes, with or without surface pegylation, have been evaluated extensively for various compounds for intravenous administration (9). Doxil® (doxorubicin HCl) liposome injection from Alza Corporation was the first pegylated liposomal doxorubicin product approved for the treatment of refractory ovarian cancer and AIDS-related Kaposi's sarcoma. However, as a depot delivery system for SC and IM use, liposomes have not proven to be the best candidates despite being biocompatible and demonstrating positive results for efficacy (132). The primary reason for this lack of success is the relatively limited drug-loading capacity and short duration of release for the entrapped drugs. This is coupled with a complex manufacturing process, and physical stability considerations (9).

Multivesicular liposomes. SkyePharma developed the DepoFoam™ [now owned by Pacira Pharmaceuticals, Inc. (133)] technology, which consists of tiny, lipid-based particles, 10 to 30 μm in size, composed of hundreds to thousands of discrete water-filled chambers containing the encapsulated drug, with each chamber separated from adjacent chambers by a bilayer lipid membrane. The bilayer is composed of synthetic phospholipids (dioleoyl phosphatidylcholine and dipalmitoyl phosphatidylglycerol), cholesterol and triglyceride (134). Drug release from DepoFoam particles is achieved by diffusion through the walls, gradual erosion of the particles, and by processes involving the rearrangement of membranes. DepoCyt® is the first approved DepoFoam product containing cytarabine for the treatment of lymphomatous meningitis, administered intrathecally every two weeks. DepoDur® is a morphine sulfate formulation for postsurgical pain relief, given epidurally every two days. DepoBupivacaine®, a sustained-release formulation of bupivacaine, is in phase III development for local anaesthesia/pain. Proteins and peptides have also been evaluated with the DepoFoam technology with regards to in vitro and in vivo release (135).

Lipid microparticles. Lipid microparticles are solid lipid-based drug delivery systems composed of a dissolved or dispersed drug in a solid lipid matrix. The low mobility of the drug in the lipid matrix and hydrophobic nature of the lipids provide the required sustained-release properties (136). A maximum loading capacity of 25% has been reported for these systems (137). Various methods of encapsulation have been utilized to produce these microparticles, such as solvent-evaporation, melt-dispersion or spray-congealing methods (138). Lipid microparticles have been evaluated for the sustained release of small molecules such as local anesthetics and antibiotics, as well as proteins and peptides (139–142).

Cochleates. Cochleates are formed by the condensation of small, unilamellar, negatively charged liposomes composed of an anionic phospholipid, such as phosphatidylserine. The small liposomes fuse to form larger lipid bilayer sheets in the presence of a cation, such as calcium. These sheets roll up into cinnamon stick-like or cigar-like structures to minimize the interactions between water and the hydrophobic surface of the sheet. The cochleates are

characterized by a tightly-packed bilayer with little or no internal aqueous phase (143). Depending on the hydrophobicity and charge of the molecule, it could either be embedded in the bilayer, or encapsulated between the bilayers (144). The characteristics of cochleates lend themselves to application via the intravenous route to increase drug circulation time (e.g., amphotericin B cochleates), and ability to penetrate and accumulate in target tissue (145,146). Recently, delivery system for vaccines (147,148) and genes (149), have utilized cochleates as well.

IMPLANTABLE DEVICE-BASED AND NONDEGRADABLE DEPOT DELIVERY SYSTEMS

One of the key aspects of an implantable, nondegradable depot delivery system is the requirement for a minor surgery for implantation, and a similar procedure for explanation of the implant once the dose has been delivered. Hence, a longer duration of drug release is required to maintain patient acceptability. Although the administration involves an invasive procedure, in the case of adverse effects, removal is straightforward. Generally, implants would not be considered where the drug dose is dependent on body weight, since the dose and release from these systems is predetermined. However, in cases where a broad therapeutic window exists and sustained drug levels are required, implants present themselves as a viable option. In this section we will briefly discuss some of the nonbiodegradable implants including polymeric systems, osmotically driven systems and other device-based systems.

Polymeric Systems

The nondegradable polymers can be processed with drug to yield depot systems of various configurations, which can then be implanted subcutaneously. Two primary categories for nondegradable polymeric systems are the encapsulated reservoir system and the matrix-loaded system. One of the leading examples of the encapsulated reservoir system is the Norplant® implant from Wyeth. Norplant is a five-year levonorgesterol implant for contraception, approved by the FDA for use in women. The implant, which consists of six flexible closed capsules, is a reservoir system with each capsule consisting of 36 mg of active in silicone rubber tubing (silastic) of 2.4 mm diameter and 34 mm length (150). Wyeth has an analogous product in the form of Jadelle®, which was designed to require fewer capsules (two) for implantation, and thus improve insertion and removal. Jadelle has been approved in the United States, but is not marketed. On the basis of the publically available prescriber's information, Jadelle is a set of two flexible cylindrical implants, consisting of a dimethylsiloxane/methylvinylsiloxane copolymer core enclosed in thin-walled silicone tubing. Each implant contains 75 mg of the progestin levonorgestrel. The implants are sealed with polydimethylsiloxane adhesive and sterilized. Each implant is approximately 2.5 mm in diameter and 43 mm in length. The implants are inserted in a superficial plane beneath the skin of the upper arm. The calculated mean daily in vivo release rate of levonorgestrel provided by the implants is about 100 μg/day at month one, followed by a decline to about 40 μg/day at 12 months, and to about 30 μg/day at 24 months, with stabilization thereafter at about 30 μg/day. One of the major drawbacks with the reservoir system is the risk of "drug dumping" if there is a rupture of the releasing membrane.

Implanon®, from Organon (now part of Merck, Sharp & Dohme Corp), is a leading example of the matrix-loaded system. Implanon is an etonogestrel implant with each implant containing 67 mg of the active embedded in an EVA (ethyl vinyl acetate) matrix, which is then surrounded by a rate-controlling EVA membrane to form a rod measuring 40 mm in length and 2 mm in diameter (150). The implant is designed to release over three years and was approved by the FDA in 2004. One of the major drawbacks with the matrix-loaded systems is a more complex release mechanism (likely diffusion controlled) which presents a significant barrier toward achieving a zero order release profile. The Hydron® implant, from Valera Pharmaceuticals (acquired by Indevus Pharmaceuticals in 2006), is a hydrogel reservoir drug delivery system designed for delivery of drugs at a predetermined rate over a one-year period. The hydrogel nature of the implant is likely to cause less discomfort when compared with metal implants. The cylindrical implant is 26 mm long, 3.5 mm in diameter and 0.5 mm in wall thickness, and is composed of a cross-linked copolymer of hydroxypropyl methacrylate and 2-hydroxyl methacrylate. The core of the implant consists of the drug (e.g., 50 mg histrelin) and

stearic acid (as in Vantas®, which is a one-year histrelin implant) (151). The implant is packaged in a glass vial containing 1.8% sodium chloride solution, which allows hydration and priming of the implant prior to insertion.

Osmotically Driven Systems

As the name suggests, these systems utilize osmotic pressure for long-term delivery of potent therapeutic agents. The Duros® implant, from DURECT, is one such example, which consists of an outer titanium cylinder, an osmotic engine (containing sodium chloride), a piston and a drug chamber. One end of the outer cylinder is capped with a semi-permeable membrane (controls the rate), and the other end has an orifice, which releases the drug using a diffusion moderator. The implant holds a maximum of 200 μL of the drug solution, and can be up to 4 mm in diameter and 44 mm in length. A brief description of the steps involved in the functioning of the Duros systems will include (1) water influx into the osmotic engine, (2) expansion of osmotic engine, (3) displacement of the piston, and (4) contraction of drug formulation-containing chamber to release the drug through the exit port (152). Because of the volume constraints, the implant usage is limited to potent compounds with high formulation concentrations. Solution formulations with various aqueous and nonaqueous solvents, and suspensions with nonaqueous solvents, have been evaluated with the Duros implants. Viadur®, from Alza Corporation, is a one-year leuprolide acetate implant, which received FDA approval for prostate cancer (153). DURECT is also developing Chronogesic®, a three-month sufentanil implant, for which the clinical trials are currently suspended to improve the device to mitigate performance issues.

Other Device-Based Systems

Besides the polymeric implants and the osmotically driven systems, there are other devices, which have been utilized for the delivery of highly potent drugs. SynchroMed pump from Medtronic and Codman 3000 from Codman and Shurtleff are representative systems. The SynchroMed pump is an implantable, programmable, battery-powered device that stores and delivers medication according to instructions received from the programmer. The various models of the pump vary in size of the reservoir and the presence of a side catheter access port. The hold volume in the refillable pump can range from 10 to 40 mL. The CODMAN 3000 implantable drug delivery system features an inexhaustible power supply obviating the need for battery and provides continuous delivery with the refillable volume ranging from 16 to 50 mL. The Codman 3000 implantable pump is divided into inner and outer chambers by accordion-like bellows. The inner chamber contains the drug to be infused while the outer chamber contains propellant permanently sealed. The patient's own body temperature warms the propellant, which exerts a constant pressure on the bellows. This causes the drug to flow out of the inner chamber through a filter and flow restrictor then slowly out of the catheter.

Both these pumps require surgical insertion and removal, and the refill of these pumps will require expertise. One of the most common applications of device-based systems is for the administration of insulin. Such insulin pumps typically consist of the pump, the disposable drug reservoir, and an infusion set, which includes a canula for SC insertion. The pumps come in various models that include Ping (Animas), Cozmo® (Deltec), Spirit® (Disetronic), Paradigm 522/722 (MiniMed), OmniPod (Insulet) and Diabecare IIS (Sooil). The insulin pumps range in hold volume from a couple of milliliters, to as much as 90 mL, and are intended to deliver rapid- or short-acting insulin 24 hours a day through a catheter placed under the skin.

DEVELOPMENT CONSIDERATIONS FOR DEPOT DELIVERY SYSTEMS

Sustained-release parenteral formulations are generally complex dosage forms, and therefore often present significant challenges during development and scaleup. These challenges include sensitivity to changing API and excipient properties, maintaining critical formulation attributes during manufacturing process development and scaleup, ensuring sterility, evaluating drug release and establishing in vitro–in vivo correlations (IVIVC), setting specifications, ensuring product quality, and managing material and manufacturing costs. Regulations, standards, and science-based guidance are generally lacking for parenteral sustained-release dosage forms (154,155), and development timelines can be long. For these

reasons, it can be advantageous to initiate development of these dosage forms early in the product lifecycle.

In Vitro and In Vivo Release from Depot Delivery Systems

Selection of in vitro release methods remains a significant challenge in the development of depot formulations, and little in the way of science-based guidance for industry exists (156). Suitable in vitro release methods can reduce the dependence on in vivo testing and speed development timelines. Key uses for in vitro release methods include assessment of drug release (including burst release) during early formulation and process development and subsequent optimization, quality control to support batch release and stability evaluation, and definition of critical product attributes and critical process parameters (154–156). In vitro release methods should be biorelavent to enable a robust IVIVC for predicting in vivo release on the basis of in vitro evaluation; validated IVIVC could potentially support formulation bridging during development. For depot formulations that are designed to release over long periods of time (e.g., months), it is often not practical to rely on real-time in vitro release evaluations, and therefore accelerated methods are required (157).

In Vitro Method Development

In vitro release methods for parenteral depot formulations have been well-reviewed (157–159). Considerations for method development include the apparatus type and design, the release media, and rational selection of experimental variables such as temperature and agitation rate. Generally, three methods are used for evaluating in vitro release from parenteral depot formulations: separation methods, flow-through cells (open system), and dialysis techniques (156–160). Each has certain advantages and limitations. There are currently no regulatory standards for in vitro release testing of parenteral depot formulations, and available compendial apparati were not designed for this purpose and are generally not suitable, with the exception of USP IV (156). Of the available methods, the separation technique is the simplest and appears to be the most widely utilized. A quantity of the dosage form is placed in a vessel, along with a specified volume of release medium, and agitated at a controlled rate and temperature. At specified timepoints, the release medium is sampled and assayed for drug content, and fresh medium is returned to the vessel. Dispersed systems must first be separated by centrifugation or filtration prior to sampling, and aggregation of dispersed systems is a concern (156). The flow-through method allows for maintenance of sink conditions, but is more complex and potentially less robust (156). The dialysis technique may provide the best approximation of the confined environment at the injection site, coupled with sink conditions in the bulk release medium, but membrane stability and drug adsorption must be evaluated (156,160).

Selection of biorelavant release media is another important aspect of method development. Many reports specify phosphate buffered saline (PBS) at 37°C as the release medium to approximate in vivo conditions, although in some cases media with different pH, ionic strength, or protein content are more appropriate. Media volume is a critical variable, particularly for drugs with solubility limitations where sink conditions may not be achieved. Media should be selected on a case-by-case basis based on the properties of the active and the formulation. Other physiological variables to consider during selection of release media and development of the method include metabolism, tissue pH and buffer capacity, vascularity, level of exercise, and volume and osmolarity of the product (156).

Accelerated In Vitro Release Methods

Accelerated methods that can promote rapid release of the depot contents over a short (e.g., few days) time period are needed for quality control (e.g., product release) and formulation development applications, particularly for very long-acting formulations (157). These methods should be capable of discriminating formulation changes that can impact bioavailability, and detecting batch to batch variability and the impact of product instability over time (155). Acceleration of release is most commonly accomplished by raising temperature (e.g., to 50–60°C), altering pH, or adding surfactants (156,157). It is important to consider the impact of

factors such as polymer transition and degradation temperatures on the release mechanism (154). To accurately assess burst release, a real-time release evaluation may be conducted in parallel to the accelerated method (154).

In Vitro–In Vivo Correlation

The need to establish IVIVC for parenteral depot formulations is well recognized (154). This has historically been difficult to achieve, presumably because of the large number of parameters influencing release from depot formulations both in vivo and in vitro, including fluid volume, viscosity, tissue barriers (e.g., fibrous encapsulation), phagocytosis, and inflammation (161–163). There are, however, increasing numbers of successful reports in the literature establishing IVIVC for parenteral depot systems, and these have been recently reviewed (156,164,165). IVIVC becomes more likely as drug release from the depot is the rate-limiting step for absorption, and as release is governed primarily by diffusion, rather than polymer degradation, which can differ in vivo and in vitro (165). Steps to establish IVIVC are similar to those for oral dosage forms, and include in vitro method development, preparation of formulation variants that are expected to have different behavior in vivo, in vitro and in vivo testing, and modification/optimization of the in vitro release method to mimic in vivo results (156). Animal models, such as the rat, are suitable for formulation development and optimization, but would not be suitable for demonstration of human bioequivalence; larger species may be needed to evaluate relevant injection volumes (154).

Development of IVIVC can be particularly challenging for local delivery, such as ocular delivery, where it may be difficult or impossible to assess the local drug concentration in humans, and plasma levels may be extremely low and not indicative of local exposure. In animal models, local tissue exposure may be determined by sacrificing animals at specified timepoints and measuring tissue concentrations or drug content of explanted dosage forms, or by microdialysis methods (29,153,166).

Manufacturing Process and Scalability

Manufacturing processes for parenteral sustained-release formulations are often complex and many involve nonconventional unit operations. Considerations during process development include ensuring that stability and activity of the drug are not compromised, optimizing process yield and drug encapsulation (e.g., for microspheres), ensuring the release profile is reproducible within specified limits and, when relevant, that particle size is controlled to specifications (31). Scaling can be a challenge for many mixing operations; scalability can be improved by utilizing continuous processes, such as in-line mixing or extrusion (for implants). Solvent-based processes present additional environmental and safety challenges, such as the need for solvent-recovery handling, and ensuring residual solvent levels conform to specifications.

Terminal Sterilization

Ensuring sterility of parenteral sustained-release formulations is a significant challenge, given the dispersed nature of many of these systems, which often precludes sterile filtration. Sterile filtration may be feasible for systems formulated as solutions, such as oily-vehicle solutions and in-situ-forming depots dosed in organic solvents. Terminal heat sterilization may be possible for suspension formulations, and cloud point modifiers can be included to improve physical stability at high temperatures. This approach is not suitable for polymeric systems such as PLGA because of the relatively low T_g of the polymer (154). γ-Irradiation has been employed for terminal sterilization of PLGA microsphere and suspension systems, but the potential for polymer and drug degradation must be evaluated. As a result of these challenges, most parenteral sustained-release formulations are aseptically processed (154).

Regulatory Considerations

Depot delivery systems are subject to the same quality control requirements that govern other parenteral drug products, including sterility, pyrogen testing, drug content, impurities and degradates. The sterility test method used depends on the nature of the depot delivery system,

with the direct transfer method typically used for suspensions that cannot be solubilized in suitable solvents, and sterilized devices, and the membrane filtration method for other depot delivery systems (167). Although particulate matter testing is not required for suspensions, these systems can be dissolved in a suitable organic solvent to test for foreign particles (154). It is important to verify syringeability and injectability with appropriately-sized needles. While there is no regulatory guidance, viscosity measurements and evaluation of needle-clogging and plunger force can be used to assess these attributes. A fundamental understanding of release mechanisms and physicochemical changes within the delivery system is an important part of quality by design.

Development Strategy and Economics

The successful development and launch of parenteral sustained-release dosage forms can be a long and expensive process, owing to their high technical complexity, nonconventional unit operations, and long duration of action. It is critical to establish an early line of sight from the concept and compound properties to the market, to minimize additional cost and lost time. This involves establishing a target product profile early, including a thorough assessment of the market and target patient populations. It is important to ensure that the properties of the active are suitable for the desired sustained-release application, and that they are properly matched to the appropriate formulation approach. Ideally, if the need for sustained release is recognized early during discovery, compound potency and physicochemical properties, such as solubility and stability, should be designed to enable formulation using specified sustained-release technologies.

Sustained-release formulations are often developed as lifecycle management opportunities for compounds already in development or launched as conventional parenteral or oral formulations. As a result, there may be a significant body of existing safety and efficacy data in humans, and depot formulations typically do not enter clinical development until a thorough understanding of PK/PD and therapeutic window is available. In vitro release data and preclinical pharmacokinetic data should be used to design the dosage form to meet the target pharmacokinetic profile. As for any new formulation, preclinical safety studies must be run prior to initiation of clinical studies. Clinical dose ranging can be supported either by administering formulations with different release rates, or by administering different doses of a single formulation. It is desirable to initiate clinical studies with a formulation composition and manufacturing process that is representative of the intended commercial product to minimize the challenge of bridging formulation changes and process changes later in development. As this is rare in practice, and given a lack of regulatory bioequivalence guidelines for sustained-release dosage forms, formulation and process changes should ideally be supported by a validated IVIVC.

Development and product costs of sustained-release formulations are typically higher than conventional formulations because of their high technical complexity, long development timelines, nonconventional excipients and manufacturing unit operations, and higher doses of active per administration. This should be planned into the overall development strategy from the beginning.

FUTURE DIRECTIONS

The future of parenteral sustained delivery promises to be an exciting one, with the potential for significant advances that will meaningfully change the way medicines are administered. Technical advances will span from incremental improvements in existing technologies, to the introduction of new excipient materials, the development of systems that offer an improved level of control over drug release, and the emergence of new applications for depot delivery. This future will require pharmaceutical and formulation scientists to broaden their already multidisciplinary backgrounds even further into areas as diverse as microelectromechanical systems (MEMS), information sciences, and cell and tissue biology (168).

Incremental Enhancements of Existing Technologies

The pace of launching new parenteral sustained-release technologies over the last several decades has been relatively slow, due in part to the major challenges and costs inherent in

commercializing new delivery modalities. It is therefore reasonable to expect that incremental improvements in existing technologies will continue to dominate the near-term future of depot delivery. These improvements may include new manufacturing process techniques, new approaches to sterilization, novel packaging technologies, and novel combinations of existing technologies. Recent examples of these include the emerging use of supercritical fluid technologies to make polymeric microspheres (169), evaluation of electron-beam and ethylene oxide as methods of sterilization (170), increasing use of delivery devices, such as the Lupron Depot-PED® dual-chamber syringe, to enhance convenience during administration, and the integration of acid-neutralizing excipients in PLGA formulations to counteract acidification by hydrolysis products (44). Further value may be extracted from these technologies if leads are optimized during discovery specifically for sustained release, emphasizing potency and stability as key criteria.

Introduction of New Excipients

The acceptability of materials for parenteral use, from both the safety and regulatory points of view, continues to be a major constraint in the development of new depot delivery technologies. The hurdles to introduction of new excipients are significant, and few companies are willing to invest the significant time and money required to bring new or novel-use excipients through development to the market. PLGA enjoys the status of being a proven and well-accepted excipient, and continues to be the most common polymer used in parenteral sustained-release systems, further entrenching it in this application. Although PLGA is attractive in many respects, new polymeric materials are needed to provide a wider range of properties and potential release profiles, and to enhance the range of actives compatible with sustained-release approaches. In the short term, the most promising new candidates for approval are likely to be copolymers of currently-approved materials, such as copolymers of PLA and PEG, which can be expected to degrade to known materials. Longer-term, one approach to speed the introduction of new excipients could be the formation of jointly-funded industrial consortia, to advance the preclinical evaluation of novel materials.

Enhanced Control over Drug Release

Despite their many advances over the years, marketed depot delivery systems continue to offer a relatively limited ability to control release rate, relying on the intrinsic properties of the formulation (e.g., matrix degradation, API dissolution or partition, osmotic pressure, etc.) to govern drug release. The ability to rationally change drug release during dosing would represent a major step forward, and continues to comprise an active area of scientific inquiry. The ultimate goal is responsive systems, or smart delivery systems, which incorporate the ability to sense their surroundings and alter their function in response to specific signals generated in the body (171). Such systems will be particularly valuable in the treatment of diabetes and other metabolic disorders, and may also be useful in chronotherapy (172,173).

Several approaches have been evaluated in the pursuit of this goal, including environmentally responsive polymers and microprocessor-based devices. Novel polymers have been synthesized, which are capable of changing their properties in response to changes in their environment, including pH, temperature, ionic strength, solvent composition or electromagnetic radiation (174–178). These include the pH-sensitive methacrylates, which change in their degree of swelling as pH changes, and temperature-sensitive systems such as poly (*N*-isopropylacrylamide) (174). Microelectromechanical solutions include an electrothermally activated implantable silicon chip, under development by MicroCHIPS (179). The device is segmented into multiple wells, which can be sealed prior to implantation and then opened on demand. Depot delivery systems of the future will likely include integrated sensing of biomarkers, metabolites, or actives, feedback-control over drug release, and real-time output of information relating to the underlying pathology and treatment (168).

New Applications

A number of new applications for depot delivery are emerging, including targeted delivery, gene delivery, and tissue engineering. Fabrication of nanoparticles from PLGA offers a new platform for targeted delivery, amenable to IV administration (180). These systems are being

developed and studied for the targeted delivery of a range of therapeutics, from small molecules to nucleic acids. Nucleic acid delivery via sustained-release systems is an increasingly active field of research given the recent advent of RNAi technology and continued interest in local gene delivery (181,182). Tissue engineering and regenerative medicine strategies often require controlled delivery of bioactive molecules, with particular sensitivity to spatial and temporal control of release (183), to a particular cell type or in a particular region of the body (184). There are many potent growth factors including nerve growth factor, bone morphogenic protein and vascular endothelial growth factor, which are under investigation (185). Approaches for regenerating nerve tissues, repairing bone defects from fractures, infections and cancers, and the ability to accelerate blood vessel formation are all areas of active research. The field of parenteral sustained release promises to be an exciting and active area of research for many years to come, offering the potential to significantly increase the value of both existing and new therapeutics and address important unmet medical needs.

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8 Biophysical and biochemical characterization of peptide and protein drug product

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INTRODUCTION

Classes of Biotherapeutics

The biotherapeutics class of drugs that are commercially available encompass a range of compounds including recombinant or purified proteins, monoclonal antibodies (also proteins), peptides, conjugated or fused peptides, antibody conjugates, protein vaccines, oligonucleotides, protein-lipid complexes, enzymes, antibody fragments (Fabs), glycosylated proteins, and carbohydrates (Fig. 1). Additional molecule types are in preclinical and clinical development.

The biotherapeutics class contains a wide variety of recombinant proteins derived from microbial, mammalian, and yeast sources (Table 1). There are few products that are extracted from natural sources. The biotherapeutics class of drugs uses a variety of technologies for extending half-life such as conjugating to polyethylene glycol (PEG), fusion with antibody or Fab, and employing the antibody itself. This is especially true for peptides and other small entities that would be cleared via the kidneys without a half-life enhancing strategy such as conjugation or fusion. Table 1 illustrates the wide variety of biotherapeutics entities on the market.

Regulatory Guidance on Structural Characterization

Regulatory approval of a biotherapeutic entity requires meeting the guidelines for chemistry, manufacturing, and controls (CMC) put forth by the relevant regulatory agency. A complete CMC package includes a description of the characterization of the biotherapeutic entity, which includes the Elucidation of Structure and Impurities sections, which, for biological entities can be quite complex. It is expected that the applicant have a detailed understanding of the structure, heterogeneity, and stability of the biotherapeutic entity using a variety of analytical methods. Regulatory guidance on the characterization of biotherapeutic molecules can be found in several sources. The U.S. Food and Drug Administration (FDA), the European Medicines Agency (EMEA), and other regulatory agencies around the world often provide guidance documents on specific topics relating to the review and approval of drugs, and these can be excellent sources of information for applicants (www.fda.gov, www.emea.europa.eu). The International Committee on Harmonization (ICH) (www.ich.org) provides guidance documentation agreed on by the regulatory agencies of the United States, Europe, and Japan. The ICH guideline Q5 deals specifically with biotechnology products, and some information concerning characterization is available in this section, particularly Q5E on comparability. Q6B deals with specifications of biotechnology products, and provides further relevant information for biotherapeutic entities.

Proof of Structure

As part of the Elucidation of Structure section of a CMC package, a detailed analysis of the structure of the biotherapeutic is required. This evaluation is in addition to the normal batch release assays used for the product which ensure the safety and efficacy of each batch. The characterization assays included in this section are used for confirmation of the predicted primary structure, higher order structures, post-translational modifications, and degradation products that may form or increase on stability. The presence and levels of variant forms needs to be measured, and their impact on the safety and efficacy of the product needs to be assessed. The attributes investigated may be assessed using multiple analytical methods for each, as discussed in some detail below.

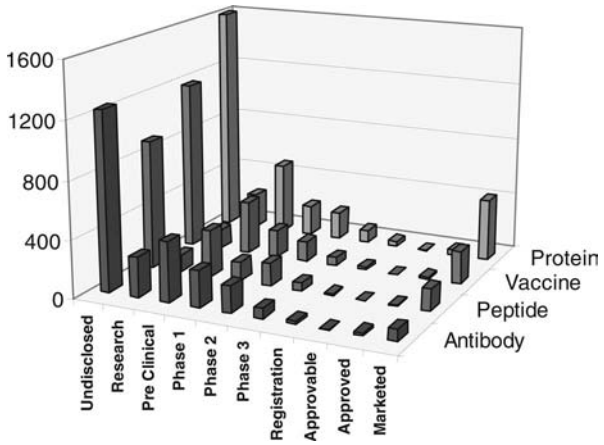

Figure 1 Portfolio of selected biotherapeutic class of drugs and drug candidates in various stages of development (data from PharmaCircle, March 2009). Numbers do not represent unique molecule types in any of the classes.

The confirmation of primary structure may include assays that demonstrate the product has the expected amino acid sequence, such as amino acid sequencing, mass spectrometry (MS), and electrophoresis. These methods ensure that there are no translation variants such as amino acid substitutions, terminal extensions, or unprocessed introns present in the product. Higher order structure may be assessed by biophysical and spectroscopic methods such as circular dichroism (CD) and fluorescence spectroscopy. This may include a determination of the disulfide bond connectivity, which can be critical for a protein to maintain its active conformation. Many post-translational modifications of proteins are possible, such as glycosylation. Other modifications may include related species formed as a consequence of degradation, such as oxidation and deamidation. For conjugated products, variants due to the conjugation process and degradation products of these need to be assessed and understood. In total, biotherapeutics may include a heterogeneous mixture due to all of the variant forms possible, and the applicant needs to demonstrate an understanding of the species present.

Potency Determination

For biologics, in most cases, a relevant potency assay for the biological entity is required for its approval. The assay needs to demonstrate "the specific ability or capacity of a product to achieve a defined biological effect." (ICH, Q6B, specifications: test procedures and acceptance criteria for biotechnological/biological products). One or more bioassays are typically included as part of batch release, and range from binding assays, cell-based assays, or in vivo animal assays. As part of characterization, it is expected that variant forms of the biological entity be assessed for potency. This involves isolation of the variant form and testing in the relevant bioassay(s) for the product. For species that form or increase on stability because of degradation, stress conditions can be used to generate sufficient material to perform potency assays.

Formulation Characterization

Most therapeutic biologics currently are administered via parenteral (intravenous or subcutaneous) route. The goal of biologics drug product formulation development is to minimize various degradation pathways to achieve a minimum shelf-life of 18 to 24 months at the intended storage condition. An emerging strategy in the biotherapeutics industry is to minimize investment in the early stages of preclinical and clinical development, and therefore, drug product formulation for early clinical trials may not be characterized in detail. Additionally, long-term stability data may be rarely available in early stage. However it is necessary to make an assessment of potential chemical and physical labilities that may impact long-term stability. A part of this assessment can be achieved by Preformulation work which is a combination of experimental and bioinformatics studies conducted in early stage prior to nominating a drug product formulation. "Formulation characterization" refers to

Table 1 Examples of Biotherapeutics Class of Molecules: Types, Sources, Technologies, and Molecules

Name of drug	Name of active substance	Class of molecule	Technology	Source	Indication	Company
Genotropin	Somatropin	Protein	Single polypeptide	*Escherichia coli* (rDNA)	Growth hormone deficiency, Turner syndrome and others	Pfizer
Somavert	Pegvisomant	Conjugated protein	Single polypeptide PEGylated at multiple sites	*E. coli* (rDNA)	Acromegaly	Pfizer
PEG-Intron Redipen	Peginterferon α-2b	Conjugated protein	Covalent conjugate of PEG to protein	*E. coli* (rDNA)	Infections, hepatitis C	Schering-Plough
Nplate	Romiplostim	Fusion protein	Fc-peptide fusion protein (peptibody)	*E. coli* (rDNA)	Thrombocytopenic purpura	Amgen
Survanta	Beractant	Lipid-protein mixture	Natural bovine lung extract containing lipids and surfactant-associated proteins, and added lipids	Bovine lung extract	Respiratory distress syndrome	Mitsubishi Tanabe; Ross (Abbott)
DigiFab	Digoxin immune Fab	Antibody fragment (Fab)	digoxin-specific Fab	Ovine serum	Digoxin toxicity or overdose	BTG International Ltd.; Nycomed
Lucentis	Ranibizumab	Antibody fragment (Fab)	Humanized IgG1 κ	*E. coli* (rDNA)	Age-related macular degeneration	Genentech and partners
Cimzia	Certolizumab pegol (CDP-870)	Antibody fragment (Fab) conjugate	Humanized antibody fragment Pegylated	*E. coli* (rDNA)	Crohn's disease	UCB and partners
Enbrel	Etanercept	Fusion protein	Dimeric fusion protein (extracellular portion of human tumor necrosis factor receptor linked to IgG1 Fc	Mammalian cell (CHO) (rDNA)	Rheumatoid arthritis; plaque psoriasis and others	Amgen, Wyeth; Takeda
Herceptin	Trastuzumab	Full length antibody	Humanized IgG1 κ	Mammalian cell (CHO) (rDNA)	Cancer (breast, stomach, pancreatic)	Genentech and partners

Vectibix	Panitumumab	Full length antibody	Humanized IgG1 κ	Mammalian cell (CHO) (rDNA)	Cancer (colorectal)	Amgen
Gardasil	Human papillomavirus quadrivalent vaccine	Protein vaccine (VLP)	Self-assembled VLP of capsid protein of HPV types 6, 11, 16, and 18—adsorbed into aluminium-containing adjuvant	*Saccharomyces cerevisiae* (yeast) (rDNA)	Prevention of several diseases caused by HPV	Merck
Prevnar	Pneumococcal 7-valent conjugate vaccine	Vaccine (glycoconjugate)	Saccharides of capsular antigens of *Streptococcus pneumoniae* serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F each conjugated to diphtheria CRM197 protein	Serotype from soy peptone broth; CRM197 from *Corynebacterium diphtheriae*	Immunisation against several diseases caused by *S. pneumoniae*	Wyeth (Pfizer)
Fragmin	Dalteparin sodium injection	Carbohydrate	Controlled depolymerization of sodium heparin	Porcine intestinal mucosa	Deep vein thrombosis and others	Pfizer
Pulmozyme	Dornase α	Enzyme (glycoprotein)	Recombinant human deoxyribonuclease I	Mammalian cell (CHO) (rDNA)	Cystic fibrosis	Genentech
Fabrazyme	Agalsidase β	Enzyme (glycoprotein)	Recombinant human α-galactosidase A	Mammalian cell (CHO) (rDNA)	Fabry disease	Genzyme

Abbreviations: IgG, immunoglobulin; Fab, antibody fragments; PEG, polyethylene glycol; VLP, virus-like particles; mAb, monoclonal antibody.

characterization of drug product formulation using biochemical and biophysical methods for adequate understanding of structural and functional correlations to stability in a stage appropriate manner. It should be noted that depending on the type of biologics candidate and its stability profile, it may be necessary to conduct additional formulation characterization studies especially when stability is poor and/or stability-bioactivity correlation is complex. In later stages of clinical development as well as for biologics license applications (BLA) it is expected that extensive formulation characterization studies are conducted.

Determination of Hot Spots

An important and first step in formulation characterization is to determine the potential liabilities in the amino acid sequence and other parts (for contents other than amino acid) of the biotherapeutic candidate. These liabilities are often referred to as "hot spots." There are some amino acids or groups of amino acids that exhibit common occurrences of chemical or physical degradation events such as oxidation and deamidation. For example, the amino acid methionine (Met) undergoes oxidation, especially in the presence of oxygen and when it is on the protein surface exposed to bulk solvent. Similarly, a surface-exposed pair of asparagine-glycine (Asn-Gly) when present in a loosely formed structural domain in the protein may be prone to deamidation under certain formulation conditions (1).

Linear sequence vs. folded structure. Determination of hot spots may not be trivial for all protein types. Prediction of lability of an amino acid based on primary structure [i.e., amino acid linkage (Table 2)] does not work well for folded proteins because surface exposure and flexibility in the three-dimensional structure are among the important criteria dictating propensity of degradation. For certain classes of biotherapeutics where adequate correlation between structural and chemical degradation is available, it might be possible to more accurately predict hot spots. For example, immunoglobulins (IgGs) of a given subtype may contain common hot spots in the conserved part of the sequence (Table 2). Similarly, degradation behavior of a nonconserved amino acid in a conserved structural motif in IgGs may be partially predicted on the basis of structural flexibility of the motif (unordered vs. helical or β sheet). While these approaches are quite useful in enlisting the common hot spots for chemical degradation, they may not predict physical degradation (aggregation) hot spots or unique chemical degradation events [e.g., tyrosine (Tyr)/tryptophan (Trp) oxidation].

The determination of hot spots needs information on folded structure but many biotherapeutic candidates will not have its crystal structure or other solution-based (e.g., NMR) structure available. In the absence of structure, homology modeling may be beneficial to derive qualitative structure using bioinformatics tools. In a recent study, Wang et al. (14) employed a novel use of bioinformatics tools to delineate common sequence segments across several antibodies and hypothesized that such segments may contribute to aggregation propensity on the basis of certain physicochemical properties of the contributing amino acids in these segments (rich in aliphatic/aromatic residues). Using full antibody atomistic molecular dynamics simulations, Chennamsetty et al. (15) identified the antibody regions prone to aggregation by using a technology called spatial aggregation propensity. Development of such bioinformatics tools is a good first step in understanding aggregation propensity, however it remains to be experimentally tested how accurately and widely such tools can be used for reliable prediction appropriate for drug development.

Physical and Chemical Degradations

Following determination of hot spots as described above, the next step in formulation characterization is to experimentally determine the major degradation pathway(s) and to understand the mechanism of degradation. Unlike small molecule drugs, protein-based biotherapeutics candidates have added complexity of several degrees of structure such as secondary, tertiary and quaternary structures that are critical to its stability and intended function. The degradations observed and/or predicted can be categorized into two types—chemical and physical degradations. Majority of the degradations cited in Table 2 are of

Table 2 Protein and Peptide Degradation Hot Spots

Labile groups	Type of degradation	Occurrence in IgG and other proteins
Asn-Gly	Deamidation, Isomerization	$NN^{386}G$ in CH3 (IgG2a) (2) $QN^{156}G$ in CL (IgG2a) (2) $LN^{316}G$ in CH2 (IgG1) (3) $SN^{385}G$ in CH3 (IgG1) (3)
Asn-Ser, Asn-Asn, Asn-Thr, Asn-Lys, Asn-His, Asn-Asp	Deamidation, Isomerization	$RN^{423}S$ in CH3 (IgG2a) (2) $PEN^{390}NY$ in CH3 (3) $VN^{30}T$ in CDR1 of LC (4) $SN^{329}K$ in CH2 (5)
Asp-Pro Asp-Gln	Clipping (peptide bond)	D^{274}-P^{275} (IgG1) (5) D-K in hinge (IgG1) (5) H-T in hinge (IgG1) (5)
Asp-Lys His-Thr		
Asp	Isomerization	$D^{102}G$ in CDR3 of HC (IgG1) (4)
Met	Oxidation	M^{34} in CDR1 of HC (IgG1) (6) M^{101} in CDR3 of HC (IgG1) (6)
Cys	Oxidation (to form disulfide)	C^{105} in CDR3 of HC (IgG2a) (2)
Trp	Oxidation	W^{54}, W^{55} in CDR2 of HC (IgG1) (6) W^{105} in CDR3 of HC (IgG1) (6)
Tyr	Oxidation	Oxidation of lens protein forms dihydroxyphenylalanine, o- and m-Tyr, and di-Tyr (7)
Pro	Proline isomerization	Trans-P^{32} isomer formation in β2-microglobulin (8)
Lys	Glycation	K^{49} in LC (IgG1) (9)
Fe-His/Asp/Tyr	Metal bond breakage	Iron loss by acidic pH, chelator in transferrin (10)
His-Fe (heme)	Metal bond breakage	Low-pH Fe-His breakage in hemoglobin (11)
Met-Fe (heme)	Metal bond breakage	Labile Fe-S (Met) bond in cytochrome c breaks under various conditions (12)
Amine and other reactive amino acids	Reaction with buffer/excipients	May form adducts such as carboxylate adduct with citrate/succinate (13)
Various hydrophobic segments	Aggregation	Potential hot spots for aggregation in IgG predicted using bioinformatics tools (14,15)

Abbreviations: IgG, immunoglobulin; LC, light chain of IgG; HC, heavy chain of IgG; Tyr, tyrosine; Met, methionine.

chemical nature, whereas physical degradation includes aggregation, particulate formation, and related structural degradation events associated with adsorption, misfolding, denaturation (by heat, chemicals, chaotropes, etc.), partial misfolding, nucleating species, and sometimes chemical degradation. Physical degradation is complex and may involve a wide variety of causative factors that may involve protein-protein interaction, native state conformational distortion, air-water interfacial tension, and conformational changes induced by solvents, additives, and processing. Therefore, a multitude of biophysical tools (in addition to biochemical characterization) is often necessary to achieve a comprehensive formulation characterization.

ASSESSMENT OF PRIMARY STRUCTURE

Simply put, the primary structure of a protein consists of its amino acid sequence. For recombinant proteins, the amino acid sequence can be predicted from the cDNA used in its production. This basic attribute of a protein determines the entirety of its biophysical and biochemical properties. The amino acid sequence of a protein determines its ability to fold properly, and thus determines its ability to maintain its function. Therefore, a small change in the primary structure, depending on its location, may have a range of effects on a protein's activity, from no effect to a very large impact. The amino acid sequence can also impact the chemical and physical stability of a protein, even when there is no measurable impact on activity. Thus, confirming the amino acid sequence of a protein is fundamental to understanding its overall structure and properties.

During production of recombinant proteins, several modifications to the primary structure are possible. These include errors in transcription or translation, generating such variant forms as amino acid substitutions, N- and C-terminal extensions, splice variants, and internal sequence extensions. Other changes to the primary structure may occur as a consequence of biochemical instability, such as deamidation or oxidation. All of these variant forms can have large impacts on the properties of the protein, and need to be detected and controlled during production and storage.

Amino Acid Composition Analysis

One of the most basic assessments of primary structure is the confirmation of the expected amino acid composition of the polypeptide. Recombinantly produced proteins have amino acid sequences predicted from the DNA sequence used in their production. The amino acid composition, therefore, is a predictable attribute, and can be confirmed using amino acid composition analysis. The technique can be broken down into three steps: complete hydrolysis of the polypeptide into its constituent amino acids, chemical labeling of the free amino acids with a chromophore or fluorophore, and separation of the amino acids by liquid chromatography (LC), with quantification of the individual amino acids by UV absorbance or fluorescence detection (16,17). Typically, overnight digestion with 6N HCl or other acids at high temperature or vapor phase hydrolysis with trifluoroacetic acid is used for complete hydrolysis. Derivatization can be achieved either prior to separation (precolumn) or after separation but prior to detection (post-column). Typical chemical labels include fluorescamine, o-phthalaldehyde (OPA), ninhydrin, and phenyl isothiocyanate (PITC). Separation can be accomplished for all twenty naturally occurring amino acids using reversed-phase or ion exchange chromatography (IEC), the former typically used with precolumn derivatization methods and the latter used in combination with post-column derivatization approaches.

The harsh conditions used for complete hydrolysis of the polypeptide can lead to destruction of particularly sensitive residues. Trp and cysteine residues are typically destroyed during acid hydrolysis, and cannot be confidently quantified using this approach. Also, amino acids with side chain amide groups, glutamine and asparagine, are modified to form their analogous amino acids with side chain acid groups. The levels of these amino acids are added to the levels for the glutamic acid and aspartic acid residues, and can be quantified as combinations of glutamine plus glutamic acid and as asparagine plus aspartic acid (Glx and Asx).

The relative amounts of the amino acids present in the protein are determined by comparison with quantitative standards. This is one of the most accurate methods for determining the protein quantity. The amino acid composition of a sample can be compared with the theoretical composition on a residue-by-residue basis. Each of the amino acid residues may have a different precision depending on the relative stability of the residue during hydrolysis and the chromatographic properties of the residue on a given system.

In combination with accurate absorbance measurements, amino acid composition analysis is commonly used for accurate determination of protein molar absorptivity, or extinction coefficient (18,19). Once an accurate extinction coefficient is determined for a given protein, the concentration of the protein in formulated solutions can be determined consistently using UV absorbance spectroscopy according to the Beer-Lambert law

$$A = \varepsilon l C \tag{1}$$

in which A is the measured absorbance at a given wavelength, ε is the molar absorption coefficient in $M^{-1}cm^{-1}$ at that wavelength, l is the pathlength used in measuring the absorbance in cm, and C is the protein concentration in M. So, after determining the molar absorptivity at, for example, 280 nm, the protein concentration can be reliably determined by measuring the absorbance at 280 nm.

N-Terminal Sequencing by Edman Degradation

Confirming the termini of polypeptides is fundamental to their characterization, and N-terminal sequencing using Edman degradation is a robust technology for achieving

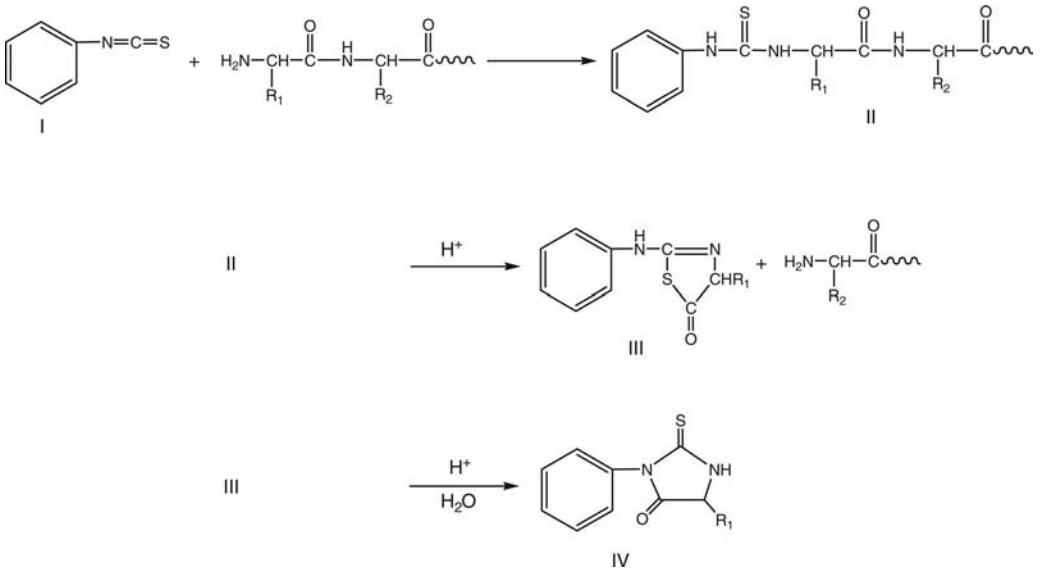

Figure 2 Edman degradation chemistry.

confirmation of the N-terminal residues (20). This technology uses amine-specific chemistry to remove the N-terminal residue, followed by chromatographic separation of the residue. By comparison of the retention time of the released residue with amino acid standards, the identity of the residue can be determined. After release of the N-terminal residue, a new N-terminal amine is generated, and the chemistry can be repeated in multiple cycles to deduce the N-terminal sequence of the protein. The reaction sequence is illustrated in the scheme below (Fig. 2). There are three steps to the Edman degradation reaction: (*i*) coupling of the Edman reagent, PITC, (*ii*) cleavage of the phenylthiocarbamyl polypeptide (*iii*) under acidic conditions to form an anilinothiazolinone (ATZ) derivative of the N-terminal amino acid, and a new N-terminus on the n-1 polypeptide, and (*iv*) conversion of the ATZ amino acid under acidic conditions to form a stable thiohydantoin (PTH) derivative of the N-terminal amino acid. The chemistry can be repeated after extraction of the PTH–amino acid to determine the next amino acid in the polypeptide, and so on. Sequencing instruments are available such that the entire process is automated.

The number of cycles that can be repeated for a protein is highly dependent on the sequence of the protein, the amount of protein in the sample, and the conditions of the reaction. Typically, up to twenty cycles is easily attainable for a recombinant protein.

In many instances, the free amine on the N-terminus of the protein may be blocked, typically by acetylation or cyclization, thus preventing the Edman degradation reaction from occurring (21). N-terminal acetylation is a common post-translational modification which can prevent Edman sequencing. There are strategies for unblocking or removing acetylated N-terminal residues using enzymatic or chemical methods, but these methods are not generally considered to be very efficient. If the N-terminal residue is glutamine, these residues undergo spontaneous cyclization, blocking the free amine to form pyroglutamic acid (22). Less common is cyclization of glutamic acid to form pyroglutamic acid (23). Pyroglutamic acid can be efficiently removed using a pyroglutaminase enzyme, generating a free N-terminus on the n +1 residue that can then be sequenced using Edman degradation (24).

Proteolytic Mapping

Proteolytic mapping of proteins is the most comprehensive method for the determination of primary structure. This method employs the use of residue-specific enzymes to cleave the protein into smaller peptides, which can then be separated using high-performance liquid chromatography (HPLC). The resulting chromatogram, or proteolytic map, can be extremely

reproducible and specific for the protein, and can be used as an identity method when compared with a reference standard of the protein. It is often used as a batch release test for this purpose (25). Proteolytic maps can be extremely efficient at detecting changes in the protein primary structure, since a single change of an amino acid at the peptide level can often generate a detectable shift in the retention time of the peptide. In combination with MS, it is often used as a characterization tool for detecting and quantifying impurities and degradants (26).

The proteolytic enzyme appropriate for use for a given protein depends on the amino acid sequence. An analysis can be performed utilizing theoretical digestion on the basis of the specificity of the enzyme to determine the most appropriate enzyme for a given protein. The goal is to generate a sufficient number of peptides that can be well separated chromatographically, typically using reversed-phase chromatography, to generate a highly specific proteolytic map.

The specificity of proteolytic enzymes suitable for mapping include trypsin (C-terminal to Arg and Lys), endoproteinase Lys-C (C-terminal to Lys), V8 protease (C-terminal to Glu and Asp), and endoproteinase Asp-N (N-terminal to Asp). There are many other less common or less specific proteases that can be used when appropriate. Trypsin is a very common enzyme used for proteolytic mapping because of its high fidelity for its substrate sites and its generation of highly specific proteolytic maps for many proteins. It has the added advantage of generating peptides with C-terminal Arg or Lys residues, which can be detected with high sensitivity when analyzed using MS because of the high ionization efficiency of basic peptides in the positive ion mode.

Mass Spectrometry

MS is a powerful method for confirmation of the primary structure of proteins and peptides (27). The use of MS for characterization of therapeutic proteins is typically performed as a part of structural elucidation for regulatory submissions, and not as a routine batch release test. Current MS instrumentation is capable of measuring the molecular mass of proteins to within 100 ppm for intact proteins, depending on the instrumentation used and the molecular mass being measured. This is sufficient mass accuracy to confirm the predicted molecular mass on the basis of the amino acid sequence and expected post-translational modifications. For example, a protein with a predicted molecular mass of 20 kDa can be measured to within 2 Da at 100 ppm mass accuracy. With this mass accuracy, many modifications of the primary structure can be detected and examined further if present. The exception to this is alterations in the sequence of amino acids, or modifications or substitutions that lead to mass changes of 2 Da or less, such as deamidation of asparagine residues (a 1 Da mass change). These types of changes require proteolytic mapping in combination with MS or other orthogonal methods for their detection.

There are many types of MS methods that can be used for analysis of biotherapeutic proteins and peptides. MS is categorized by the type of ionization method and the type of mass analyzer used. For proteins and peptides, either electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI) are used almost exclusively as ionization methods. ESI is more commonly used, as it is directly compatible with LC/MS as long as volatile mobile phase components are used for the separation (28,29). For analysis of intact proteins, either ionization method can be used. There are many types of mass analyzers used for therapeutic proteins. MALDI is typically coupled with time-of-flight (TOF) mass analyzers, and is characterized by very good sensitivity and a fairly high tolerance of salts and other buffer excipients (30). Compared with ESI coupled with TOF analyzers, however, the resolution of MALDI-TOF is significantly lower, meaning that the mass accuracy is not as good and the ability to detect variant forms is diminished. ESI is very intolerant of salts and buffer components; adducts of alkali metal ions are common for impure samples, which can lower the effective sensitivity and lead to an inability to accurately determine the molecular mass. Therefore, significant sample preparation to desalt the sample is required. However, when coupled with LC/MS, the separation effectively ensures that a pure sample is introduced into the ionization source so that high-quality spectra can be acquired. An efficient approach to analyzing intact proteins is to use LC/MS in which a de-salting column is used prior to introduction of the sample into the

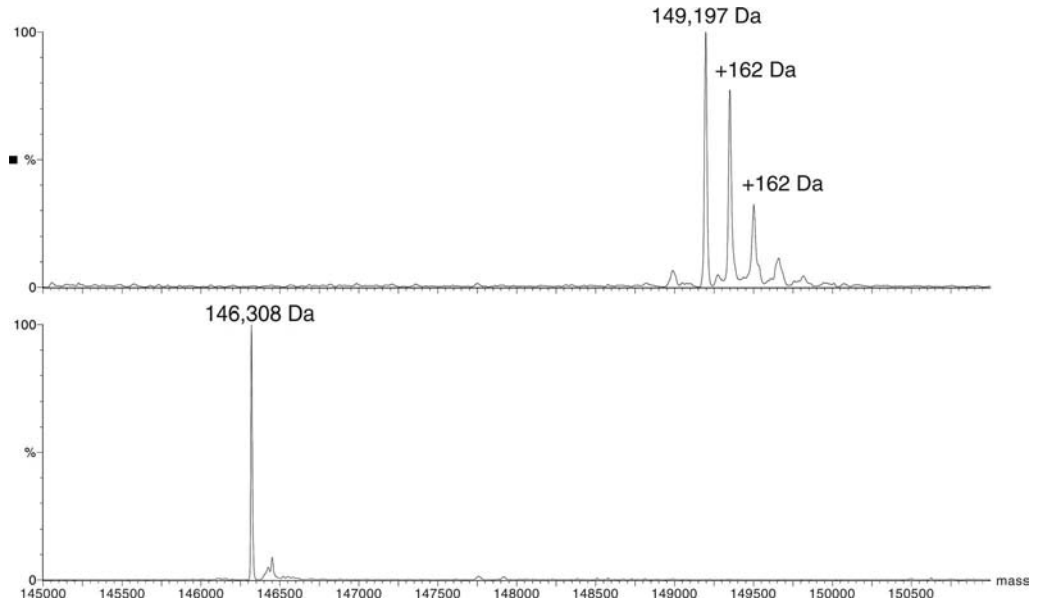

Figure 3 Mass spectra of an IgG (*top*) and the same IgG treated with PNGaseF to remove the N-linked glycans (*bottom*).

ionization source. A common approach for characterizing proteins and peptides using MS is to first analyze the molecule intact, and then perform analyses on samples which have been purposefully degraded in some way to generate smaller species which can be thoroughly characterized. For example, a multichain glycoprotein can be analyzed intact, reduced, deglycosylated, reduced and deglycosylated, etc., with proteolytic mapping as the final "degradation" approach. An example of this is shown below in Figure 3, which shows a therapeutic monoclonal antibody (mAb) before and after deglycosylation analyzed using LC/MS. In the top spectrum, the glycan heterogeneity is evident from the multiple signals observed in the spectrum, most of which differ by the mass of a single hexose moiety, 162 Da. The mass difference between the untreated and deglycosylated samples yields the molecular mass of the N-linked glycans removed from the mAb, in this case 2889 Da. This difference corresponds to the molecular weight of two G0 glycans, each with a monosaccharide composition of four *N*-acetylglucosamine residues, three mannose residues, and one fucose residue. This is a common type of glycan for IgG molecules. On the basis of this analysis, the molecular mass of the expected primary structure can be confirmed, and information concerning some of the modifications, in this case glycosylation, can be determined as well.

When used in combination with proteolytic mapping, MS is invaluable for detecting unpredicted modifications to the primary structure and post-translational modifications (26). By digesting the protein into smaller pieces, more detailed information can be obtained for any modifications to the primary structure. For a tryptic peptide of molecular mass 1000 Da, for example, a mass change of 1 Da from the predicted molecular mass can be easily determined. An example is shown in Figure 4, which shows an expanded version of the 214 nm UV absorbance chromatograms of a tryptic digest of an IgG (top) compared with the same molecule subjected to a pH of 8 for three days to induce deamidation (bottom). The control sample shows a peak, labeled Peak A, which has a molecular weight consistent with an expected tryptic peptide with a sequence of GFYPSDIAVEWESNGQPENNYK. Two new peaks show up in the stressed sample, labeled B and C. Figure 5 shows the mass spectra of these peaks. Peaks B and C show a 1 Da difference relative to Peak A. This is consistent with deamidation of an asparagine residue. The predicted peptide contains three asparagine residues, making this a reasonable interpretation of the data. Tandem MS, in which an ion

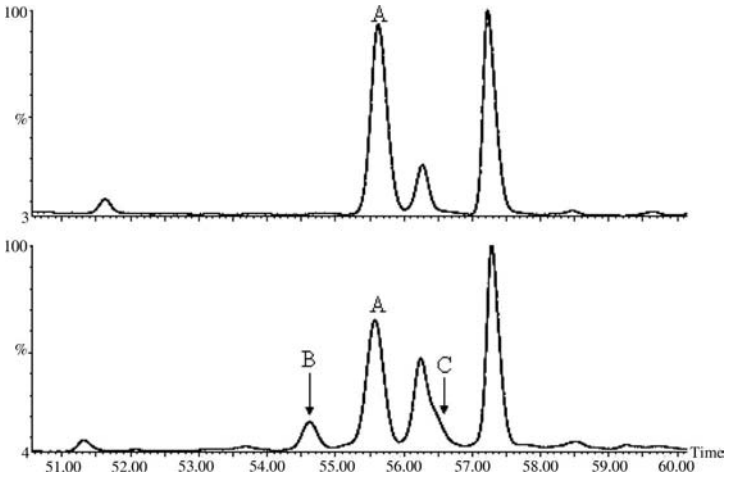

Figure 4 Expanded views of the tryptic maps (214 nm) of an IgG sample (*top*) and the sample treated with pH 8 for three days (*bottom*).

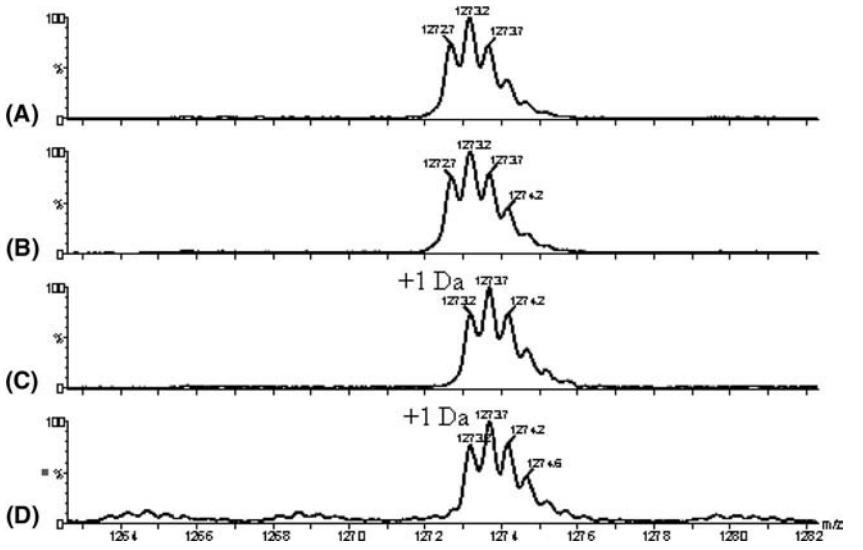

Figure 5 Mass spectra of the peaks shown in Figure 3. (**A**) IgG peak A. (**B**) Stressed IgG peak A. (**C**) Stressed IgG peak B. (**D**) Stressed IgG peak C.

formed in the ionization source is subjected to fragmentation and the resulting fragment ions are measured, is a powerful tool for determining the sites of modifications. In the example above, the precursor ion for the putatively deamidated peptide can be subjected to tandem MS to determine which asparagine in the peptide is the site of deamidation for each of the peaks.

Tandem mass spectrometry (MS/MS) can be accomplished using multiple modes of fragmentation. Most commonly used is collisionally activated dissociation (CAD), in which the precursor ion is accelerated in a collision cell in the mass spectrometer which is filled with a collision gas, such as argon, to impart internal energy into the ion, leading to fragmentation. For peptides, fragmentation tends to occur along the peptide backbone at the amide bonds. This leads to fragment ion spectra which differ in mass by the residue mass of the amino acids present in the peptide. In this way, the sequence of the peptide and the site of any modifications to the peptide can be determined. Fragmentation of the peptide can also be generated using other means, including electron transfer dissociation (ETD) in ion trap

instrument, or electron capture dissociation (ECD) or multiphoton dissociation (MPD) in ion cyclotron resonance (ICR) instruments.

ASSESSMENT OF SECONDARY STRUCTURE

Circular Dichroism

CD spectroscopy measures differences in the absorption of left-handed and right-handed circularly polarized light which arises from an optically active (chiral) molecule. The amide bonds in a protein absorb in the far ultraviolet (far UV), approximately 180 to 250 nm, where the peptide contributions dominate. The intrinsic CD of a protein in the far-UV region is influenced by the asymmetric environment as a consequence of the three-dimensional structure adopted by the molecule, and therefore is sensitive to the secondary structure (e.g., α-helical, β-sheet, β-turn) of the protein. This gives rise to characteristic CD profiles for each secondary structure type (31,32). Therefore, any changes in protein secondary structure due to unfolding or structural conversion can be conveniently monitored by CD spectroscopy.

In the wavelength range at greater than ~240 nm, typically ~240 to 300 nm (near UV), the amino acids Cys (at >240 nm and >320 nm), Phe (peaks at ~262 nm and ~268 nm), Tyr (maxima at ~275–282 nm) and Trp (~280–293 nm and ~265 nm) exhibit CD bands that are characteristic of the tertiary structure of the biomolecule. These bands can be used to monitor local conformational changes as well as large scale structural changes in the protein (31,32).

The biomolecules that contain non–amino acid groups in their active site (ligand- or substrate-binding site) such as porphyrin, heme, metal centers (Fe, Mo, Cu, etc.) coordinated to amino acids, Fe-S cluster, and many other groups may display characteristic CD bands depending on the local structure of the chromophore and its chiral properties. Such CD bands can be extremely useful probes for studying structure-function relationship in these proteins, especially the redox-induced events.

CD of protein-based biologics such as human growth hormone or monoclonal antibodies is measured in aqueous buffered solutions. A protein solution of approximately 0.1 to 1.0 mg/mL can be used, depending on protein molecular weight, its CD strength, and pathlength of measurement cell to measure far-UV CD spectra. Because the near-UV CD signal of proteins is far less intense than in the far UV, typically a ~10× higher concentration is needed for the near-UV range. Alternatively, with higher protein concentration, various pathlengths of sample cell (e.g., cuvette) can be used to accommodate CD measurements in wider wavelength ranges. One can push the measurable limit of high protein concentrations using conventional CD instrumentation by reducing the cell pathlength to much less than 1 mm. However, concerns of surface denaturation of protein due to interfacial tension, artifacts of solution drying, and inaccuracy of pathlength need to be considered to ensure the quality of CD data. To consistently acquire good quality CD spectra down to ~190 nm, the spectrometer along with the UV lamp and mirrors must be carefully maintained and purged with high quality nitrogen flow.

Far-UV and near-UV CD data are often used for assessment of secondary and tertiary structure, respectively, of a biologics candidate—for analytical reference material characterization as well as drug product formulation characterization. It is also used to establish comparability of drug substance between campaigns and/or batches. It should be noted that the near-UV CD spectral signature by itself generally does not point to any particular tertiary structural type of a protein, but instead can be used to compare changes between batches of recombinantly produced protein.

The CD spectrum of proteins in the far-UV range has distinct signatures for α-helical and β-sheet structures. For example, a majority α-helical content (e.g., human growth hormone) displays strong negative bands at ~208 nm and ~222 nm, and a positive band at ~192 nm, while a majority β-structure content (e.g., mAb) shows a negative peak at ~216 nm and a positive peak at ~200 nm depending on the mix of β-sheet (parallel or antiparallel β-sheet) and β-turn components, and any α-helical contributions. Because α-helical structure contributes much stronger to the CD spectrum in the far UV, the presence of even a small percentage of α-helical structure content can significantly change the CD peak positions of a majority β-sheet protein. Unordered (random coil, e.g., unfolded protein) structures can exhibit a strong negative band at ~195 to 200 nm (32). The CD spectrum in the far-UV range can be used to

make an empirical estimate of secondary structure using several algorithms including least squares fitting, singular value decomposition, and self-consistent method (SELCON) (31,33). SELCON is quite popular for secondary structural estimates, and it deconvolutes decent structural information for both α-helical and β-sheet/turn structural components. However, depending on the quality of CD spectra, the estimation of structure can vary significantly, and therefore such estimates should not be used for comparability purposes. A better protocol for drug substance comparability is to compare and overlap normalized CD spectra of protein samples of which accurate protein concentration data (of the identical samples that are used in CD measurements) are available. Normalized CD values can also be expressed in molar residue ellipticity (i.e., also normalized for number of amino acid residues) that is useful to compare CD value/spectra between different proteins belonging to the homologous structural class.

Although CD spectra in far and near UV are very useful in assessing as well as comparing the secondary and tertiary structures of a protein, it is very challenging to reproducibly detect small structural changes. Because of uncertainties introduced by the measurement protocol as well as interference from the drug product formulation matrix, it is difficult to determine an accurate limit of quantitation of the method.

Fourier Transform Infrared

Fourier transform infrared (FTIR) spectroscopy is another tool for probing secondary structure of protein- and peptide-based biologics candidates (34,35). The vibrational motions in a molecule when coupled with a change in dipole moment can be observed, in principle, in an FTIR spectrum. However, several factors including overlap with rotational motions result in significant band broadening under normal conditions relevant to biologics formulations. Additionally, the changes in dipole moment need to be sufficient for actually observing a vibrational frequency. For peptides and proteins, typically the amide region is tracked for secondary structure determinations (34,35). The amide region has multiple frequencies but practically three of them (Amide I, Amide II, and Amide III) are most useful. Modern FTIR spectrometers are capable of producing high quality spectra in the mid-IR range of approximately 1000 to 1800 cm^{-1} that is useful for protein secondary structures. The low frequency range (<1000 cm^{-1}), if desired for detecting out-of-plane bending modes in polypeptides, can be studied using accessories with appropriate IR grade materials (crystal). For example, an attenuated total reflectance (ATR) accessory with diamond crystal and compatible optics can go down to approximately 200 cm^{-1}. In addition to protein-related vibrational bands, one can choose to probe signature bands from excipients (e.g., sucrose) and other additives present in biologics formulations. Finally, the CO stretching vibration of carboxylic acid-containing side chains and other vibrational modes from polar and aromatic side chains of amino acids also can be seen in FTIR spectra of proteins, but these are typically much weaker than amide I and II bands (35).

Protein FTIR spectra show a strong amide I band in the 1600 to 1700 cm^{-1} range arising from primarily C = O stretching of the polypeptide backbone. Amide II ($\sim$1480–1580 cm^{-1}) and III ($\sim$1230–1300 cm^{-1}) bands are comprised of CN stretching and NH bending modes. The amide bands are sensitive to type of secondary structure (e.g., α-helical, β-sheet, β-turn) and therefore the band pattern (intensity and frequency) in the amide region of an FTIR spectrum can be used to distinguish protein structural types. The origin of this sensitivity (frequency and intensity pattern) is attributed to hydrogen bond strength of amide CO and NH groups, and associated dipole orientations (collectively) present in a particular secondary structure type.

Unfortunately, interference from water (water bending frequency at $\sim$1645 cm^{-1} overlaps with amide I) is a major issue for most biologics formulations, especially for aqueous solutions. Water being the major component ($\sim$55 M) in aqueous formulations gives rise to a strong band that requires careful subtraction by a reference spectrum. Obviously, the water issue is minimized when the biologics formulation is freeze-dried to make lyophilized powder with low water content. Water interference as well as other measurement errors can lead to erroneous assignment of secondary structure types. Several practical measures have been proposed to avoid some of the artifacts (36). These include ensuring (a) appropriate amide I/II ratio (1.2–1.7), (b) presence of amide III bands, (c) presence of C-H stretching modes,

Table 3 Fourier Transform Infrared Frequencies of Amide I Band in Polypeptides

Structure type	Amide I frequency, cm^{-1}
α-Helix	~1654 (range 1640–1660)
β-Sheet[a]	~1633 (range 1620–1641)
	~1684 (range 1670–1695)[a]
β-Turn[b]	~1672 (range 1650–1690)
3_{10}-Helix	~1660–1670
Unordered structure (random coil)	~1654 (range 1640–1660)
Denatured aggregate[c]	~1615
	~1695

[a]β-sheet amide I is often characterized by a shoulder at ~1670 to 1695 cm^{-1} in addition to the major band at ~1620 to 1640 cm^{-1}.
[b]Assignment of amide I for β-turn is highly variable and should therefore be used with caution.
[c]Aggregates formed by native state or nearly native state of proteins may not exhibit amide I frequencies similar to denatured aggregates.
Source: From Refs. 35–38.

(d) appropriate subtraction of vapor bands, (e) no artifact from protein adsorption on sample cell or ATR crystal, (f) appropriate baseline of spectrum, and (g) mismatch of pathlength between sample and reference spectra. An ATR accessory is particularly useful for versatile applications including lyophilized powder, suspensions, liquid, etc., that provide adequate surface contact on the crystal. Diamond crystal is scratch resistant and may help avoid excessive protein adsorption, therefore eliminating some of the artifacts noted above.

Determination of secondary structure is often achieved by examining the amide I frequency or group of frequencies (Table 3). This is possible when a protein or peptide has predominant helical or β structure. However, if structure content is mixed, it is difficult to readily assign a structural type. Additionally, as seen in Table 3, the range of amide I frequencies of multiple structure types overlap significantly (e.g., frequency overlap of α-helix and unordered structure). For an unknown structure, one can get a qualitative estimate by using various algorithms including curve fitting, and pattern recognition such as factor analysis. The derived numbers for content of structural component types are only qualitative, and they can be quite sensitive to the quality of an FTIR spectrum. Therefore structure content determinations may not be suitable for QC (quality control) environment.

Collecting FTIR spectra of low-concentration protein formulations (1 mg/mL or less) can be challenging for the detection of amide bands. However, many of the commercial and clinical biologics formulations employ relatively high active concentrations; therefore FTIR can be suitably used. In fact, for very high concentration protein formulations, FTIR is one of the very few techniques that do not require sample dilution. To prepare samples for FTIR measurements, KBr pelleting has been extensively used. This could be a problem for some sensitive proteins. The modern applications (using ATR and other state-of-the-art accessories), however, do not require sample manipulation, and therefore enables higher throughput as well as application to a wide variety of biologics samples.

ASSESSMENT OF TERTIARY STRUCTURE

Disulfide Bond Determination

The tertiary structure of a protein is often highly dependent on the formation of disulfide bonds. Disulfide bonds confer physical stability to the protein as well as ensuring that it maintains its active form. For recombinantly produced proteins, the confirmation of disulfide bonds is a fundamental part of the elucidation of structure, and any variants present because of incorrectly paired disulfides needs to be assessed. The number and arrangement of cysteine residues in a protein can lead to significant complexity for the determination of the disulfide connectivity.

A typical approach for the determination of disulfides in a protein involves proteolytic mapping under nonreducing conditions, followed by detection of the resulting disulfide-bound

peptides formed, often using mass spectrometric detection (39). For small proteins with few cysteines, this may be straightforward. For larger proteins with many cysteine residues, the complexity may require additional analyses to map all of the disulfides. A parallel analysis, in which all of the disulfides are reduced, with a comparison of which peaks have changed upon reduction, can aid in the detection of which peptides are involved in disulfide bonding. IgG molecules, which are a major class of biotherapeutics in the form of monoclonal antibodies (mAbs), have several disulfides predicted in the constant and variable regions of the molecule. These disulfides serve to connect the heavy and light chains together and to form the intrachain loops necessary for the IgG to maintain its functions. Most of the commercial therapeutic mAbs are IgG1 molecules, which is the major subclass of the IgG class of molecules. The disulfide bonding of IgG1 molecules has been well established. IgG2 molecules have been under development as biotherapeutic entities for some indications because of their low level of secondary activity, such as antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). One IgG2 molecule, panitumumab, which is an antiepidermal growth factor receptor (EGFR) mAb, has been approved for use for the treatment of metastatic colorectal carcinoma (40). It was recently discovered that IgG2 molecules have an intrinsic heterogeneity in their disulfide connectivity, which leads to a mixture of at least three forms of disulfide isomers (41). These disulfide mediate isomers differ in the interchain disulfide bonds. As therapeutic entities, the levels of each form and their relative activities and properties are attributes that need to be determined.

Protein Intrinsic Fluorescence

Fluorescence spectroscopy is a powerful and widely used tool to monitor higher order structures in proteins (42). Most proteins have intrinsic fluorescence that originates primarily from Trp residues. Tyr and Phe residues also contribute to total protein fluorescence, although quantum yield of Tyr is much less than for Trp, and Phe is the weakest among the three. Fluorescence may also originate from other cofactors present in a protein such as flavin, porphyrin, etc. For most therapeutic proteins, Trp is widely used as a fluorescence probe because of its frequent presence in proteins as well as ease of use and wide applicability in formulation screening and characterization studies. The advanced uses of fluorescence include fluorescence lifetime measurement (time-correlated single photon counting method, phase modulation method in frequency domain), fluorescence resonance energy transfer (FRET), fluorescence correlation spectroscopy (FCS), single-molecule fluorescence, rotational correlation time by time-resolved anisotropy, decay-associated spectrum (DAS), and others. Discussion of these advanced uses is generally out-of-scope for this section.

When a fluorophore (e.g., Trp) is excited using a light source matching its absorption band (excitation wavelength) electrons are promoted from ground electronic state (S_0) to excited states (S_1, S_2, etc.). The cascade of events following excitation is often described by the Jablonski diagram (42). Fluorescence emission occurs from the lowest vibrational level of the excited state (S_1), and exhibits a red shift because of loss of energy in the process.

Trp(s) in proteins exhibit a relatively broad absorption band at approximately 280 nm. When excited at 280 nm, Trp emission occurs over a range of wavelengths up to approximately 450 nm and appears as a very broad band. Most folded proteins show Trp fluorescence emission maxima in the 320 to 350 nm wavelength range. Exceptions include azurin in which the Trp located in a highly hydrophobic environment exhibits an emission maximum at 308 nm—the most blue-shifted spectrum known of Trp in a protein. Typically, protein unfolding causes exposure of buried Trp to bulk solvents, and hence a red shift of the emission maxima to approximately 350 nm is observed.

A typical steady-state (i.e., not a fluorescence lifetime study) fluorescence measurement is quite straightforward. However, several precautions should be taken to avoid artifacts. An appropriate concentration of the protein or peptide in solution should be chosen to ensure that absorbance at 280 nm or the chosen excitation wavelength is not far greater than approximately 0.1 OD. High absorbance at or following the excitation wavelength causes nonlinearity and an artificial reduction of emission intensity called “inner filter effect” (loss of emitted photons due to absorption). Inner filter effect is caused by high absorbance of any

component in solution including protein, excipients and other additives if it overlaps with the emission wavelengths, and may lead to incorrect conclusions from fluorescence data, reported in literature [caused by sodium dithionite absorbance, (43,44)]. It is possible to collect partial emission spectra of higher concentration protein solutions (up to $\sim$0.5 OD at 280 nm) at $>$310 nm emission range and using a higher wavelength excitation (e.g., at 295 nm). However data interpretation must be conducted with great care keeping in mind that emission intensity may not be proportional to lower protein concentrations.

Appropriate baseline correction should be performed by subtraction of a reference spectrum of matching solvent. This is particularly important for low-concentration protein solutions and when quantum yield of Trp is very low (such as quenched by heme/metal, or Trp is oxidized). In these cases, the relative intensity of the water Raman band may appear as a prominent shoulder or peak in the emission spectrum. Location of the Raman band ($\sim$3450 cm^{-1}) in a fluorescence spectrum depends on the excitation wavelength (for 295 nm excitation, it appears at $\sim$329 nm).

Choice of excitation wavelength depends on what fluorophore is used as a probe. If a protein contains both Trp and Tyr residues, one can use either 280 nm or 295 nm to collect fluorescence contribution, respectively, from Trp plus Tyr or Trp only. If there are multiple Trps present in a protein, they all contribute to the emission spectrum. Therefore, if a change in fluorescence intensity and/or emission maximum is observed in a multi-Trp protein (such as a mAb), it is not easy to interpret the data because of the large number of possibilities as causative factors including local conformational change, global structural change, solvent effect (if relevant), quenching due to charge, quenching by oxygen/additives/side chain/bound groups/disulfide bond, change of quenching efficiency of quenchers present in native state, and many others.

Measurement of fluorescence lifetime is generally recognized as providing a more quantitative estimate of some of the fluorescence events. For example, if a fluorescence dye partitions itself between hydrophobic and solvent-exposed environments, simplistically it may yield two distinct lifetimes and one can determine the percentage population of each of the components. Measurement of Trp lifetime may not always help because each single Trp displays two prominent lifetime components arising from two rotamers (42,45,46). Therefore multi-Trp proteins are comprised of (theoretically) several lifetime components; however, there are practical difficulties of how many discrete lifetimes can be retrieved from fluorescence decay data. Analysis involving more than four lifetime components is unreliable, but one can employ lifetime distribution analysis aided by sophisticated mathematical algorithms such as Maximum Entropy Method (47). Trp lifetime data can sometimes help in understanding the impact of solvent relaxation and dynamic quenching.

The sensitivity of Trp fluorescence emission maximum in proteins is generally interpreted as excited Trp (indole ring) interacting with its microenvironment (45,46). For example, in azurin (also noted above), Trp side chain is surrounded by a nonpolar environment, whereas if the excited state interacts with a polar solvent or charged/polar side chains, it emits in the red. Emission maxima as well as quantum yield are also influenced by intramolecular quenching (for example, Fe-porphyrin in cytochrome c, Cu in hemocyanin).

For practical applications of Trp fluorescence in formulation characterization as well as for comparability purposes, steady-state fluorescence studies are quite sufficient to probe conformational changes or unfolding of a therapeutic biological candidate because of high sensitivity of fluorescence signal to local environment of Trp and high signal-to-noise ratio of fluorescence signal. The major goal in the application of Trp fluorescence spectroscopy in a comparability study is to interpret the fluorescence properties such as emission maxima and fluorescence intensity in terms of changes in protein structure. In other words, it is expected that comparison of fluorescence spectra will detect any significant changes in folding and structure of a biologics candidate arising from changes in manufacturing and process. Fluorescence quenching studies using acrylamide and sodium iodide provide valuable information on surface exposure of Trp. A conformational transition may change the exposure of Trp to solute quenchers (acrylamide, iodide, or CsCl), hence can be monitored by measuring Trp quenching (45). Steady-state fluorescence anisotropy is another fluorescence protocol that can be used to study rigidity (or lack of) of a protein segment and relative size of a protein.

Anisotropy value can change upon unfolding of a protein or complexation of a protein (e.g., aggregation, antigen binding).

Tyr fluorescence is less commonly studied because of its weaker fluorescence relative to Trp. Tyr absorption band appears at ~277 nm (tyrosinate at ~294 nm) and the corresponding fluorescence emission maximum is at ~303 nm (~340 nm for tyrosinate emission). Although the microenvironment of Tyr may have a strong effect on its emission intensity, the emission maximum of Tyr is relatively insensitive to local environment (48), in sharp contrast to the behavior of Trp.

Use of external fluorescence probes is very popular in all areas of biology, biological chemistry, and protein chemistry. There are literally thousands of fluorescent dyes for various purposes. For formulation characterization, a few of them are worth noting in this section. ANS (8-anilino-1-naphthalenesulfonic acid) and bis-ANS are used traditionally to probe hydrophobicity and change in surface exposure of hydrophobic groups in a protein. Thioflavin T and Congo red are generally used to look for the presence of amyloid-like structure (aggregate). Nile red is also known to be sensitive for aggregate detection.

POST-TRANSLATIONAL MODIFICATIONS

Most proteins are modified in some way after translation of the polypeptide chain. These modifications may impart specific function to the protein and can be integral to the protein activity or stability. For biotherapeutic proteins, common post-translational modifications include disulfide bond formation, N-terminal acetylation, or glycosylation. Degradation of amino acid residues can be considered as post-translational modifications, but are typically discussed separately as part of stability. However, the tools used for analysis of many types of post-translational modifications are the same. The types and propensity of these modifications are dependent on both the protein and the expression system used for its production. Some of the most common modifications and degradation products observed for biotherapeutic proteins are discussed below.

Glycosylation Analysis

Glycosylation of proteins is a common post-translational modification which can affect the physical properties and activity of the biotherapeutic protein. Glycosylation has been shown to affect the activity, *in vivo* clearance, immunogenicity, and stability of biotherapeutic proteins (49,50). For these reasons, the levels and types of glycosylation need to be determined and controlled for biotherapeutic proteins.

Glycoproteins can be either N-linked or O-linked, depending on the type of covalent modification of the glycan to the protein. The type of glycosylation is dependent on both the protein sequence and the expression system used to produce it. Glycosylation may commonly occur for proteins expressed in mammalian or yeast expression systems, but is not observed for proteins expressed in bacterial systems. N-linked glycosylation occurs only at asparagine residues in the consensus sequence of Asn-Xxx-Ser or Asn-Xxx-Thr, where Xxx is any amino acid except proline. While the presence of this sequon does not guarantee glycosylation, it makes N-linked glycosylation a predictable attribute. The amino acid sequence can be easily scanned for this sequon to determine if N-linked glycosylation is a possibility for a given biotherapeutic protein. Analysis of N-linked glycosylation, therefore, begins with an assessment of the site occupancy levels of any possible N-linked glycosylation sites in molecule, referred to as the macroheterogeneity. This can be accomplished using analytical methods which can distinguish size variants, such as electrophoretic or chromatographic separations, or MS. For glycoproteins with multiple glycosylation sites, macroheterogeneity can lead to complex mixtures. For example, the therapeutic glycoprotein interferon γ (IFN-γ) has two sequons for N-linked glycosylation. Therefore, there are four theoretical forms on the basis of occupancy alone: unoccupied, two different singly occupied forms, and one fully occupied form.

The identities of the glycans at a specific site can be extremely varied as well, contributing to additional complexity termed microheterogeneity. Microheterogeneity can be assessed by isolating the glycans associated with a given site and determining the glycan identity. There are a wide variety of analytical methods and approaches for assessing the levels

and identities of glycans present in a biotherapeutic protein. The methods used depend highly on the specific molecule being analyzed, the type of instrumentation and skill available in the laboratory performing the analyses, and the level of detail required for regulatory approval. For routine batch release of a glycoprotein, profiling for consistency may be appropriate, while more detailed structural characterization may be required to satisfy Elucidation of Structure expectations.

For N-linked glycans, there are enzymes such as PNGaseF, which are efficient at removing glycans, which can then be identified using orthogonal methods. Typically, chemical labeling of released glycans is necessary, since they lack a chromophore and thus a sensitive detection method. A common method for quantifying released N-linked glycans, termed glycan size profiling, employs enzymatic release of the glycans, removal of the protein by precipitation or filtration, labeling of the glycans with a fluorophore, and separation of the labeled glycans using normal-phase HPLC (NPLC) with fluorescence detection (51). This method is highly quantitative, since each glycan has one fluorescent label. For this reason, it can be used for routine batch release to ensure consistency in the types and levels of glycans.

Charge profiling is a common method for the determination of the relative amount of charged, or sialic acid containing, glycans. In this method, the glycans are prepared identically to size profiling: enzymatic release of the N-linked glycans followed by fluorescent labeling. The glycans are then separated by anion exchange chromatography, which separates neutral from singly charged from doubly charged glycans. This yields the relative levels of sialic acids in the glycan population.

There are several types of sialic acids possible, and these types depend on the production cell line. For example, murine cell lines such as NS0 produce mainly *N*-glycolylneuraminic acid, while CHO cell lines produce mainly *N*-acetylneuraminic acid. These sialic acid types can be distinguished using sialic acid typing, in which the sialic acid residues are removed from the glycans by acid hydrolysis, labeled with a fluorescent tag, and separated by reversed-phase HPLC. The identities of the sialic acids are determined by comparison of the retention times to a sialic acids reference panel of standards.

Glycan structure determination includes the assessment of monosaccharide composition, the sequence of the monosaccharides, the branching heterogeneity, and the linkage heterogeneity. While known structures can be confirmed using authentic standards, unknowns require a combination of methods, including MS and linkage-specific enzymes. The identities of the glycans can be determined using MS or by analysis of authentic standards. MALDI-TOF or electrospray MS of the released, labeled glycans yields accurate masses, which can be compared with the theoretical masses for confirmation of identity. For detailed structural information, tandem MS may be performed, which can be used for the determination of the sequence, linkage, and branching (52). In combination with chemical labeling methods such as permethylation or peracetylation, linkage information can be determined as well. The types of MS instruments utilized for tandem MS experiments include quadrupole time-of-flight (Q-TOF) hybrid instruments, which have an advantage of yielding accurate mass of product ions, or ion trap instruments, which are capable of multiple stages of fragmentation (MS^n) for potentially increased structural information. The degree of characterization performed is dependent on the nature and requirements of the molecule being developed.

Charge Heterogeneity

Biotherapeutic proteins may have intrinsic heterogeneity based on charge variants. These variants may be due to a variety of sources, including but not limited to, glycosylation with acidic or basic glycans, variably processed or modified N- or C-termini, degradation due to deamidation or cyclic imide formation, other modifications to basic or acidic residues, or peptide bond hydrolysis.

Deamidation may be a major degradation pathway for peptides and proteins containing asparagine residues. The mechanism for asparagine deamidation, shown in Figure 6, involves loss of NH_3 via a cyclic intermediate. The cyclic imide intermediate can be hydrolyzed to yield two potential products: an aspartic acid or an isoaspartic acid, which is a β amino acid. Both of these products are acidic variants of the original polypeptide and can be separated using charge-based separation methods, and both generate a change in mass of 1 Da relative to the

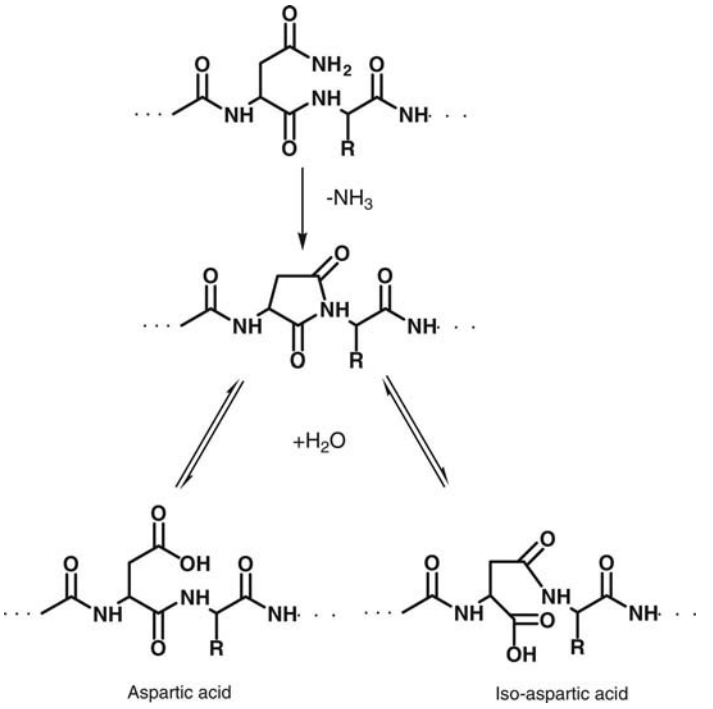

Figure 6 Mechanism for deamidation of an asparagine residue to form aspartic acid and isoaspartic acid via a cyclic imide intermediate.

original polypeptide. The degree of degradation is dependent on many factors, including neighboring residues, surface accessibility and conformation, and the pH of the formulation. The susceptibility and rate of deamidation of a given asparagine residue has been shown to be greatly influenced by the N+1 residue (1). Glycine in the N+1 position has been shown to give the highest rate of deamidation, followed by His, Ser, and Ala. A similar mechanism may occur for aspartic acid residues, in which cyclization followed by hydrolysis to yield either the starting material or its isomer, the isoaspartic acid residue may form. In this case, there is no difference in charge or mass relative to the original molecule. Exceptions to this are examples in which the cyclic imide intermediate is stable. For these species, the cyclic imide has a net basic shift in charge relative to the aspartic acid starting molecule, and is 18 Da lower in molecular mass.

Pyroglutamic acid formation is a common modification for proteins, and occurs spontaneously when the N-terminal residue is a glutamine, or less commonly, a glutamic acid. The formation of pyroglutamic acid from an N-terminal glutamine residue generates a net acidic shift and a loss of 17 Da. This is due to cyclization with the N-terminus with the loss of NH_3 from the side chain, which blocks the N-terminal amine. For monoclonal antibodies, N-terminal glutamine and glutamic acid residues are common for both heavy and light chains, and pyroglutamic acid formation is a very common post-translational modification for IgG molecules (22).

For monoclonal antibodies, variable levels of C-terminal lysine on the heavy chains lead to charge heterogeneity as well. The conserved heavy chain sequence of IgG molecules predicts a C-terminal lysine residue. This residue has been observed to be removed as a post-translational modification, and is thought to be due to proteolysis in the cell leading to a heterogeneous population (53). Typically, a mixture of species exists in which zero, one, or two heavy chains have the lysine removed.

Other modifications leading to charge heterogeneity include glycation of lysine (acidic shift), carbamylation of lysine (acidic shift), C-terminal amidation (basic shift), and N-terminal acetylation (acidic shift). These potential modifications of biotherapeutic proteins need to be detected, quantified, and controlled using analytical methods appropriate for their detection and quantification.

There are a variety of methods that are useful for detecting, characterizing, and quantifying charge variants in proteins. These include isoelectric focusing (IEF), capillary IEF, and ion exchange chromatography (IEC). The advantages of these methods are that they can separate and quantify overall charge heterogeneity. However, they give little or no information concerning the types or sites of charge heterogeneity present in the molecule. For monitoring stability, inherent charge variability may interfere with the ability to monitor degradation using these methods. An example would be the assessment of deamidation in a glycoprotein in the presence of significant heterogeneity in sialic acid levels. So, while these methods may be appropriate for routine batch release and monitoring of consistency, more detailed characterization is required to gain information on the presence of specific modifications leading to charge heterogeneity. Site-specific information can be assessed using approaches involving proteolysis and LC/MS. This approach can be used to characterize and quantify, for example, deamidation at a specific site in the presence of inherent heterogeneity elsewhere in the molecule.

IEF is a gel-based method which separates analytes in an immobilized pH gradient (54). Proteins will migrate in an electric field to their isoelectric point (pI), which is the pH at which the overall charge is net neutral. Charge variants can be well separated using this technique, with resolution as high as 0.01 pH units. The resolution can be determined by the gradient used in the separation. For high resolution separations a very narrow pH gradient may be used with long focusing times. IEF offers a reproducible method for establishing consistency of batches with regard to charge variants, as well as a powerful method for monitoring stability of protein therapeutics. This method is tried and true, but is not highly quantitative because of the need for general protein staining and densitometry, both of which may be highly variable.

Capillary IEF offers the possibility of high resolution with a more reproducible quantitation (55). In capillary IEF, the species are focused in a capillary to their pI, and then migrate to the detector using either electroosmotic flow or differential pressure. Detection can be performed by UV absorbance, offering reproducible and automated quantitation. For more sensitive detection, laser-induced fluorescence (LIF) detection can be used. Another mode of capillary IEF, termed imaged capillary electrophoresis (iCE), offers detection in the capillary without a mobilization step (56). This leads to increased resolution because no band broadening occurs as a consequence of the mobilization step. Also, detection is based on imaging of the entire capillary, so quantitation is generally more reproducible. This method offers high quality, quantitative data for charge variants. The acidic and basic species can be reproducibly quantified using this method.

IEC is a powerful method for separating charge species in an HPLC format (57). IEC separates charged species on the basis of electrostatic interactions of the analyte with a column resin. Anion exchange resins are positively charged, and bind negatively charged analytes, while cation exchange resins are negatively charged, and bind positively charged analytes. In an anion exchange separation, more acidic, or negatively charged analytes, will be retained more strongly and will elute later than less acidic species. Ion exchange resins can be considered strong or weak, depending on the type of resin used. A typical strong anion exchange resin contains a quaternary amine, which has a fixed positive charge or other strong basic species, and tightly binds negatively charges species. Weak anion exchange resins have basic species such as diethylamine functional groups, which bind negatively charged species, but not as tightly as strong anion exchange resins. Conversely, cation exchange resins are either strong acids, such as sulfate groups, or weak acids, such as carboxymethyl groups. Elution of analytes from ion exchange resins can be obtained using a salt gradient to compete with the charge on the resin, or by changing the charge on the analyte by changing the pH of the mobile phase over the course of the separation. As a consequence of IEC being an HPLC method, it has advantages over IEF methods in terms of throughput, ease of use, and quantitative precision. HPLC methods are extremely valuable in that individual fractions of separated species can be collected and characterized further. In an IEC separation, for example, an acidic variant can be separated and quantified, collected, assessed for potency relative to the parent molecule, and further characterized using orthogonal methods to determine the specific site(s) of modification in the molecule. This type of further characterization would be difficult or impossible using capillary electrophoretic methods.

Size Heterogeneity

Size heterogeneity of recombinant proteins may refer to truncated variants because of peptide bond hydrolysis or to the formation of aggregates.

Truncated Species

For truncated variants due to peptide bond cleavage, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a common method used for analysis. SDS-PAGE is based on the migration of a protein in an electric field. The proteins are first treated with SDS, which coats the proteins with a polyanion. In this way, the proteins are coated to similar size to charge ratios, and migration through a polyacrylamide gel is dependent on the size of the protein, with smaller proteins migrating faster through the gel, and larger proteins migrating a shorter distance. By comparison with standards, the molecular weight of the analytes can be estimated on the basis of the migration distance through the gel. Just about any protein analysis laboratory is set up to perform routine SDS-PAGE analysis as a first step in characterization. It gives a visual assessment of the quality of the material in terms of the purity and size heterogeneity. It can be extremely flexible in the type of detection used. General protein stains such as Coomassie blue are reliable and give a visual readout for the detection. For more sensitive detection, silver stain or some of the fluorescent stains such as Sypro Ruby offer the highest sensitivity. Finally, gels offer the possibility of immunoblotting or immunostaining of analytes once they are separated, which provides some additional functional information of the species being separated. Like other gel methods, SDS-PAGE is labor intensive and suffers from a difficulty in reliable quantitation. For routine characterization, SDS-PAGE is a reliable method for assessing the size heterogeneity of a formulated protein product, and is a powerful method for comparison of batches or stability in different formulations.

Capillary gel electrophoresis (CGE), also referred to SDS-CE, has the ability to resolve proteins from 10 to 200 kDa. It offers similar resolution to gel-based separations, but is more easily quantifiable because of the detection methods used (UV absorbance). Like SDS-PAGE, the proteins are coated with SDS and are separated on the basis of migration through an electric field, although in this case through a capillary. Since this method utilizes UV absorbance detection, the sensitivity may be limited for low levels of size variants that in gels could be detected with sensitive staining techniques such as silver stain. LIF detection can help overcome this limitation when applied to CGE. In combination with fluorescence labeling, LIF can lead to sensitive detection of separated species, including low level impurities and truncated variant species (58).

Aggregates and Particulates

Aggregation is a process in which one or more drug molecules combine physically and/or chemically to form nonnative oligomers which may remain soluble or become insoluble depending on their size and other physical properties. Protein aggregates and particulates form in a wide range of sizes (nanometer to centimeter, thereby spanning nearly million-folds in dimension) and shapes making it extremely challenging to comprehensively characterize particulates in a biologics formulation (59,60). Dimers and other smaller size aggregates are soluble in nature and typically range in size from few nanometers to tens of nanometers. The aggregate species that are in the size range of hundreds of nanometers may still remain soluble in the sense that they may not exhibit any change in appearance of the formulation. Some of the multimers can grow huge in size and may eventually appear as visible particulates. Although all multimeric species (referring to degradation products only and not a purposefully created multimeric therapeutic candidate) are generally termed as aggregates, particulates refer to the large size aggregates in the size range of tens of microns or larger species that are visibly detected. Subvisible particulates range in size from few microns to many tens of microns. An approximate size boundary for a particulate to be visibly detected is 100 μm (59).

Characterization of aggregates typically includes detection of soluble aggregates on the basis of size, and determination of physicochemical properties and nature of aggregated

species such as covalent, noncovalent, reversible, irreversible, etc. Covalent aggregates are generally irreversible. Example of a covalent aggregate is disulfide scrambled species, which often are dissociable by a reducing agent. Noncovalent aggregates can be held together either by strong association (i.e., not dissociated by simple dilution or mild treatments) or weak association (i.e., may be reverted to monomer by dilution). Aggregates in both of these structural categories can cover a wide range of sizes. Therefore, it may be more convenient to classify the aggregates in terms of their size in reference to the capability of various biophysical and particle analysis technologies. Insoluble aggregate is also referred to as particulate (subvisible and visible—as noted above), and precipitate (large size species that easily sediments). Insoluble aggregates require somewhat specialized protocols including enumeration (using light obscuration, light scattering, and light microscope) and characterization (imaging-based techniques, and spectroscopic methods such as FTIR or Raman), depending on the types of aggregates observed. Finally, finding the root cause of aggregation may involve all of the above and additional custom-designed protocols.

Formation of aggregates may occur under conditions such as storage, shipping, handling, manufacturing, processing, and freezing-thawing. One of the most challenging areas in aggregation, lately, is studying aggregate formation induced by freezing and thawing of biologics. It should be noted that the freeze-thaw induced aggregation phenomenon should not be confused with cold denaturation. Cold denaturation classically refers to denaturation induced by thermal factors per se without a change in the state of the bulk, and is linked to thermodynamically favored hydration of the hydrophobic core at low temperature (61). Freeze-thaw induced aggregation has been linked to secondary factors such as ice surface denaturation, freeze-induced change in solute concentration and pH, etc. (62), but theoretically may also include effects of cold denaturation. The study of freeze-thaw-induced protein denaturation and aggregation requires specialized equipments and protocols that can probe events in the frozen state.

Several aggregate separation methods are available depending on the type of information sought. Separation methods may either detect the presence of various species in a drug formulation [such as dynamic light scattering (DLS) and analytical ultracentrifugation (AUC)], or fractionate various species [such as size exclusion chromatography (SEC) and asymmetric flow field flow fractionation (aFFF)]. Fractionated species, if desired, may be collected for further analysis.

SEC is considered a "work horse" technique, especially for biologics, and major advantages include high throughput, automation, amenability to several detection systems, reproducibility, reliability, and operational compatibility in both the development and QC environments. Major applications include separation of drug monomer from higher molecular weight species that might accumulate during storage stability and processing. Disadvantages of SEC include concern for potential alteration/dissociation of aggregated species as a result of column/mobile phase interactions. Also, for a given biologics, the dynamic range for separation of various aggregated species is rather limited leaving large aggregates unfractionated or lost.

All four methods noted above (SEC, aFFF, AUC, DLS) are used in formulation characterization to monitor aggregate formation and to delineate the aggregation mechanism. Only SEC is used in a QC environment such as in GMP stability studies. Use of the other three techniques (AUC, aFFF, and DLS) in a QC environment is quite challenging because of the difficulty in adequately validating the methods and/or their low throughput. More detail of these techniques is covered in the last section of this chapter.

Although a relatively smaller number of techniques are available to study large particulates including protein precipitates, additional characterization can be accomplished by solubilizing the particulates using dispersing/denaturing solvents. Multiple biochemical assays can be utilized with solubilized particulates including SDS-PAGE or CGE. This characterization approach can be employed to estimate aggregate size after solubilization and determine if there are covalent linkages between protein molecules. However, the influence of hydrodynamic size may result in an inaccurate estimate of molecular mass for certain molecules, such as conjugated or pegylated proteins.

FORMULATION CHARACTERIZATION METHODS

Selected formulation characterization methods are described below. Use of these methods depends on the type of formulation (e.g., liquid, lyophilized powder, etc.), stage of clinical development, and type of information sought (e.g., to solve a process-related issue, to characterize a degradant, or delineate a stability issue).

Analytical Ultracentrifugation

AUC is an orthogonal method for size-based separation of high and low molecular weight species that employs centrifugal principles to determine size and shape (60). Two principal types of experimentations are conducted in AUC—sedimentation velocity and sedimentation equilibrium. Sedimentation rate (velocity) of the protein species (monomer, dimer, etc.) present in solution is measured as sedimentation coefficient which is governed by several factors including molecular mass, conformation and solvent properties.

AUC measurement does not involve any matrix (column, membrane) interactions, does not dilute the measured sample, and covers a wide size range, especially when multiple measurements are conducted using various centrifugal speeds. AUC typically uses absorbance as a probe. Additionally, interference (for higher concentration) and fluorescence (low-concentration) probes are also available. Therefore, AUC offers an independent confirmation of the presence of any aggregate species in a biologics formulation measured by SEC.

Equilibrium studies employ low centrifugal force to achieve a diffusion-controlled equilibrium, and are typically used to determine molecular mass as well as equilibrium binding constants (e.g., monomer-dimer reversible transition). One of the important applications of equilibrium studies in biotherapeutics is to detect any self-association (reversibly aggregated species).

AUC suffers from low throughput of measurement, lack of robustness, and artifacts from solvent and high concentration formulations. The majorities of the commercial as well as clinical biologics formulations cannot be studied "as is" with the absorbance probe, and require dilution. Although the concentration limit can be pushed higher by the use of an interference probe, several sources of "nonideality" (high concentration, sensitivity to excipients, protein shape factor) can cripple data interpretation. Because the sedimentation profiles by themselves do not provide an estimate of the protein species present, one needs good data analysis software to derive relative quantity of individual species. This is in contrast to SEC quantification, which relies on relative UV absorbance. Several instrument configuration parameters (rotor, cell, loading, probe alignment, wavelength, etc.) add sensitivity to analyzed data, and therefore it can be quite challenging to achieve consistent quantitative results.

Asymmetric Flow Field Flow Fractionation

aFFF uses cross flow onto a membrane in a channel with parallel flow where the smaller particles are transported more rapidly along the channel than the larger particles, hence achieving separation. Prior to migration of particles is initiated, the injected sample is focused onto a narrow area. aFFF is considered an orthogonal method of aggregate separation, using no column (a difference from SEC), and to achieve a wider dynamic range. However, potential interactions with membrane and concern of aggregate formation during its focusing step make aFFF rather unreliable (63). Like SEC, aFFF can also use one or more probes such as light scattering, UV, and refractive index to detect and characterize the fractionated species by hydrodynamic size, molecular weight, and conformational difference.

Light Scattering (Static and Dynamic)

Static Light Scattering

Static Light Scattering measures time-averaged value of scattered light intensity from a sample, typically over many seconds. SLS is used typically in conjunction with separation techniques such as SEC or aFFF. The intensity of the scattered light depends on protein concentration as well as scattering angle, and it is related to radius of gyration, hence molecular mass. SLS provides quite reliable determination of molecular mass of protein monomers and aggregates. For large size particles (such as protein aggregates larger than $\sim$60 nm, depending on wavelength of incident laser) angular dependence is significant, and measurement at several

angles can produce useful data on size. For most protein monomers ($< \sim 10$ nm) such angular dependence is diminished, and measurement at a 90° angle can be used to determine mass.

Dynamic Light Scattering

DLS relies on measuring fluctuations (microsecond and longer time scale) of the scattered light caused by Brownian motion of molecules in solution, and therefore relates to diffusion coefficient (63). With spherical approximation, hydrodynamic radius (Rh) can be extracted from diffusion coefficient values. DLS provides a relatively easy and fast measurement of size (Rh), and covers a large dynamic range (~ 1 nm to ~ 1 μm) in one single measurement. Additionally, the measurement can be done with liquid/suspension of formulated API or drug product without any alteration/dilution (unless strength is greater than approximately 0.3 mM). However, it can resolve species of various sizes only if their hydrodynamic sizes differ by more than 2-fold to 5-fold. This is a serious disadvantage because, for example, monomer and dimer cannot be separated by DLS, instead an average value of size will be measured.

DLS is also referred to as photon correlation spectroscopy (PCS) or, quasielastic light scattering (QELS). Some of the DLS equipments are also configured to measure zeta potential.

Imaging (Static and Dynamic)

Microscopy is an established technique for studying protein particulates. Typically, it requires the particulates to be filtered and examined in static mode. Microscope images can be used for enumeration (pharmacopeia method) as well as for directly visualizing size and shape. Advances in imaging technology enable analysis in dynamic mode where the particles remain suspended in fluid either in stationary or flow modes (59). Digital images of particulates are collected and analyzed to provide a digital archive of particle parameters such as Feret diameter, aspect ratio, circularity, and intensity. Also, if particulate formation in a protein formulation is relatively slow, the dynamic nature of size distribution can be tracked over time. Such data are valuable to characterize particulate formation during biologics formulation development as well as to find potential prevention strategies. Disadvantages include the inherent complexity in determining a true size distribution from imaging data for biologics particulates because of their often extreme irregularity in size and shape. Finally, the size distribution and particulate count from dynamic imaging cannot be directly compared with such information obtained from light obscuration or laser-diffraction analyses (63).

Raman Spectroscopy

Raman spectroscopy, discovered by C.V. Raman (64), is a powerful tool to record the vibrational frequency pattern of a molecule that can be used as a fingerprint for identification. Raman spectroscopy uses laser excitation in modern applications, and frequency shifts caused by the probed molecule relative to the excitation frequency are recorded to generate a Raman spectrum. It is a powerful tool for small molecule pharmaceutical applications that include API identification, determination of tablet depth, and study of polymorphs. Unlike FTIR, Raman spectra have minimal interference from water and therefore the technique is quite suitable for studying aqueous biologics formulations. But protein fluorescence is a significant problem, and Raman bands might completely disappear with elevated background from highly fluorescing proteins, especially in the near-UV region. For proteins, amide bands can be conveniently detected in the ~ 1200 to 1700 cm^{-1} spectral range (analogous to FTIR spectrum) for secondary structure determination. A more advanced application of Raman spectroscopy is Resonance Raman that uses a laser frequency excitation overlapping with a particular protein absorption band (chromophore). This enables detailed structural analysis (including local tertiary structure) of the desired chromophore (e.g., Tyr).

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) is capable of measuring thermally induced transitions, and particularly the structural transitions of biological macromolecules, such as between the folded and the unfolded structure of a protein. DSC measures the excess heat capacity of a protein solution (Cp) as a function of temperature and the structural transition is recognized as

a sharp endothermic peak centered at the melting temperature (T_m). Generally, DSC is useful to study the energetics of protein thermal unfolding. The T_m of liquid protein formulations is often used as a probe for protein physical stability, that is, higher T_m value may indicate greater physical stability. However, it is recognized that comparisons of physical stability of different classes of proteins by T_m may not hold true. T_m values are also known to be sensitive to the solution matrix such as excipients, pH, buffer and surfactants. However, no clear correlation exists. While some of the stabilizing components may increase the T_m value, some of the stabilizers (e.g., surfactants) may actually result in lower T_m values. Because T_m cannot reliably predict physical stability, establishment of critical parameters in formulation screening and characterization should not be based solely on DSC data. Finally, for monoclonal antibodies, quite often multiple T_m values are observed which are typically assigned arbitrarily to structural domains on the basis of available T_m data of isolated domains in similar protein class. This makes structural interpretation of changes in T_m values as a function of pH or other additives very challenging.

Isothermal Titration Calorimetry

ITC measures heat change from binding interactions, such as antibody-antigen binding or receptor-ligand binding. It is quite versatile and can be applied to a wide variety of molecules in solution without any pretreatment (such as fixation of matrix). ITC can also detect weak interactions with dissociation constants in the sub-millimolar range. Appropriate control experiments must be conducted as several sources of heat change (e.g., heat of dilution) can introduce artifacts.

Near-Infrared Spectroscopy

A near-infrared (NIR) spectrum (12,000–14,000 cm^{-1}) represents combination and overtone bands that are harmonics of absorption frequencies in the mid-infrared region. Because each material has a unique NIR spectrum, NIR spectroscopy can be used as a positive identification of material. NIR is a versatile technique with reduced or eliminated sample preparation, decreased cost and analysis time, and the ability to record spectra through glass and packaging materials.

NIR measures vibrational spectra of a wide variety of materials including solids, liquids, powders, pastes and tablets. NIR has a variety of applications in the area of microbial and cell culture system monitoring and control. An important pharmaceutical application in injectables development is moisture analysis of freeze-dried samples without opening the vials. Determination of water content employs the strong water absorption bands in the NIR region, most prominently the first overtone of OH stretching at around 6800 to 7100 cm^{-1} and the combination band of OH stretching and bending at around 5100 to 5300 cm^{-1}. Karl Fischer method is the most commonly used method for measuring moisture content but it is a destructive method, may need method development, and requires careful handling of sample to not allow additional moisture when a vial is opened. On the other hand, NIR offers increased efficiency in measurement time (higher throughput than Karl Fischer) and reduced cost (especially for expensive biologics products) because the vials can be reused to conduct other assays following NIR measurement. It should be noted however that a standard curve must be generated and requires method development to establish a robust NIR protocol for moisture analysis. If formulation composition is changed, NIR spectrum may also change and additional method development may be necessary.

Powder X-Ray Diffraction

The primary use of powder X-ray diffraction (XRD) in characterizing biologics formulation is to probe the presence of amorphous and any crystalline states in the freeze-dried form. It also can detect the presence of polymorphs of certain excipients such as mannitol. Additionally, the X-ray diffraction can be used to study the phase behavior of the frozen state of protein solutions as well as placebos, using low-temperature accessories (65). Low-temperature XRD is a powerful tool to identify the phases that crystallize during cooling and annealing of frozen solutions.

Freeze-dried powder is often characterized for its crystalinity or amorphousness by the presence or absence of sharp diffraction peaks in the XRD profile. If formulations contain

mannitol, it is important to establish if any of the mannitol polymorphs are present. Mannitol is known to crystallize in multiple forms such as α, β, and δ forms. It also forms a metastable hydrate form that might negatively impact the stability of the freeze-dried formulation.

SUMMARY

Biotherapeutic entities include a variety of macromolecular compounds, each with distinct biochemical and biophysical properties. Extensive structural characterization must be performed for these molecules to be approved as drugs by worldwide regulatory agencies. Characterization should typically include assays to demonstrate that the molecule has the expected primary, secondary, tertiary structure, as well as the expected bioactivity. Any post-translational modifications or variant forms need to be adequately described in terms of the levels and any therapeutic effects such as potency or in vivo half-life. Similarly, the sponsor must demonstrate that the molecule has adequate chemical and physical stability for the proposed shelf-life in its formulation, and that the degradation pathways are well-understood. The analytical toolbox required for these investigations can be extensive, and a suite of assays specific for the biotherapeutic entity can be tailored to provide the required information.

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9 Formulation of protein- and peptide-based parenteral products

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INTRODUCTION

Since the early 1970s, scientific advances in molecular biology and genetic engineering have led to enormous success in protein- and peptide-based therapeutics for the treatment of many human diseases. They cover almost all therapeutic categories, including cardiovascular hemostasis, antineoplastic, diabetes and endocrinology, anti-infective, neuropharmacological, enzyme replacement, wound healing, respiratory, and bone cartilage. Protein-based therapeutics are emerging as a major class of new molecular entities in the pharmaceutical industry. Over 200 biotechnology and pharmaceutical companies are developing protein-based therapeutics. More than 150 biologics are currently marketed, and over 400 are in advanced stages of testing and clinical trials (1).

Unlike small molecules, which are typically synthesized through chemical processes, proteins are produced in living systems. The main technology used to produce proteins utilizes recombinant DNA techniques to produce protein molecules in a host cell. Several types of host cells have been employed, including *Escherichia coli*, yeast, mammalian cells [e.g., Chinese hamster ovary (CHO) cells and human fibroblasts], and plant-derived cells. Several other technologies are also used to produce therapeutic proteins. Small proteins and peptides, such as calcitonin, may be produced by chemical synthesis. Most human serum albumin is sourced from human blood, urokinase from urine, and streptokinase from fungi. Recombinant human antithrombin (ATryn®), a new product approved by the FDA in 2009, is produced by transgenic animals.

CHARACTERISTICS OF PROTEINS AND PEPTIDES

Compared with small-molecule drugs, protein-based pharmaceuticals are not only larger in molecular weight, but they also contain more complex compositions and higher order structures. Intrinsically, most proteins have poor stability and a very short half-life in vivo. Because of their poor oral bioavailability, most proteins require parenteral administration routes. In some cases, they require specific delivery systems targeting the specific site of action to achieve sufficient efficacy. Therefore, formulating these proteins as therapeutic agents with proper efficacy and safety profiles has been a challenging task. For successful product development, one needs to have a thorough understanding of the protein's physicochemical and biological characteristics, including stability, immunogenicity, and pharmacokinetic properties. The characterization of proteins is therefore an important step in formulation development.

Molecular Composition, Structure, and Heterogeneity

A protein, or polypeptide, is formed through the linkage of peptide bonds of amino acids. Generally, protein structures are described at four levels: primary, secondary, tertiary, and quaternary. Details about these can be found in the preceding chapter of this volume.

Because of their complex manufacturing process, from cell culture to downstream purification, protein products generally contain multiple species in terms of molecular weight or size, which could be due to various modifications to the polypeptide side chains or glycans, reversible or irreversible formation of oligomers by either noncovalent or covalent linkages, and formation of large soluble and/or insoluble aggregates. It is important to characterize and quantify all species, as they may directly affect product efficacy, safety, and immunogenicity.

Depending on its size and the nature of its associations, several analytical techniques can be used to characterize a protein's size. Routinely, electrophoretic and chromatographic (with multiangle light-scattering detector) techniques have been used to estimate protein size up to

oligomers. By combining a denaturing electrophoretic technique (sodium dodecyl sulfate polyacrylamide gel electrophoresis [SDS-PAGE]) with size-exclusion high-performance liquid chromatography (HPLC) or a native electrophoretic technique (Native PAGE), the size of proteins and the nature of their associations (covalent vs. noncovalent) in native and denatured states can also be estimated. To more accurately determine the size of proteins, mass spectrometry, such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) or liquid chromatography mass spectrometry (LC-MS), is often used. However, because of the matrix effect and the high energy applied, the molecular weight or size determined by this technique may not be the true size in solution.

To measure the size of a protein in solution up to 100 nm, several biophysical techniques may be feasible, including analytical ultracentrifugation (AUC), field flow fractionation (FFF), and dynamic light scattering. It should be noted that the size distribution of proteins in solution, especially for reversible association, may be highly dependent on the solution properties, including pH, salt concentration, and protein concentration. Therefore, the mobile phase used in these analyses is preferably the same as the formulation vehicle, and the impact of the dilution factor during analysis should be assessed.

Insoluble aggregates or particles larger than 100 μm can be observed by visual inspection with the unaided eye. Their size can be estimated by microscopy. Subvisible insoluble aggregates between 10 and 100 μm in size can be quantified and sized either by a light obscuration test or by a microscopic particle count test per USP method <788>. It is still technically challenging to accurately quantify and size particles between 0.1 and 10 μm. A technique using Micro-Flow™ imaging (MFI) has been used for particles as small as 0.75 μm (2).

Isoelectric Point

Proteins that contain both positively and negatively charged amino acids are amphoteric molecules. One property that characterizes a protein's charge profile is its isoelectric point, or pI. The pI of a protein is the pH at which it carries no net electrical charge. At a pH below its pI, a protein is positively charged; above its pI, it is negatively charged.

The pI may be approximately calculated from the amino acid composition data, that is, $pI = (pK_1 + pK_2 + pK_3 \ldots + pK_n)/n$ for n ionizable groups. However, because the dielectric constant in the immediate vicinity of an ionizable group depends on protein structure, and because hydrogen bonding may alter dissociation constants (K_a), the true pI can differ significantly from the calculated one. Several websites provide theoretical estimations of pI for proteins (e.g., http://www.scripps.edu/~cdputnam/protcalc.html, http://www.expasy.ch/tools/pi_tool.html, and http://www.nihilnovus.com/Palabra.html).

Some proteins have multiple species with different charge profiles, and each species has its own pI, so these proteins appear to have more than one pI. Some glycoproteins in particular exhibit complicated pI patterns because of the heterogeneity in their glycan composition. Also, some proteins comprise multiple deamidation species, which also results in complicated charge profiles that could be characterized by several techniques, including isoelectric focusing (IEF), ion exchange chromatography (IEC), and capillary electrophoresis (CE).

Proteins show a broad range of pIs, mostly in the range of 2 to 12. The pI of a protein may play an important role in solubility and stability. In general, protein solubility is at its minimum when the pH is near its pI. Also, because zero net charge at pI should presumably allow maximum interaction between salt bridges and exert the least interaction between protein molecules, it could be expected to be the most stable condition for conformation. However, studies have shown that the optimal pH for conformational stability can be quite different from the pI and in many cases is found at a pH corresponding to a large net charge of the protein (3).

Solubility

The varieties of functional groups (charged, hydrophobic, etc.) on the side chain of amino acids and glycans (for glycoproteins) make protein solubility dependent on the pH, salt concentration, and polarity of the solvent. The overall size of the protein does not necessarily influence solubility. For example, antibodies, which have molecular weights of approximately 150 kDa, can often achieve aqueous solubility greater than 100 mg/mL.

The aqueous solubility of a peptide or protein is not easy to determine because peptides and proteins at high concentrations may form gels, or may develop aggregates upon concentrating, thus making solubility assessment difficult. In addition, solubility varies significantly depending on the conformation. The solubility determined by most methods is apparent solubility, because the true solubility of a protein as a hydrocolloid is difficult to define. A common approach is to concentrate a protein solution using a semipermeable membrane with centrifugation until the highest protein concentration is reached. Another approach is to lyophilize a protein or peptide and then add water to the point where undissolved material is barely present. When a limited amount of protein is available, one approach is to determine solubility in polyethylene glycol (PEG) solution (typically 1–9%) and then extrapolate the solubility to 0% PEG to determine aqueous solubility (4).

The factors that determine a protein's solubility include its intrinsic properties and the composition of the solvent. The intrinsic properties are the composition of amino acids, the folded structure, and for glycoproteins, the composition and structure of glycans. Generally, a protein made of a large proportion of hydrophobic amino acids such as Phe, Tyr, and Trp will have low water solubility, and adding glycans increases water solubility. The solvent properties, including pH, salt concentration, and specific ligands, can also significantly affect the solubility. Protein solubility as a function of pH is typically in the shape of a U or V, where the minimum is at the pI. However, there are exceptions. The solubility of a protein at low ionic strength generally increases with the salt concentration, which is called the salting-in effect. As the salt concentration increases, the additional counter-ions shield the ionic charge and thereby increase the protein solubility. As salt concentration continues to increase, protein solubility decreases (the salting-out effect). At high salt concentration, the salts begin to compete with the ionic moieties of the protein for the solvation of the polar solvent, which results in decreasing solubility. A specific ligand or stabilizer that binds to the protein may also influence solubility. For example, increased solubility of fibroblast growth factor was observed in the presence of heparin or heparin-like substances (5). Also, alteplase solubility was increased by the addition of arginine (6). However, one needs to assess whether the ligand or excipient is acceptable for the intended clinical use before adding it into the final formulation.

Thermal Transition Midpoint

Because native proteins exhibit folded structure in solution, they can undergo transition from native form to unfolded or denatured form with increasing temperature. The thermal transition midpoint (T_m), defined as the temperature at which equal amounts of native and denatured forms exist in equilibrium, is an important characteristic of proteins, measuring their thermal stability. Generally, a higher T_m value indicates better thermal stability.

The most commonly used technique to determine T_m is differential scanning calorimetry (DSC), as this method not only provides an accurate measurement of T_m but also can assess reversibility of transition and estimate apparent enthalpy. Temperature-controlled spectrometry, including circular dichroism (CD), fluorescence, and ultraviolet (UV) absorbance spectroscopy, is also sometimes used to differentiate the transitions by tertiary structure from those by secondary structure.

Measurement of T_m has been widely used in preformulation and formulation development. The profile of T_m as a function of pH provides important information in selecting the optimal pH for formulation. This method has also been used in screening different stabilizers, as an excipient that elevates T_m is expected to be a potential stabilizer (7). However, it should be noted that in choosing the formulation, one also needs to consider other information, as T_m alone is only indicative of thermal stability.

Proteins in solid state also exhibit thermal transitions upon heating. These are typically determined by DSC. However, it is difficult to measure the true thermal transitions of solid protein, because in most cases other components present in the solid dosage form also contribute to the overall thermal transition. Recently, glass transition temperatures (T_g) for proteins have been estimated by extrapolating excipient concentration to zero using T_g values measured at a very fast scanning rate in binary mixtures of protein and another glass form excipient, such as sucrose, over a range of excipient concentrations (8).

Instability: Key Degradation Pathways

The structural complexity of proteins makes them susceptible to processing and handling conditions that can result in structural and functional modifications. A protein can undergo a variety of covalent and noncovalent reactions or modifications, which may be generally classified into two main categories: (*i*) physical or non–covalent bond degradation pathways and (*ii*) chemical or covalent bond degradation pathways. Common physical degradation pathways include denaturation or unfolding, adsorption, and aggregation due to noncovalent forces. Chemical degradation pathways include covalent-bonded aggregation, disulfide exchange, deamidation, isomerization, racemization, fragmentation, oxidation, β-elimination, Maillard reaction, diketopiperazine formation, and so on. Oftentimes, physical degradation facilitates chemical degradation, and vice versa. The fundamentals of these degradation pathways have been extensively described in several review articles and book chapters (9–14). A brief description of each degradation pathway, the factors responsible for degradation in some proteins, and remedies are presented below.

Denaturation

Denaturation is the process of altering protein structure (i.e., secondary, tertiary, or quaternary structures) from its native folded state. Denaturation may result in an unfolded state, which could further facilitate other physical and chemical degradations. Because a specific structure is required for proteins to exert physiological and pharmacological activities, denaturation causes loss of efficacy and incurs the risk of safety such as immunogenicity.

Many times, the denaturation process can be described as $N \leftrightarrow I \leftrightarrow D$. The folded native structure (N) unwinds and passes through a partially unfolded or intermediate state (I) to a denatured state (D). This process may be reversible or irreversible, depending on conditions. For reversible denaturation, the unfolded protein will regain its native state once the denaturing condition is removed.

Many factors can cause denaturation, including heat, freezing, extreme pHs, organic solvents, high salt concentration, lyophilization, surface adsorption, and mechanical stress. These denaturing conditions disrupt a protein's higher order structure, which is held together by intramolecular forces including hydrogen bonding, salt bridges or electrostatic forces, hydrophobic interactions, and van der Waals forces.

Hydrogen bonds are critical in determining overall protein conformation, since they are the major forces that stabilize the secondary α-helices and β-sheets, as well as the overall folded structure. Water, the nearly ubiquitous medium for proteins, contributes to this hydrogen bonding. Cosolvents such as ethanol and acetone and chaotropic agents such as urea and guanidine hydrochloride disrupt the hydrogen bonds and thus readily denature proteins.

The ionic side chains of aspartic acid, glutamic acid, lysine, arginine, and histidine, normally found on the surface of the protein, contribute to the stability of the native conformation by forming salt bridges. The pH of the solvent will determine the charge of the side chains on these amino acids and the extent of ionic bonding. Therefore, an extreme pH shift can disrupt these salt bridges and lead to denaturation. Furthermore, organic solvents will reduce dielectric constant and increase ionic forces or salt bridges, so inappropriate exposure to organic solvents can also result in denaturation.

Because hydrophobic side chains (i.e., phenyl, indole, and hydrocarbon chains) are usually tucked inside the protein's globular structure, significant stabilizing effects result from their hydrophobic interactions. These interactions, too, are sensitive to the effects of solvents. Disruption of hydrophobic interactions is also considered the mechanism of denaturation by surfactant, extreme temperature, and mechanical stress, all of which commonly occur during manufacturing processes.

Adsorption

Proteins are amphiphilic polyelectrolytes, so they tend to adsorb at liquid-solid, liquid-gas, and liquid-liquid interfaces. When adsorption of proteins occurs, the molecules exchange their interactions with the solvent and other solutes for interactions with the surface. Two mechanisms are primarily responsible for protein adsorption. One mechanism is charge-charge

or electrostatic interaction. For example, salmon calcitonin, as a positively charged protein, strongly binds to the negative potential of a glass surface through electrostatic interaction (15). The other mechanism is hydrophobic interaction. One example is bovine serum albumin, which near its isoelectric point has shown the highest affinity to the hydrophobic surface of polystyrene through hydrophobic interactions (16). Other interactions, including charge-dipole, dipole-dipole, and van der Waals forces, may also contribute to the adsorption.

These interactions may lead to altered structures, including secondary, tertiary, and quaternary structures, which could further facilitate other physical and chemical degradation, including aggregation and covalently bonded modification. Therefore, depending on the nature of the protein and of the contact surface, interfacial adsorption can significantly impact a protein drug's potency, stability, and safety, particularly in a low-concentration dosage form.

The key strategy to minimize or inhibit protein adsorption is either to adjust formulation parameters or to modify or avoid certain contact surfaces. The formulation parameters that potentially control adsorption include protein concentration, pH, ionic strength, and addition of specific excipients such as surfactants or albumin. For example, modification of the contact surface of siliconized vials has minimized interferon adsorption on the glass surface (17). When these approaches do not prevent significant adsorption, alternative contact surfaces should be considered during process or storage. In some cases, when the level of adsorption can be predicted, overage is required in the vials.

Aggregation by Noncovalent Linkage

Non–covalently linked aggregation often results from some degree of denaturation of proteins, since unfolding leads to the exposure of hydrophobic moieties previously buried in the protein interior, which is followed by the association of unfolded molecules via noncovalent interactions to form aggregates. Non–covalently linked aggregation can be a reversible or an irreversible process, depending on conditions.

Reversible aggregation is highly dependent on protein concentration, pH, salt concentration, and other formulation components. Generally, proteins tend to form high–molecular weight species (HMWS) at high protein concentration. Upon dilution, these HMWS or oligomers may dissociate into monomers or dimers. This self-association phenomenon may be characterized by AUC (18) or by static light scattering (19).

Irreversible aggregates can be soluble or insoluble, depending on the size and nature of the molecules. Generally, these aggregates can be induced by single or multiple stress conditions, including heat, extreme pH, mechanical pumping, high pressure, shaking or agitation, freezing, and freeze-drying. For example, acidic pH and a temperature of 37°C have resulted in irreversible aggregation of albumin (20).

To minimize aggregation, besides tight control of the process parameters, adjusting formulation parameters, such as adding sucrose, should be assessed. Sucrose and other polyols maintain protein molecules in a native compact form, so as to be resistant to external stress.

Aggregation by Covalent Linkage

The most commonly observed protein aggregation by covalent linkage occurs through intermolecular disulfide linkage, also called disulfide bond formation and scrambling. This intermolecular aggregation may occur to any protein containing cysteine or cystine.

Generally, proteins with a free thiol group tend to form aggregates more easily through disulfide bonds, especially when the free thiol group is solvent-exposed on the surface of the protein. Free thiol groups buried within the tertiary structure are less reactive. The formation of disulfide bonds in protein aggregates with free thiol groups can take place either through the two free thiol groups available on the surface of each of two protein molecules, or through thiol-disulfide exchange, whereby a reactive thiol group in one molecule attacks an existing disulfide bond in another molecule to form a new disulfide bond between the two molecules.

Proteins without free thiol groups may still form aggregates by disulfide bond scrambling through intermolecular disulfide exchange, especially in alkaline conditions. A cystine or disulfide bond in one molecule can be reduced into two free cysteines, which can react with cysteine or cystine in another molecule to form a new disulfide bond.

Aggregates formed through disulfide bonds may, through multiple disulfide scrambling reactions, result in high-molecular-weight aggregates, which could eventually precipitate from solution. In addition, the formation of a new disulfide bond may change the native conformation to a denatured form, which could further aggregate through noncovalent hydrophobic interactions due to exposed hydrophobic residues.

In neutral or alkaline pH conditions, disulfide-bonded aggregation generally becomes more severe as the thiol group becomes more reactive. However, extremely acidic pH may also cause disulfide-bonded aggregation (21).

To prevent or minimize disulfide-bonded aggregates, the main formulation parameter is pH. A slightly lower pH (e.g., pH 5) may significantly reduce aggregation. The addition of reducing agents such as cysteine, or of stabilizers that alter conformation such that free cysteine or reactive cystine becomes more buried into tertiary structure, may also minimize aggregation. For proteins with severe disulfide aggregation, lyophilized formulation should be considered, as reactivity in the solid state is reduced significantly.

Nonreducible aggregates through nondisulfide linkages have also been reported. The reactions involving these covalent linkages include (*i*) oxidation-induced reactions through Trp or Tyr linkage (22); (*ii*) reaction through transamidation, whereby an amino group of amino acids (e.g., lysine residue or N-terminal of a protein) in one molecule forms an isopeptide bond with the carbonyl group of either Asn or Gln in another molecule [examples are insulin (23) and lyophilized ribonuclease A (24); and (*iii*) reaction through a reactive dehydroalanine generated from β-elimination at alkaline pH, which forms nonreducible cross linkages with other amino acids such as Tyr, Lys, His, Arg, and Cys.

Intramolecular Disulfide Exchange

Disulfide exchange can also take place within a protein molecule when a cystine (disulfide) bond is reduced into two cysteines; one of the cysteine residues then reacts with another cysteine to form a new disulfide bond. Improper linkages of disulfide bonds were responsible for a reduction in biological activity of interleukin-2 (IL-2) (25). There are three cysteines in IL-2 at positions 58, 105, and 125. The native protein forms a disulfide linkage between the two cysteines at 58 and 105. The cleavage of this disulfide in IL-2 and the subsequent formation of two less active isomers with disulfide bonds at incorrect positions (Cys^{58}-Cys^{125} and Cys^{105}-Cys^{125}) are promoted by high pH and copper ions (25). Intramolecular disulfide exchange has also been reported for monoclonal antibodies.

To minimize this type of degradation, it is important to select a low formulation pH and minimize any impurities, such as peroxides or heavy metals, known to promote redox reactions.

Deamidation/Isomerization

Deamidation refers to the removal of ammonia from the amide ($RCONH_2$) moiety of an Asn or Gln side chain, resulting in a carboxylic acid. Deamidation is a major cause of instability of proteins and peptides in aqueous solution. In lyophilized solid state, the deamidation rate is slower than in solution.

Deamidation occurs through different pathways at different pH levels. In an acidic pH of 2 to 5, deamidation occurs by direct hydrolysis, which causes Asn or Gln residue to change into Asp or Glu residue, respectively. The type of neighboring amino acids does not affect the deamidation rate. Hydrolytic mechanisms in neutral or alkaline pHs are more complex, however. Under these conditions, the side chain carbonyl group on the Asn or Gln residue reacts with the nitrogen atom on the peptide backbone to form a cyclic imide (succinimide) intermediate (Asu). Depending on which bond in the cyclic imide breaks, the reaction product can be (*i*) the des-amido peptide (Asp), (*ii*) the isopeptide (IsoAsp), or (*iii*) D-isomers. The formation of isopeptides is called isomerization, or sometimes referred to as transpeptidation, because an extra methylene group is inserted to the peptide backbone. When deamidation occurs, the IsoAsp to Asp ratio is typically 3. Detailed descriptions of deamidation and isomerization can be found in a review by Wakankar and Borchardt (26).

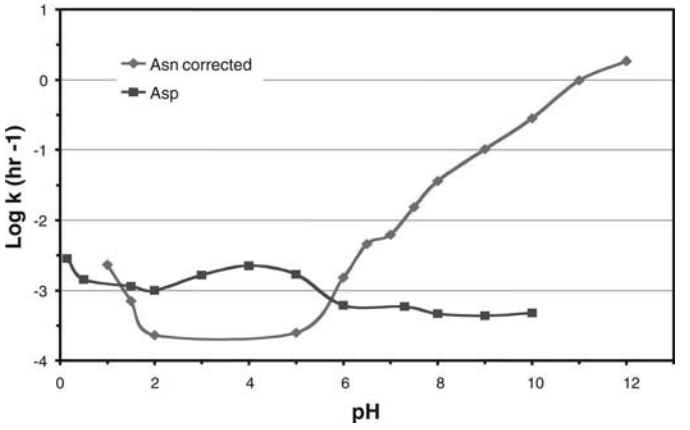

Figure 1 Comparison of Asn deamidation (*diamond*) and Asp dehydration (*square*) rates in a model peptide (Val-Tyr-Pro-Asx-Gly-Ala) at 37°C. Dehydration is the rate-limiting step for isomerization. *Source*: From Refs. 104 and 105.

At neutral to alkaline pHs, the rate of deamidation is significantly affected by the size of the amino acid on the C-terminal side of the Asn or Gln residue. In general, Asn is more labile than Gln and is most labile when adjacent to glycine, which is least obstructive to the formation of a cyclic imide. Since Asn-Gly is most susceptible to deamidation, protein engineers make significant efforts to avoid constructing a protein drug candidate with such a hot spot.

The deamidation rate profile as a function of pH is V-shaped, usually with a minimum rate at a pH of about 4 to 5 (Fig. 1). In a number of synthetic peptides, the half-life of deamidation reactions of Asn residues at 37°C in pH 7.2 phosphate buffer ranges from two days to nine years (27). Not all Asn residues are equally labile; those buried within the interior portion of a protein are inaccessible to water and thus less reactive. Secondary and tertiary structures play an important role in determining the site and the rate of deamidation. In insulin, for example, there are three asparagines. At acidic pH, the prevailing deamidated species was monodesamido-(A21)-insulin. At neutral pH, deamidation occurred to Asn at the B3 position (28). For growth hormone containing nine asparagines, deamidation occurred primarily at the Asn-149 position (29). These proteins and others—pramlintide (30), epidermal growth factor (31), IgG (32), IgG1 (33)—represent a small fraction of proteins that have shown deamidation.

Isomerization at Asp goes through the same cyclic imide intermediate, Asu. Because of the effect of pH on the leaving group (-OH), the rate-pH profile is significantly different from that of deamidation of Asn. Examples of protein drugs that undergo IsoAsp formation include insulin aspart (34), hirudin (35), and porcine somatropin (36). On the basis of the study of two monoclonal antibodies (37), the degradants from isomerization were detected by hydrophobic interaction chromatography.

The best way to avoid deamidation and isomerization is to mutate Asn-Gly or Asp-Gly sequence in the solvent-exposed region of the protein, if the mutation at these sites does not affect the biological activity. Otherwise, an appropriate pH (in the range of 5–6) should be selected to minimize the degradation rate. As the formation of cyclic imide intermediates does not depend on water, these reactions may occur even in anhydrous conditions such as 100% dimethyl sulfoxide (DMSO) (38)

Racemization

The racemization reaction is catalyzed by both acid and base. Racemization of peptides and proteins results in the formation of diastereomers. Racemization under basic conditions is hypothesized to proceed by abstraction of the α-proton from an amino acid in a peptide to yield a negatively charged planar carbanion. A proton can then be returned to this optically inactive intermediate, thus producing a mixture of D- and L-enantiomers for the individual amino acid. Since a peptide is composed of multiple chiral centers, the product formed is a diastereomer. Racemization is biologically significant because a peptide composed of D-amino acids is generally metabolized much more slowly than a naturally occurring peptide made

only of L-amino acids. For this reason, many new synthetic peptides, both agonists and antagonists, incorporate D-amino acids. A pH dependency for racemization was demonstrated in an aqueous degradation study of a decapeptide, RS-26306 (39), which found that at neutral and alkaline pHs, racemization contributed to more degradation than did deamidation.

Fragmentation

The peptide bond (RNH—CO—R) can undergo hydrolysis, resulting in peptide fragments. Generally, most peptide bonds are relatively stable. For example, oxytocin injection was reported to be stable at room temperature for five years (40). Protirelin, a tripeptide (PyrGlu-His-Pro), is stable for 20 hours at 80°C at both pH 3.3 and pH 6 (41).

The formulation factor that most influences the hydrolytic rate is solution pH. The rate of hydrolysis is in direct proportion to the activity of hydronium or hydroxide ions, when in acidic or alkaline pHs, respectively. Generally, the reaction becomes much faster in either extremely acidic or extremely alkaline conditions. Fragmentation of therapeutic peptides, including nafarelin (42), secretin (43), captopril, (44), and urokinase (45), has been reported in various pH conditions.

The type of neighboring amino acids also affects the susceptibility of the linkage to fragmentation. For example, the bond between aspartic acid and proline is sensitive to acid hydrolysis. A cleavage at Asp-Pro was found in basic fibroblast growth factor (bFGF) (46) and macrophage colony-stimulating factor (47). The resultant products are peptides with aspartic acid at the C-terminus. The C-terminal peptide bond adjacent to serine is also a reactive one, because of the neighboring-group effect of the alcohol on serine (48).

Another example of fragmentation is the cleavage at hinge regions of antibodies resulting in a Fab fragment. The hinge region in IgG1 heavy chain comprises about eight amino acids, including two cysteines responsible for the two disulfide linkages between the two heavy chains. Cleavage takes place at multiple sites, with the majority between Asp and Lys and between His and Thr (49). The extreme flexibility at the hinge region and the solvent exposure are the driving forces for such cleavage. The rate of hydrolysis at hinge region is minimal at pH 6 (50).

Besides chemically induced fragmentation, protein hydrolysis can also be mediated by some residual proteases remaining from production. The cleavage site in this case is dependent on the type of proteases present. As the proteolytic activity of proteases is typically pH dependent, the degradation rate is also dependent on solution pH. For example, a cathepsin D protease derived from CHO was identified as being responsible for degradation of an Fc-fusion recombinant protein. This protease belongs to an aspartic protease family and is preferentially active at acidic pH (51).

Oxidation

Several amino acid residues including Cys, Met, Trp, His, and Tyr are potential sites of oxidation. These residues can be oxidized by atmospheric oxygen or by peroxide impurities from a number of raw materials including formulation excipients such as polysorbates. Oxidation can also be induced by exposure to light or catalyzed by transition metal ions such as Cu^{2+} and Fe^{3+}. The most commonly observed oxidations in therapeutic peptides and proteins are described below.

Oxidation of cysteine. Under neutral or basic conditions, the free thiol (-SH) group of a cysteine is the most reactive moiety of all amino acid components. The disulfide (-S—S-) bond formed from the oxidation of two thiol groups results in significant changes in conformation both intramolecularly and intermolecularly.

Oxidation of the thiol group is promoted at both neutral and basic pH. The rate-pH profile for captopril, a quasi-dipeptide, shows an increase in oxidation rate starting at pH 5 (44). This reaction can also be catalyzed by heavy metals. For example, this reaction was effectively retarded by the addition of a metal chelating agent such as EDTA in FGF formulation (5).

Oxidation of methionine. The thiolether ($-CH_2-S-CH_3$) moiety on methionine is susceptible to oxidation to form sulfoxide ($-CH_2-SO-CH3$) derivatives. The susceptibility of methionine to

oxidation is highly dependent on its site in a protein. For example, of the three methionines in human growth hormone, Met-125 is most reactive, Met-14 is less, and Met-170 is not reactive at all (52). The reactive methionine is likely to be the one exposed on a protein surface, and the unreactive one buried within the core. Air in the headspace of formulated and freeze-dried growth hormone can cause 40% of the growth hormone molecules to be oxidized during a six-month storage period (53). Examples of other pharmaceutical proteins exhibiting Met oxidation are listed below.

- Interleukin-2 (54)
- Murine antibody (Orthoclone OKT3) (55)
- Herceptin® (trastuzumab) (56)
- Recombinant interferon γ (Actimmune) and recombinant tissue plasminogen activator (alteplase, Activase®) (57)
- Recombinant human granulocyte colony-stimulating factor (58,59)
- Parathyroid hormone (60)

As methionine can be easily oxidized by atmospheric oxygen, replacing oxygen with nitrogen or argon during manufacturing or in the headspace of the final product container is a common practice to minimize oxidation. In addition, adding free methionine as an antioxidant is also an effective approach to reducing oxidation (61).

Oxidation of tryptophan. Oxidation of tryptophan can generate multiple oxidized species. Stability studies of Trp amino acid alone in aqueous solution (62) and Trp residues in small peptides and lysozymes (63) and in bovine α-crystallin (64) clearly identified the main degradants as 5-hydroxy-Trp, oxy-indole alanine, kynurenine, and *N*-formylkynurenine. There are very few articles reporting the oxidation of Trp in therapeutic proteins. Davies et al. reported oxidized bovine serum albumin with oxygen radicals generated from cobalt radiation (65); Trp oxidation in monoclonal antibodies was recently reported by Yang et al. (66) and Wei et al. (67), and ozone and UV irradiation were used as stress conditions in these studies. That Trp oxidation has not been studied in depth is perhaps due to the fact that no model oxidizing condition has been adopted, and a system that promotes Trp oxidation is not easy to reproduce. In the case of Met oxidation, tert-butyl hydroperoxide (tBHP) and H_2O_2 are commonly used reagents to generate oxidized Met species. Most recently, a free radical generator, AAPH (2,2′-azobis(2-amidinopropane) dihydrochloride) was found to effectively oxidize tryptophan in peptides or large proteins (68). By using AAPH as an oxidation stressing agent, one can predict the vulnerability of a protein or the specific Trp residue in a protein.

As Trp oxidation appears to be mediated through oxygen radicals because of light exposure or peroxide residues from excipients, it is important for mitigation of Trp oxidation to limit direct light exposure and to use high-quality excipients.

β-Elimination

The disulfide bond between two peptide chains can be cleaved disproportionally, catalyzed by hydroxide ions to produce dehydroalanine and thiocysteine through the mechanism of β-elimination. The thiolate ion (HS-) is very reactive and will continue to react with other disulfide bonds, causing chain reactions. This is one of the operative mechanisms for the covalent aggregates in solid phase for bovine serum albumin (69) and ANP, a 25–amino acid peptide (70). β-elimination has also been attributed to the fragmentation of hinge regions in antibodies (71).

Maillard Reaction

The first substantial investigation of the reaction of reducing sugars with amino acids was carried out about 75 years ago by Maillard. The chemical reactions involved are, first, the reversible formation of a Schiff base between the aldehydic function group of reducing sugars (e.g., glucose) and the amino group of lysine residues in proteins, followed by a relatively

slow, but essentially irreversible, Amadori rearrangement, with the formation of ketoamines, which forms the hemiketal structure. As the Schiff base may also be involved in cross-linking, this reaction commonly leads to nonenzymatic browning, also called a browning reaction. This reaction occurs most readily in neutral to weakly alkaline conditions.

In protein formulation, reducing sugars such as glucose, maltose, fructose, and lactose should be avoided to prevent potential Maillard reactions. In addition, one needs to be concerned with acidic pH, which may cause hydrolysis of sucrose into glucose and fructose, leading to glycation of the protein. One example is freeze-dried bFGF. When the cake collapsed at elevated temperature, the acidity from its buffer, citric acid, caused hydrolysis of sucrose, which resulted in glycation of the protein with glucose (46).

Diketopiperazine Formation

Rearrangement of the N-terminal dipeptide results in the splitting off of a cyclic diketopiperazine at high pH. Proline and glycine in the N-terminal promote the reaction. Aspartame degrades through a similar mechanism (72), with the cyclization of aspartame minimal at a pH of 6 to 7, moderate at 7 to 8.5, and rapid above 8.5. Diketopiperazine formation has also been reported in the case of vascular endothelial growth factor (73).

PHARMACEUTICAL DEVELOPMENT PRINCIPLES

Because of their poor permeability to epithelium cell membranes and instability in gastrointestinal tract, proteins and peptides have very poor oral bioavailability and are therefore primarily formulated as injectable or parenteral products for intravenous (IV) infusion or subcutaneous (SC), intramuscular (IM), or intrathecal (IT) administration. Some proteins are also delivered through noninvasive administration routes, such as nasal and pulmonary formulations, to improve patient compliance. For local therapeutic effect, proteins such as growth factors are formulated for topical application and bone matrices.

Most protein products are in aqueous solution or in solid, freeze-dried form. Some are also formulated as suspensions in a crystalline form or in other lipid- or polymer-based delivery systems such as microspheres, liposomes, and nanoparticles. Rarely, some proteins, such as bovine growth hormone, are formulated in oleaginous vehicle.

The formulation development process for products that involve lipids or polymers as carriers is more complicated than the process for simple conventional dosage forms of liquid and lyophilized formulations. The principles described below focus mainly on the development process for the conventional dosage forms, some of which have also been discussed in several book chapters and review articles (74–79).

General Formulation Development Process in Industry

Generally, the manufacturing process for most protein products consists of two steps. One is the manufacture of drug substance, *aka* the active pharmaceutical ingredient (API), which is typically stored in bulk containers, such as plastic or stainless steel containers, in frozen condition for long-term storage purposes. The other step is the manufacture of the final drug product from drug substance. This is the final presentation to patients and healthcare professionals, typically stored in glass vials, prefilled syringes, or injection devices, at refrigerated condition for convenient use and distribution.

Formulations for drug substance and drug product may not be the same. Typically, the drug substance formulation is developed earlier, as it is the first step of the manufacturing process. However, drug substance should ideally be in a formulation vehicle that can be readily further formulated into drug product. Therefore, it is important to develop the drug product formulation as early as possible, so that the manufacturing process from drug substance to drug product will be harmonized and straightforward.

Prior to formulation development, it is important to conduct preformulation development activities, which serve to identify the key degradation pathways of the molecule and to develop stability-indicating assays to support formulation development studies. In general, preformulation activities should evaluate the biophysical and biochemical properties of the molecule under pharmaceutically relevant stress conditions, which include chemical-related

factors (such as extremes of pH, salts, buffers, oxygen, and peroxides) and process-related factors (such as high temperature, freezing, thawing, light, agitation, pressure, and shear stress). In these studies, biophysical characterization assays such as UV-visible spectroscopy, DSC, CD, Fourier transform infrared spectroscopy (FT-IR), AUC, and fluorescence spectroscopy are often used to determine which conditions result in the highest thermal stability and the least conformational changes. Biochemical methods such as size-exclusion HPLC, reversed-phase HPLC, hydrophobic interaction chromatography, capillary electrophoresis, peptide map, gel electrophoresis, ion-exchange HPLC, and potency assays are used to identify the key chemical degradation pathways, primarily through covalent bond changes, under these stress conditions.

Results from preformulation studies should identify the potential sources of the instability of the molecules and the key stability-indicating assays to monitor the degradation products, as these are needed for formulation screening and optimization studies. Once the leading formulation(s) are identified from the screening studies, appropriate container closure for final product presentation should be evaluated. In addition, the manufacturing process should be developed.

The choice of formulation, dosage form, and final product presentation is dependent on several factors. These choices not only need to offer the best stability and safety profiles, but they must also be easy to scale up for manufacturing and convenient to use during administration. The decisions are also dependent on the development stage of the product. At preclinical and early clinical stages, the goal of the project is to evaluate the proof of concept in product efficacy and safety as early as possible, so the shelf-life of early clinical products may only need to be 12 months or even less, and the storage or shipping conditions may not require the refrigerated or ambient temperature that is typically used for commercial products. Therefore, frozen or lyophilized formulations are typically chosen for preclinical and early clinical studies. At late clinical and commercial stages, there are more stringent requirements. At these stages, not only stability but also cost and marketing competitiveness are very important in making the final choice of the formulation. A very critical factor in a field of many competing, similar biotech products is patient compliance and acceptance, so prefilled syringes and auto-injection devices are in many cases essential for the success of the product's introduction to the market. As a result, evaluation of these dosage forms and devices must be initiated during phase III or pivotal clinical trials.

Overall, formulation development is a critical and evolving process step in product development. It is important to initiate the development effort as early as possible. It is also important to have input from multiple functional areas before finalizing the formulation choice.

Evaluation of Critical Formulation Parameters

To prepare a stable formulation suitable for patient use, the critical formulation parameters should be evaluated, including protein concentration, pH, buffers, stabilizers, tonicity modifiers, bulk agents (for lyophilized products only), and preservatives (for multidose products only). A discussion of evaluating each formulation parameter follows.

Protein Concentration

Protein concentration not only serves as a critical parameter in finalizing product presentation, but is also a key parameter in product stability. The following considerations are important in the selection of a viable protein concentration in a formulation:

- It should be below the protein's solubility in the selected formulation vehicle. Since protein solubility varies in different formulation vehicles and at different temperatures, the selected protein concentration should be below the "true" or thermodynamic solubility in the vehicle at the intended long-term storage temperature. However, measurement of true protein solubility is challenging, so this is an empirical exercise where real-time data needs to be evaluated on potential precipitation of the protein at the selected concentration and stored for the desired duration. This

assessment should be made for both drug substance and drug product before a protein concentration is finalized.

- It should maintain the protein's stability during long-term storage. Generally, proteins at high concentrations may lead to aggregation and precipitation. On the other hand, low protein concentrations may lead to significant loss of protein content due to adsorption onto various product-contacting surfaces (e.g., container closures during storage, filter membranes and silicone tubing during processing). Also, a higher relative ratio of any reactive impurities (such as traces of heavy metals, peroxides from surfactants, and sterilants used in the aseptic filling process) to the protein could cause degradation.
- It should be operable under manufacturing conditions. High protein concentrations achieved at lab-scale may not be operable at manufacturing scale. For example, a formulation with a high protein concentration may have high viscosity, requiring an undesirably long processing time for filtration or the ultrafiltration and diafiltration (UFDF) step. On the other hand, at low concentrations there may be significant protein loss from the filtration membrane due to the larger surface area at manufacturing scale. Therefore, it is critical to assess the scalability of the manufacturing process before choosing a protein concentration.
- It should minimize product waste during the manufacturing process, testing, and clinical use. Material losses during manufacturing (e.g., line loss), sample testing, and dose preparation for clinical use (e.g., residual in containers) are all volume based, so a high protein concentration will result in a large amount of protein waste. Therefore, the lowest protein concentration that delivers the required dose and maximizes production yield should be identified.
- It should meet the requirements for product presentation. Product presentation is selected on the basis of factors such as dose (size and frequency), administration route, convenience of dose preparation, patient weight distribution (for weight-based dosing schemes), number of manufacturing lots desired or manageable per year, and commercial considerations of cost and product differentiation. From these analyses, the amount of protein per vial is specified, and subsequently, the optimal volume and corresponding protein concentration is determined on the basis of factors such as solubility, stability, minimal protein waste, and manufacturability.
- It should take into account manufacturing process cycle time and cost. For lyophilized products, the major contributor to manufacturing cost is the lyophilization time. Reducing the fill volume by increasing protein concentration can significantly reduce the lyophilization time.

In summary, protein concentration, as a critical formulation parameter, should be chosen on the basis of multiple factors, including solubility, stability, manufacturability, cost, product presentation, and commercial considerations.

pH

As proteins containing both positively and negatively charged amino acids are amphoteric molecules, solution pH has a direct impact on the overall stability of proteins. When solution pH is far from the pI of the protein, electrostatic repulsions between like charges in the protein increase, causing a tendency to unfold. In addition to electrostatic interactions, pH also affects other interactions within proteins. Therefore, changing pH directly affects the conformational stability of proteins and their solubility in aqueous solution. In addition, since certain chemical reactions are highly pH dependent, solution pH also affects the chemical stability of proteins. The following areas should be assessed when selecting an optimal pH for a protein formulation:

- Profiles of conformational stability, chemical stability, and solubility as functions of pH in solution may not overlap each other for a given protein. The pH at maximum solubility may not be the same as the pH at maximum conformational stability or

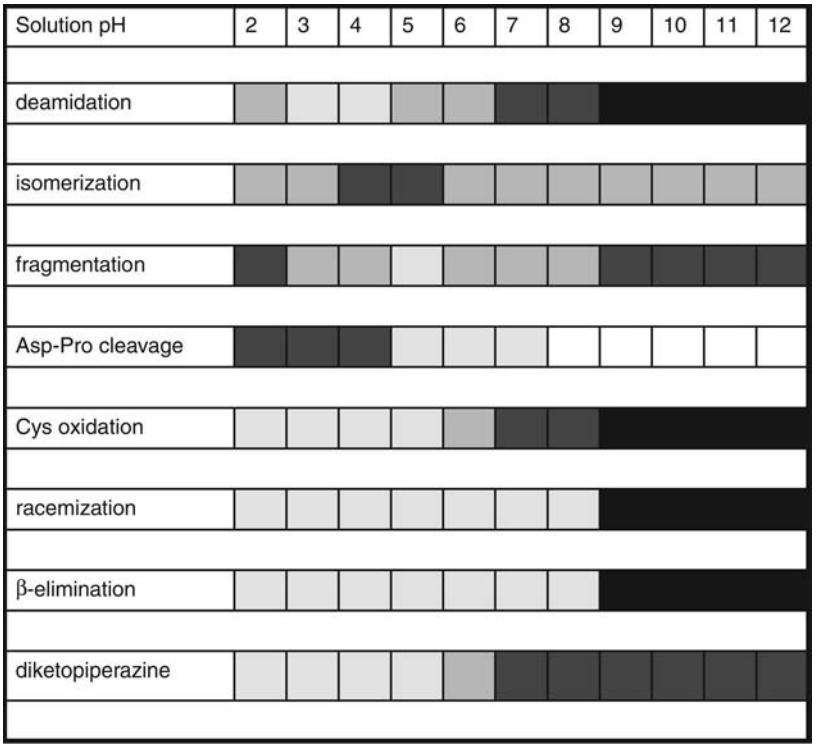

Figure 2 Reaction rates as a function of pH. (Darker color indicates a faster reaction rate at designated pH range.)

chemical stability. It is important to define an optimal pH that is a good compromise among all these characteristics.

- As chemical stability involves several different degradation pathways and each degradation pathway may have a different stability profile as a function of pH in solution (Fig. 2), it is important to balance all the degradation profiles before finalizing the optimal pH for the formulation. The degradation pathways that lead to significant loss in potency or biological activity or that cause immunoreactivity should be kept to a minimum when selecting the pH.
- The optimal pH selected in solution may not be optimal for proteins in solid dosage forms, so it is important to reassess the effect of pH when the protein dosage form changes from liquid to lyophilized form. For example, opposite trends in pH-dependent stability were observed for lyophilized and liquid formulations (80).
- The selected pH should not have any impact on the stability of other excipients in the formulation. Certain excipients may not be stable in a certain pH range under long-term storage conditions. For example, acidic pH has caused the hydrolysis of sucrose, a commonly used stabilizer in protein formulations (81,82).
- The selected pH should be compatible with product-contacting surfaces during manufacturing and storage. It has been reported that acidic pH caused corrosion of stainless steel in the presence of chloride ions, which generated iron ions that catalyzed methionine oxidation in a monoclonal antibody (83). Also, high or low pH may cause more leachables from stoppers, which serve as primary container closure.
- The selected pH needs to be safe to use for its intended administration route. A certain pH range may be suitable for IV injection but cause side effects when used for SC, IM, or IT injection.

For most protein formulations, the selected pH is in the range of 6 to 7, as this range is close to the physiological pH and also provides the optimal stability for most proteins. For

peptides, formulation pH is mostly in the acidic range of 4 to 5, as this pH range provides better aqueous solubility and less adsorption.

Buffers

Buffers are often used to control the formulation pH, keeping it within a narrow range to prevent small changes that can affect the stability and solubility of proteins. The selection of a proper buffer type and concentration for proteins should be based on the following considerations:

- The buffer species and concentration should not cause protein instability. For example, sodium phosphate buffer may result in a significant pH drop upon freezing, which has been found to cause instability of some proteins, particularly at high buffer concentration or low protein concentration conditions (84).
- The buffer species should have a pK_a near the target pH, preferably within one pH unit. Table 1 lists the pH control ranges for some commonly used buffer species.
- When buffer concentration has no effect on the protein stability, it should be kept to a minimum, but high enough to provide sufficient buffering capacity to control the formulation pH. High buffer concentration may cause some pain or discomfort upon SC or IM injection. In addition, for lyophilized product, a high concentration of buffer species such as sodium phosphate or citrate may lead to a lower glass transition temperature, which would require a longer lyophilization cycle.
- Buffers, like salts of ionic compounds, contribute to the overall ionic strength of the formulation solution. Therefore, buffer concentration also influences other properties that are dependent on ionic strength, such as protein solubility and stability.
- Certain buffer species, besides controlling solution pH, may also serve as a stabilizing agent in some protein formulations. For example, citrate may serve as a chelating agent to remove any heavy metals that potentially catalyze oxidation. Histidine also has an antioxidant effect (85).
- The buffer species and concentration should be safe to use for its intended administration route. Some buffer species and concentrations may be suitable for IV injection but may not be compatible when used for SC, IM, or IT injections. For example, citrate was found to cause more pain than histidine as a buffer when administered by SC injection (86).

It should be noted that as zwitterions, proteins have their own buffering capacity, especially at high concentration, so a buffer may not be required in formulations if the pH can be maintained by the protein itself. It has been reported that monoclonal antibodies at 60 mg/mL have a strong self-buffering capacity and that the long-term stability of self-buffered formulations is comparable to that of conventionally buffered formulations (87).

Ionic Strength/Salt Concentration

As proteins carry both negatively and positively charged groups, ionic strength in formulation solution may directly affect the solubility and stability. Ionic strength in parenteral formulation

Table 1 Buffers for Protein Formulations

Buffer	Acid	Base	pH range control	Examples
Phosphate	Monosodium phosphate	Disodium phosphate	5.8–7.8	Elaprase®, Remicade®
Acetate	Acetic acid	Sodium acetate	3.8–5.8	Avonex®, Neupogen®
Citrate	Citric acid	Sodium citrate	3.0–7.4	Amevive®, Rituxan®
Succinate	Succinic acid	Sodium succinate	3.3–6.6	Actimmune®
TRIS	TRIS HCl	TRIS	7.1–9.1	Wellferon®, Enbrel®
Histidine	Histidine HCl	Histidine	5.1–7.0	Xolair®, Raptiva®
Carbonate	Sodium bicarbonate	Sodium carbonate	5.4–7.4, 9.3–11.3	Fuzeon™

is mainly adjusted using NaCl. NaCl affects electrostatics in a protein either by nonspecific (Debye-Huckel) electrostatic shielding or by specific ion binding to the protein. At low concentration, salts affect electrostatic shielding and weaken ionic repulsion/attraction as counter-ions, so this shielding effect may be stabilizing when there are major repulsive interactions leading to protein unfolding, or could be destabilizing when there are major stabilizing salt bridges or ion pairs in the proteins. At high concentrations, electrostatic shielding is saturated. The dominant effect of salt, as of other additives, is on the solvent properties of the solution. The stabilizing salts seem to increase surface tension at the water-protein interface and strengthen hydrophobic interaction by keeping hydrophobic groups away from water molecules, inducing preferential hydration of proteins (13). Therefore, ionic strength or salt concentration affects both the solubility and the stability of proteins. The following areas should be assessed when selecting a proper ionic strength or salt concentration:

- Similar to the effect of pH, the profiles of solubility and stability as functions of ionic strength may not overlap each other for a specific protein, so the optimum ionic strength at maximum solubility may not be the same as at maximum stability. It is important to define an optimal ionic strength that is a good compromise between these two characteristics.
- Ionic strength or NaCl concentration has been reported to have an impact on the viscosity of formulations, especially at high protein concentrations (88). It may therefore serve an important factor in adjusting the viscosity of the product. Viscosity is an important parameter for syringeability of high–protein concentration formulations used in SC and IM administration.
- Ionic strength or NaCl concentration should be compatible with other excipients in the formulation and with product-contacting surfaces. It has been reported that high salt concentration combined with acidic pH may cause rusting of stainless steel, resulting in an elevated level of iron ions responsible for oxidation of the protein (83).
- Ionic strength or NaCl is certainly a key contributor to the overall tonicity of the formulation, and it is important to keep the concentration or tonicity suitable for the intended administration routes.

Stabilizers

When adjusting the parameters discussed above—protein concentration, pH, buffer, and ionic strength—still does not result in sufficient protein stability, the addition of stabilizers should be considered. Several types of stabilizers, listed in Table 2, are commonly used in protein formulations to stabilize proteins against various stresses. It is important to consider the following aspects when choosing a stabilizer:

- The choice of stabilizer type and concentration should be rational. An experimental laboratory-scale model should be developed to screen the stabilizer type and concentration for specific degradation against specific stress. For example, to identify a stabilizing excipient against shaking stress, surfactants and concentration ranges should first be tested through an established shaking model. To find a stabilizer against freezing/thawing stress, an appropriate freeze/thaw stress model should be used to screen various cryoprotectants and their concentration ranges.
- The number of stabilizers in a single formulation should be minimal and based on needs. An ideal stabilizer inhibits or minimizes multiple degradation pathways. For example, conformational stabilizers, such as sucrose, which enhance conformational stability, minimizes not only the aggregation but also other chemical degradations such as oxidation and fragmentation that occur when the reactive sites are exposed in absence of sucrose.
- The type and concentration of stabilizers should be compatible with other excipients in the formulation. For example, Ca^{2+} may be a good stabilizer, but if the buffer is phosphate and the pH is above neutral, precipitation of calcium phosphate may occur.
- Any stabilizers that may cause interference with protein assays should be avoided. For examples, polymers and albumins used as stabilizers in formulations may interfere

Table 2 Commonly Used Stabilizers in Protein Formulations

Type	Hypothesized stabilizing mechanism(s) (reference number)	Examples
Sugars	Stabilize proteins by preferential hydration in solution; serve as cryoprotectant and/or lyoprotectant; certain sugars such as glucose may chelate heavy metals, thus serving as antioxidants (89).	Sucrose: Follistim®, Panglubulin®, Ovidrel®, Xigris™ Trehalose: Advate, Herceptin® Lactose: Factrel®, Glucagon® Glucose: Gammagard® S/D Maltose: Bexxar®, Gamimune®
Polyols	Stabilize proteins by preferential hydration in solution; may serve as cryoprotectant and/or lyoprotectant; certain polyols such as mannitol may also serve as antioxidants by chelating metal ions (89).	Mannitol: DigiFab™, Fabrazyme®, Cerezyme® Sorbitol: Digibind®, Neulasta™, Neupogen® Glycerol: Humalog®, Humulin®R
Surfactants	Reduce agitation-induced aggregation by reducing surface tension; facilitate refolding by specific or nonspecific binding to protein; minimize adsorption and prevent other degradation by preferentially binding to interfaces (air-liquid, ice or solid surfaces).	Polysorbate 20: Neulasta™, Replagal®, Raptiva® Polysorbate 80: PEG-Intron, Remicade®, WinRho SDF® Poloxamer 188: Elitek™
Amino acids	Suppress protein aggregation and protein-protein or protein-surface interactions; arginine increases the surface tension of water, thus favorably interacting with most amino acid side chains and peptide bonds (90).	Glycine: Gamunex®, Synagis®, Neumega® Arginine: Activase®. TNKase® Cysteine: Acthrel®, SecreFlo® Histidine: BeneFIX®
Metal chelators	Chelate heavy metals to prevent metal-ion-catalyzed oxidation of cysteine and methionine residues in proteins.	EDTA: Kineret™, Ontak®
Divalent metal cations	Stabilize protein conformation by specific binding to certain sites of protein.	Ca^{++}: Pulmozyme®, ReFracto®, Kogenate® Zn^{++}: Nutropin Depot™, Aralast™
Polymers or proteins	Stabilize proteins by mechanisms similar to those of surfactants; serve as cryoprotectant or lyoprotectant.	Albumin: Intron®A, Rebif®, Zevalin™, Procrit® PEG: Autoplex®T, Hemofil®M, Monarc-M™, Prolastin® Carboxylmethylcellulose: Plenaxis™ Heparin: Thrombate III®, Autoplex®T Dextran: Mylotarg™

with protein assays, particularly in UV, HPLC methods and gel electrophoresis, creating complications for release and stability testing.

- Any stabilizers that may introduce potential contaminants, especially animal or human derived, should be avoided. Albumin, for example, is an excellent stabilizer for many therapeutic proteins and was widely used in early products; however, because of concerns about blood source contamination, it has seldom been used as a stabilizer in recent products. However, availability of pharmaceutical grade recombinant human serum albumin may change this dynamics.
- Stabilizers of high quality from a reputable vendor should be used for lab screening studies. Like any excipients, stabilizers may contain different levels and types of impurities when they are made from different sources or processes. These impurities

could cause inconclusive results from screening studies, as they may result in instability even while the stabilizer itself has a stabilizing effect. A case in point is the peroxide level in polysorbates: varying peroxide levels in polysorbates used in formulation studies often confound the study results.
- The type and concentration of stabilizers chosen should be safe to use for intended administration routes. It is important to assess the safety and toxicity of any new excipient prior to clinical studies.

As shown, choosing the proper type and concentration of stabilizer is a challenging process. It is important to consider all aspects, including solubility, stability, compliance, safety, and operational challenges (such as posing analytical difficulties).

Tonicity Modifiers

For parenteral administration, the final product is generally formulated to be isotonic or iso-osmotic, which is equivalent to 0.9% or 150 mM NaCl with an osmolality of 289 mOsm/kg. The following aspects should be considered when finalizing the type and concentration of tonicity modifiers in the final product:

- Commonly used tonicity modifiers in protein formulations include NaCl, mannitol, and sorbitol. An excipient already selected for the formulation, such as salt or stabilizer, is the preferred choice when increasing concentration does not have an effect on the overall properties of the formulation.
- The requirement of isotonicity depends on the administration route and dose preparation. If the product is diluted into IV fluid such as normal saline solution prior to administration, the formulation may not be required to be isotonic. However, if the product is directly injected without any dilution, isotonicity is preferred, particularly for SC, IM, and IT injections.
- For lyophilized product, the formulation prior to lyophilization may not be required to be isotonic even when the reconstituted solution is required to be isotonic. Isotonicity in final reconstituted solution can be achieved by choosing the proper type and volume of diluent to reconstitute the lyophilized product.

It should be noted that some recent studies have shown that infusion of solutions with iso-osmolality but hypotonicity may cause some adverse effects (91). This suggests that although the terms "isotonic" and "isoosmotic" have been used interchangeably, they may have different effects on safety, particularly for products that will be infused in large quantity.

Bulking Agents (for Lyophilized Product Only)

For lyophilized product, bulking agents are required to provide enough solids to maintain good cake structure during lyophilization and long-term storage. To choose the proper type and amount of bulking agent, the following aspects should be considered:

- The type and amount of bulking agent added to the formulation should be compatible with the protein and other excipients. It should not cause significant protein instability during lyophilization or storage. An excipient already selected for the formulation, such as a stabilizer (sucrose or trehalose), should be preferred as the bulking agent when increasing the concentration does not have an effect on the overall properties of the formulation (e.g., high sucrose concentration may require an extremely long lyophilization cycle or result in partially collapsed cakes).
- Mannitol and glycine are commonly used bulking agents as they provide better cake appearance and do not require a longer lyophilization cycle. However, because of their crystalline nature, they may cause phase separation during storage, leading to stability issues. In addition, a high content of mannitol may lead to vial breakage during freezing (92) due to volume expansion during crystallization. This vial breakage phenomenon has been also observed with NaCl crystallization during lyophilization (93).

Table 3 Preservatives for Protein Formulation

Type	Example(s)	Comments
Phenol	Antivenin Aplisol® Nutropin AQ®	Air and light sensitive; may act as a reducing agent.
Benzyl alcohol	Epogen® Nutropin® Pegasys® Factrel®	Usually in diluent for reconstitution of lyophilized product.
m-Cresol	Humatrope NovoLog®	Used in both liquid multiuse products and diluents for lyophilized products.
Thimerosal	Antivenin	Not commonly used for recent products because of mercury-related toxicity.
Chlorobutanol	Desmopressin	Widely used preservative in pharmaceuticals, including injectables. Typically used at 0.5%.

- Polymeric bulking agents [e.g., hydroxyethyl starch (HES) or dextran] and proteins (e.g., albumin) may significantly reduce the length of lyophilization cycle by raising T_g', however, some of them may interfere with certain protein assays. Therefore, potential complications in analytical testing should be considered when choosing this type of bulking agents.
- For high-protein-concentration formulations, bulking agents may not be required, as a protein itself serves as a bulking agent to provide good cake structure.

Preservatives (for Multidose Products)

Most injectable protein products are intended for single-dose injection, which does not require inclusion of antimicrobial preservatives in the formulation. However, some products are intended for multidose administration, which requires preservatives in the formulation to prevent any microbial growth from the time the product is opened for use to the time the last dose is administered.

Table 3 lists the preservatives that have been used for protein formulations. To choose the appropriate type and concentration of preservatives in a formulation, the following aspects should be considered:

- The type and concentration of preservatives selected for a formulation should make the final product meet the antimicrobial effectiveness testing required by USP and BP/EP at the time of product release and at the end of shelf-life and last dosing. One needs to be aware that requirements in BP/EP are more stringent than those in USP (94).
- Adding preservatives generally results in protein instability. This is not surprising, as the bactericidal or bacteriostatic effect is derived from the preservative's interaction with proteins or DNA in microorganisms. It is important to screen for a compatible type of preservative for specific formulations or proteins.
- Minimizing the contact time between preservative and protein is a general approach to reducing the preservative's stability impact. In this approach, preservatives are typically added to the diluent (for lyophilized product) or to the product upon preparation for the first dose.

General Strategies for Formulation Screening and Optimization

As discussed above, protein formulation has multiple parameters, including the protein itself, buffers, pH, stabilizers, and other excipients, and each parameter has its own functions. Some of these parameters may interact with each other; for example, pH and stabilizers both affect

Table 4 Formulation Parameters, Evaluation Models, and Critical Attributes for Design of Experiments

Formulation parameters	Evaluation models	Critical attributes
Protein concentration	Thermal stress	Appearance
pH	Freeze/thaw cycle	Content
Buffer type/concentration	Peroxides exposure	Aggregation
Stabilizer type/concentration	Light exposure	Fragmentation
Tonicity modifier type/concentration	Shaking/agitation	Oxidation
Preservative type/concentration		Deamidation
		Potency

the stability of the molecule. In addition, the product has to be exposed to multiple process-related stresses during manufacturing, storage, and handling, such as extreme temperatures, freeze/thaw cycles, agitation, and pressure. Therefore, it is challenging to develop a stable formulation containing many parameters against various process-related stresses.

Design of experiments (DOE) has proven an effective tool in dealing with such a complicated development process involving multiple variables. DOE, as part of the concept of quality by design (QbD) recently introduced by regulatory agencies for pharmaceutical development, is a tool to establish the design space through statistical analysis. The design space forms a link between development and manufacturing design (ICH Q8, Pharmaceutical Development). For formulation development, the design space refers to the defined range of formulation parameters and quality attributes that have been demonstrated to provide assurance of quality.

Formulation development using a DOE approach typically has two stages: formulation screening and optimization/robustness studies. The goal of the screening study is to identify the key formulation parameters, while the optimization/robustness study defines the optimal or robust range of the selected key parameters.

To design a proper space for statistical DOE studies, it is important to collect all the information from preformulation development activities and any prior knowledge on the protein. This information helps in identifying the critical formulation parameters and the key degradation pathways that potentially affect the critical quality attributes (CQAs) of the product for the design space, and in selecting a proper evaluation model that can be used for screening or optimization of formulations. In addition, the target product profile (TPP) should also be established prior to DOE studies.

Table 4 lists the parameters and attributes for DOE studies. Once the study is completed and data are collected, statistical analysis should be performed to establish the design space. This established design space can not only justify the choice of formulation ranges and help identify the robust region of the formulation, but can also enable study of the interactions between each formulation parameter. Several case studies using the DOE concept have been described in a book chapter written by Ng and Rajagopalan (95).

Choice of Container and Closure System

Because proteins may interact with the contact surfaces, the compatibility of immediate packaging material with protein product needs to be evaluated during selection of the container closure system.

As described in section "General Formulation Development Process in Industry," most protein products are formulated as drug substance and drug product, which are typically stored at frozen and refrigerated conditions, respectively. In choosing an appropriate container closure system for drug substance, the following aspects should be considered:

- Commonly used container closure systems for drug substance include plastic bottles or bags and stainless steel vessels. Various types of plastics have been used in packaging protein drug substance, including Teflon, polyolefin, glycol-modified polyethylene terephthalate (PETG), polypropylene (PP), polycarbonate (PC), polyvinylchloride (PVC), polyvinyl alcohol (PVA), and polyethylene (PE). It is important to

evaluate whether the product remains stable under intended storage conditions. For example, hydrophobic proteins tend to adsorb more on hydrophobic polymers such as Teflon. In addition, the material's gas permeability and leachables should also be evaluated. Plastics with high gas permeability may affect product stability during long-term storage if the product is oxygen sensitive. Plastics such as PVC that contain a substantial amount of plasticizer may generate more leachables than other plastics. When stored in a stainless steel vessel, acidic pH and chloride ions may cause corrosion. With an increased amount of dissolved metal ions, protein oxidation is a concern (83).

- The material should retain its function at the intended storage condition. Since most protein drug substances are stored frozen, it is important to assess the brittleness point of the plastics at the intended storage temperature. If the brittleness point is above the intended storage temperature, container closure integrity may be compromised. In addition, breakage of container closure may occur upon impact, such as an accidental drop. Therefore, PC and Teflon are preferred because of their lower brittleness points.
- The size of the containers should be selected on the basis of product stability, potential expansion of product, and cost effectiveness. For example, a sufficient amount of headspace should be allowed to accommodate volume expansion upon freezing for frozen drug substance. If the product is stored as a liquid, then minimal headspace should be considered to minimize the potential instability caused by agitation upon handling and shipping. With large containers, it may be difficult to control the freezing process. Stainless steel cryo-vessels with temperature-controlling systems have many advantages over plastic containers, such as controlled freezing and thawing rates and nonbreakable characteristics. However, they are expensive and need to undergo cleaning validation for multiuse purposes. In addition, they may need frequent passivation to retain resistance to potential corrosion.

To choose an appropriate container closure system for drug product, the following aspects should be considered:

- Unlike drug substance, most drug products are stored in glass vials with rubber stopper systems. Some products are also packaged in prefilled syringes, cartridges, and dual chamber Lyo-Ject® syringes, which all consist of glass barrels or tubing and rubber stoppers. Plastic vials or tubing have also recently been introduced. It is important to evaluate the product's compatibility with various types of glass, plastics, and rubber stoppers, as protein adsorption and other degradations may occur.
- Container closure integrity should be retained to ensure the product's sterility throughout its shelf-life.
- Most glass vials are washed and then depyrogenated prior to use. It has been reported that siliconized vials may minimize adsorption of the product (17). However, the possibility of silicone oil causing protein aggregation also needs to be examined. For prefilled syringes, the glass barrels should be siliconized for proper syringeability. It is important to recognize, however, this step may affect not only syringeability but also product stability. For lyophilized product, the stoppers may need to be dried in an oven or autoclave following steam sterilization, because retained moisture may be released to the lyophilized cake during long-term storage and cause instability issues, which are critical for moisture-sensitive products.
- The type of container closure system should be decided on the basis of the product's stability, the development stage, the intended use (indication and administration route), and marketing competitiveness. For an early clinical development stage, a vial and stopper system is often chosen, as it involves less technical complexity and thus requires less development time. For late development or commercial stages, the performance of a container closure system—such as improving ease of administration, minimizing drug wastage, and conforming with patient needs (e.g., self-administration)—becomes more important. Prefilled syringes, self-injection devices using cartridges, and dual chamber lyo-ject syringes may help the drug product to gain a greater market share because of their convenience of use, which leads to better patient compliance.

- The size of container closure should be decided on the basis of several factors, including dosing regimen, product stability, cost effectiveness, and compliance for patient use. For example, vial size affects the headspace, which is a critical parameter for most liquid products as it may impact product stability upon agitation during shipping and handling. For prefilled syringes, the size of the syringe also affects the headspace and the movement of the stoppers upon exposure to reduced pressure during airplane shipping. Cartridges tend to have less headspace.

For both drug substance and drug product, the suppliers of the container closure system should be reputable and well established. Suppliers that have established Drug Master Files (DMFs) for the packaging components should be preferred. In addition, for commercial products, a second source for the container closure system may need to be established in case issues arise with the primary source.

Manufacturing Process Development

The next step after selection of an appropriate formulation and container closure system is to develop a manufacturing process that maintains protein stability under process conditions such as mixing, filtration, filling, and lyophilization. Instability of proteins under these conditions is often observed, and therefore it is important to evaluate and define the optimal process conditions prior to cGMP manufacturing. From these studies, the critical process parameters and acceptable operation ranges should be defined using a DOE approach similar to that used in formulation development. The CQAs impacted by these process conditions should be evaluated. The following aspects should be considered when developing a robust and suitable manufacturing process:

- All product-contacting surfaces during manufacturing should be compatible with the formulation. Generally, the product-contacting surfaces during drug product manufacturing include silicone tubing, a Teflon-coated stir bar, a stainless steel tank or impeller mixer, rubber gaskets, plastic connectors, plastic housing and filter membranes, and other glass or plastic containers. It is important to evaluate the compatibility of the protein with these contact surfaces prior to the start of manufacturing using these materials.
- The container closure system should be compatible with the fill line at the manufacturing site. Typically, machinability needs to be conducted prior to cGMP manufacturing to ensure that the filling and stoppering operations run smoothly, with low rejection rates, and that the final container closure system meets the container closure integrity test criteria (integrity is typically tested by dye leak or vacuum decay method).
- The mixing condition should not result in product degradation. If mixing by a magnetic stir bar causes protein precipitation, alternative mixing methods such as an impeller should be considered.
- If the product is sensitive to dissolved oxygen in solution, several manufacturing process steps should be designed appropriately. Degassing the solutions and overlaying inert gas (nitrogen or argon) in the headspace of vials may be required to minimize oxidation due to dissolved oxygen in the product.
- The filter size for sterile filtration should be large enough not to give high back pressure during filtration. For aqueous protein formulations, a polyvinylidene fluoride (PVDF) membrane is the most commonly used. A filter sizing study should be performed to define the proper size of filter for cGMP manufacturing.
- The filling conditions should be compatible with the product. For solution filling, several filling machines are commonly used, including peristaltic pump, stainless steel piston syringe, ceramic piston syringe, rolling diaphragm, and rotary time pressure filling systems. While a syringe-filling system typically provides better accuracy, it applies high shear stress between the piston and barrel during movement, which could lead to precipitation of proteins. In addition, the filling speed needs to be controlled to avoid foaming or splashing during filling.

- The lyophilization parameters developed at laboratory scale should be robust enough to produce consistent product quality. Sometimes when the lyophilization cycle developed for the laboratory-scale lyophilizer is directly transferred to the production-scale lyophilizer, product quality may not be the same. This could be caused by the poor robustness of lyophilization cycle, as different lyophilizer designs may lead to different levels of heat and mass transfer, which could cause changes in product quality, such as cake collapse, if the selected lyophilization parameters are on the edge of the process design.
- Hold conditions (time and temperature) for all process intermediates should be established to meet the needs of routine production operations and to support potential excursions. An operation deviation may result in longer hold time for the process intermediate than during routine manufacturing; examples could be deviations prior to the formulation step or filling into final product, or after lyophilization. Supporting data for defining acceptable hold conditions need to address product quality from both chemical stability and microbiological perspective. Assessment of the microbiological acceptability of a process intermediate hold time is tied to manufacturing process operations and to the microbial growth potential of the intermediate composition. Support for extended or cumulative hold times generally comes from development-scale studies, while support for microbiological properties comes from manufacturing-scale studies.

Stability Studies

After the manufacture of drug substance and drug product, the following stability studies are generally conducted to support the shelf-life during long-term storage and the product quality during distribution and use at clinics:

- *Long-term stability studies to support shelf-life*. Several ICH guidelines outline the content and testing requirements for stability studies supporting shelf-life at long-term storage conditions (ICH Q1A, Q1C, Q1D, Q1E, and Q5C). Depending on the intended long-term storage condition, accelerated and stress conditions are often required for the clinical stability program and lots made during process validation. For postapproval commercial stability programs, one lot of drug substance and one lot of drug product are generally required to be placed on stability annually, and only at the long-term storage condition.
- *Temperature cycling studies to support excursions during distribution*. From completion of manufacturing to the time when the product is dosed into patients, the drug product experiences exposure to various temperatures, different from the intended long-term storage temperature range, which is typically the refrigerated temperature. It is important to conduct studies to evaluate the effect of these temperature variations on product stability. The design of these studies has been recommended in the Parenteral Drug Association (PDA) Technical Report No. 39 (96).
- *Shipping studies to support the exposure to vibration and reduced pressure during air and ground transportation*. It is important to recognize that shaking studies conducted at laboratory scale may not be representative of the actual shipping conditions to which the product is exposed, since the shaking studies may not have vibration amplitudes and frequencies similar to those generated during shipping. In addition, reduced pressure occurs during air shipment. This is a concern particularly for prefilled syringes, as stopper movement has been observed during multiple cycles of reduced pressure, which may affect the sterility of the product (97). Therefore, it is important to demonstrate that the product remains intact or within the designed space using actual or simulated shipping conditions, including representative secondary packaging and product orientation.
- *Confirmatory photostability studies to support exposure to light*. Sensitivity to light is highly dependent on the composition, structure, and formulation of the product. Most proteins and peptides are sensitive to intense light, particularly UV light, which

typically results in oxidation. However, they are relatively stable under normal indoor light. In addition, protein products are typically stored in refrigerated condition, in which there is no direct exposure to light. Commercial products normally have secondary packages, which prevent the product's exposure to light. To demonstrate that the product remains stable, it is important to conduct a confirmatory photostability study using representative commercial packaging per the ICH guideline Q1B.

- *In-use and compatibility studies with the administration system to support product stability and to assess compatibility with product-contacting surfaces and stresses during administration.* For IV infusion, some products may need to be diluted in an IV bag and then infused into patients through an IV infusion apparatus by a pump or other mechanism. It is critical to assess product stability after dilution prior to dosing, as well as the compatibility of the product in contact with the surfaces of the administration apparatus and infusion system, such as pumps. For lyophilized products, the stability of reconstituted solution should be evaluated to ensure the product remains stable during the ambient exposure period after reconstitution.

FORMULATIONS OF MARKETED PROTEIN PRODUCTS

In the United States, by law all marketed injectable products must disclose quantitative formulation, so the details on each marketed product are public. Sources on prescription information include the Physicians' Desk Reference (PDR) and numerous websites, including the FDA's http://www.accessdata.fda.gov/scripts/cder/drugsatfda and websites for specific products (which commonly take the form www.*tradename*.com—e.g., www.simponi.com). The formulations of discontinued products can be found in older editions of the PDR.

The excipients used in parenteral products were first reviewed and collated by Wang and Kowal in 1980 (98). Subsequently, there were reviews by Nema et al. (99) and Powell et al. (100). Specifically for biotech products, they were first reviewed by Wang and Hansen (101), and recently by Gokarn et al. (102).

The types of excipients used in protein formulations have evolved over time. For example, in the early years (1980s–1990s), human serum albumin (HSA) was commonly used as a stabilizer in many protein formulations, particularly low-concentration, high-potency products such as interferons, Factor VIII, and other growth factors. However, because of concerns about potentially contaminated blood that might compromise the quality of albumin, most products have been reformulated into HSA-free formulations. For example, Eprex® (epoetin α), originally containing albumin, was reformulated to use polysorbate 80 as an HSA-free product in prefilled syringes. Avonex® (interferon β), originally having albumin in lyophilized form, was reformulated to a polysorbate-20-containing liquid formulation in prefilled syringes.

Another example of evolving parenteral formulation excipients is the type of polysorbate. Because of concerns about potential auto-oxidation of the unsaturated double bond in polysorbate 80 (103), used predominantly in the early days of protein formulations, the trend seems to be moving toward the use of polysorbate 20. For example, Neupogen® is formulated with polysorbate 80, but the surfactant in its newer version, PEGylated protein (Neulasta®) was changed to polysorbate 20. Polysorbate 80 included in the Activase® formulation was also changed to polysorbate 20 in its newer variant TNKase®.

To show how formulations have evolved over time, examples of recombinant human growth hormones and monoclonal antibodies (including Fab, Fab-PEG, and Fc-fusion proteins) are listed in Tables 5 and 6, respectively. For human growth hormone, changes of formulation have been minimal since its first approval in 1985 to one recently approved in 2008. For lyophilized monoclonal antibodies, the buffer species used has changed from phosphate in early approved products to histidine in recently approved products. Sucrose is the most commonly used sugar in lyophilized formulations, and only a few products use trehalose or maltose. In addition, amino acids including arginine and glycine are used in both liquid and lyophilized formulations. For liquid monoclonal antibodies, sodium phosphate appears to be the most commonly used buffer, likely because of its good buffering capacity

Table 5 Formulations for Human Growth Hormone (in order of approval year)

Trade Name	Approval year	Dosing route	Presentation	Dosage form	Formulation
Protropin®	1985	SC	Vial/stopper	Lyophilized	5 mg vial[a]: 40 mg mannitol, 0.1 mg monobasic sodium phosphate, 1.6 mg dibasic sodium phosphate, pH 4.5–7.0
Humatrope®	1987	SC/IM	Vial/stopper	Lyophilized	5 mg/5 mL vial: 25 mg mannitol, 5 mg glycine, 1.13 mg dibasic sodium phosphate, pH 7.5
			Pen cartridge	Lyophilized	6 mg/2 mL cartridge[a]: same formulation as in vial/stopper
Norditropin®	1987	SC	Pen cartridge	Liquid	5 mg/1.5 mL cartridge[a]: 1 mg histidine, 4.5 mg Poloxamer 188, 4.5 mg phenol, 60 mg mannitol
Nutropin®	1993	SC	Vial/stopper	Lyophilized	5 mg/1 mL vial[a]: 45 mg mannitol, 0.4 mg sodium phosphate monobasic, 1.3 mg sodium phosphate dibasic, 1.7 mg glycine, pH 7.4
			Vial/stopper	Liquid	10 mg/2 mL vial: 17.4 mg sodium chloride, 5 mg phenol, 4 mg polysorbate 20, 10 mM sodium citrate, pH 6.0
			Pen cartridge	Liquid	5 mg/1 mL cartridge[a]: same formulation as in vial/stopper
Tev-Tropin®	1995	SC	Vial/stopper	Lyophilized	5 mg/5 mL vial: 30 mg mannitol, pH 7–9
Genotropin®	1995	SC	2-chamber cartridge	Lyophilized	5 mg/1 mL cartridge[a]: 5.8 mg somatropin, 2.2 mg glycine, 1.8 mg mannitol, 0.32 mg sodium dihydrogen phosphate anhydrous, 0.31 mg disodium phosphate anhydrous, pH 6.7
Saizen/Serostim®	1996	SC	Vial/stopper	Lyophilized	4 mg vial[a]: 27.3 mg sucrose, 0.9 mg phosphoric acid, pH 6.5–8.5
Zorbtive®	2003	SC	Vial/stopper	Lyophilized	4 mg vial[a]: 27.3 mg sucrose, 0.9 mg phosphoric acid, pH, 7.4–8.5
Omnitrope®	2006	SC	Vial/stopper	Lyophilized	1.5 mg/1.13 mL vial[a]: 0.88 mg disodium hydrogen phosphate heptahydrate, 0.21 mg sodium dihydrogen phosphate dihydrate, 27.6 mg glycine
			Pen cartridge	Liquid	5 mg/1.5 mL cartridge[a]: 1.3 mg disodium hydrogen phosphate heptahydrate, 1.6 mg sodium dihydrogen phosphate dihydrate, 3.0 mg poloxamer 188, 52.5 mg mannitol, 13.5 mg benzyl alcohol
Valtropin®	2007	SC	Vial/stopper	Lyophilized	5 mg vial/1 mL: 10 mg glycine, 45 mg mannitol, 0.22 mg monobasic sodium phosphate, 2.98 mg dibasic sodium phosphate, pH 6.0
Accretropin®	2008	SC	Vial/stopper	Liquid	5 mg vial: 0.75% NaCl, 0.34% phenol, 0.2% Pluronic F-68, 10 mM sodium phosphate, pH 6.0

[a]Indicates a product that has multiple dose strengths, but only the lowest strength is listed

Table 6 Formulations for Antibodies, Fc Fusion, and Fab Conjugates (in order of approval year)

Trade name	Nonproprietary name	Approval year	Dosing route	Presentation	Dosage form	Formulation
Orthoclone OKT-3®	Muromonab-CD3	1986	IV	Ampule	Liquid	5 mg/5 mL ampule: 2.25 mg monobasic sodium phosphate, 9.0 mg dibasic sodium phosphate, 43 mg sodium chloride, 1.0 mg polysorbate 80, pH 6.5–7.5
Reopro®	Abciximab (Chimeric Fab)	1994	IV	Vial/stopper	Liquid	10 mg/5 mL vial: 0.01 M sodium phosphate, 0.15 M sodium chloride, 0.001% polysorbate 80, pH 7.2
Rituxan®	Rituximab	1997	IV	Vial/stopper	Liquid	100 mg/10 mL vial[a]: 9 mg/mL sodium chloride, 7.35 mg/mL sodium citrate dihydrate, 0.7 mg/mL polysorbate 80, pH 6.5
Enbrel®	Etanercept	1998	SC	Vial/stopper	Lyophilized	25 mg/0.5 mL vial: 40 mg mannitol, 10 mg sucrose, 1.2 mg tromethamine, pH 7.1–7.7
				PFS	Liquid	25 mg/0.5 mL PFS[a]: 1% sucrose, 100 mM sodium chloride, 25 mM L-arginine hydrochloride, 25 mM sodium phosphate, pH 6.1–6.5
				Autoinjector	Liquid	Same as Liquid in PFS
Remicade®	Infliximab	1998	IV	Vial/stopper	Lyophilized	100 mg/10 mL vial: 500 mg sucrose, 0.5 mg polysorbate 80, 2.2 mg monobasic sodium phosphate monohydrate, 6.1 mg dibasic sodium phosphate dihydrate, pH 7.2
Simulect®	Basiliximab	1998	IV	Vial/stopper	Lyophilized	10 mg/2.5 mL vial[a]: 3.61 mg monobasic potassium phosphate, 0.5 mg disodium hydrogen phosphate (anhydrous), 0.80 mg sodium chloride, 10 mg sucrose, 40 mg mannitol, 20 mg glycine
Synagis®	Palivizumab	1998	IM	Vial/stopper	Liquid	50 mg/0.5 mL vial[a]: 1.9 mg histidine, 0.06 mg glycine, 0.2 mg chloride, pH 6.0
Herceptin®	Trastuzumab	1998	IV	Vial/stopper	Lyophilized	440 mg/20 mL vial: 400 mg α,α-trehalose dihydrate, 9.9 mg L-histidine HCl, 6.4 mg L-histidine, 1.8 mg polysorbate 20, pH 6.0
Zenapax®	Daclizumab	1999	IV	Vial/stopper	Liquid	25 mg/5 mL vial: 3.6 mg sodium phosphate monobasic monohydrate, 11 mg sodium phosphate dibasic heptahydrate, 4.6 mg sodium chloride, 0.2 mg polysorbate 80, pH 6.9
Mylotarg®	Gemtuzumab ozogamicin (calicheamicin)	2000	IV	Vial/stopper	Lyophilized	5 mg/5 mL vial: dextran 40, sucrose, sodium chloride, monobasic and dibasic sodium phosphate[b]
Campath®	Alemtuzumab	2001	IV	Vial/stopper	Liquid	30 mg/1 mL vial: 8.0 mg sodium chloride, 1.44 mg dibasic sodium phosphate, 0.2 mg potassium chloride, 0.2 mg monobasic sodium phosphate, 0.1 mg polysorbate 80, 0.0187 mg disodium edetate dihydrate, pH 6.8–7.4

Humira® (United Stataes) Trudexa (European Union)	Adalimumab	2002	SC	PFS	Liquid	40 mg/0.8 mL in PFS: 4.93 mg sodium chloride, 0.69 mg monobasic sodium phosphate dihydrate, 1.22 mg dibasic sodium phosphate dihydrate, 0.24 mg sodium citrate, 1.04 mg citric acid monohydrate, 9.6 mg mannitol, 0.8 mg polysorbate 80, pH 5.2
Zevalin®	Ibritumomab tiuxetan (^{90}Y)	2002	IV	Vial/stopper	Liquid	3.2 mg/2 mL vial: 0.9% NaCl, 50 mM sodium acetate
Amevive®	Alefacept	2003	IV/IM	Vial/stopper	Lyophilized	7.5 mg/0.5 mL vial[a]: 12.5 mg sucrose, 5 mg glycine, 3.6 mg sodium citrate dihydrate, 0.06 mg citric acid monohydrate, pH 6.9
Bexxar®	Tositumomab (IgG and IgG-^{131}I)	2003	IV	Vial/stopper	Liquid	35 mg/2.5 mL vial[a]: 10% maltose, 145 mM sodium chloride, 10 mM phosphate, pH 7.2
Xolair®	Omalizumab	2003	SC	Via/stopper	Lyophilized	202.5 mg/1.4 mL vial: 145.5 mg sucrose, 2.8 mg L-histidine hydrochloride monohydrate, 1.8 mg L-histidine, 0.5 mg polysorbate 20
Raptiva®	Efalizumab	2003	SC	Vial/stopper	Lyophilized	150 mg/1.3 mL vial: 123.2 mg sucrose, 6.8 mg L-histidine hydrochloride monohydrate, 4.3 mg L-histidine, 3 mg polysorbate 20, pH 6.2
Avastin®	Bevacizumab	2004	IV	Vial/stopper	Liquid	100 mg/4 mL vial[a]: 240 mg α,α-trehalose dihydrate, 23.3 mg sodium phosphate (monobasic, monohydrate), 4.8 mg sodium phosphate (dibasic, anhydrous), 1.6 mg polysorbate 20, pH 6.2
Erbitux®	Cetuximab	2004	IV	Vial/stopper	Liquid	100 mg/50 mL vial[a]: 8.48 mg/mL sodium chloride, 1.88 mg/mL sodium phosphate dibasic heptahydrate, 0.41 mg/mL sodium phosphate monobasic monohydrate, pH 7.0–7.4
Tysabri®	Natalizumab	2004	IV	Vial/stopper	Liquid	300 mg/15 mL vial: 123 mg sodium chloride, 17.0 mg sodium phosphate monobasic monohydrate, 7.24 mg sodium phosphate dibasic heptahydrate, 3.0 mg polysorbate 80, pH 6.1
Vectibix™	Panitumumab	2005	IV	Vial/stopper	Liquid	100 mg/50 mL vial[a]: 29 mg sodium chloride, 34 mg sodium acetate, pH 5.6–6.0
Orencia®	Abatacept	2005	IV	Vial/stopper	Lyophilized	250 mg/10 mL vial: 500 mg maltose, 17.2 mg monobasic sodium phosphate, 14.6 mg sodium chloride, pH 7.2–7.8
Lucentis®	Ranibizumab	2006	Intravitreal	Vial/stopper	Liquid	0.5 mg/0.05 mL vial: 10 mM histidine HCl, 10% α,α-trehalose dihydrate, 0.01% polysorbate 20, pH 5.5

(*continued*)

Table 6 Formulations for Antibodies, Fc Fusion, and Fab Conjugates (in order of approval year) (*continued*)

Trade name	Nonproprietary name	Approval year	Dosing route	Presentation	Dosage form	Formulation
Soliris®	Eculizumab	2007	IV	Vial/stopper	Liquid	300 mg/30 mL vial: 13.8 mg sodium phosphate monobasic, 53.4 mg sodium phosphate dibasic, 263.1 mg sodium chloride, 6.6 mg polysorbate 80, pH 7.0
Cimzia®	Certolizumab pegol	2008	SC	Vial/stopper	Lyophilized	200 mg/1 mL vial: 100 mg sucrose, 0.9 mg lactic acid, 0.1 mg polysorbate, pH 5.2
Arcalyst®	Rilonacept	2008	SC	Vial/stopper	Lyophilized	160 mg/2.3 mL vial: histidine, arginine, polyethylene glycol 3350, sucrose, glycine, pH 6.2–6.8[b]
Nplate®	Romiplostim	2008	SC	Vial/stopper	Lyophilized	250 μg/0.72 mL vial[a]: 30 mg mannitol, 15 mg sucrose, 1.2 mg L-histidine, 0.03 mg polysorbate 20, sufficient HCL to bring pH to 5.0
Simponi®	Golimumab	2009	SC	PFS	Liquid	50 mg/0.5 mL in PFS: 50 mg golimumab antibody, 0.44 mg L-histidine and L-histidine monohydrochloride monohydrate, 20.5 mg sorbitol, 0.08 mg polysorbate 80, pH 5.5
Ilaris®	Canakinumab	2009	SC	Vial/stopper	Lyophilized	180 mg/1 mL vial: sucrose, L-histidine, L-histidine HCL monohydrate, polysorbate 80[b]

[a]Indicates a product that has multiple dose strengths, but only the lowest strength is listed
[b]Quantitative formulation is not disclosed, not consistent with 21 CFR 201.100 (b) (5) iii.
Abbreviations: SC, subcutaneous; IM, intramuscular; IV, intravenous.

around pH 6 to 7, the pH at which most monoclonal antibodies are formulated. Polysorbate 80 or 20 is also present in many monoclonal antibody formulations.

CONCLUSION

In this book chapter we have attempted to provide an overview of formulation development for peptide- and protein-based therapeutics. For successful formulation development, it is important first of all to understand and characterize the unique characteristics of the protein or peptide, including molecular composition, structures, size, charge profile (pI), solubility, thermal transition midpoint, and key degradation pathways. Preformulation activities are also critical in identifying the key instability issues and potential pharmaceutically relevant sources responsible for specific degradation pathways. This chapter provides general principles and examples of major pharmaceutical development activities, including evaluation of critical formulation parameters, selection of container closure, development of the manufacturing process, and stability studies to support shelf-life and clinical use conditions. The results from these development activities are generally required in completing the pharmaceutical development sections of regulatory filings. Finally, trends in the evolution of formulation development since the early 1980s are described on the basis of several examples, including human growth hormone and monoclonal antibodies.

ACKNOWLEGMENTS

The authors would like to thank Drs. Zahra Shahrokh and Jamie Tsung (both at Shire Human Genetic Therapies, Inc., Cambridge, MA) and Dr. Wei Wang (Pfizer, St. Louis, MO) for their valuable comments, and also Mr. Dusan Djordjevich for editing the manuscript.

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10 | Development of ophthalmic formulations

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INTRODUCTION

Ophthalmic formulations are those that are intended for treating conditions of the eye; they may be intended to alleviate the signs or symptoms associated with a certain disease state, to provide relief from minor discomfort and irritation, or for treatment of the cause of a disease of the eye itself. In general, the best way to treat ophthalmic diseases is with a local treatment such as a topically administered eye drop. However, the biological design of the eye is optimized to keep the surface of the eye clear of all foreign substances and to provide a substantial barrier to transport of materials into or out of the eye. As a result, ophthalmic drug delivery presents a significant technical challenge. The ophthalmic formulator, therefore, must begin with a good understanding of the physiology of the eye and understand what ophthalmic drug delivery possibilities are available. The formulator must also understand the nature of the drug substance that needs to be delivered to the eye and its limitations. The ultimate job of the ophthalmic formulator is to discover the best way to bring the drug and the eye together in a fashion that will provide the optimal benefit to the patient.

This chapter will focus on the anatomy and physiology of the eye and the challenges in drug delivery to this organ. The goal will be to familiarize the reader with the biopharmaceutical aspects of drug delivery to the eye, the various strategies for targeting different tissues within the eye, and to provide a guide to a rational approach to formulation development for ophthalmic drug delivery. We will also provide a brief overview of ophthalmic formulation preservation, manufacturing and packaging, and regulatory pathways for bringing a formulation to market. Finally, the chapter will discuss some recent advances in drug delivery and the future of ophthalmic drug delivery.

STRUCTURE AND FLUID COMPOSITION OF THE EYE

The eye globe is continually cleansed and hydrated by the secretions of the nasolacrimal system. The eye globe can be divided into three concentric tunics: the fibrous tunic comprising the cornea and sclera, the vascular tunic (consisting of the iris, ciliary body, and choroid), and the retinal tunic. Other internal components of the eye include the lens, aqueous humor, and vitreous humor. Figure 1 illustrates the relative locations of these tissues in the eye. When a drug is delivered to the outer surface of the eye, it may need to diffuse through many of these tissues before it can reach the target tissue. The following is a very basic review of the main components of the eye relevant to the drug delivery:

Nasolacrimal System

The nasolacrimal system consists of three parts: the secretory system (lacrimal glands, meibomian glands, and goblet cells), the distributive system (eyelid movements and blinking), and the excretory system (lacrimal puncta; superior, inferior and common canaliculi; lacrimal sac; and nasolacrimal duct). The nasolacrimal system plays a major role in protecting and hydrating the eye surface. It also has significant impact on the amount of drug absorbed to the eye from topical administration.

Tear Fluid Secretion and Volume

Tears are continuously secreted by the lacrimal glands and the goblet cells. The normal (basal) secretion rate is about 1.2 mcL/min (1), however, under reflex tearing the secretion rate may increase to as high as 300 to 400 mcL/min (2). The normal volume of tear fluid on the eye is about 6 to 7 mcL. When additional fluid is added to the eye surface, the eye can hold about 25 mcL of fluid but will appear "watery" because of the added liquid. With greater additions of fluid, the excess tear fluid will immediately overflow at the lacrimal lake (1) or be splashed into the eyelashes by reflex blinking (3).

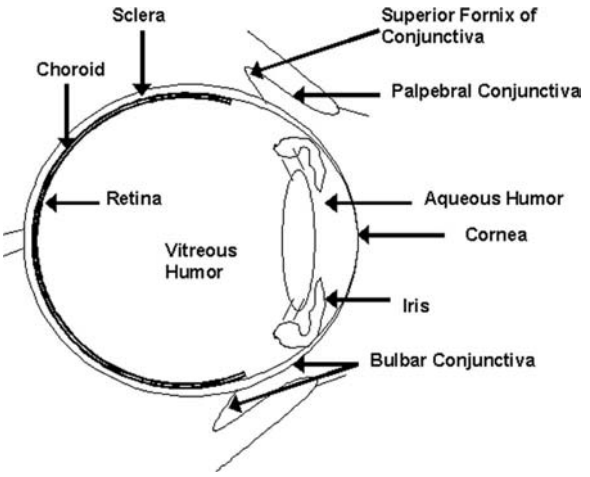

Figure 1 Schematic cross section of human eye.

Tear Fluid Lipid Content

The lipid layer of the tear film is secreted by the meibomian glands. The lipid layer of the tear film serves many functions including reducing evaporation from the ocular surface, lubricating the ocular surface, controlling the surface energy of the tear film, and providing a barrier function at the lid margin to inhibit the flow of skin lipids into the eye and tear fluid out of the eye (4,5). The meibomian secretions are primarily wax esters and sterol esters (about 59%), phospholipids (about 15%), and the remainder is diglycerides, triglycerides, free fatty acids, free sterols, and hydrocarbons (4,5). The polar lipids (phospholipids) are primarily phosphatidylcholine (40%) and phosphatidylethanolamine (18%) (6). The meibomian secretions are produced at a rate of about 400 mcg/hr and are excreted onto the lid margin and the anterior surface of the tear film by the normal blinking action. The thickness of the oil film on the tear fluid has been measured by various interference techniques giving values of 32 to 80 nm in thickness (5). From this thickness, the steady state amount of oil present on the surface of the tear fluid is calculated to be about 9 mcg per eye (5).

Tear Fluid Proteins and Enzymes

Tear fluid contains proteins in high concentration (about 8 μg/μL). Major components include lysozyme (an antibacterial enzyme), lactoferrin (which sequesters iron), secretory immunoglobin A (an antibody important for mucosal immunity), serum albumin, lipocalin and lipophilin (7). In addition, over 400 other proteins have been identified that serve various roles in the tear fluid (7).

Tear Fluid Mucus Layer

The mucus layer is secreted onto the eye surface by the goblet cells. Mucus consists of glycoproteins, proteins, lipids, electrolytes, enzymes, mucopolysaccharides, and water. The primary component of mucus is mucin, a high-molecular-weight glycoprotein that is negatively charged at physiological pH. The mucus layer forms a gel layer with viscoelastic properties which protects and lubricates the eye. The mucus gel traps bacteria, cell debris, and foreign bodies. The mucus layer may hinder drug delivery by forming a diffusional barrier to macromolecules, but it may also bind other substances (i.e., cationic or mucoadhesive) and prolong residence on the surface of the eye.

Tear Fluid pH and Buffer Capacity

The pH of normal tear fluid is 7.4 ± 0.2 (8). The primary buffering components present in the tear fluid are bicarbonate and proteins (8,9). The buffering capacity of the tear fluid is not symmetric around the mean pH. Rather, the tear fluid has more than twice the buffer capacity to resist drops in pH than it has to resist increases in pH (9). As a result of this asymmetric

buffering capacity, unbuffered solutions in the pH 4.0 to 8.5 range will cause minimal shift of the pH on the surface of the eye and will be easily neutralized by the tear fluid (9). Solutions with higher buffer capacities, particularly if greater than that of the tear fluid, may be uncomfortable to the eye if they result in a significant shift of the tear fluid pH.

Tear Fluid Osmolality

The tear fluid osmolality normally ranges from about 300 to 320 mOsm (3,8) and most of the osmolality in the tear fluid can be attributed to the salt content of the lacrimal fluid which is primarily sodium chloride, sodium bicarbonate, potassium chloride, calcium chloride, and magnesium chloride (3). Normally, there is about five times more sodium than potassium in the tear fluid and the levels of calcium and magnesium are less than 1/200th of the sodium levels (3). Higher than normal osmolality in the tear fluid is often seen in patients with dry eye syndrome. Abnormally high evaporation of tear fluid increases the salt levels and results in higher osmolality. As a result, many products for treatment of dry eye are often formulated with lower than normal osmolality.

Tear Fluid Viscosity and Surface Tension

The viscosity of the tear fluid would be expected to be primarily controlled by the higher molecular weight proteins dissolved in the lacrimal fluid. The viscosity of human tears has seldom been determined because of the difficulty of collecting enough sample for a determination. Schuller, et al., (10) found the viscosity of human tears ranges from 1.3 to 5.9 cps with a mean value of 2.9 cps. The viscosity of ophthalmic solutions may be increased in an effort to improve retention on the ocular surface. Hung, et al., (11) estimated that a painful sensation would be elicited if the tear fluid viscosity is increased above 300 cps at the shear conditions of the closing eyelid (shear rate of 20 000/sec). The surface tension of the tear fluid depends on the presence of soluble mucins, lipocalins, and lipids. The mean surface tension value is about 44 mN/min (12).

Fibrous Tunic

Cornea

The cornea is a transparent structure responsible for the refraction of light entering the eye. It forms the anterior one-sixth of the eyeball. The cornea is thinnest at its center (0.5–0.6 mm) and thicker in the periphery (1.2 mm) (13). The cornea is an avascular tissue that is supplied with oxygen and nutrients via the lacrimal fluid, aqueous humor, and the blood vessels at the cornea/sclera junction. The cornea is composed of five layers.

1. The epithelium—a stratified squamous epithelium made of 5 layers of cells (10 layers at the corneoscleral junction, i.e., the limbus) that has total thickness of around 50 to 100 μm. At the limbus, the corneal epithelium is continuous with the bulbar conjunctiva. The epithelial cells are connected through tight junctions which limit drug permeability significantly.
2. Bowman's membrane—lies between the basement membrane of the epithelium and the stroma, and is composed of acellular interwoven collagen fibers.
3. The stroma—accounts for 90% of the cornea thickness and is mainly composed of water and collagenous lamellae that gives the strength and structure for this layer and yet allows the penetration of light. Generally, it does not significantly limit drug permeability.
4. The Descemet's membrane—composed of collagen fibers, it lies between the stroma and the endothelium.
5. The endothelium—composed of a single layer of flattened cells that are connected via tight junctions. It controls the hydration of the cornea by limiting access of water from the aqueous humor and by active transport mechanisms.

The cornea provides a limited surface area of about 1 cm^2 for drug diffusion, and is a significant barrier to both hydrophilic and lipophilic compounds. Lipophilic molecules will

diffuse more easily through the epithelium and the endothelium, but hydrophilic molecules will diffuse more easily through the highly aqueous stroma.

Conjunctiva

The conjunctiva is a thin mucus secreting membrane that lines the posterior layer of the eyelids (palpebral conjunctiva), the anterior sclera (bulbar conjunctiva), and the superior and inferior conjunctival fornices (joining areas between the palpebral and bulbar conjunctiva). The conjunctiva is composed of two layers: an outer epithelium layer (which is continuous with the corneal epithelium) and an underlying stroma layer.

The conjunctival epithelium is made of 5 to 15 layers of stratified epithelial cells that are connected at the apical side with tight junctions and it plays a major role in limiting drug penetration (14). Nevertheless, the human conjunctiva is 2 to 30 times more permeable to drugs than the cornea (15).

The stroma layer of the conjunctiva contains the nerves, lymphatics, and blood vessels and it attaches loosely to the sclera. The conjunctiva contributes to the tear film formation by secreting electrolytes, fluid, and mucin (14).

Sclera

The sclera covers five-sixths of the eyeball surface and has a mean surface area of 16.3 cm^2 (16). It connects to the cornea anteriorly at the limbus. The sclera is mainly composed of collagen fibers with varying sizes and orientation that are embedded in a glycosaminoglycan matrix. Scleral thickness varies by location; the mean thickness is 0.53 mm near the limbus, is 0.39 mm near the equator, and is about 0.9 to 1.0 mm near the optic nerve (17). The sclera is composed of three main components.

1. Episclera—the outermost layer made of loosely arranged collagen fibers that is connected to the eyeball sheath (Tenon's capsule).
2. Stroma—composed of larger collagen fibers and elastic tissue.
3. Lamina fusca—the innermost layer of sclera that forms the uveal tract with the choroids. It is composed of loosely coherent collagen bundles and melanocytes.

Aqueous Humor

Aqueous humor is a clear fluid that is secreted by the ciliary body via the filtration of blood passing the through the ciliary body capillaries. It has several functions including maintaining the shape of the eye by controlling its pressure, providing nutrition to the cornea and lens, and providing transport of waste materials away from surrounding tissues. The aqueous humor is composed mainly of water, high concentrations of ascorbic acid, glucose, amino acids, and limited levels of proteins. Aqueous humor flows from the ciliary body in the posterior chamber (behind the iris) into the anterior chamber (between the iris and the cornea). Aqueous humor flows out of the eye through the trabecular meshwork (a network of collagen fibers and endothelial-like trabecular cells) into Schlemm's canal, and through the uveoscleral route (18). The entire volume of the aqueous humor is about 0.2 mL and is replaced every one to two hours (13). Maintaining the intraocular pressure (IOP) of 10 to 20 mmHg is a balancing act of production and drainage.

Vitreous Humor

Vitreous humor is a gel-like material that occupies the space between the lens and the retina. The vitreous humor is composed mainly of water (98–99.7%), collagen fibrils and hyaluronic acid (19). It supports the posterior surface of the lens and helps keep the neural part of the retina in place. The normal aging process can lead to liquefaction of the vitreous (>50% by age 80–90) and posterior vitreous detachment. The close proximity of the vitreous to the retina and choroid makes this cavity a direct place for drug delivery to the posterior tissues.

Vascular Tunic

Blood-Retina Barrier

The blood-retina barrier is composed of two parts which regulate the transport to the retina: the outer retina barrier formed by retinal pigment epithelium (RPE) and the inner retina barrier formed by the endothelial cells of the retinal vessels.

Two vascular beds supply the retina. Retinal vessels supply the inner two thirds, while the outer retina is avascular and receives oxygen and nutrients from the choriocapillaris. The choriocapillaris is fenestrated to enhance nutrients transport to the underlying retina. Plasma leaks from the choriocapillaris and diffuses through the Bruch's membrane and through the RPE to the outer retina. RPE tight junctions constitute the outer blood-retinal barrier.

Retinal vessels are supplied by the central retinal vessel. Retinal capillaries are composed of a single layer of endothelial cells surrounded by a basement membrane and pericytes. The endothelial cells are attached to each other by tight junctions forming the inner blood-retina barrier. These narrow tight junctions, similar to those present in the brain vessels, impair the paracellular transport of hydrophilic compounds and necessitate their passage through the intracellular routes (20).

Choroid

The choroid is a highly vascularized tissue between the retina and the sclera. It consists of: the vessel layer, the choriocapillaris, and Bruch's membrane (which is in direct contact with the RPE). Between the sclera and the choroid there is the suprachoroidal, or perichoroidal, space. This is a very thin space consisting of various connective tissue lamellae and is characterized as sponge tissue. Substantial amounts of the aqueous humor that leaves the eye via the uveoscleral route ends up in the suprachoroidal space and is finally drained out from the eye through porosites in the sclera.

The choriocapillaris is found in the inner portion just below the RPE and it provides nutrition to the RPE and the outer one third of the retina. Between the RPE and the choriocapillaris is the Bruch's membrane. Bruch's membrane is composed of five layers: the basement membrane of the RPE, an inner collagenous layer, the elastic layer, the outer collagenous zone, and the basement membrane of the capillary endothelial cells.

Retinal Tunic

The retina is composed of neural retina and RPE. The inner surface of the neural retinal is facing the vitreous humor while the outer border is next to the RPE. The neural retinal is composed of nine layers containing the nerve fibers and the photoreceptors responsible for light detection. The RPE is composed of a single layer of cells connected by tight junctions.

Biopharmaceutics and Routes of Administration

Drug penetration into the eye is a challenging task and can follow different pathways to reach the ocular target tissues depending on the route of administration and the drug's physicochemical properties. The target tissue within the eye is different for each drug and indication. In general, the focus of drug delivery to the eye can be divided into delivery to the anterior segment of the eye and to the posterior segment of the eye.

Delivery to the Anterior Segment of the Eye (Topical)

The anterior segment includes the conjunctiva, the cornea, the anterior sclera, the iris, the ciliary body, and the aqueous humor. Topical administration of drugs is considered the most common and acceptable route of administration for these target tissues. Drugs applied topically as an eye drop of a solution or suspension or as an ointment are easy to administer and noninvasive. However, drug penetration via this route is inefficient—bioavailability is generally less than 5% of the administered dose. Accordingly, the majority of the dose will end up in the systemic circulation and may have systemic effects (21,22). In certain conditions, particularly when sustained prolonged drug release is preferred, a subconjunctival injection or implant may be used to target these tissues. Drug penetration to the anterior tissues from topical administration faces significant barriers that limit its ocular bioavailability (Fig. 2).

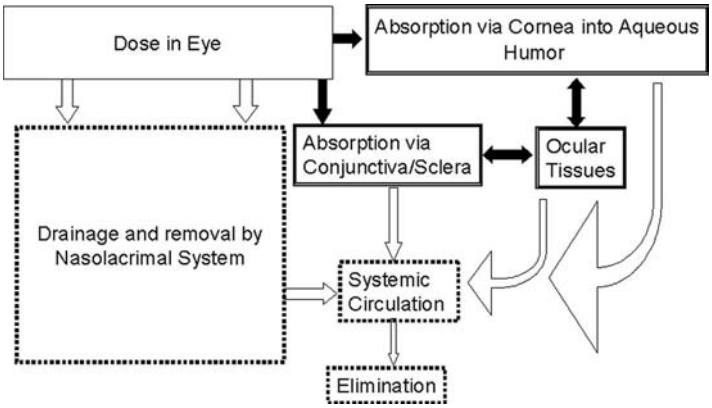

Figure 2 Schematic representation of compartmental drug penetration and elimination from topical administration.

Nasolacrimal Drainage

One of the most important attributes of a good ophthalmic topical formulation is that it needs to remain on the surface of the eye long enough to deliver a therapeutic amount of the medication. This necessarily means that the formulation needs to mix with or replace a portion of the natural tear fluid and should be as comfortable on the surface of the eye as the natural tear fluid. If the formulation evokes discomfort on the eye in any way (pH, osmolality, viscosity), it will lead to reflex tearing and blinking as the body attempts to flush the offending agent from the surface of the eye. Therefore the first step to understanding how to formulate an ophthalmic formulation is to understand the characteristics of the tear film (discussed above) that should be appropriately mimicked by the formulation. In addition, the formulation should be designed so that it does not adversely interact with the components in the natural tear fluid.

Most of the topical dose is lost through the nasolacrimal drainage before it can reach the eye. The limited volume that the eye surface can accommodate (30 μL), the high tear turnover rate (0.5–2.2 μL/min), and blinking rate are all natural ways of the eye to protect itself and limit penetration through its surface. The introduction of an eye drop (average volume of 39 μL), and possibly its composition will induce more tear secretion and increased blinking that will enhance the drainage out of the eye surface and reduce amount of drug available for absorption (23).

Corneal Absorption

The cornea offers the major site of drug diffusing into the anterior chamber of the eye, especially for small molecules. Drug penetration through the cornea can be by passive diffusion or by active transport mechanisms. The two main factors influencing the passive diffusion are lipophilicity and molecular size. Small lipophilic compounds generally penetrate through the epithelium via the intracellular route, while small hydrophilic compounds are limited to the paracellular route (partitioning of small lipophilic compounds into the cornea causes it to act as a depot). Large hydrophilic compounds (5000 Da) are generally excluded by the epithelium tight junctions (24). The fraction of a lipophilic compound penetrating through the cornea is 20 times more than a hydrophilic molecule of similar molecular size (25). A logD value of 2 to 3 for β-blockers was reported to provide optimal corneal permeation (26). Molecular size is also an important factor for small hydrophilic and lipophilic compounds. Increasing the molecular size from 0.35 nm to 0.95 nm reduces the permeability through the cornea, and conjunctiva significantly (25).

Active transport in the cornea can carry drug molecules from the eye surface into the aqueous humor and vice versa. However, saturation of the active transporter is possible and may limit the significance of this route during the limited residence time of the formulation on the surface of the eye. A prodrug approach targeting certain transporters in the cornea to enhance the permeation of acyclovir has been recently reported (27,28). Mannermaa, et al., (29) has provided a detailed review on the emerging role of transporters in ocular delivery.

Conjunctival and Scleral Absorption

Permeation through the conjunctival epithelium is limited by the tight junctions. However, the pore size of 5.5 nm in the conjunctiva allows larger molecular weights up to 38 600 Da to passively diffuse (30). The high surface area of diffusion of the conjunctiva compared with the cornea (17:1) contributes the importance of this route especially for hydrophilic compounds and large molecules (31). Compounds penetrating through the conjunctiva can continue the penetration into the eye through the sclera. Scleral permeation does not depend on the compound lipophilicity, but depends on the molecular radius (32). The presence of blood vessels in the conjunctiva can act as a sink condition that limits drug penetration to the sclera, carrying drug instead to the systemic circulation. As with the cornea, active transporters in the conjunctiva have been reported and reviewed (33).

Elimination from the Anterior Segment of the Eye

Drug molecules reach the aqueous humor though the corneal route or the iris/ciliary body through the conjunctiva/sclera route can be cleared through the aqueous humor drainage and through the blood vessels penetrating the eye to the systemic circulation.

Delivery to the Posterior Segment of the Eye

Posterior drug delivery may target the retina, choroids, and vitreous humor. Targeting the posterior tissues of the eye has gained significant interest in recent years with the advent of new agents for treatment of age-related macular degeneration and diabetic retinopathy.

Several routes can be used to direct drug molecules to the posterior tissue (Fig. 3). The following is a summary of these administration and possible penetration routes:

Topical

Several compounds have been reported to reach the posterior segment of the eye from topical administration (34,35). As with the delivery to the anterior segment of the eye, there are two main pathways for drugs to reach the posterior segment of the eye: the corneal route, and the conjunctival/sclera route. Once the drug molecules reach the anterior segment tissue it can penetrate to the rest of the ocular tissues via several routes as explained in Figure 4. Penetration through the lens into the vitreous is limited and generally observed with lipophilic compounds (36). Alternatively drug molecules can diffuse against the aqueous humor outflow to into the vitreous, or through the uveoscleral route. Drug penetration through the conjunctiva/sclera route is believed to be most significant in reaching the retina and choroids. Drugs reaching the sclera can diffuse laterally around the orbit and into the choroids and retina. Systemic recirculation plays a role in reintroducing the drug molecules lost to the systemic circulation back to the ocular tissue as observed with the effect of topical β-blockers on the contralateral eye (37).

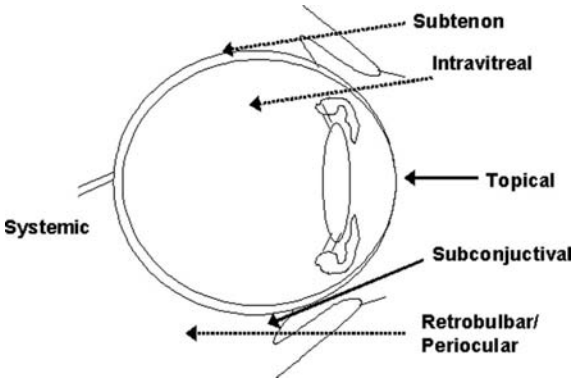

Figure 3 Different routes of ocular administration.

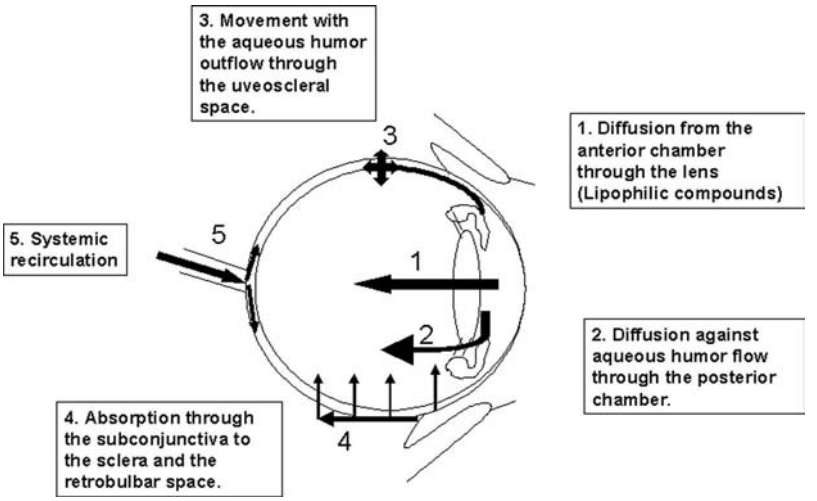

Figure 4 Schematic representation of drug penetration pathways to the posterior segment of the eye.

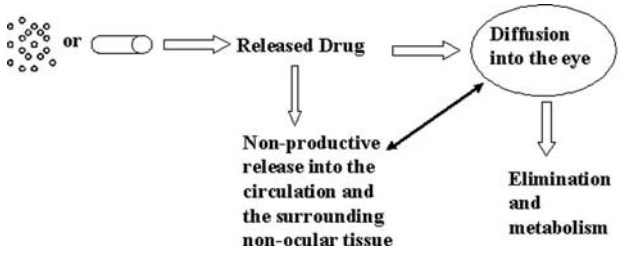

Figure 5 Schematic representation of drug release and diffusion into the eye from controlled delivery system (microsphere or implant) after periocular administration.

Subconjunctival/Transscleral Delivery

This is an important and promising route of delivering compounds to the back of the eye. It includes subconjunctival, peribulbar, retrobulbar, and subtenon injections. In all these injections the major permeability and loss to the systemic circulation limitations through the conjunctiva is avoided. Additionally, the drug has more time to diffuse through the sclera to the choroids and retina than that with topical administration. Scleral permeability, as discussed before, is not affected by lipophilicity of the compound but with the molecular radius. Large molecules up to 70 kDa are still able to penetrate the sclera (38). The large surface area of the sclera offers great potential for both small and large molecules to diffuse into the choroids, retina, and vitreous. In the periocular delivery, drug release from various delivery system and elimination can be depicted as in Figure 5. Once drug molecules diffuse through the sclera, it has to diffuse through the suprachoroidal space to the choriocapillaris, and then through Bruch's membrane to the RPE (outer retina-blood-barrier). The major limitation of drug to diffuse to the retina is the RPE. The majority of drug dissolved or released will be lost to nonocular tissue and eventually to the systemic circulation. Minimal loss to the choroidal circulation is expected (39). The ability of the nanoparticles to penetrate through the sclera-choroid-retina has been recently reported to have nonsignificant transport across these tissues with the majority of the nanoparticles being lost to periocular circulation and lymphatics (40). Differences between the various injections (subconjunctival, peribulbar, retrobulbar, and subtenon) exist with regard to penetration into posterior tissues (41). More drug is available in the vitreous and subretinal fluid when given as a subconjunctival injection compared with peribulbar injection (42,43). This can be due to the close proximity to the eye in case of subconjunctival injection. Subtenon injection also utilized for the delivery of active compounds behind the macula for effective delivery to the choroids and retina. The advantage of this

injection is the potential ability of Tenon's capsule to capture the delivery system (suspension, microspheres, or nanoparticles) in place where drug release/dissolution will continue for extended period of time. The transscleral route is most promising and less invasive route when compared with intravitreal delivery, especially with the advancement in the controlled release delivery systems.

Intravitreal Injection/Implant

Delivery through the intravitreal route is the most direct way to the retina. When delivered via this route, drug molecules only need to the diffuse through the vitreous to reach the retina, and through the RPE to reach the choroids. The low systemic exposure with intravitreal injection is a major advantage for this route. However, repeated injections of medication are often required which may lead to increased risk of endophthalmitis, damage to the lens, and retinal detachment. Intravitreal injections are typically administered in the inferotemporal quadrant, approximately 4 mm from the limbus (44). Controlled release formulations and implants can be used to decrease the frequency of administration required. Drug elimination and distribution from intravitreal delivery is controlled by the position of an intravitreal injection, and the lipophilicity and molecular size of the drug (45). Disposition from the vitreous humor can be through retinal absorption (retina/choroid/sclera) or via the posterior chamber (annular gap between lens and the ciliary body) then through the aqueous humor. Compounds with high lipophilicity are believed to be cleared via the retina pathway, while small hydrophilic and macromolecules are cleared anteriorly through the aqueous humor flow. Clearance and localization of polymeric nanoparticles after intravitreal injection was reported to depend on the size of the particles (46,47).

Ophthalmic Indications and Diseases

Table 1 lists several examples of marketed ophthalmic formulations used to target disease conditions in both the anterior and posterior tissues. While it is beyond the scope of this chapter to provide a comprehensive listing of ophthalmic diseases and indications, we will briefly discuss the most common indications.

Anti-infective Agents

There are many drugs available to treat bacterial, viral, and fungal infections of the eye. The antibiotic drugs that are available are generally broad spectrum. Ophthalmic formulations in this category are in the form of ointments and suspensions in addition to conventional solution eye drops. Many of the products are combinations of drugs and the relative efficacy of the formulations is judged on the frequency of instillation and duration of treatment.

Broadly the following are the major types of ocular infections that are treated by antibiotics or a combination of antibiotics and anti-inflammatory agents (48):

> Conjunctivitis (viral, bacterial, neonatal); episcleritis; keratitis (viral, bacterial, keratitis due to light exposure); uveitis (anterior, intermediate, posterior, and retinitis); hordeolum and chalazion; dacryocystitis; and periorbital and orbital cellulitis.

During the day, patients are usually treated using eye drops (sometimes up to several times a day) and at night they may be additionally directed to use an ophthalmic ointment (e.g., in the case of severe infections and blepharitis).

Anti-inflammatory Agents

Inflammation is the manifestation of vascular and cellular response of the host tissue to injury. Injury to the tissue may be inflicted by physical or chemical agents, invasion of pathogens, ischemia, and excessive (hypersensitivity) or inappropriate (autoimmunity) operation of immune mechanisms. In ocular tissues, inflammatory reactions are mediated by arachidonic acid cascade products formed via the cyclooxygenase pathway. There are two types of anti-inflammatory agents: corticosteroids and nonsteroidal anti-inflammatory drugs (NSAIDs). Both corticosteroids and NSAIDs may be administered orally as well as topically, but topical

Table 1 Examples of Marketed Ophthalmic Formulations for Treatment of Anterior and Posterior Indications

Indication	Active ingredient	Class/mechanism	Formulation type	Product example
Anterior drug delivery examples				
Acute infection—blepharitis	Bacitracin	Miscellaneous antibiotic	Ointment	Ciloxan (Alcon)
Acute infection—conjunctivitis	Tobramycin	Aminoglycoside	Suspension	Tobrex (Alcon)
Acute infection—conjunctivitis	Azithromycin	Macrolide	Mucoadhesive solution	AzaSite (Inspire Pharma)
Acute infection—keratitis	Ofloxacin	Quinolone	Solution	Ocuflox (Allergan)
Acute pain/inflammation	Prednisolone acetate	Corticosteroid	Suspension	Pred-Forte (Allergan)
Acute pain/inflammation	Diclofenac sodium	NSAID	Solution	Voltaren (Novartis)
Acute pain/inflammation	Flurbiprofen sodium	NSAID	Solution	Ocufen (Allergan)
Acute pain/inflammation	Loteprednol etabonate	Soft steroid	Suspension	Lotemax (Bausch & Lomb)
Allergy, OTC	Ketotifen fumarate	Antihistamine	Solution	Alaway (Bausch and Lomb)
Allergy, OTC	Naphazoline HCl, pheniramine maleate	Decongestant/vasoconstrictor	Solution	Opcon-A (Bausch & Lomb)
Allergy, Rx	Azelastine HCl	Antihistamine	Solution	Optivar (MedPointe)
Allergy, Rx	Olopatadine HCl	Antihistamine	Solution	Pataday (Alcon)
Allergy, Rx	Loteprednol etabonate	Soft steroid	Suspension	Alrex (Bausch & Lomb)
Dry eye, OTC	PEG400, propylene glycol	Aqueous tear-fluid replacement/ stabilizer	In situ gelling solution	Systane (Alcon)
Dry eye, OTC	Glycerin, propylene glycol	Aqueous tear-fluid replacement/ stabilizer	Mucoadhesive solution	Soothe (Bausch & Lomb)
Dry eye, OTC	Light mineral oil, mineral oil	Ocular lipid replacement	Emulsion	Soothe XP (Bausch & Lomb)
Dry eye, Rx	Cyclosporine	Immunomodulator/anti-inflammatory	Emulsion	Restasis (Allergan)
Glaucoma	Brimonidine tartrate	$\alpha 2$ adrenergic agonist	Mucoadhesive solution	Alphagan P (Allergan)
Glaucoma	Betaxolol HCl	β-blocker, $\beta 1$	Mucoadhesive, complexed solution	Betoptic S (Alcon)
Glaucoma	Timolol maleate	β-blocker, $\beta 1$ and $\beta 2$	In situ gelling solution	Timoptic XE (Merck)
Glaucoma	Dorzolamide HCl	Carbonic anhydrase inhibitor	Solution	Trusopt (Bausch & Lomb)
Glaucoma	Latanoprost	Prostaglandin	Solution	Xalatan (Pfizer)
Posterior drug delivery examples				
"Wet" AMD	Ranibizumab injection	Monoclonal antibody fragment/VEGF inhibitor	Solution, intravitreal injection	Lucentis (Genetech)
Wet AMD	Pegaptanib sodium	Oligonucleaotide/VEGF inhibitor	Solution, intravitreal injection	Macugen (Pfizer)
Chronic uveitis	Fluocinolone acetonide	Corticosteroid	Implant	Retisert (Bausch & Lomb)
Cytomegalovirus retinitis	Ganciclovir	Antiviral	Implant	Vitrasert (Bausch & Lomb)

Abbreviations: NSAID, nonsteroidal anti-inflammatory drug; VEGF, vascular endothelial growth factor.

administration is the preferred route for management of ocular inflammation as it provides high ocular drug concentrations and reduces the systemic side effects.

Corticosteroids work by blocking the enzyme phospholipase A2 to inhibit arachidonic acid production, thereby preventing the synthesis and release of prostoglandins, thromboxanes, and eicosanoids. Some concerning side effects of corticosteroid treatment are an increase in IOP, suppression of the immune system response to pathogens, slowed wound healing, and formation of cataracts. Steroids have been used extensively before and post surgery as a result of their broad effects and are generally more potent than NSAIDs for treatment of severe inflammation. Recently, soft steroids have been introduced in an effort to maintain the potent efficacy of the cortiosteroids while reducing the undesirable side effects. One of these soft steroids is loteprednol etabonate, which has less effect on IOP because of its rapid metabolic deactivation. Most of the steroids have low aqueous solubility and, hence, most are formulated as suspensions or emulsions.

NSAIDs exert their anti-inflammatory action by inhibiting the cyclooxygenase enzymes (viz., COX-1, COX-2, and COX-3). NSAIDs are commonly used to treat postoperative inflammation, in the prevention and treatment of cystoid macular edema and for relief in allergic conjunctivitis. Treatment with NSAIDs is preferred because of the lower occurrence of side effects associated with steroidal drugs; however, most of the NSAIDs for ophthalmic use are weakly acidic compounds (49) which have a tendency to lower the pH of the formulations making the formulations somewhat irritating. In addition, many of them have poor water solubility; thus, they are often used in the form of their more soluble salt forms (e.g., sodium, potassium, tromethamine, or lysine salts) or are formulated with solubilizers like surfactants or cyclodextrins.

Antiallergy Agents

Ocular allergic disorders include seasonal allergic conjunctivitis (SAC), perennial allergic conjunctivitis (PAC), vernal keratoconjunctivitis (VKC), giant papillary conjunctivitis (GPC) and atopic keratoconjunctivitis (AKC). The treatment of acute and more chronic forms of allergic conjunctivitis has been mainly focused on symptomatic relief of symptoms such as, redness, itching, and burning. They are primarily antihistamines, that is, H1 blockers. In some cases the allergic condition may require the use of topical corticosteroids as well. Some of the antiallergy eye drops are available OTC, but many are still only available as prescription medications.

Dry Eye Treatments

Dry-eye syndrome results from problems originating in the nasolacrimal system resulting in inadequate quantity and quality of tears or ocular surface abnormalities. Signs and symptoms of dry eye include itchiness, redness, foreign body sensation, and grittiness. Most treatments alleviate the signs and symptoms of dry eye rather than treating the cause. Most OTC dry-eye treatments are designed to replace and stabilize the aqueous portion of the tear film. A couple of OTC treatments are also available to replace the lipid portion of the tear film which may be inadequate to prevent evaporation of the aqueous portion of the tear film. A pharmaceutical approach to treating dry eye may involve the use hormones (or analogs) to increase the lipid production of the meibomian glands or to treat inflammation of the lacrimal glands to increase the secretion of the lacrimal glands (50). Regardless of the type of treatment, most dry-eye products are often dosed several times a day and therefore need to be mild and contain little or no preservatives or preservatives that are nonirritating.

Antiglaucoma Agents

Glaucoma is a sight-threatening optic neuropathy. The disease is characterized by increased IOP, excavation of the optic nerve head, reduction in the number of retinal ganglion cells, and a resultant progressive loss of visual field. Elevated IOP is a major risk factor and available antiglaucoma drugs treat this facet of the disease. The most common form of the disease is open-angle glaucoma in which IOP rises as a result of decreased outflow of aqueous humor through the trabecular meshwork and Schlemm's canal. Antiglaucoma drugs may act by decreasing aqueous humor production or increasing aqueous humor outflow (via the

trabecular meshwork or the uveoscleral pathway) (51). Drugs that affect aqueous humor production include β2-adrenergic receptor agonists, β1-adrenergic receptor agonists, α2 adrenergic receptor agonists and carbonic anhydrase inhibitors. The newest category of drugs used in the treatment of glaucoma is the prostaglandin analogs which affect aqueous humor outflow (52,53). Most of these products need to be dosed once or twice daily. The prostaglandin analogs however, have certain side effects associated with them namely, iris hyperpigmentation and change in the length, color and thickness of eyelashes, hyperemia and pruritis.

Posterior Indications

"Wet" age-related macular degeneration is a condition where blood vessels behind the retina start to grow and leak blood and fluid. This causes damage to the macula (the center of the retina) and results in central vision loss that can occur quickly. The medications available to treat wet AMD work by inhibiting the action of vascular endothelial growth factors (VEGF). Delivering these actives to the retina involves an intravitreal injection every 6 to 12 weeks.

Intravitreal implants are available for the treatment of posterior diseases as well and offer the ability to reduce the dosing frequency for these posterior treatments to once per year. However, there are two significant issues with intravitreal implants. Firstly, current intravitreal implants require a surgical procedure that is more invasive than an intravitreal injection. Secondly, a formulation that delivers drug over the course of a year requires a much longer (and, hence, more costly) clinical trial. Therefore, the use of intravitreal implants is limited.

Formulations for Ophthalmic Delivery

The following section discusses the various components and factors to be considered in the development of the different types of formulations for ophthalmic medications.

Excipients for Use in Ophthalmic Formulations

A suitable ophthalmic formulation must include excipients to control the osmolality, pH, and stability of the formulation. Control of the formulation stability includes chemical, physical, and antimicrobial stability. In addition, some excipients may be added to a formulation to enhance the drug delivery of the formulation by modifying the solubility of the active ingredient or increasing the retention of the active ingredient on the surface of the eye. When a formulator begins to design a new formulation for an active ingredient, they must be aware of the additional development effort or risk that might be associated with the use of novel formulation ingredients. In some cases, the risk of using novel ingredients will be warranted in an effort to gain patent protection or overcome difficult drug delivery issues. In other cases, the risk may not be warranted as it could lead to longer and more costly development programs. In either case, the formulator should always begin their formulation development efforts by selecting ingredients from those that have previously been used in ophthalmic formulations. In the U.S. market, the Food and Drug Administration (FDA) inactive ingredients database provides a convenient listing of these materials. A listing of these ophthalmic excipients and their potential use is provided in Table 2. No such database is currently available from the other primary regulatory agencies.

Solutions

An ophthalmic solution formulation is always the first choice if a drug substance has suitable aqueous solubility and stability in the range from pH 5 to 8. A good example of the simplest approach to a topical solution formulation is the marketed latanoprost formulation. The formulation is a neutral pH, phosphate-buffered saline preserved with 200 ppm benzalkonium chloride (BAK). This formulation example demonstrates that even the simplest formulation should provide control of pH and osmolality, and provide antimicrobial stability.

The osmolality and pH of the formulation should always be matched as closely as possible to that of the tear fluid; however, significant ranges for both of these variables have been found to be acceptable in practice. The eye is better able to tolerate pH excursions on the acidic side rather than on the basic side, hence, the range of acceptable pH values is skewed more to the acidic side of the mean tear fluid pH. There are many topical formulations in the pH 5.5 to 7.5 range, and a few that go as low as 4.0 and as high as 8.0. This asymmetry of the

Table 2 Excipients Listed in Food and Drug Administration Inactive Ingredients Database

Category of excipient	Ingredient name	Use level in database	Compendial listing(s)
Wetting and solubilizing Agents/emulsifying agents	Benzalkonium chloride	2%	NF, PhEur, JP
	Benzethonium chloride	–	USP, PhEur, JP
	Benzododecinium bromide	0.01%	–
	Carbomer 1342	0.05%	NF, PhEur, JPE
	Cetyl alcohol	0.5%	NF, PhEur, JP
	Cholesterol	–	NF, PhEur, JP
	Cocamidopropyl betaine	0.002%	–
	Glyceryl monostearate	0.5%	NF, PhEur, JP
	Lanolin alcohols	10%	NF, JPE
	Lauralkonium chloride	0.005%	–
	N-lauroylsarcosine	0.03%	–
	Nonoxynol 9	0.12%	USP, JPE
	Octoxynol 40	0.01%	JPE
	Poloxamer 188	0.1%	NF, PhEur, JPE
	Poloxamer 407	0.2%	NF, PhEur, JPE
	Polyoxyl 35 castor oil	5%	NF, PhEur, JPE
	Polyoxyl 40 hydrogenated castor oil	0.5%	NF, PhEur, JPE
	Polyoxyl 40 stearate	7%	NF, PhEur, JP
	Polysorbate 20	0.05%	NF, PhEur, JPE
	Polysorbate 60	15%	NF, PhEur, JPE
	Polysorbate 80	4%	NF, PhEur, JP
	Sorbitan monolaurate	–	NF, PhEur, JPE
	Tyloxapol	0.3%	USP
Suspending and/or viscosity-increasing agents	Carbomer 1342	0.05%	NF
	Carbomer 934P	0.45%	NF, JPE
	Carbomer 940	4%	NF, JPE
	Carbomer 974P	0.5%	NF, PhEur, JPE
	Carboxymethylcellulose sodium	0.5%	NF, PhEur, JP
	Gellan gum	0.6%	NF
	Hydroxyethyl cellulose	0.5%	NF, PhEur, JPE
	Hypromellose 2906	0.5%	USP, PhEur, JP
	Hypromellose 2910	0.5%	USP, PhEur, JP
	Methylcellulose	0.5%	USP, PhEur, JP
	Polycarbophil	0.9%	USP
	Polyethylene glycol 8000	2%	NF, PhEur
	Polyvinyl alcohol	1.4%	USP, PhEur, JPE
	Povidone K30	2%	USP, PhEur, JP
	Povidone K90	1.2%	USP, PhEur, JP
	Xanthan gum	0.6%	NF, PhEur, JPE
Acidfying agents/alkalizing agents (pH adjustment)	Acetic acid	0.2%	NF, PhEur, JP
	Ammonium hydroxide	–	–
	Citric acid	0.2%	USP, PhEur, JP
	Diethanolamine	–	NF, JPE
	Hydrochloric acid	1.06%	NF, PhEur, JP
	Nitric acid	–	NF, PhEur
	Phosphoric acid	–	NF, PhEur, JPE
	Sulfuric acid	0.02%	NF, JPE
		1.1%	NF, PhEur, JP
		–	–
		1%	NF, PhEur, JP
		0.1%	NF, PhEur, JP
	Sodium borate		
	Sodium bisulfate		
	Sodium carbonate		
	Sodium hydroxide		
Buffering agents (pH control)	Acetic acid	0.2%	NF, PhEur, JP
	Boric acid	37.2%	NF, PhEur, JP
	Citric acid	0.2%	USP, PhEur, JP

(*Continued*)

Table 2 Excipients Listed in Food and Drug Administration Inactive Ingredients Database (*Continued*)

Category of excipient	Ingredient name	Use level in database	Compendial listing(s)
	Phosphoric acid	–	NF, PhEur, JPE
	Potassium acetate	4%	USP, PhEur, JPE
	Potassium phosphate, monobasic	0.44%	NF
	Potassium sorbate	0.47%	NF, PhEur, JPE
	Sodium acetate	1.27%	USP, PhEur, JP
	Sodium borate	1.1%	NF, PhEur, JP
	Sodium carbonate	1%	NF, PhEur, JP
	Sodium citrate	2.2%	USP, PhEur, JP
	Sodium phosphate, dibasic	1.4%	USP, PhEur, JP
	Sodium phosphate, monobasic	0.78%	USP, PhEur, JPE
	Sorbic acid	0.2%	NF, PhEur, JPE
	Tromethamine	0.93%	USP
Humectants/tonicity agents/salts (ionic strength and osmolality control)	Calcium chloride	0.04%	USP, PhEur, JP
	Glycerin	2.6%	USP, PhEur, JP
	Magnesium chloride	0.03%	USP, PhEur
	Mannitol	23%	USP, PhEur, JP
	Polyethylene glycol 300	–	NF, PhEur, JPE
	Polyethylene glycol 400	4.99%	NF, PhEur, JP
	Potassium chloride	22.2%	USP, PhEur, JP
	Propylene glycol	10%	USP, PhEur, JP
	Sodium chloride	55%	USP, PhEur, JP
	Sodium nitrate	1.18%	–
	Sodium sulfate	1.2%	USP, PhEur, JPE
	Sorbitol	40%	NF, PhEur, JP
Ointment base	Lanolin	3%	USP, JP
	Light mineral oil	–	NF, PhEur, JP
	Mineral oil	59.5%	USP, PhEur, JP
	Petrolatum	85%	USP, PhEur, JP
	Petrolatum, white	89%	USP, PhEur, JP
Antioxidants/chelating agents/sequestering agents (chemical stability control)	Citric acid	0.2%	USP, PhEur, JP
	Creatinine	0.5%	NF, JPE
	Divinylbenzene styrene copolymer	0.75%	USP, JP
	Edetate sodium	10%	USP, PhEur, JP
	Sodium bisulfite	0.1%	JP
	Sodium citrate	2.2%	USP, PhEur, JP
	Sodium metabisulfite	0.25%	NF, PhEur
	Sodium sulfite	0.2%	NF, PhEur, JPE
	Sodium thiosulfate	5%	USP, PhEur, JP
	Tocophersolan (Vit E TPGS)	0.5%	NF
Antimicrobial Preservatives	*Quaternary ammonium compounds*		
	Benzalkonium chloride	8.8%	NF, PhEur, JP
		–	USP, PhEur, JP
		0.01%	–
	Benzethonium chloride	0.0005%	–
	Benzododecinium bromide		
	Polyquaternium-1	0.2%	NF, PhEur, JP
		37.2%	NF, PhEur, JP
		4%	USP, PhEur, JPE
		0.47%	NF, PhEur, JPE
		1.27%	USP, PhEur, JP
		1.1%	NF, PhEur, JP
		0.2%	NF, PhEur, JPE
		0.65%	NF, PhEur, JP
		0.5%	USP, JPE
		0.0008%	NF, PhEur
		0.002%	NF, PhEur
		1%	USP, PhEur
		0.05%	NF, JP
		0.01%	NF, JP

(*Continued*)

Table 2 Excipients Listed in Food and Drug Administration Inactive Ingredients Database (*Continued*)

Category of excipient	Ingredient name	Use level in database	Compendial listing(s)
		0.005%	–
		0.0025%	USP, PhEur, JP
	Acid/base compounds		
	Acetic acid		
	Boric acid		
	Potassium acetate		
	Potassium sorbate		
	Sodium acetate		
	Sodium borate		
	Sorbic acid		
	Alcohols		
	Chlorobutanol		
	Phenylethyl alcohol		
	Organic mercurial compounds		
	Phenylmercuric acetate		
	Phenylmercuric nitrate		
	Thimerosal		
	Parabens		
	Methylparaben		
	Propylparaben		
	Oxidizing agents		
	Sodium chlorite		
	Metal salts		
	Zinc chloride		

Note: Compendial listings for ingredients are also noted.

acceptable pH range is partially due to the buffering capacity of the tear fluid, but is also a result of the fact that excursions to high pH can result in saponification of lipids in the tissues and immediately compromise the barrier properties of the tissues (2). to minimize the discomfort caused by a formulation, at the extreme low or high end of this pH range, it is advisable to minimize the buffer capacity of a formulation. For example, a 50 mM pH 5 acetate buffer would be more easily neutralized by the tear fluid than a 50 mM pH 5 citrate, but a 5 mM pH 5 acetate buffer would be even better if it could provide sufficient pH stability for the formulation. For osmolality, reflex tearing is generally not seen in the range of 200 to 400 mOsm/Kg (2,3), but some studies suggest that hypotonic formulations may be better tolerated than hypertonic formulations and have been shown to enhance drug delivery in some instances (3). The osmolality of the formulation may be controlled equally well using electrolytes or nonelectrolytes and a comfortable formulation may be designed using either. In general, it may be preferable to rely on nonelectrolytes for osmolality control and target osmolalities slightly less than that of the tear fluid to avoid aggravating the hyperosmotic conditions that afflict the population of people with evaporative dry eye.

Additional formulation components that may be beneficial for solution formulations include surfactants and viscosity increasing agents. Even if not required for its solubilization, the addition of a small amount of surfactant may help with the wetting and spreading characteristics of the formulation on the surface of the eye. The addition of a viscoelastic polymer to the formulation can also be beneficial in prolonging the retention of the formulation on the surface of the eye. Increasing the viscosity of a solution formulation to 12 to 15 cps was shown to provide optimal benefit—higher viscosities show diminishing improvements in slowing the drainage rate (54).

Ointments

After solution formulations, the next most complicated formulation to design and manufacture is an ointment. Ointment formulations are generally suspensions of drugs in a base of mineral

oil and petrolatum. These formulations are generally suspensions because mineral oil and petrolatum are not good solvents for most drug compounds. The ointment base of petrolatum softens at body temperature and melts between 38°C to 60°C. The melting and softening behavior of the petrolatum base may be modified by the addition of mineral oil or light mineral oil. Ophthalmic ointments typically contain from 40% to 90% petrolatum with 60% to 10% mineral oil. The simplest ointment formulation will have only the active ingredient suspended in this mineral oil/petrolatum base. The active ingredient will need to be suitably controlled with respect to its particle size; hence, the drug substance is typically micronized before addition to the ointment base. The mineral oil/petrolatum base is not supportive of microbial growth (i.e., due to the low water activity), and does not affect the pH or osmolality of the eye, hence, additional excipients are not required.

This simple petrolatum and mineral oil base is the best option for drug substances that are hydrolytically unstable, but the drug delivery from such an ointment may suffer because of the fact that the ointment base is not readily miscible with the tear fluid. To improve the drug delivery characteristics of an ointment, a formulation may include a water-in-oil surfactant such as lanolin. This creates what is termed an absorption ointment base and is intended to improve the drug absorption from the ointment as well as improve the ability to incorporate hydrophilic drugs. The absorption ointment base is more likely to require the addition of a preservative such as chlorobutanol or parabens.

Suspensions

Suspensions are a necessary formulation option for cases where the aqueous solubility of the drug substance is extremely low or when the stability of the drug substance is significantly enhanced by keeping it as a suspended particle rather than dissolved (e.g., to reduce hydrolysis). Suspension formulations have the additional concerns of particle size distribution, sedimentation and resuspendability, and content uniformity of the delivered formulation.

Particle Size Distribution

The particle size distribution in an ophthalmic suspension must be controlled to assure the comfort of the formulation on the surface of the eye as well as assure that the drug delivery characteristics are consistent. Particle size of the active agent also plays a key role in physical stability of the drug product. The rate of sedimentation, agglomeration and resuspendability are affected by particle size. Table 3 lists the current compendial requirements for ophthalmic suspensions.

The most common method for controlling the particle size is mechanical comminution of previously formed larger, crystalline particles (e.g., by grinding with a mortar-pestle, air-jet micronization, or wet-milling with ceramic beads). Another method is the production of small particles using a controlled association process (e.g., spray drying, precipitation from supercritical fluid, or controlled crystallization). The process used to obtain the desired particle size distribution may have significant effects on the properties of the drug product. For example, comminution methods may generate heat that can create amorphous regions or polymorphic changes in the active pharmaceutical ingredient (API) particles which can, in turn, affect dissolution and drug delivery characteristics. In addition, if a change is made from one comminution method to another during the course of development, the API behavior may change significantly. For example, jet-air micronization can result in triboelectrification

Table 3 Summary of Compendial Requirements for Suspension Particles Size Distributions

USP	"It is imperative that such suspensions contain the drug in a micronized form to prevent irritation and/or scratching of the cornea. Ophthalmic suspensions should never be dispensed if there is evidence of caking or aggregation."
EP	NMT 20 particles $>$ 25 μm/10 mcg solid NMT 2 particles $>$ 50 μm/10 mcg solid No particles $>$ 90 μm/10 mcg solid
JP	No particles $>$ 75 μm

(i.e., charging) of the API particles. This charging of the particles may impact the aggregation and processing requirements of the formulation. Because is not practical to fully optimize the method for controlling the particle size independent of the formulation, a formulator must keep in mind how the process may change during the planned development and scale-up activities and be prepared for those necessary changes. The ultimate goal is to develop the formulation and particle size control method that will be used for manufacturing the marketed drug product and, therefore, the earlier this compatibility can be tested and verified, the better.

When formulation research is started, the formulation scientist typically has very little API available for evaluation of particle size methods. Some simple, small-scale experiments may help indicate what particle size control methods are viable options. For example, grinding a small amount of drug substance with a mortar-pestle to evaluate how easily a material can be ground (brittleness) and evaluation of the crystallinity of the drug substance before and after grinding may indicate if comminution methods are viable. Likewise, small-scale experiments with dissolving and precipitating the drug may indicate if a controlled precipitation process will produce a suitable crystalline particle.

Physical Stability (Sedimentation and Resuspendability)

It is important to understand that suspensions are kinetically stable but thermodynamically unstable systems. When left undisturbed for a long period of time the suspension particles will aggregate, sediment, and eventually cake. When a suspension is very well dispersed (i.e., deflocculated), the particles will settle as small individual particles. This settling will be very slow and will result in a low-volume, high-density sediment that may be difficult or impossible to redisperse. When the particles are held together in a loose open structure, the system is said to be in the state of flocculation. The flocculated particles will settle rapidly and form a large-volume, low-density sediment that is readily dispersible. Relative properties of flocculated and deflocculated particles in suspension are provided in Table 4.

The flocculation state of a suspension product is primarily controlled by the nature of the surface of the suspended particles. The surface charge (i.e., zeta potential) of the particle may be adjusted to move between a flocculated and deflocculated state. Also, adsorption of surface active polymers or surfactants can stabilize suspensions by preventing the removal of water from between the particles. A textbook example (55) illustrates how to modify the zeta potential of a suspension to switch between a deflocculated and a flocculated state. First, the adsorption of a cationic surfactant (e.g., BAK) to the surface of a suspended particle provides charge-charge repulsion resulting in a deflocculated suspension. Then, an oppositely charged flocculating agent (e.g., phosphate) is added at increasing levels to shield these surface charges and reduce the zeta potential close to zero, at which point flocculation is observed. A list of the formulation factors that can be adjusted to affect the physical stability of a suspension formulation includes the following:

- Flocculation/deflocculation: (*i*) add charged surface active polymer or surfactant, (*ii*) add an oppositely charged flocculation agent, (*iii*) add a nonionic surface active

Table 4 Relative Property of Flocculated and Deflocculated Particles in Suspension

Deflocculated	Flocculated
Little to no aggregation. Particles are present as primary particles.	Particles form loose aggregates (flocculants).
Sedimentation is slow.	Sedimentation is fast.
Sedimentation volume is small as particles may pack more efficiently.	Sedimentation volume is typically large.
Sediment may become a hard cake that is difficult or impossible to redisperse. Resuspendability is typically poor.	A dense cake does not form. The sediment is easy to redisperse, so as to reform the original suspension. Resuspendability is typically excellent.

polymer or surfactant, (*iv*) adjust the ionic strength of vehicle, and (*v*) if drug has a pKa, adjust pH to modify the surface charge.
- Sedimentation rate: (*i*) increase the viscosity of the vehicle, (*ii*) decrease the particle size of the drug, and (*iii*) develop a structured vehicle, which does not settle.

Content Uniformity in Delivery from the Selected Container/Closure

Another difference between a suspension formulation and a solution formulation is that when a suspension drop is delivered from the controlled-tip dropper bottle, it is not guaranteed to be uniform. Several factors, which may affect the uniformity of the drop delivered to the patient's eye, include compatibility between the formulation and the package, resuspendability of the formulation in the selected package, and the patient's ability to properly resuspend the formulation within the selected package. Typically, patients are not willing to vigorously shake a bottle of suspension for more than a few seconds. In addition, the resuspendability of a suspension formulation may be significantly affected by the material of the container (e.g., polyethylene vs. glass). It is advisable that careful, early evaluation of the resuspendability of suspension formulations be performed under simulated use conditions in the selected container/closure system to identify and fix physical stability issues as early as possible. An evaluation like this should indicate that a drop delivered from the selected package will have the appropriate potency (e.g., 90–110% of label) when delivered according to the label instructions.

Emulsions

Although emulsion formulations are not very novel and have been used extensively in topical (dermatological) and oral delivery routes, there are currently only two marketed formulations for ophthalmic use [Restasis® (cyclosporine emulsion in castor oil) and Durezol® (difluprednate emulsion in castor oil)]. The potential advantages of emulsions for ophthalmic drug delivery include being able to provide a greater driving force for drug delivery of low solubility compounds and being able to eliminate many of the quality control issues associated with suspended drug particles. The disadvantages of the emulsion formulations are that they have proven to be difficult formulations to preserve and difficult to manufacture under sterile conditions. These disadvantages are being overcome and there will undoubtedly be many new ophthalmic emulsions brought to the market over the next several years.

The emulsion formulation has an aqueous continuous phase that must comply with the same requirements as the solution formulations discussed above. In addition to the aqueous continuous phase, the emulsion formulation contains an oil (lipid) phase, which is dispersed in the continuous phase with suitable emulsifiers. The oil phase for an emulsion should be selected to provide adequate solubilization of the drug substance. The oil-in-water emulsifiers may include surfactants (e.g., polysorbate 80 or polyoxyl 35 castor oil), Carbomer 1342, or both.

The difficulty in preserving an emulsion formulation is evident from the fact that most of the antimicrobial preservatives readily available for ophthalmic use are incompatible with some aspect of the emulsion formulation. The emulsion formulation generally contains either high levels of surfactants or Carbomer 1342. High levels of surfactant can deactivate parabens, BAK, alcohols, and organic acids. Carbomer 1342 is an anionic polymer that may interact strongly with the quaternary amines. In addition, any surface active or lipophilic preservatives may partition into the oil phase and become unavailable for preservation of the aqueous phase. In the Restasis formulation, the preservative problem was solved by designing a preservative-free, single-dose formulation. For the Durezol formulation, the use of a combination of three water-soluble antimicrobial acids (sorbic, acetic, boric) provided sufficient preservative efficacy.

Enhanced Drug Delivery Systems

After topical administration, typically less than 5% of the applied drug penetrates the cornea and reaches intraocular tissues. The primary problem for topical delivery of ophthalmic drugs is the rapid and extensive precorneal loss caused by drainage and high tear fluid turnover. A major portion of the formulation efforts have been aimed at maximizing ocular drug absorption through prolongation of the drug residence time in the cornea and the conjunctival sac. Improved ocular residence of liquid formulations has been accomplished through the use

of viscosity-increasing and mucoadhesive agents, *in situ* gelation of the formulation, and use of charge-charge interactions between cationic components in the formulation and the anionic surface of the eye. Even greater residence improvements can be made by using polymeric inserts for drug delivery. Enhanced drug delivery from these formulations may allow the treatment of posterior indications with topical administration. In addition, various polymers may be used to produce prolonged-delivery systems which allow less frequent injections for posterior treatment.

High-Viscosity Liquid Formulations

A high-viscosity formulation can improve the retention of a drug substance on the surface of the eye, however, if the viscosity is too high under the shear conditions of the closing eyelid (about 20,000/sec) it may cause discomfort and reflex tearing (56). Many commonly used viscosity-increasing agents result in Newtonian viscoelastic behavior so that the viscosity increases similarly at both low and high shear. Polymers that thicken this way include hydroxypropylmethyl cellulose (HPMC), hydroxyethylcellulose (HEC), polyvinylpyrrolidone (PVP), and polyvinyl alcohol. Patton, et al., (54) found that increasing the viscosity to about 12 to 15 cps using either HPMC or PVA resulted in significant improvement in ocular retention whereas further increases in viscosity resulted in only small improvements. Other polymers may be used to produce non-Newtonian viscoelastic fluids that are either shear thinning or thixotropic. Polymers resulting in shear-thinning behavior include Carbomers and sodium carboxymethylcellulose. Polymers that shear thin more dramatically and can be considered thixotropic include polycarbophil and xanthan gum. In the AzaSite® (polycarbophil suspension of azithromycin) formulation, the polycarbophil creates a low-shear viscosity of over 2000 cps, but the formulation is still well tolerated in the eye because the viscosity of the formulation during the eye blink is much less (i.e., less than 300 cps).

Mucoadhesive Liquid Formulations

Mucoadhesion refers to the tendency of a polymer to specifically bind with the mucins of mucus membranes and lead to enhanced retention or viscosity as a result of the polymer-polymer interactions. The mucoadhesive performance of the ophthalmically-used polymers can be qualitatively ranked as follows (12): carbomers, polycarbophil > hyaluronan > carboxymethylcellulose sodium > sodium alginate > poloxamers, HPMC, methylcellulose, PVA, PVP.

Examples of formulations taking advantage of the ability of mucoadhesion to enhance the retention of a formulation include the Pilopine HS® (Carbomer 940 gel of pilocarpine HCl), and Alphagan® P (NaCMC solution of brimonidine tartrate).

In Situ Gelling Liquid Formulations

In situ gelation can be induced on the surface of the eye because of the change in pH, temperature, or ionic strength that occurs after the formulation is administered and mixes with the tear fluid. The change in pH can be used to induce *in situ* gelation between borates and polyol-containing polymers. The OTC dry-eye treatment Systane® takes advantage of the gelation between borates and HP-Guar as the pH is increased after administration. *In situ* thermal gelation with poloxamers has also been investigated, but is not currently applied in any marketed products. The gelation induced by interaction with the salt content of the tear fluid is used by the Timoptic-XE® (timolol maleate solution in gellan gum) product.

Cationic Liquid Formulations

Because the surface of the eye is generally anionic, the application of cationic drugs or drug delivery systems should interact electrostatically with the mucins on surface of the eye and lead to enhanced retention. Some formulations demonstrating this approach include cationic nanoparticles, cationic emulsions, and formulations using of cationic suspending or mucoadhesive agents. Nanoparticles may enhance delivery of poorly water-soluble drugs, but without improved retention on the eye nanoparticles are unlikely to result in delivery

superior to a solution. Preparation of cationic nanoparticles can be accomplished using either cationic Eudragit® polymers, chitosan polymers, or by incorporating cationic surfactants into solid-lipid nanoparticles (57). Chitosan polymers and cationic cellulosic polymers (e.g., polyquaternium-10) have also been used as cationic suspending agents and have been shown to provide good mucoadhesion properties (12). Cationic emulsions have been prepared by incorporating cationic surfactants at the solid-liquid interface of the emulsion to enhance drug delivery (58).

Prolonged-Delivery Polymeric Systems

Topical eye drop administration is mainly suitable for treatment of ocular conditions in the anterior segment of the eye. Targeting the posterior segment of the eye presents a far greater challenge and represents an area of unmet medical needs. Many of the newer drugs aimed at treating conditions such as diabetic retinopathy and age-related macular degeneration are administered via repeated intravitreal injections. Alternative approaches that would improve patient acceptance such as biodegradable inserts or micro- and nanoparticulate delivery systems present a growing field in the area of ophthalmic drug delivery.

Controlled release of drugs can be obtained by encapsulating the drug in micro-(1–10,000 μm) or nano(1–1000 nm)particles. These are usually given as intravitreal injections. They can provide sustained delivery over few weeks up to several months (59). However, the intravitreal injections of these particulates can cause vitreal clouding. Microparticles tend to sink to the lower part of the vitreal cavity, while nanoparticles are more susceptible to cause clouding in the vitreous (59).

Biodegradable and biocompatible polymers such as polylactide and PLGA [poly-(lactic-co-glycolic acid)] (both approved by the FDA) are typically used. In these materials, the drug is released by bulk erosion of the matrix following cleavage of the polymeric chains via autocatalytic acid/base and/or enzymatic hydrolysis; the products lactic and glycolic acids, are metabolized to carbon dioxide and water. Low molecular weight polymers tend to degrade rapidly; copolymers such as PLGA degrade faster than the corresponding homopolymers. Some microsphere formulations have shown promise in preclinical studies but have yet to undergo clinical trials. A microsphere formulation of PKC412 (protein kinase C inhibitor + receptors for VEGF) was administered via pericoular injection to treat choroid neovascularization. The studies showed a significant suppression of neovascularization using this delivery system.

Poly(anhydrides) and poly-(ortho-ester)s are also promising polymers for drug delivery; their release properties are regulated mainly by surface erosion rather than diffusion (60). Poly (orthoester)s have shown excellent ocular biocompatibility and have been used to demonstrate the sustained release of 5-fluorouracil (61).

Nanoparticulates are of importance since colloidal delivery systems are particularly suitable for poorly water-soluble drugs. However, the major impedence to the use of nanoparticles has been the availability of a universally acceptable method of making the nanoparticles especially on large scale and the stabilization and sterilization of the formulations. Some nanosytems based on surface-charge segregated particles containing chitosan or polyethylene glycol have been found to be stable and also in overcoming preclinical barriers.

Intraocular implants are usually placed intravitreally, at the pars plana of the eye and therefore, requires minor surgery. However, the use of implants have the benefit of by-passing the blood-ocular barriers to deliver constant therapeutic levels of drug at the site of action, avoidance of repeated administration and use of smaller doses of drugs (62). Implants may be nonbiodegradable or biodegradable depending on the material from which they are fabricated. Biodegradable implants of a poly (DL-lactic-co-glycolic acid) implant containing a novel aldose reductase inhibitor, fabricated with 50% drug loading have been shown to give sustained drug release in vitro and in vivo in rats (63). Nonbiodegradable implants provide more accurate/reproducible dosing lasting over longer periods of time than biodegradable inserts (62). The nonbiodegradable implants however, require surgical removal after completion of therapy. Vitrasert® and Retisert® (Bausch and Lomb) are two clinically used nonbiodegradable implants for the treatment of CMV retinitis (AIDS-related) and chronic uveitis, respectively.

Other implant systems in different phases of clinical trials include Medidur® (Alimera Sciences) for treatment of diabetic macular edema; Surodex® and Posurdex® (Allergan, U.S.A.) containing dexamethasone.

Transporter-Mediated Drug Delivery

Transporter-mediated drug delivery involves targeting of drug molecules to the membrane transporters to enable efficient passage across the cell membranes. Various transporters may be utilized to facilitate the passage of drugs across cell membranes (64); these include nutrient transporters for peptides, aminoacids, monocarboxylic acids, folates and organic anion and cation transporters, etc. Various peptide and amino acid transporters have been utilized for retinal drug delivery. Majumdar et. al. have studied the role of various dipeptide prodrugs of gancyclovir to improve its ocular bioavailability after topical administration and found good corneal permeability with a Val-Val dipeptide gancyclovir prodrug (65). The approach of using various transporter mechaninsms in the eye for improved intraocular delivery following topical administration, is interesting and provides newer opportunities for ophthalmic drug delivery.

Intraocular Irrigation Solutions

An ophthalmic irrigation solution is used for the application on the external surface of the eyes topically and in ocular surgeries to rinse, as well as to keep the operated ocular tissues moist. Replacement of the aqueous or vitreous humors with the irrigation solution occurs as the consequence of ocular surgeries including corneal transplant (penetrating keratoplasty), cataract extraction, intraocular lens implantation and vitrectomy. In these instances, the irrigation solution remains in the eyes after surgery until the components are either deprived by the surrounding tissues or the solution is eventually equilibrated with body fluids, with subsequent clearance through the circulation. Thus, it is essential that the irrigation solution used should be physiologically compatible, including tonicity and pH, and desirably should also contain components enabling the cells to sustain their viability and capability to perform physiological functions.

Irrigation solutions used during and after surgery are of particular importance to the cornea and the lens. Both organs are avascular. The cornea obtains its nourishment mainly from the fluid in the anterior chamber, and to a lesser extent, from the tear. The lens obtains its nourishment from fluids, both in the anterior chamber and in the vitreous. The retina, ciliary body and iris are vascularized tissues; they obtain their nourishment through the circulating plasma of the blood vessel network. Therefore, the components of the irrigation solution may not exert an effect on these tissues as significant as that on the cornea and the lens. A proper electrolyte balance as well as addition of certain nutrients such as glucose, amino acids, etc., may add to the beneficial nature of an irrigation solution. Often irrigation solutions are used to simply bathe and soothe the eye and help wash away impurities and contaminants from the environment. There are two intraocular irrigation solutions presently being used in ophthalmic surgeries. These two irrigation solutions are BSS and BSS Plus (both by Alcon Labs Inc.). BSS is a balanced salt solution that incorporates a sodium citrate of a balanced salt solution with a bicarbonate buffering system, with Dextrose added as an additional osmotic agent and energy source. An additional component, oxidized glutathione is reduced by the ocular cells and serves as an antioxidant. In addition, some intraocular irrigation solutions may contain viscoelastic components or viscosity enhancers such as sodium hyaluronate, chondroitin sulfate, hydroxypropylmethyl cellulose, and polyacrylamide. However, the use of these agents may lead to an elevation of IOP (66).

PRESERVATION OF OPHTHALMIC FORMULATIONS

Ophthalmic formulations must not only be sterile products but need to be adequately preserved from microbial contamination once the package is opened. Most ophthalmic products are multidose products packaged in semi-permeable containers. The repeated opening and closing of the containers as well as frequent contact with the ocular surface (e.g., for dropper tips) exposes the contents of the package to a variety of microorganisms from

the external environment. Many of the microorganisms can cause severe reactions (inflammation, itching, pain, loss of visual acuity, etc.) including, in the most severe cases, blindness. The choice of the preservative is dictated by the nature of the formulation itself, whether it is a suspension, solution or gel system. Often the choice of buffer/vehicle composition will also affect preservative efficacy. It is well known that the borate buffer system itself has good antimicrobial properties (67) and can help boost the antimicrobial efficacy of some preservatives. Additionally it is known that high salt concentrations can decrease preservative efficacy. The specific composition of the formulation not only affects the efficacy and stability of the preservative system but, may also alter the tolerability of the preservative system. For example, incorporation of viscosity-increasing agents can increase the irritation potential of a preservative because of increased residence on the eye—this has been demonstrated in BAK-containing systems with hydroxyethylcellulose (68). In addition the incorporation of surfactants and polymers that bind the preservative(s) will result in decreased antimicrobial efficacy. The use of preservatives in chronic-use products such as antiglaucoma and dry-eye medications is of concern because of the cumulative toxicity of certain agents on the corneal epithelium (69). Thus, such medications should ideally be preservative-free or contain preservatives that have little to no chance of accumulating in ocular tissues.

Antimicrobial Effectiveness Testing

Antimicrobial effectiveness testing (AET) is used to ensure that a product is adequately protected from microbial contamination during patient use. The AET method is described in the major compendia—the USP in chapter <51> (70), the PhEur in chapter 5.1.3, and the JP in chapter <19>. The bacterial challenge organisms used in the AET are *Escherichia coli* (ATCC8739), *Pseudomonas aeruginosa* (ATCC9027) and *Staphylococcus aureus* (ATCC6538), and the yeast/mold challenge organisms are *Candida albicans* (ATCC10231) and *Aspergillus brasiliensis* (ATCC16404). Criteria for the effectiveness of a preservative system are expressed as the percentage of reduction in viable cells in a specific amount of time. At this time, there is not one harmonized criteria that is accepted globally for product preservation. Table 5 summarizes the criteria that are most widely used for antimicrobial preservative testing—from the USP, PhEur, and JP. The PhEur criteria are the most stringent among the three and guide the development of globally-acceptable pharmaceutical formulations.

Preservatives Used in Ophthalmic Formulations

There are a wide variety of agents that alone or in combination with each other can act to effectively reduce the chances of contamination of a formulation by microbial growth. The section below addresses some of the more widely accepted ophthalmic preservatives that are used today. Many previously used preservatives such as the organic mercurial compounds (e.g., thimerosal) have seen a decline in use because of evidence of hypersensitivity and ocular toxicity upon long-term use (71,72).

Table 5 Criteria of Acceptance for Antimicrobial Effectiveness Testing (USP Category "1" Products, PhEur Parenteral and Ophthalmic Formulations, and JP Category IA Products)

		Log_{10} reduction				
	Innoculum (CFU/mL)	6 hr	24 hr	7 days	14 days	28 days
USP: bacteria	10^5–10^6	–	–	1.0	3.0	No increase[a]
JP: bacteria	10^5–10^6	–	–	–	3	No increase
PhEur-A: bacteria	10^5–10^6	2	3	–	–	No recovery
PhEur-B: bacteria	10^5–10^6	–	1	3	–	No increase
USP: yeast/mold	10^5–10^6	–	–	No increase	No increase	No increase
JP: yeast/mold	10^5–10^6	–	–	–	No increase	No increase
PhEur-A: yeast/mold	10^5–10^6	–	–	2	–	No increase
PhEur-B: yeast/mold	10^5–10^6	–	–	–	1	No increase

[a]No increase implies no decrease in the log reduction values for microbial growth from previous time point.

Quaternary Ammonium Compounds

Quaternary ammonium compounds are small, positively-charged molecules. It is believed that they act by perturbing the cell membrane of gram-positive and gram-negative bacteria specifically via intercalating into the lipid bilayers and displacing ions, such as calcium and magnesium, that play a crucial role in stabilization of the bacterial cytoplasmic membrane (73). These agents can interact with the teichoic acid and polysaccharide elements in gram positive bacteria and the lipopolysaccharide element in Gram-negative bacteria. It is believed that chelating agents such as ethylenediamine tetraacetic acid or EDTA and ethylene glycol tetraacetic acid or EGTA (used in concentrations from 0.01–0.1% w/w), further potentiate the antimicrobial effect of these agents. The most commonly used agent in this category is benzalkonium chloride (BAC or BAK) followed by cetyltrimethyl ammonium bromide (cetrimide). These agents are usually used in concentrations from 20 to 200 ppm, have good ocular tolerability profiles, good stability, and a long history of use. They are incompatible with high concentrations of anionic components or surfactants in a formulation. Although widely used, it is generally agreed that there may be concern regarding the cumulative toxicity of these agents when present in chronic use products such as dry-eye medications and antiglaucoma medications. Therefore, there is a growing preference for other, more gentle antimicrobials.

Polyquaternary Ammonium Compounds

Polyquaternium is the International Nomenclature for Cosmetic Ingredients designation for several polycationic polymers that are used in the personal care and pharmaceutical industry. Polyquaternium is a generic term used to emphasize the presence of multiple quaternary ammonium centers in the polymer. INCI has approved at least 37 different polymers under the polyquaternium designation. Because of their large size, they are generally thought to be less permeable across the corneal epithelium and, hence, pose less risk of accumulation in ocular tissues leading to chronic toxicity issues. Their mode of action is similar to the monoquaternary compounds in that they also destabilize the outer membrane of bacteria and cause leakage of intracellular components leading to cell death (73).

Two commonly used polyquaterniums are polyquaternium-1 (PQ-1) and polyquaternium-42 (PQ-42) and the molecular weight of these compounds can go up to several thousand Daltons. Chemically, PQ-1 is ethanol, 2,2′,2″-nitrilotris-, polymer with 1,4-dichloro-2-butene and *N*,*N*,*N*′,*N*′-tetramethyl-2-butene-1,4-diamine; typically, it has an average molecular weight of around 6 kDa. It can be used in concentrations of 1 to 10 ppm and its efficacy against yeast and fungi is improved at higher pHs. Chemically, PQ-42 is [polyoxyethylene(dimethylimino) ethylene-(dimethylimino)ethylene dichloride]. It has been used in ophthalmic formulations such as Freshkote, Dwelle, and Dakrina eye drops and Nutra-tear. It is also used in a lens care solution for rigid gas-permeable (RGP) lenses (Total Care CLS by AMO) at a concentration of 6 ppm by weight. PQ-1 is a more potent antimicrobial agent than PQ-42.

Biguanides and Polymeric Biguanides

Biguanides refer to the class of compounds that are derivatives of imidodicarbonimidic diamide. The most commonly known biguanide is chlorhexidine [1,6-bis(4′-chloro-phenyl-biguanide)hexane; usually used a its digluconate salt] which has a broad spectrum of activity. However, its action is pH dependent and greatly reduced by the presence of organic matter. It can only be used in very low concentrations in ophthalmic formulations because of its irritation potential. Chlorhexidine is believed to exert its action by membrane destabilization leading to the leakage of intracellular components; at high concentrations it can cause protein and nucleic acid precipitation (74). It is generally used at concentrations of 5 to 10 ppm by weight. Because of its weak activity against yeast, fungi, and *Serratia marcescens*, it is usually used in combination with other agents such as EDTA, BAK, etc.

Polymeric biguanides are also available, the most widely used one being polyaminopropyl biguanide or PAPB (also known as polyhexamethylene biguanide or PHMB, or polyhexanide) and is commercially available under the trade names of Cosmocil and Vantocil. PAPB has a broad spectrum of activity and can be used in concentrations as low as 0.5 ppm up

to 5 ppm. Lower concentrations may be used in combination with other antimicrobial agents. PAPB activity is reduced by anionic polymeric agents such as hyaluronic acid, carboxymethylcellulose, alginates, etc., and cellulosic polymers.

Alcohols

Phenylethyl alcohol and chlorobutanol are antimicrobial alcohols. Phenylethyl alcohol (up to 0.5%) is usually used in combination with another preservative but is limited in its application because of its volatility and tendency to permeate through plastic packaging. Chlorobutanol is a commonly used ophthalmic preservative and is generally considered to be quite safe (75). It is mostly used in ophthalmic ointments because it has good solubility in petrolatum. It can be used at concentrations up to 0.5%, but it is unstable at pH > 6, high temperature, susceptible to absorption into packaging components and may be lost through the headspace of semi-permeable packaging because of its volatility.

Parabens

Parabens are esters of p-hydroxybenzoic acid. They have been widely used in pharmaceuticals and as ophthalmic preservatives. They have a well established safety and tolerability profile. The useful concentration is typically limited by the water solubility, and therefore a combination of parabens can be used together to enhance their activity (e.g., 0.05% methylparaben + 0.01% propylparaben). Parabens are effective in the pH range of 4 to 8, but they are more susceptible to hydrolysis at high pH. Parabens may permeate into packaging components, and may be inactivated by high concentrations of surfactants or polymers.

Acids

Antimicrobial acids have a useful pH range around the pKa of the acid and the optimal antimicrobial activity will typically occur very close to this pKa. The most commonly used acid for preservation of ophthalmic formulations is sorbic acid (or potassium sorbate) which has a pKa of 4.76. Sorbic acid is primarily antifungal, but does have antibacterial activity. Sorbic acid is useful in the range of pH 4.5 to 6 and is usually combined with EDTA or other preservatives for broad-spectrum preservation. Sorbic acid is sensitive to oxidation, which results in discoloration of the product, and is more rapidly degraded at temperatures above 38°C. Boric acid is another useful acid for preservation in ophthalmic formulations; however, its activity is classified as bacteriostatic rather than biocidal.

Oxidizing Agents

Oxidizing agents are generally deemed much safer and well tolerated than most other preservatives because the preservatives "disappear" over time and pose little or no chance of accumulation in ocular tissues over repeated use. The two most widely used preservative systems in this category are stabilized hydrogen peroxide systems and hypochlorites. Hydrogen peroxide provides its antimicrobial action via generation of the hydroxyl radical which can readily attack bacterial cell membrane lipids and intracellular DNA (76). Hydrogen peroxide is effective against a wide variety of microorganisms and relatively unaffected by pH. Aside from hydrogen peroxide itself, other peroxide-generating compounds that are useful include sodium perborate, percarbonates and carbamate peroxide. The use of hypochlorites in ophthalmic formulations was introduced in 1996. The stabilized oxychloro complex (SOC) (i.e., Purite) is a hypochlorite preservative consisting of 99.5% chlorite; 0.5% chlorate and a trace amount of chlorine dioxide. The formation of chlorine dioxide in the microbial acidic environments leads to disruption of protein synthesis. However, the components of the preservative system dissipate readily in the eye into components already found in human tears (Na^+, Cl^-, O_2, and H_2O).

CONTACT LENS CARE SOLUTIONS AND REWETTING DROPS

Contact lenses may be rigid gas-permeable lenses (RGP) or soft contact lenses. to properly use contact lenses, they must be kept clean and free from microbial contamination when stored. Contact lens solutions are mainly multipurpose solutions (MPS) that achieve

cleaning, disinfection, and lubrication (for insertion comfort) all in one step. The development of new contact lens multipurpose solution compatible with an increasing array of soft contact lens materials on the market is very challenging. In addition to being able to effectively clean and disinfect the contact lenses, solutions are required to provide patient comfort when the cleaned lens is inserted back into the eye. The products must also maintain their ability to effectively clean and disinfect when stored in unopened containers over a period of 18 months to two years. An even greater challenge is designing a product that is robust enough to counter noncompliance of patients in their contact lens cleaning regimens (77) where compliance requires discarding opened solutions after three months; changing the contact lens cleaning case and never resoaking lens in previously used solution. The market needs are constantly evolving such that there is a continuous need for newer and better products. Biocidal efficacy is tested against five organisms (three bacteria: *Pseudomonas aeruginosa*, *S. aureus*, and *S. marcescens*; one yeast: *C. albicans*; and one fungus: *Fusarium solani*) in the presence of organic soil (required for United States and not for Europe) with a defined (e.g., four hours) exposure time. At least a 3 log reduction in CFUs per mililiter for the bacteria and 1 log reduction in CFUs per mililiter for the yeast and fungi are required (initially and throughout shelf-life) to be considered as passing the biocidal efficacy testing (78).

The key components of MPS are: surfactants or cleaning agents (such as the block copolymers Tetronic 1107 or 1304), lubricating agents (e.g., hyaluronic acid, hydroxypropyl guar, cellulosic polymers), disinfectants (viz., PQ-1, PAPB, SOC, sodium perborate, PQ-42), chelators (e.g., EDTA or hydroxyalkylphosphoates) and other agents that help in moisture retention (e.g., dextran, glycerin). In addition, buffers, electrolytes and stabilizers (e.g., antioxidants) are also included. The ionic strength of the formulations is of particular importance in the formulations because of the significant effect on lens shrinking and swelling. Preservatives can also be taken up by the lenses resulting in changes in lens dimensions.

Contact lens rewetting drops contain a suitable wetting agent (surfactant), an ocular demulcent, a preservative system in a suitable vehicle containing buffers, electrolytes and stabilizers. Rewetting drops help relieve symptoms of ocular discomfort (dryness, foreign body sensation, itching, blurry vision, etc.) in contact lens wearers during use.

MANUFACTURING AND PACKAGING OF OPHTHALMIC FORMULATIONS

Sterile Manufacturing

In 1953, the FDA announced that all ophthalmic products must be manufactured sterile (79). The sterility requirements for ophthalmic formulations first appeared in USP XVIII, third supplement, 1972. In general, ophthalmic formulations are described in the USP as "sterile dosage forms essentially free from foreign particles suitably compounded and packaged for instillation in the eye" (80). The formulations should be terminally sterilized by autoclaving whenever possible. As an alternative to steam sterilization, formulations may be sterilized by sterile filtration through 0.22-μm filters. If neither steam sterilization nor filtration is an option, then aseptic processing of presterilized components is required (81). The requirements and guidance for the compounding of sterile preparations is outlined in detail in USP <797>. In addition to the quality of raw materials and packaging components, and the condition of manufacturing components, a major factor in ensuring the quality of the final product is the environment in which it is manufactured and filled. For ophthalmic formulations, manufacturing must be carried out in an ISO class 5 (previously class 100) environment. There are also high standards that are described with regard to personnel garbing and gloving; personnel training and testing in aseptic manipulations, environmental quality specifications and monitoring and disinfection of gloves and surfaces. Formulation compounding may involve several steps rather than the simplified idea of putting all ingredients into a sterilized mixing vessel and mixing. In many cases the manufacturing may consist of a multistep process where the thermostable portion of the formulation is autoclaved and then the heat sensitive components are added aseptically (through a sterile filter) to the autoclaved portion (after cooling down to acceptable temperatures). When

developing a formulation it is very important to focus on the following general points to establish a manufacturing process that will be scaleable, reproducible, and cost and time efficient.

1. The order of addition of the components in the formulation.
2. The time (and temperature) required for mixing and type of mixing that may be desirable.
3. Possible interaction of formulation components with the manufacturing components, including tubing, filters, filter housing, cleaning agents that may be used to clean manufacturing components, sources of trace metal contamination, etc.
4. Filter choice is of particular importance when dealing with potent drugs and preservatives since these are prone to significant loss because of binding by the filter. In such cases several developmental batches may be necessary to determine the flush and discard volume (prior to beginning of the filling operation) to saturate the filters and minimize losses to the filter. Often a certain overage is included in the formulation to account for losses during manufacturing.

Other things to focus on are formulation specific, that is, different manufacturing requirements for suspensions/emulsions, regular solutions versus viscous formulations (gels), ointments, etc. Below are two examples of formulations requiring special compounding procedures for manufacturing and filling.

Manufacturing Example 1: an Aqueous Solution Sterilized by Filtration
The active ingredient, a lipid-soluble drug substance, was weighed into a glass vial with a calculated overage to compensate for loss to filters and the process surfaces during manufacturing. A cationicpreservative, which also serves as a solubilizer for the drug, was added as a concentrate solution to a glass beaker. The drug was transferred from the glass vial into the glass beaker with rinsing and this drug/preservartive concentrate was mixed thoroughly for a sufficient length of time. In a large stainless steel manufacturing vessel the other formulation ingredients were dissolved with constant stirring in $\sim$80% of the water for injection (WFI) for the batch. After all ingredients were dissolved, the concentrated premix solution was quantitatively transferred to the manufacturing vessel and the solution in the vessel was stirred continuously. In-process pH adjustment was performed and the formulation brought to its final weight with WFI. The filling operation was performed in an ISO class 5 environment. The filling line had a 5-μm pore size clarifying filter followed by two, serial, 0.22-μm sterilizing filters. Several liters of formulation were purged through the filling lines and filter assembly to saturate the filters with drug and preservative before the formulation was filled into presterilized plastic bottles. Presterilized tips were inserted and presterilized caps were applied in the ISO class 5 environment. Additional labeling and packaging was performed in an ISO class 7 manufacturing environment.

Manufacturing Example 2: Sterile Addition of a Drug Suspension to an Autoclaved Gel
A drug having very low solubility was suspended in a Carbomer gel to enhance drug delivery. The Carbomer was first dispersed, in an ISO class 7 manufacturing environment, at a high concentration in a clean compounding vessel using high speed homogenizers. The Carbomer phase was then transferred using a diaphragm pump to the manufacturing vessel in an ISO class 5 manufacturing environment. The Carbomer phase was autoclaved in the manufacturing vessel with continuous mixing and then cooled to $\sim 40^{\circ}$C. The micronized drug substance, which was sterilized by γ irradiation, was aseptically added, with continuous mixing, to the Carbomer phase in the ISO class 5 environment. Approximately 50% of the water in the formulation was used to dissolve the chlorobutanol preservative and this solution was sterile filtered through a 0.22-μm filter into the manufacturing vessel. The pH was adjusted to 4.5 using autoclaved sodium hydroxide to produce a thick gel. The final formulation was aseptically filled into presterilized tubes and closed. The final labeling and packaging operations were completed in an ISO class 7 manufacturing environment (82).

Table 6 Cap Color Coding for Ophthalmic Products

Therapeutic class	Color
β-blockers	Yellow, blue, or both
Mydriatics and cycloplegics	Red
Miotics	Green
Nonsteroidal anti-inflammatory drugs	Grey
Anti-infectives	Brown, tan
Carbonic anhydrase inhibitors	Orange
Prostoglandin analogs	Teal

Packaging

Packaging of ophthalmic formulations is very important since the shelf-life of a product is inherently tied in with packaging choice in many cases. The vast majority of ophthalmic formulations (except the injectable and specialized delivery systems) are packaged in polyolefin containers predominantly high-density polyethylene (HDPE), LDPE (low-density polyethylene), polypropylene (PP) and may also include materials such as polyethylene terephthalate (PET). Topical eye drops are typically packaged in 5 to 15 mL LDPE or HDPE bottles with tips that can be of linear low-density polyethylene (LLDPE) or HDPE or PP and caps that are usually HDPE or PP. LDPE is generally preferred for eye drop bottles because of their pliability which affects the ease with which a drop can be dispensed. The quality of the product may be affected by additives in the polymer which may interact with formulation components (e.g., binding of preservatives and actives, formation of insoluble complexes resulting in haze over time, etc.) or they may appear as contaminants in the form of extractables and leachables. Extractables and leachables may also be contributed by labels and secondary packaging components such as cartons and package inserts. The FDA is highly sensitive to the presence of extractables and leachables in ophthalmic products. To ensure the best quality of the product, bottles have some form of tamper evident seal. All primary packaging components must be sterile. Sterilization of plastics may be by ethylene oxide vapors (typically for LDPE and PP) or by γ irradiation (HDPE, LLDPE) and the sterilization method for packaging components must be validated. In blow-fill-seal (BFS), or form-fill-seal, operations product is filled into the bottle as it is being formed (in a sterile environment); because of the high temperature of the polymer as it is molded, it is assumed to be sterile and no further sterilization of the end product is generally required. The dropper tips may be molded as part of the operation or separate preformed, presterilized tips may be inserted followed by capping. There are specific color-coding requirements for different ophthalmic drugs as outlined in Table 6.

Preservative-free Multidose Devices

Although the great majority of eye drops are available as preserved multidose formulations in traditional LDPE dropper bottles, there are some patient populations that are sensitive to the presence of preservatives in formulations. That is the reason many formulations are also packaged as "unit-dose" or single-use vials. These are usually small volumes (0.5 mL or less) in LDPE form-fill-seal containers with twist off caps. Once opened these containers can not be stored beyond a single day of use and need to be discarded because of risk of contamination. The unit-dose presentations are more expensive to manufacture and as such are more costly for patients. As a result the market has seen the advent of preservative-free multidose devices (PFMD). The ABAK® system is a patented preservative free multidose eye drop dispenser (Fig. 6). It contains a 0.2 nylon fiber micro membrane that filters the solution. The pressure exerted causes the solution to pass through the antibacterial filter in the ABAK® system, forming a drop that falls from the tip of the dispenser. When pressure is released, the solution is reabsorbed and filtered from bacteria and air, ensuring the protection of the solution throughout its use. The ABAK® system filter provides a double protection: without using preservatives, it protects the solution inside the bottle from microbial contamination. The system has been used for the delivery of preservative-free timolol formulations to the eye and

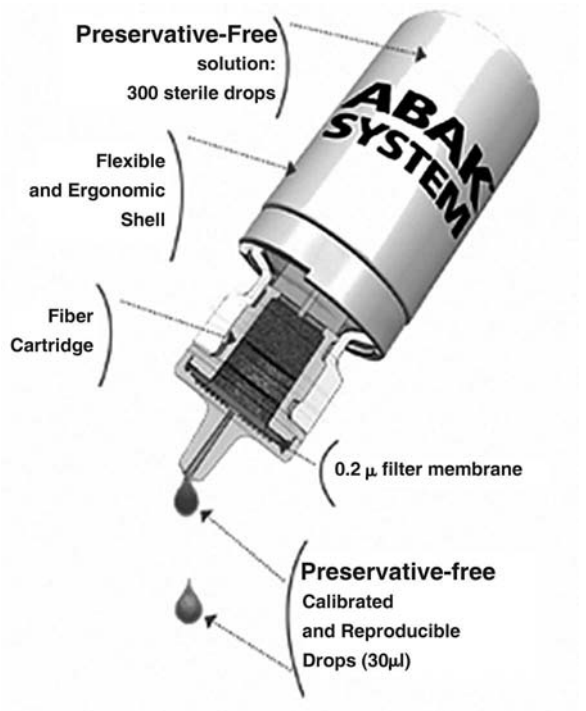

Figure 6 A preservative-free delivery device: ABAK®. *Source*: From Ref. 85.

is available in certain markets under the trade name of Timabak (Thea, France; Nitten Pharmaceutical Ltd., Japan). Administration of timolol via Timabak® showed marked decrease in cytotoxicity in human corneal epithelial cells when compared with preserved formulations containing 0.2% benzalkonium chloride (83). Alternatively, devices which employ a valve-mechanism that prevents the suction of air back into the container, which could contain bacteria, can also be employed for multiuse applications of preservative-free formulations. PFMDs have recently been used for several dry-eye therapies including Artelac-advanced-MDO and Hycosan® (both marketed by Bausch and Lomb) and Hylo-Comod® (Ursapharm). With growing regulatory and safety concerns regarding the use of preservatives in ophthalmic formulations, especially those intended for chronic use, PFMDs are likely to gain more popularity.

Regulatory Pathways for Ophthalmic Formulations

Ophthalmic New Drug Applications

The cost, monetarily and in time, for developing a new ophthalmic formulation will be determined primarily by the number and complexity of in vivo studies required. These regulatory requirements, as well as the potential market exclusivity of the new formulation, may influence whether a decision is made to develop a generic formulation, a new formulation, or to not develop a formulation at all. The benefit of developing a generic formulation is that a product may be developed without any in vivo studies, or, possibly, with one small in vivo study. Typical development time for a generic formulation is two to four years. Once approved, the generic formulation may then be prescribed for any indications for which the reference-listed drug (RLD) is approved. This pathway has very little risk with regards to safety/efficacy of the active, little risk with regards to clinical efficacy, and moderate risk with regards to regulatory approval. However, this pathway results in no market exclusivity except for the potential 180-day market exclusivity granted to the "first-to-file" generic developer when the patent has expired. Alternatively, if faced with the prospect of performing in vivo studies to demonstrate clinical efficacy of a new formulation in a particular indication, it may make more sense to produce a new, nonequivalent formulation of a

Table 7 Summary of Regulatory Pathways, Risks, and Costs for New Formulation Development

	Generics	Supergenerics	Proprietary
Filing	ANDA 505(j)	NDA 505(b)(2)	NDA 505(b)(1)
Exclusivity	None or 180 days	3 yr	Composition or application patent
Cost	Low	Medium	High
Time	2–4 yr	3–5 yr	10–12 yr
Development	0.5–1.5 yr	0.5–1.5 yr	1–3 yr
Animal safety	–	0–0.5 yr	3 yr
Human safety	–	0–1 yr	1 yr
Human efficacy	0–1.5 yr	0.5–1.5 yr	3 yr
Regulatory review	1–2.5 yr	1–2.5 yr	2.5 yr
Risks			
Safety/efficacy risk	Low	Low	High
Clinical risk	Low	Medium	High
Regulatory risk	Low	Medium	High
Revenue/margins	Low	Medium	High
Commercial advantage	Price	Benefit to patient/price	Benefit to patient
Required stability data	3 mo real time on 1 batch, accelerated 3 mo	12 mo real time on 3 batches, accelerated 6 mo	12 mo real time on 3 batches, accelerated 6 mo

previously approved drug. Typical development time for this pathway is three to four years, but involves more costly in vivo studies. This regulatory pathway also has little risk with regards to the safety/efficacy of the active, but does have moderate risk with regards to clinical efficacy and regulatory approval. The potential benefit of this development pathway is three-year market exclusivity for the new formulation or new indication. In addition, there is always the potential that the new formulation may be patentable and provide additional exclusivity for the formulation composition that is developed. The longest and most costly development pathway is for a new pharmaceutical ingredient. This pathway has the same clinical and regulatory risks as developing a new formulation for an approved drug, but this is compounded with significant risk with regards to the safety/efficacy of the active ingredient. The typical development time for this pathway is 10 to 12 years. Table 7 summarizes the differences between these regulatory/development pathways.

Ophthalmic Medical Devices [510(k)]

Section 510(k) of the Food, Drug and Cosmetic Act requires device manufacturers who must register to notify FDA, at least 90 days in advance, of their intent to market a medical device. A medical device, according to the U.S. FDA, is an instrument, apparatus, implement, machine, contrivance, implant, in vitro reagent or other similar or related article, including component part or accessory. Thus, certain nonmedicated OTC products (e.g., contact lens rewetting drops), irrigation solutions and contact lens solutions would generally be filed in this category. There are three classes of medical device class I (general controls are sufficient to show safety and efficacy); class II (general and special controls are required; special controls may include labeling requirements, requirements for postmarketing surveillance, etc.); and class III (general controls and premarket approval (PMA) are required to demonstrate safety and effectiveness). Most of the ophthalmic products that enter the market through the 510(k) filing fall into class II and III medical devices. The process involves detailed scientific review by FDA for PMA including clinical studies protocol that has been agreed by FDA. There is no regulatory time-limit for PMA review but, the FDA does target completion of approval within 180 working days of receipt, if it can be approved as received, or 320 days if additional information is required. The filing of a 510(k) requires identification of a "predicate" device (with very similar composition, packaging, and use) and a detailed comparison with the predicate device.

Stability Storage and Testing of Ophthalmic Formulations

The stability testing requirements for various types of ophthalmic products (eye drops, eye ointments, ophthalmic inserts, injections, irrigating solutions, lens care products, etc.) are not always straightforward. The International Conference on Harmonization (ICH) guidelines do not address all of the stability requirements for the diverse array of products. For the large number of ophthalmic formulations that are packaged in semi-permeable containers, "stress conditions" are present at high temperatures and low humidity. Thus, accelerated testing of these products is carried out under these conditions as per ICH guidelines. The specific conditions include long-term stability testing at 25°C/40%RH; intermediate accelerated (if 40°C fails) testing at 30°C/40% RH (FDA guidelines) or 30°C/60%RH (ICH guidelines) and accelerated testing at 40°C/15%RH (84). For specialized formulations and packaging systems, a well-planned, customized stability protocol will have to be written that properly addresses important product characteristics during use and storage. To increase the chances of product approval it is important to develop a well-defined stability protocol that aims to address all international, regional and local requirements that is approved by the regulatory authorities prior to start of stability studies.

FUTURE DIRECTIONS

Many new ophthalmic drug delivery technologies which offer potential advantages are currently available, and more will be discovered in the near future. However, the development of a new ophthalmic drug or new ophthalmic drug delivery technology is an expensive and time-consuming project. Therefore, the decision of whether or not to develop a new formulation will continue to be based on the added value that a new product will offer to the patient. A new technology may offer more comfortable, less invasive treatment of a disease, less frequent dosing of a product, or safer, more effective treatment of a particular indication.

The advances in drug delivery technology that promise to reduce the dosing frequency of a drug substance (e.g., moving from four times per day to twice per day), or offer more comfortable formulations will likely be developed for the treatment of chronic indications, such as glaucoma and dry eye. The benefits of lower dosing frequency and offering more comfortable treatment are not outweighed by the cost increase for treatment of acute indications, but over years of treatment, a patient will be willing to pay more for an improved formulation technology. It is also likely that combination products, which offer the convenience of delivering a single drop rather than multiple drops, will continue to be developed for chronic indications, but will not likely be developed for acute indications.

Although noninvasive methods such as use of an oral tablet or a topical formulation for posterior treatment (rather than an intravitreal injection) will always be of significant interest for any ophthalmic indication, they will most likely be of greatest use in the treatment of acute indications. Invasive technologies involving implants and intraocular injections will be of importance in treating chronic indications and where patient compliance is likely to be low because of a frequent dosing schedule (e.g., in elderly glaucoma patients).

Whenever feasible, new drug substances will continue to be brought to market, at least initially, in simple, low-risk formulations like solutions and suspensions.

In the future, the ophthalmic formulator will need to continue to have a firm understanding of the structure of the eye, the nature of the drug substance that needs to be delivered to treat the eye, and the options for how to bring the two together in the best way possible. The final choice of drug substance, formulation type, delivery method and manufacturing and packaging for the final product will need to take into account the overall market potential of the product as well as the cost and benefit to the patients.

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11 Glass containers for parenteral products

Robert Swift

INTRODUCTION

Glass containers have a long history as packaging materials for foods, beverages and medicinal products. Among other properties, glass compositions suitable for use as pharmaceutical containers offer sufficient inertness to minimize product interactions, impermeability to prevent ingress of contaminants, dimensional stability at temperatures needed for sterilization or depyrogenation as well as for lyophilization or frozen storage and transparency to allow product inspection. Where needed, coloration for light protection is possible. From the business side, a wide range of glass container styles and sizes is readily available in large quantities at reasonable cost. Increasingly, many manufacturers also offer preinspection, sterilization, barrier coatings or other specialized services needed for specific applications.

Despite general familiarity with glass in everyday life, detailed knowledge about the chemistry and manufacture of glass containers—and, specifically, glass containers used for parenteral medications—is limited. To provide basic information about glass, this chapter explores the characteristics of the glassy state, the broad range of industrial glass compositions and applications, the function of the various types of constituents that are included in commercial glasses and the manufacturing process steps that are common to the production of virtually all glass articles. This is followed by a more specific discussion of types of glass compositions used for pharmaceutical applications, how they are categorized and tested in the major pharmacopoeia, the various design families of containers used for parenteral products and the manufacturing processes by which they are produced. Some key aspects of quality control also are mentioned. The chapter concludes with a series of topics that are relevant to pharmaceutical formulation development, pharmaceutical filling, inspection and packaging operations and the quality of parenteral drug products that are filled into glass containers: the chemical, thermal and physical properties of containers and an overview of some quality blemishes and defects that can arise at various points throughout the supply chain.

When the first edition of this work was published in 1984, molded bottles for both small volume parenterals (SVPs) and large volume parenterals (LVPs) were in widespread use. By the time the second edition was published, in 1992, a significant proportion of LVPs had shifted to flexible containers. The LVP container information in the earlier editions is largely unchanged and still may be relevant in some markets. However, recent market trends for SVPs have increased interest in single dose vials, prefilled pens and prefilled syringes. This edition addresses these containers more fully.

THE GLASSY STATE

Glassy materials have been described or defined several ways by numerous authors and organizations. For example, Boyd (1) quotes Morey, ASTM C162 and Shelby, while Pfaender (2) provides three popular answers to the question "What is glass?" Some common themes can be summarized as follows:

- A supercooled liquid that has solidified or frozen without crystallization
- A solid material with amorphous, liquid-like structure
- A liquid with such high viscosity at room temperature that it behaves as a solid
- A material which lacks long-range molecular order but exhibits the stress-strain characteristics of a brittle, elastic solid.

While a wide range of materials, including organic molecules can be induced to form glasses, commercial container glasses are inorganic silicates produced by melting. With this

restriction, one can say that glass is an inorganic material or mixture of materials that has been heated to a molten liquid state then cooled without crystallization to a solid state.

The backbone of any glass formulation is a network former. There are several metallic oxides that readily cool without crystallization to form glasses. Special purpose glasses are produced using oxides of boron (B_2O_3), phosphorus (P_2O_5) or germanium (GeO_2) as the network former (3). However, the primary network former in glass formulations for commercial applications—including parenteral containers—is silica (silicon dioxide, SiO_2).

The basic network building block for silicate glasses is a tetrahedral form of silica, (SiO_4) (Fig. 1). Ideally, each silicon atom has shared bonds with four oxygen atoms and each oxygen atom has shared bonds with two silicon atoms. This configuration leads to a cross-linked, 3-D network (Fig. 2) of shared covalent bonds. The spatial interaction of these bonds causes viscosity to increase rapidly with decreasing temperature and inhibits the molecular reordering needed for the material to make the transition from a randomly ordered structure of the liquid state to the regular, long-range order of a crystalline solid. As a result, the network cools to rigidity in the glassy state. When processed under the appropriate conditions silica will crystallize as quartz (Fig. 3).

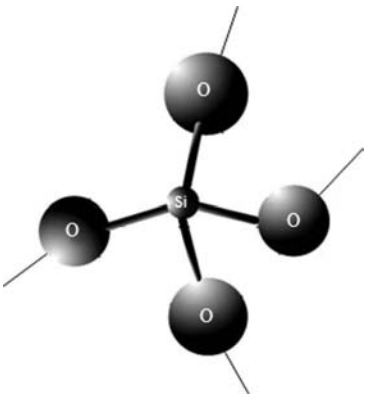

Figure 1 The SiO_4 tetrahedron is the basic network building block for silicate glasses. Each oxygen atom binds to the central silicon atom and either bridges to an adjacent silicon atom or exists as a nonbridged oxygen anion.

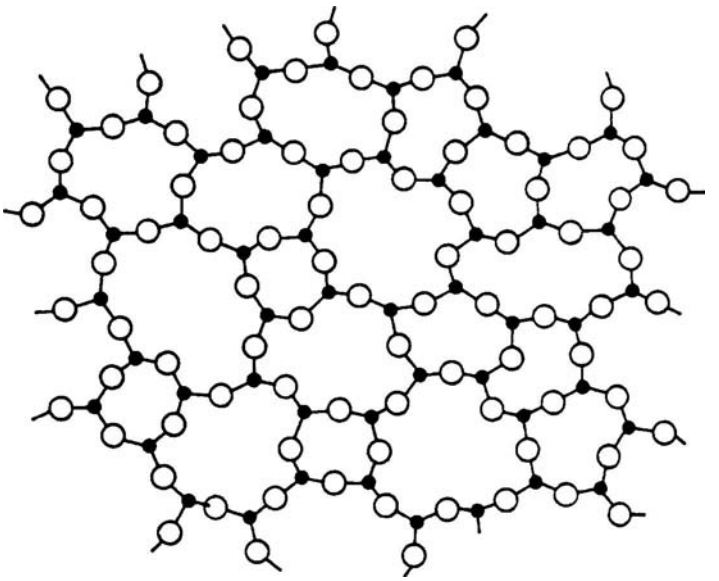

Figure 2 Two-dimensional schematic representation of glassy silicon dioxide in a random 3-D network of tetrahedral silica. *Source*: From Ref. 5.

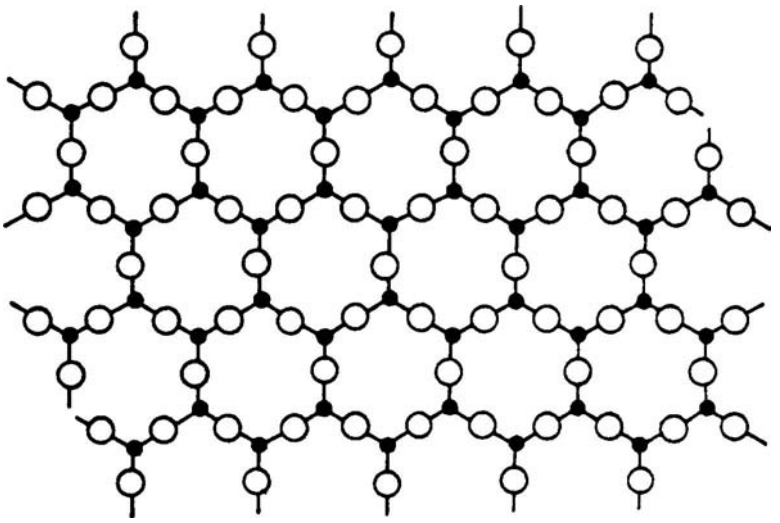

Figure 3 Two-dimensional schematic representation of 3-D crystalline quartz with long-range structure composed of tetrahedral silica. *Source*: From Ref. 5.

SILICATE GLASS FORMULATION FAMILIES

Both vitreous (glassy) silica and crystalline silica (quartz) are found in nature. However, commercial glass melting techniques require the viscosity of the melt to be in the range of 1 dPa-sec. For pure silica, this viscosity corresponds to about 2300°C, which is not practical for industrial production. Similarly, upon cooling, the viscosity of molten silica increases too quickly to be formed into containers using conventional production processes. As a result, practical glass formulations for containers are mixtures of silica and other minerals that lower the melting point and modify the properties of the glass to improve workability.

In fact, the network modifiers have the greatest influence on the physical and chemical properties of the glass and resulting finished glass articles. For this reason, glass formulations can be divided into broad families on the basis of the primary network modifiers used. The following sections describe the glass families used for containers and the role of the various network modifiers.

Soda-Lime-Silicate Glasses

The oldest and most widely melted glasses are known as soda-lime-silicate glasses. In the raw material mixture, or batch, these oxides typically are supplied as soda ash (sodium carbonate) and limestone (calcium carbonate)—hence, the common description "soda-lime" glass (4). In a glass formulation, soda and lime refer to sodium oxide and calcium oxide, which are the primary network modifiers and comprise roughly 25% of the composition by weight. Glasses in this family may include some magnesium oxide by the addition of dolomite (calcium magnesium carbonate). Potassium oxide, supplied as potash (potassium carbonate) may also be used. Within the silica matrix, the monovalent cations, sometimes called alkaline oxides, (Na^+ and K^+) satisfy the charges of nonbridged oxygen atoms (Modifier cation M_1 in Fig. 4). This reduces the extent of cross-linking in the silica backbone, which lowers the melting point. However, the sodium or potassium cations are relatively mobile and can be leached from the surface which limits chemical durability of the glass. The bivalent cations, also known as alkaline earth oxides, (Ca^{2+} and Mg^{2+}) interact with the silica matrix in a similar way occupying locations adjacent to two nonbridged oxygen atoms (Modifier cation M_2 in Fig. 4) and are more resistant to leaching. Usually, 2% to 3% aluminum oxide (Al_2O_3) is added to facilitate melting and to improve chemical durability. The aluminum cations (Al^{3+}) are able to form covalent bonds within the silica matrix (Modifier cation M_3 in Fig. 4) and, thus, are much more resistant to leaching. When light protection is needed, ferric oxide (Fe_2O_3) is added to produce amber glasses, which absorb ultraviolet wavelengths more effectively than colorless

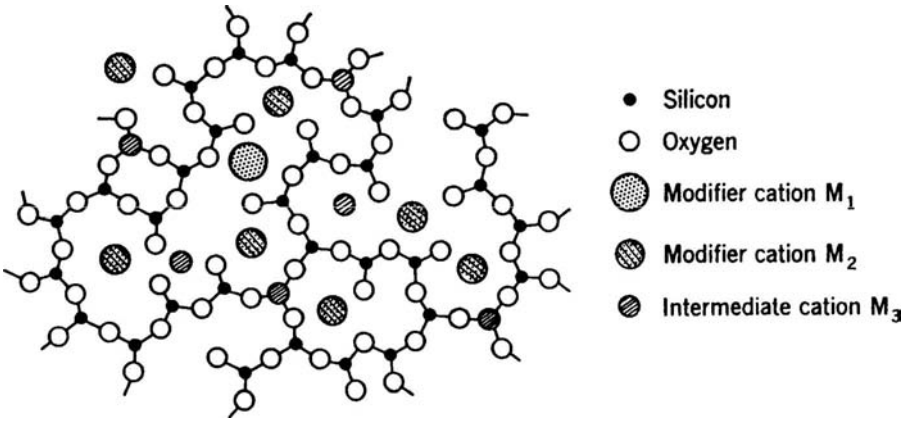

Figure 4 Two-dimensional schematic representation of the 3-D structure of a multicomponent glass. Monovalent and divalent cations exist in interstitial space and balance the negatively charged nonbridged oxygen atoms. Trivalent cations integrate into the silica network. At surfaces, nonbridging oxygen atoms are dominant and yield a net negative charge. *Source*: From Ref. 5.

glasses. The nominal compositions and properties of several soda-lime container glass formulations are shown in Table 1.

Borosilicate Glasses

In the late 19th century, Otto Schott, a German chemist and glass researcher, conducted systematic research to investigate the effects of various minerals and oxides on the optical, chemical and thermal properties of silicate glasses. He discovered that replacing some of the sodium and calcium with boron oxide (B_2O_3) resulted in glasses with exceptional chemical durability and heat resistance—including resistance to abrupt temperature changes, or thermal shock (7). Over time, a wide range of borosilicate glasses and other special glasses (8) have been developed for various applications including pharmaceutical containers as well as the familiar Duran®, Kimax®, and Pyrex® brands of laboratory glassware. Thermal shock and the related property of the thermal expansion coefficient will be developed more fully in the section Mechanical and Thermal Properties later in this chapter.

Borosilicate glasses require higher melting and forming temperatures than soda-lime glasses. However, with roughly ten-fold improvement in durability, as measured by extractable alkali, and high tolerance for thermal processes such as depyrogenation, lyophilization and terminal sterilization, borosilicate glasses now account for nearly all containers used for small volume parenterals. Ferric oxide (Fe_2O_3) and titanium oxide (Ti_2O_3) or manganese oxide (MnO) can be added to produce amber borosilicate glasses for protection from ultraviolet light. The nominal composition and properties of representative borosilicate container glasses also are shown in Table 1.

Compendial Classifications and Test Methods

Pharmacopoeias around the world acknowledge these two families of glass compositions as suitable materials for drug product containers. In fact, the compendia designate glass "types" based on these composition categories and reference the composition family in the general description of each type. For example, in the USP (9), type I containers have the description "Highly resistant, borosilicate glass" while type III containers are described as "soda-lime glass." Test methods used to differentiate between borosilicate and soda-lime containers and classify them according to type rely on the substantially lower quantity of alkaline ions that can be extracted from borosilicate glass or containers.

For example, the USP "powdered glass" test assesses the intrinsic chemical resistance of the glass formulation by crushing containers to obtain powder of a defined grain size and performing an extraction from the powdered glass into water by autoclaving. The alkali

Table 1 Nominal Compositions and Properties of Representative Soda-Lime and Borosilicate Glasses Used for Blow-Molded and Tubular Containers for Parenterals

Chemical composition (weight %)	Borosilicate							Soda-lime	
	Tubing					Molded		Molded	
	Clear			Amber		Clear	Amber	Clear	Amber
Silicon (SiO_2)	81	75	74.7	69	70	69	66	73	72
Boron (B_2O_3)	13	10.5	11.1	10	7	12	11		0.5
Sodium (Na_2O)	4	7	7.3	6	7	10	8	14	14.2
Aluminum (Al_2O_3)	2	5	6.1	6	6	6	6	2	2
Calcium (CaO)		1.5	0.4	0.5	< 1	1	1	10.5	10
Potassium (K_2O)			0.8	2	1				
Magnesium (MgO)				0.5					
Barium (BaO)				2		2	1		
Titanium (TiO_2)				3	5				
Manganese (MnO)							5		
Iron (Fe_2O_3)					1	<0.1	1	<0.05	0.3
Zinc (ZnO)				1					
Chloride (Cl^-) and fluoride (F^-)			0.4						
Sulfate (SO_3)				0.2				0.2	
Physical properties									
Thermal expansion 0–300°C ($\times 10^{-7}$)	33	49	51	54	55	60	62	88	91
Softening point (°C) ($10^{7.6}$ dPA-s)	825	785	783	765	770	739	745	729	713
Annealing point (°C) (10^{13} dPa-s)	560	565	566	558	560	567	530	548	536
Strain point (°C) ($10^{14.5}$ dPa-s)	525	Not published	518	520	Not published	528	515	510	496
Density (g/cm^3)	2.23	2.34	2.32	2.39	2.42	2.41	2.48	2.48	2.5

Source: From Ref. 6.

content of the resulting extract solution is determined by titration with acid. Although the test details differ, other compendia include similar methods. These methods can differentiate between type I borosilicate glass and type III soda-lime glass because of the significantly higher quantity of alkali that can be extracted from soda-lime glass. This is expected given the much higher levels of sodium, calcium and other alkaline and alkaline earth oxides present in soda-lime glass. The USP glass powder test and similar methods assume that the fresh surface exposed by crushing the container is representative of the inner surface of the container which will contact the drug product. This assumption is not always justified, as will be discussed in the section "Glass Chemistry" later in this chapter.

There are chemical treatments that can be applied to the inner surface of freshly formed containers to react with the alkaline ions at or near the surface. Sulfur dioxide (SO_2) or sulfur trioxide (SO_3) gas, or, more conveniently, ammonium sulfate [$(NH_4)_2SO_4$] pellets or solution may be injected into the containers before annealing. At elevated temperature and in the presence of water vapor, these substances produce sulfuric acid which reacts with alkaline ions on the glass surface to form various salt residues that are readily removed by rinsing prior to use. The removal of alkaline ions from the inner surface in this way significantly reduces the level of alkali available for leaching into the drug after filling. When type III containers made from soda-lime glass are treated in this way, the surface resistance is improved to such an extent that the pharmacopoeias recognize them separately as type II glass or containers. For example, USP <660> (9) designates Type II glass and provides the general description "Treated soda-lime glass." Similar classifications and descriptions are found in the European Pharmacopoeia (10).

Since only the surface resistance is improved by the treatment process, glass powder test methods cannot differentiate between treated or nontreated containers or assess the effectiveness of the treatment process. For treated containers, alternative test methods such as the USP Water Attack at 121° Test, the USP Surface Test, the Ph. Eur. Test for Surface Hydrolytic Resistance, or similar method must be used. In these tests, the extraction into water is performed using intact, filled containers rather than glass powder. As with glass powder methods, the results usually are determined by titration of the extract with acid. Some methods allow the use of spectroscopy to quantify directly the concentration of extracted alkaline ions.

If the composition family of the container glass is known (e.g., soda-lime glass), one may perform any of the surface test methods, apply the corresponding limit values and confirm the use and effectiveness of a chemical treatment process. When neither the glass formulation family nor use of chemical treatment is known, it may be necessary to perform both a surface test and a glass powder test to classify the containers correctly. However, many pharmaceutical companies confirm the container type on the basis of the supplier's test results and certificate of conformance.

As will be explained in section "Surface Chemistry," later in this chapter, there is another reason that the chemical resistance of the inner surface may be different from the intrinsic resistance of the glass formulation. The container forming process can cause degradation of the physical and chemical properties of the inner surface even when borosilicate glass is used. The Ph. Eur. test for surface hydrolytic resistance, the USP Surface Test or other similar methods may be used to evaluate residual surface alkalinity of containers made from borosilicate glass to confirm that the inner surface retains the level of chemical resistance expected in type I containers. The compendia are silent on test methods for and classification of containers made from borosilicate glass that are subsequently chemically treated to reduce alkaline surface residues deposited during forming. Users of "treated" borosilicate containers are advised to consult with their supplier to understand how the forming and "dealkalization" processes are controlled to ensure consistent results.

There is a tendency to assume that the terms soda-lime and borosilicate, especially as used in the pharmacopeias, refer to specific glass formulations. In fact, within the broad categories of soda-lime and borosilicate glasses, a wide range of glass formulations have been developed for specific applications. This point is especially relevant to borosilicates where two major subfamilies are important for parenteral containers. Within the industry, these subfamilies are often identified as "33 expansion" and "51 expansion." These terms are derived from the thermal expansion coefficient of some typical formulations in each group.

The American Society for Testing and Materials has published ASTM E-438-92 (11) defining nominal composition ranges and physical properties for glassware used in laboratory apparatus. This standard differentiates between the two borosilicate subfamilies by the designations "Type I, Class A" and "Type I, Class B." Although these designations are not used in the pharmacopeias, they are understood by glass manufacturers. Thus, for example, a specification defining the material requirement as ASTM E-438 Type I, Class A ensures that a 33-expansion borosilicate glass will be used.

GLASS PRODUCTION

Regardless of the glass composition, production of all glass containers begins with the transformation of inorganic raw materials into molten glass in large furnaces lined with refractory brick. A simplified cross section is shown in Figure 5.

The conversion of granular high purity silica sand, alumina, various carbonates and, for borosilicates, sodium borate into molten glass suitable for forming involves a series of complex physical and chemical reactions well beyond the scope of this chapter. However, the main process steps can be summarized as follows. The raw materials, or batch, are weighed, blended and conveyed continuously to the melting furnace. Typically, the batch includes a controlled percentage of internally recycled crushed glass known in the industry as cullet, which facilitates melting of the other batch materials. As the materials are heated and the melting process begins, carbon dioxide is liberated by decomposition of the carbonates and dissolved water is released. A substantial portion of the overall melting process is the refining process during which the CO_2, H_2O, and other gases coalesce, rise through the molten glass and escape into the furnace atmosphere. Bubbles that do not escape can be carried through to the forming process as seeds or blisters in molded bottles or as air lines in tubular containers. During refining, convection currents within the glass serve to homogenize the melt. Finally, the refined, homogenized molten glass must be slowly and uniformly cooled to reach the viscosity needed for the forming process which follows.

Glass composition is controlled primarily through careful monitoring of the raw material composition and corresponding minor adjustments to the batch proportions. Complete chemical analysis of glass composition is difficult and time consuming. Therefore, day-to-day monitoring of the melting process is accomplished by measuring physical properties such as density and thermal expansion coefficient that are extremely sensitive to changes in composition. Homogeneity and relative absence of bubbles are monitored by quality control inspections of the molded containers or tubing.

Figure 5 Longitudinal cross section of a large industrial glass melting furnace. Raw materials are added continuously at the batch feeder (1). Melting (2), refining (3), and homogenization (4) occur gradually as the melt progresses through the main furnace chamber. The molten glass flows under the bridge wall (5) into the conditioning section (6) before passing into the forehearth (7) for transfer to the blow-molding or tube draw process. *Source*: From Ref. 2.

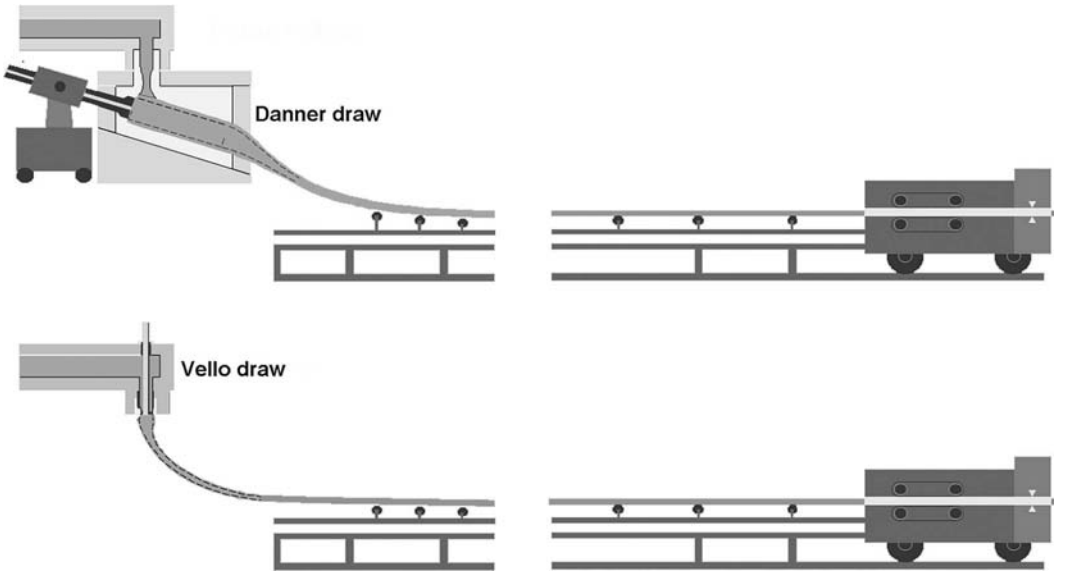

Figure 6 Glass tubing production for small volume parenteral containers uses either the Danner process of the Vello process. Both processes receive a continuous vertical stream of molten glass at the appropriate viscosity and transform it into discrete lengths of glass tubing with precisely controlled outer diameter and wall thickness. *Source*: From Ref. 4.

For both types of forming processes, the continuous output of the melting process is molten glass that has been cooled to reach a suitable viscosity. For blow-molded containers, a reciprocating plunger in the forehearth pumps the glass so that it can be cut into discrete charges, or "gobs," of molten glass that are guided through chutes to the forming machine. For all other styles of parenteral containers, the molten glass flows continuously from the furnace and is drawn into tubular form. The diameter and wall thickness of the tube are as needed for the body of the container into which the tube will later be formed. Glass tubing for parenteral containers is produced using either the Danner process or the Vello process (Fig. 6).

In the Vello process, a mandrel with the approximate shape of a bell is positioned in the stream of molten glass flowing from the orifice ring located in a bowl-shaped "drain" in the bottom of the forehearth. The molten glass flows out of the furnace and over the bell. Compressed air is blown through the center of the bell to form and maintain the stream as a tube.

The Danner process is similar except that the molten glass streams from the furnace onto a ceramic mandrel as a ribbon. The mandrel rotates slowly and is inclined slightly downward. As the glass flows down the length of the mandrel, it cools to the appropriate viscosity. Compressed air is blown through the center of the mandrel to form the tube.

With either process, the tractor belts of the drawing machine, located up to 120 m (~400 ft) downstream, provide a pulling motion which redirects the glass stream into a horizontal orientation. As the continuously moving glass tube cools and solidifies, it is supported on carbon rollers or air beds. The diameter and wall thickness are controlled by a delicate balance of the glass flow rate out of the furnace, the pressure of the blowing air and the speed of the drawing machine. The glass flow rate cannot be controlled directly but is the result of precise control of forehearth temperature, glass level within the furnace and, for Danner, the mandrel temperature or, for Vello, the relative dimensions and positions of the bell and ring. For either process, just after the drawing machine, the continuous tube is cracked off into discrete lengths, the ends are flame-smoothed or trimmed and fire-polished to prevent chipping and cracking and the tubes are packaged for shipment to the container producer.

In state of the art production facilities for pharmaceutical grade glass tubing, tubing diameter and melting defects such as knots, stones, and air lines are continuously inspected on

the tubing alley between the furnace and the drawing machine using laser or camera-based instruments. Additional checks of all other tubing dimensions and attributes are performed through automated or visual inspection of finished tubes. In general, acceptable quality level (AQL) sampling plans are used.

The next sections will discuss the various styles of container designs, some advantages and disadvantage of each and provide some details about the container forming processes.

CONTAINER DESIGNS AND MANUFACTURING PROCESSES

There are four main categories of container designs used for parenteral medications. Each is available in a range of sizes and shapes from multiple manufacturers.

Ampoules

An ampoule is a complete one-piece container system made entirely of glass and produced from tubing. The most common capacity range for ampoules is 0.5 to 2 mL. Capacities up to 20 mL or larger are possible for special applications. Some sizes and configurations have become de facto standards in certain markets. In addition, the International Organization for Standardization (ISO) has defined materials, dimensions, capacities, performance and packaging requirements for glass ampoules for injectable products in ISO 9187-1 (12).

The main steps of the forming process are illustrated in Figure 7. The wall of the tip or stem is thin and tightly controlled in the forming process. Similarly, the wall of the constriction is also tightly controlled. The constriction generally is preweakened by scoring or by the application of a color-break band to facilitate breaking the ampoule in the constriction at the time of use. Color-break bands are ceramic enamels with a slightly different thermal expansion coefficient. The mismatch prestresses the constriction to reduce the force needed to open the ampoule. An improved ampoule opening system called "One Point Cut" (OPC) has been developed. In this system, a small score of precise width and depth is cut at a single point of the constriction. OPC is claimed to provide more consistent opening force and fewer glass particles. ISO 9187-2 describes the requirements for ampoules using this design.

Quality control for ampoule manufacturing may include online 100% gauging of critical dimensions. State of the art producers use feedback control of the flames to maintain tight

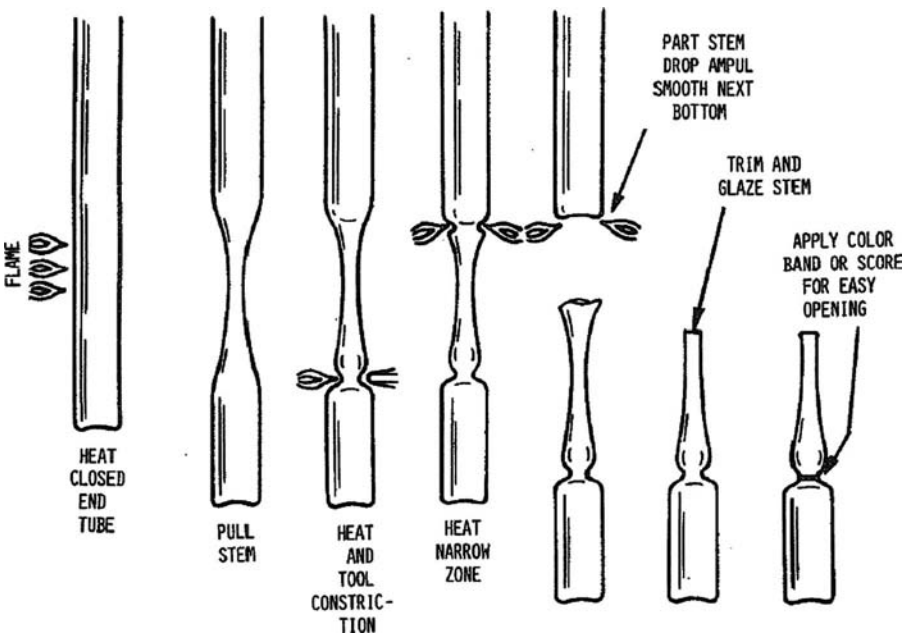

Figure 7 Typical process steps to form ampoules from glass tubing. *Source*: From Ref. 6.

control over the diameter and wall thickness of the stem. This minimizes variability in the sealing process during pharmaceutical processing. Some manufacturers offer supplemental 100% camera-based inspection to eliminate minor cosmetic blemishes at the point of manufacture to reduce container-related rejection of ampoules after filling and sealing. Additional quality control checks for dimensional and cosmetic attributes and breaking strength are performed periodically on finished ampoules. In general, AQL sampling plans are used.

After filling by the pharmaceutical producer, the stem of the ampoule is melted and usually pulled to seal the container. The combination of geometry and preweakening at the constriction allows the user to snap off the tip at the time of use and withdraw the contents of the ampoule into a disposable syringe so that the dose can be administered.

The main advantage of the ampoule container system is the simplicity of a single product contact material, highly inert borosilicate glass, throughout the shelf life of the drug product. However, breaking glass to gain access to the contents is not considered to be user-friendly. One also must consider the safety aspects of the sharp edges created when opening an ampoule and the possible need to use a filter when transferring the dose to a disposable syringe for administration. Therefore, while ampoules still are widely used for generic drugs and in developing countries, it is rare for new products to be developed in ampoule format.

Bottles and Vials

The most recognizable container system for parenteral products is a glass bottle or vial that has been closed with an elastomeric stopper and aluminum crimp seal. The glass container may be produced from glass tubing. Tubing vial capacities generally are limited to 30 mL. ISO has defined the materials, shape, dimensions, capacities and performance requirements for injection vials made from glass tubing up to 30 mL in ISO 8362-1 (13). A wide range of other heights, diameters and wall thickness also are produced. In addition, with specially designed forming machines and tubing up to 50 mm (~2 in) in diameter, it is possible to produce tubing vials up to 100 mL capacity or larger.

The production steps to form a vial from a glass tube are shown in Figure 8. The dimensions of the container body are unchanged by the forming process and retain the diameter and wall thickness of the original tube. As such, the wall thickness and diameter are uniform and well-controlled. This may allow higher filling and packaging line speeds and facilitate the use of high speed, camera-based inspection of filled containers. Forming the shoulder and bottom of the vial can cause occasional slight dimensional variation which may affect processing efficiency. In addition, the lighter weight of tubing vials may cause handling problems on lines orginally designed for heavier molded bottles.

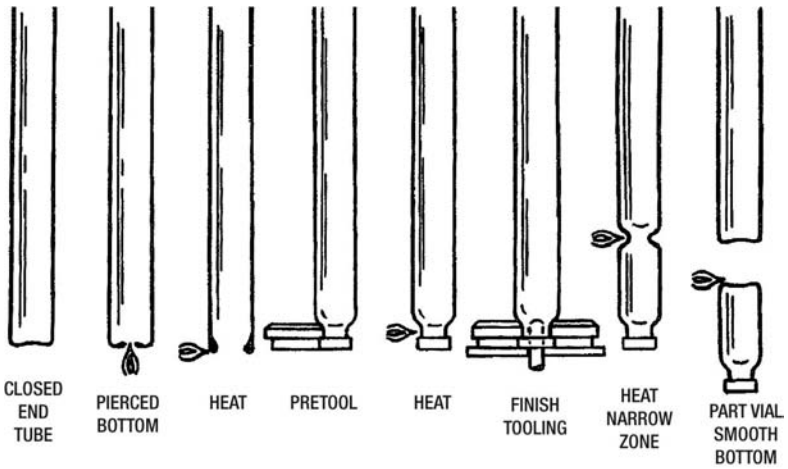

Figure 8 Typical process steps to form vials from glass tubing. *Source*: From Ref. 6.

Leading producers generally use camera-based systems directly after forming to perform 100% inspection of dimensions affecting the interface with stopper and seal. As with ampoules, some manufacturers offer supplemental 100% camera-based inspection to eliminate minor cosmetic blemishes at the point of manufacture to reduce container-related rejection of vials after filling and sealing. Additional quality control checks for dimensional and cosmetic attributes are performed periodically on finished vials using AQL-based sampling plans.

Containers for use with elastomer stoppers and aluminum seals also may be blow-molded bottles. Molded bottles for parenterals are available with capacities from 2 mL to 1 L or more. ISO has defined materials, shape, dimensions, capacities and performance requirements for injection vials made of molded glass in ISO 8362-4 (14). As with tubing vials, a wide range of other sizes and shapes are available. Typical process steps are shown in Figure 9. Compared with forming lines for tubular vials, molded bottle production lines have higher tooling costs longer changeover times and also must be located adjacent to the melting furnace. As such, production campaigns for molded bottles may be longer but less frequent. Production planning and inventory levels can be adjusted accordingly. Quality control steps for molded bottles are similar to those for tubular vials.

The nature of the blow-molding process is such that the wall thickness of a molded bottle will be heavier and more variable than the wall thickness for a tubing vial of similar capacity. Optical distortion caused by wall thickness variation can complicate inspection of the contents, especially when using automated, camera-based inspection systems. To accommodate the longer overall working time needed, borosilicate glass formulations suitable for blow-molding tend to have slightly higher sodium and boron content when compared with similar tubing glass formulations. On the other hand, heavier wall molded bottles may be more resistant to breakage caused by accidental abuse or mishandling.

For bottles and vials intended to be used with elastomeric stoppers and aluminum seals, the bottle or vial is only part of the overall container-closure system. Three-dimensional parameters of the mouth or finish are of particular functional importance at the interface with the stopper and seal. The neck inner diameter must ensure an appropriate interference fit with the plug of the stopper. Similarly, the outer diameter and thickness of the lip or finish must be suited to the diameter and skirt length of the aluminum seal. Other details of angles and radii also are important in matching the three components and the sealing equipment to create a robust container-closure system. While all of these parameters matter, by convention, the size designation is based on the nominal outer diameter of the finish. For small volume parenterals, typical container systems use finishes with either 13 mm or 20 mm nominal flange diameter.

ISO 8362-1 and ISO 8362-4 standards for injection vials provide dimensional details for the finish area as well as design parameters for complete containers, that is, diameter, total height, wall thickness, capacity, etc. ISO 8362—parts 2, 3, 5, and 6 are companion standards for elastomeric closures, aluminum caps and aluminum-plastic combination caps. This family of standards is intended to facilitate suitability of components from different suppliers in different but related industries. The roots of these ISO standards can be traced to German DIN standards. As such, the nominal dimensions and tolerances were developed in millimeters.

Historically, in the United States, container finish dimensions and matching closures have been based on the "2710 Biological Finish" standard developed in the 1940s by the Glass Container Manufacturer's Institute (GCMI), now known as the Glass Packaging Institute (GPI). The dimensions and tolerances of the GPI 2710 standard (15) are in inches but the size designations also are based on the nominal outer diameter of the finish in millimeters. As a consequence, both the ISO family of standards and the GPI 2710 standard include finish designs having finish outer diameters of about 13 and 20 mm. The important dimensions are similar but not identical. When selecting components, one must be aware, for example, that a "20-mm" stopper and seal from a U.S. producer may not be optimized for use with a 20-mm vial from a European producer. Discrepancies of this nature may also exist in published or de facto standards that may be widely used in other markets. Care must be taken to ensure the selected components are suitable for use as an integrated container-closure system.

As pharmaceutical filling line speeds have increased, container and closure manufacturers have worked with their customers to optimize processing efficiency. When stoppers are inserted into filled vials, the stopper plug often seals the neck of the vial before the stopper is

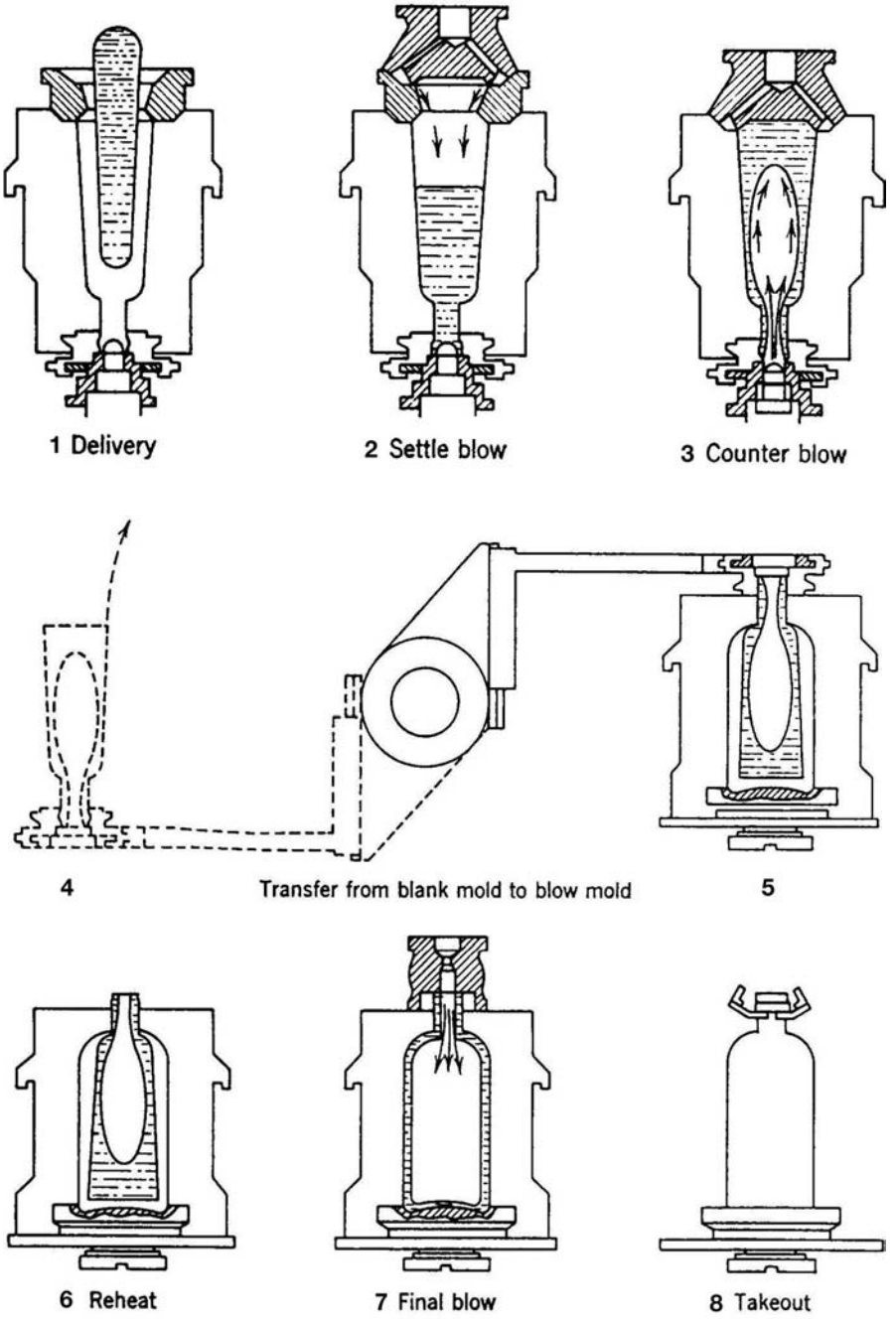

Figure 9 Typical process steps in the production of molded bottles by the blow-blow process. A charge or gob of molten glass is delivered by a chute from the furnace to the preform mold (**1**). Compressed air blows the glass into the mold to form the container mouth and neck (**2**). Compressed air then counterblows to shape the preform (**3**). The preform mold retracts (**4**), allowing preform to be transferred to the final mold which closes around it (**5**). The outer surface of preform that has been cooled by the preform mold reheats from residual heat in the molten core (**6**). Compressed air blows the glass out to the shape of the final mold (**7**). After some cooling time, the finished bottle is removed from the mold (**8**) and conveyed to the annealing (stress relief) tunnel. *Source*: From Ref. 5.

fully inserted. Completing the insertion creates a slight overpressure in the headspace resulting in a tendency for stoppers to "pop up" slightly after insertion. To address this, a "no-pop" ring can be molded into the stopper plug and a corresponding "blowback" ring can be formed into the neck of the vial. The intention is to provide additional mechanical interference to help retain the stopper in the seated position until the aluminum overseal is positioned and crimped. Here also, care is needed to ensure that the design details of each component are appropriately sized and positioned. The container system designer is advised to work closely with the component manufacturers to ensure compatibility.

The blowback feature originally was developed for smaller containers, for example, a vial with a nominal fill capacity of 2 cm^3 having a fill volume of 2 mL plus overage. In this situation, the volume of the stopper plug can be a significant percentage of the total headspace volume which increases the likelihood of pop-out because of pressurizing the headspace. Pharmaceutical companies producing lyophilized products also recognized the possibility for the blowback feature to improve the control over the position of the partially inserted stoppers during transfer of filled vials between the filling suite and the lyo chamber. Thus, vials and stoppers for lyophilization also often incorporate blowback rings.

Prefilled Cartridges

Glass cartridges are tubular glass containers that are open on one end to receive a suitable elastomeric plunger stopper. The opposite end has been tooled to form a neck and flange. After filling, the tooled end is closed with an aluminum cap which is lined with a suitable elastomeric septum. Just before use, a double-ended needle is attached. When the needle is attached, the end of the needle at the aluminum seal pierces the septum allowing the medication to be administered. Dental anesthetics and insulin therapy are two important markets for prefilled cartridge systems. For ease of use, the systems often are combined with reusable holders or, increasingly, adjustable multidose pen devices. Compared with a vial of equal capacity, a cartridge-based system will be longer, smaller in diameter and have little or no headspace gas. ISO has defined materials, dimensions, performance, and test methods for the product contact components of such systems in ISO 11040. Parts 1 and 4 (16,17) of the standard are glass cylinders, while parts 2, 3, and 5 address plungers, septa (disks) and aluminum caps. Additional requirements for components used in pen-injector systems are defined in ISO 13926 (18)—parts 1 through 3.

The glass forming process for the finish of a pen cartridge is similar to that used to form the neck and flange of a tubular vial. Online 100% inspection and off-line quality control checks also are similar. Cartridges are produced from tubing and can be formed using either one of two basic process concepts. The neck and flange may be formed, as with tubular vials, on the end of the tube. After forming the finish, the cartridge is separated from the tube using thermal shock and the open end is flame polished. Alternatively, full length tubes may be first cut into blanks using thermal shock and flame polished. On a separate forming line, the flange and neck are formed on one end of each blank. The smoothness and uniformity of the open end can have an important effect on the ability of the finished cartridge to endure the rigors of packaging and distribution.

In addition to its role as a drug product container during shelf life, at the time of use, the cartridge also plays a functional role as part of the drug delivery system. To fulfill this function, the body of the cartridge must be lubricated to reduce and control the static and dynamic friction between the glass cylinder and the elastomeric plunger. Generally, the lubricant is an emulsion of polydimethylsiloxane that is added to the final WFI rinse prior to depyrogenation using dry heat. The depyrogenation process drives off the residual water leaving behind the lubricating silicone layer. The interaction between the glass surface, the silicone fluid, the drug product and the elastomer plunger is complex. The processes affecting this interaction should be characterized thoroughly, validated and monitored to ensure consistent functional performance throughout shelf life. This is especially important for pen-injector systems where precise dosing is required. Cartridges for injection devices also may have additional dimensional requirements related to dose accuracy or to fit and function within the device.

Prefilled Syringes

In some ways, prefilled syringes can be considered an extension of the cartridge concept. Prefilled syringes also are formed from glass tubing. With a cartridge, one end is open to receive a suitable elastomeric plunger stopper. Unlike cartridges, the open end of a prefilled syringe is tooled to form a finger flange by which the syringe is held during administration of the dose. The opposite end of the syringe may be tooled to the shape of a male luer taper or to accept a plastic luer lok adapter or a small channel may be formed at the inner diameter of the tip into which a cannula is later inserted and glued. In each case, prior to filling, the syringe tip is fitted with a suitable elastomeric luer tip cap or needle shield. Prefillable syringes can be supplied as "bulk" (unprocessed) containers intended to be rinsed, siliconized and sterilized just prior to filling. Luer tip and Luer Lock syringe barrels can tolerate dry heat depyrogenation and the tip cap or tip cap and adapter are assembled under aseptic conditions in the filling suite. The adhesives typically used on syringes with glued in cannulae cannot tolerate dry heat. "Bulk" staked needle syringes are sterilized by autoclaving rather than by dry heat.

As with cartridges, prefilled syringes are produced from tubing and can be formed using either one of two basic process concepts. The tip may be formed, as with tubular vials, on the end of the tube. After forming the tip, the syringe body is separated from the tube using thermal shock and the open end is flared and tooled to form the finger flange. Alternatively, full length tubes may be first cut into blanks using thermal shock and flame polished. On a separate forming line, the finger flange is formed on one end of each blank and the tip is formed on the other end. The flange forming process may occasionally reduce the inner diameter at the flange opening. This may affect processing when mechanical plunger setting tubes are used.

Numerous dimensional and functional attributes of the glass barrels and various in-process assembly steps for prefilled syringes are 100% inspected using camera-based systems. Other process control and quality checks are performed at the appropriate stages of production using both time-based and AQL-based sampling plans.

In addition to bulk, unprocessed syringe barrels, there also is a significant and growing market for prefillable syringes that have been rinsed, siliconized, suitably packaged and then sterilized by the syringe manufacturer. These ready to fill systems are sterilized by ethylene oxide using validated cycles. Sterility testing is routinely performed on each sterilization batch.

As with pen cartridges, prefilled syringes serve double duty as the container-closure system during shelf storage of the drug product and as an integral part of the drug delivery system at the time of use. In prefillable syringes, the lubricant generally is applied as an aerosol mist of silicone fluid. The processes affecting this aspect of the syringe system should be well understood and controlled to ensure consistent functional performance.

For prefilled syringes, there is an additional level of complexity in that the tip cap or needle shield also serves a dual purpose. During shelf storage, this product contact interface is an integral part of the container-closure system. Yet, at the time of use, the tip cap or needle shield must be easily removed. And, for a luer tip or luer lok syringe, system performance requirements include the ability to form a leak-tight seal with the injection needle or delivery system adapter. Prefilled syringes also are increasingly being incorporated into automatic injection devices. Additional specification requirements and quality control tests may be required to ensure consistent drug delivery performance of prefilled syringes and auto-injectors.

While the focus of this chapter is on glass containers for parenterals, it is important to recognize that from the perspective of drug product compatibility, prefilled cartridges and prefilled syringes have added complexity compared with vial-stopper-seal systems. At a minimum, these systems include a second elastomer in the septum, tip cap or needle shield in addition to the plunger stopper. These systems also include the silicone fluid lubricant on the barrel and generally on the plunger stopper as well. Finally, for syringes with preattached needles, the stainless steel cannula and adhesive are in direct contact with the drug product throughout shelf life. The potential effects of each of these additional product contact materials needs to be assessed during qualification of the container-closure system.

Specialty Items

Other special purpose container systems, such as dual chamber vials, cartridges and syringes, threaded vials for infusion systems and high-strength capsules for needle-free injection systems also are available. An exhaustive review of these systems is beyond the scope of this chapter. The interested reader is encouraged to contact glass container manufacturers to learn about speciality products and new developments.

SURFACE CHEMISTRY

There are two fundamental mechanisms of chemical attack that can occur when an aqueous solution is in contact with the surface of a glass container (19). Through ion exchange, H_3O^+ ions in the solution can replace Na^+ ions in the glass. Once the sodium ions have been removed from the near surface layer, the rate of diffusion of sodium ions from within the bulk glass slows the process considerably. Ion exchange is the dominant mechanism of attack for most acidic and neutral formulations.

By contrast, hydroxyls and other alkaline species attack the silica network itself by breaking Si-O bonds. The rate of attack is highly dependent on the glass formulation and the solution pH. Surprisingly, several investigators (20–23) have shown that, at the same pH, different buffer systems can have markedly different rates of attack. It has been speculated that chelating agents are more aggressive toward glass because they are able to pull the various metal ions out of the surface. The resulting voids are then more susceptible to the other mechanisms of attack. Unfortunately, this means that simple formulation guidelines based on pH alone are not adequate.

In addition, the chemical resistance of the container surface also may vary. As mentioned earlier, the forming process can alter the composition, morphology and physicochemical characteristics of the container surface. During forming, especially when making the bottoms of ampoules and tubular vials, the temperature of the inner surface can exceed the boiling point of the more volatile ingredients of the formulation, primarily sodium and boron. These elements can vaporize from the hotter surface of the bottom and subsequently condense on the cooler sidewall as sodium borate. Then, as the finished container passes through the annealing oven, the deposits can be partially reintegrated into the underlying silica network. As a result, the alkaline deposits may not be completely removed by the pharmaceutical company's rinsing process but remain as less durable regions of the surface that is in contact with the drug product. This phenomenon will occur to some extent in the production of any container from glass tubing. For molded borosilicate glass bottles, vaporization and condensation of alkaline ingredients is generally not significant since the peak temperature of the glass is inherently lower. The resulting quantity of alkaline residue can be controlled by production speed, heating rate and maximum glass temperature. Residual alkalinity can be monitored by testing the surface resistance of the finished containers.

The alkaline residues can affect the drug product through three separate but related mechanisms. Firstly, the locally alkaline region or leached ions may react directly with the formulation. Secondly, by ion exchange with Na^+ ions in the glass, the loss of H_3O^+ ions from the solution can increase the pH of unbuffered or weakly buffered solutions. Thirdly, in extreme cases, the interaction can trigger the formation of an unstable layer of silica gel which can slough off as delaminated glassy particles.

Chemical dealkalization of borosilicate containers, for example, by the introduction of ammonium sulfate solution into the containers just before annealing, has been used, especially in the United States, as a means to control or minimize these effects. This process has been shown to be highly effective in reducing extractable alkali and the related effect on pH. Some users have found that the combination of controlled alkalinity in the forming process plus chemical dealkalization yields precise pH control for unbuffered products. However, studies by Ennis (24) showed that ammonium sulfate treatment without proper forming process controls did not eliminate delamination. In fact, in those studies, higher quantities and concentrations of treatment solution increased the formation of glass flakes.

Unpublished studies with which the author is familiar showed that delamination resulted from an interaction between excessive residual alkali on the vial surface, the parameters of the rinsing and depyrogenation processes, and the pH and composition of the

drug product vehicle. Anecdotally, acidic residues from excessive dealkalization also have been reported to have caused a reduction in drug product pH and long term damage to washers and deypryogenation tunnels.

Phenomena such as these highlight the importance of evaluating the chemical durability of the inner surface of the finished container using, for example, the USP Surface Test, the Ph. Eur. test for surface hydrolytic resistance, ISO 4802-1 (25) or similar quantitative spectroscopic surface extraction test methods such as ISO 4802-2 (26).

MECHANICAL AND THERMAL PROPERTIES

The preceding section addressed the chemical properties of the product contact surface, which can be of vital importance to the physical and chemical stability of drug products stored in the containers. Physical integrity of the container as a means to maintain product sterility is another equally important requirement of containers for parenterals. In this respect, the mechanical and thermal characteristics of glasses must be considered. Earlier in this chapter, glasses were described as amorphous materials exhibiting the stress-strain characteristics of a brittle, elastic solid. Describing glass as a "brittle" material is perhaps consistent with the general perception that glass is fragile. By contrast, the notion that glass is "elastic" seems contradictory. However, as material science terms, brittle and elastic have more precise meanings both of which apply to glasses.

In this context, brittle refers not to the strength of the material but to the failure mode when local stress exceeds local strength. Most metals, when overloaded, will deform in a permanent way, technically, "plastic deformation," before breaking. Brittle materials, such as glasses, are unable to undergo plastic deformation and therefore break abruptly (27). Intrinsically, glasses are very strong materials in response to compressive loads. However, surface damage significantly reduces the effective strength under tensile stress. A compressive load squeezes the margins of a surface flaw or discontinuity together and has little effect. By contrast, a tensile load pulls a surface flaw or discontinuity apart and concentrates the stress at the bottom of the discontinuity. Thus, the flaw or discontinuity significantly reduces the practical strength of the material as elucidated by Griffith (28).

Similarly, as a material science term, elastic refers to the response of a material to the application and removal of a mechanical load that does not exceed the strength of the material. Elastic materials deform when loaded then return to the original shape when the load is removed. The stiffness of a material can be characterized by its elastic modulus, also known as Young's modulus, which is the ratio between the applied unit load, or stress, and the resulting unit deformation, or strain. In this respect, glasses are relatively stiff. Typically, the elastic modulus of glass is about the same as aluminum (29). Jiang (30,31) attached strain gages to the outer surface of glass vials to observe in real time the physical deformations of and corresponding stresses in the vials during freezing, frozen storage and subsequent rewarming and thawing of various buffers and formulated drug products. Although it was not the objective of the studies, the work demonstrates the elastic deformation of the glass in response to the changing physical dimensions of the contents.

Because of the combination of stiffness, brittle behavior and reduction in strength at surface flaws, one does not usually observe directly the elastic deformation that occurs in glass containers before catastrophic brittle failure occurs. Indirectly, when failure occurs, the energy stored by elastic deformation may be observed in the form of rapid fracture propagation and dispersion of the glass fragments.

Stress in glass containers can result from forces exerted on the container, either externally or internally. Stress also can be the indirect result of nonhomogeneous composition or other imperfections from the melting process or from thermal effects. Thermally induced stresses may be either permanent artifacts from the glass forming process or a transient response to temperature gradients within the glass. Moreover, stress in the glass is additive. The total stress at a given point is the sum of the stresses at that point regardless of the source.

Silicate glasses have relatively low thermal conductivity. As a consequence, heating or cooling results in a steep temperature gradient between the heated or cooled surface and the underlying glass core. This is the reason that the coefficient of thermal expansion of the glass composition is important in determining the thermal resistance of a container. When a

container is cooled, the outer surface tries to contract. The contraction at the surface is resisted by the warmer core resulting in tensile stress at the outer surface. While this phenomenon is the principle behind "cutting" glass by thermal shock, it also can lead to unintended cracks during container production as well as during pharmaceutical processing.

For a given temperature difference, the stress level is proportional to the thermal expansion coefficient and the modulus of elasticity of the glass composition (32). Thus, all other conditions being equal, a 33-expansion borosilicate glass container can withstand a temperature difference on the order of three times larger than a container of identical size, shape and geometry made from a "90-expansion" soda-lime glass. It should be noted that, in addition to the properties of the glass, the cooling rate, the geometry of the container and the presence of surface flaws caused by handling all contribute to thermal resistance.

QUALITY ATTRIBUTES

Several aspects of quality control already have been mentioned in the discussions of the manufacturing processes. These described the process points where quality control checks are performed rather than the quality attributes being examined. A detailed discussion of the full range of possible container defects and cosmetic flaws is beyond the scope of this chapter. Nevertheless, it is worthwhile to point out that certain types of flaws can occur only in specific process steps. As such, some basic knowledge can be helpful when investigating container defects and failures. For example, glass flaws known as knots, stones, cord, seeds, blisters and airlines all originate in primary glass melting and tubing manufacture. Certain types of surface blemishes can occur only during blow-molding or conversion of tubing into ampoules, vials, cartridges or syringes. Finally, there are blemishes and defects that are more likely to be the result of interactions between containers and fill-finish equipment or processes. On the other hand, scratches, scuffs, bruises, and metal marks may occur at any process or handling step. Even in these cases, though, detailed examination may yield clues pointing to the root cause. For example, a scratch running the full length of the body of a tubing vial and fading into the heel and shoulder may indicate that the scratch was present on the tube prior to forming the container. Similarly, the location and orientation of a scuff or metal mark may eliminate most potential points of contact. The interested reader is advised to explore these topics with container producers. In addition, the Parenteral Drug Association (PDA) has published lexicons of attributes for tubular vials and molded bottles (33). Similar lexicons are being developed for ampoules, cartridges and prefilled syringes.

In some situations, the use of more sophisticated analytical tools may be warranted. Glass fracture analysis is the science of determining the origin of the breakage and the nature, direction and relative magnitude of the force that caused the breakage. Scanning electron microscopy with X-ray diffraction analysis or similar methods can be used to determine the elemental composition of surface flaws or of foreign materials that may be present.

ACKNOWLEDGMENTS

The author is indebted to Frank R. Bacon, R. Paul Abendroth and Robert N. Clark, the authors of Glass Containers for Parenterals in the First and Second Editions of Pharmaceutical Dosages Forms: Parenteral Medications. Years ago, their chapters provided a solid foundation of knowledge about glass and glassware for pharmaceutical products. More recently, in preparing this edition, their examples have guided the work.

The author also gratefully acknowledges the support and guidance of many coworkers and former colleagues as well as the editors. In particular, Ron Forster has been a constant source of encouragement. Finally, Nick DeBello, Peter Gassmann, Carlo Pantano, Jochen Pfeifer, and Randy Thackrey provided numerous suggestions that improved the content and readability.

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12 Plastic packaging for parenteral drug delivery

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INTRODUCTION

Driven by the development of biotechnology products, newer drug therapies, and reformulation of poorly soluble drugs, parenteral delivery is expected to provide strong growth in years to come. Routes of administration include subcutaneous, intramuscular, intradermal and intravenous injections. Drug products have been almost exclusively dispensed in glass containers, primarily because of the clarity, inertness, barrier property and thermal resistance of these containers. With the development of plastic polymer technology over the last 30 years, plastics have become logical alternatives for small-volume parenteral (SVP) and large-volume parenteral (LVP) packaging. Although plastic containers have become well-established as containers for LVP products, plastics have been, until recently, used on a limited scale for SVPs.

Glass vials are the primary container of choice because of their excellent gas and moisture barrier properties. More importantly, there is an extensive knowledge base on processing, filling, regulatory review and commercial availability of glass containers. Glass, however, may not be the best solution for all chemical or biological drug candidates. Glass contains free alkali oxides and traces of metals. Depending on the characteristics of the drug being packaged, it is likely that delamination could occur for high pH products over time, thereby affecting the shelf-life of the drug product. Proteins and peptides can be readily adsorbed onto the glass surface and can be denatured or become unavailable for treatment. With a glass prefillable syringe (PFS), potential leachables such as silicone, tungsten and adhesive can affect the stability of biopharmaceutical products. Glass may break during processing or transportation and when stored at low frozen temperatures. In these and other areas, plastic containers have made clear in-roads in the parenteral drug delivery market.

With the proliferation of new polymers and newer process technologies, most of the less-desirable characteristics of plastic containers have been overcome and the use of plastic packaging as vials and syringes is increasing. This chapter will discuss the role of plastic in pharmaceutical parenteral drug delivery. The discussion will provide insights on the following areas:

- Advances in plastic resins for SVP packaging with an emphasis on cyclic olefins as well as other plastics used: The properties of these plastics, applications and challenges will also be discussed.
- Plastic vial systems: This section will discuss in detail the development activities in this area including the use of plastic vials in lyophilization and the use of reconstitution devices.
- Plastic PFS systems: As more biopharmaceutical drugs and higher viscosity formulations are delivered in a PFS, there is the need for a break-resistant, high-quality, plastic PFS. Challenges with glass include breakage, reactivity of glass and leachables, such as silicone, tungsten and adhesive. Discussion will include how plastic PFS offer options to solve these challenges.
- IV bags and disposable bags: Following a brief overview of use of plastics for IV bags for LVPs, discussion will focus on new developments in the use of plastics for disposable bags in the packaging of biologics, including considerations for selection of disposable bags.
- Quality and regulatory considerations: U.S. Pharmacopeia (USP), European Pharmacopoeia (Ph.Eur.) and Japanese Pharmacopoeia (JP) compendial requirements will be discussed and referenced for plastic containers.

This chapter provides the reader with adequate information on recent developments, availability and use of various plastic packaging systems for pharmaceutical drug products, including suitable references to commercialized drugs products.

ADVANCES IN PLASTICS

Plastic resins are the most widely used raw materials in global pharmaceutical packaging, accounting for 61% of consumption compared with glass, paper products and aluminum foil. The worldwide demand for plastics for packaging was estimated at $25.8 billion or 2.3 billion lbs. of material consumed in 2006 (1). High-density polyethylene (PE) is the most widely used plastic with 1.2 billion lbs. consumed, followed by polypropylene (PP) at 0.4 billion lbs. However, the fastest growth is expected with the newer resins, the cyclic olefins growing at a compound annual growth rate of 5.5% by 2011 (Fig. 1). The growth is expected to penetrate specialty fields such as pharmaceutical drug delivery. This is driven by a need for clear, highly transparent, biocompatible packaging systems with improved quality and improved barrier protection.

Cyclic olefins: Compared with the traditional plastic resins, the development and application of cyclic olefins in parenteral drug delivery is relatively new. Cyclic olefins are prepared by additional polymerization of monocyclic olefins, cyclobutane or cyclopentane or bicyclic olefins such as norbornene. The resulting product has improved chemical and physical properties, such as glass-like transparency, excellent chemical resistance and improved moisture barrier. Mitsui Petrochemical Industries produced copolymers of ethylene and other cyclic olefins. Starting in the 1980s, Mitsui and Hoechst (2,3) began using single-sited metallocene catalysis in the polymerization of cyclic olefins that led to the development of the cyclic olefin copolymer (COC) Topas® by Ticona. In this process, 2-norbornene was reacted with ethylene in the presence of a metallocene catalyst to produce a series of copolymers whose properties can be modified by varying the norbornene percentage in the material. Another commercially viable route is through a two-step process based on the ring-opening metathesis polymerization (ROMP) of dicyclopentadiene followed by complete hydrogenation of the double bonds to form cyclic olefin polymers (COP) (Fig. 2). Using this process, the Zeon Corporation developed the Zeonex® and Zeonor® line of COP. A similar process also resulted in another clear COP plastic, called Daikyo Crystal Zenith® (CZ) that is available only in a finished container format from Daikyo Seiko, Ltd.

COP and copolymers (COC) possess many excellent properties, including glass-like transparency. This glass-like transparency of the polymers permits visual inspection of

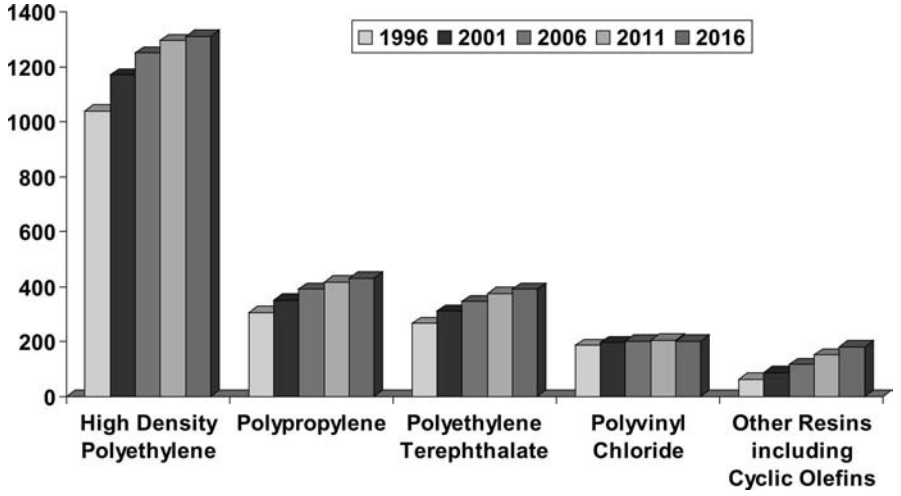

Figure 1 (*See color insert*) World pharmaceutical packaging plastics demand by resin (million pounds by weight).

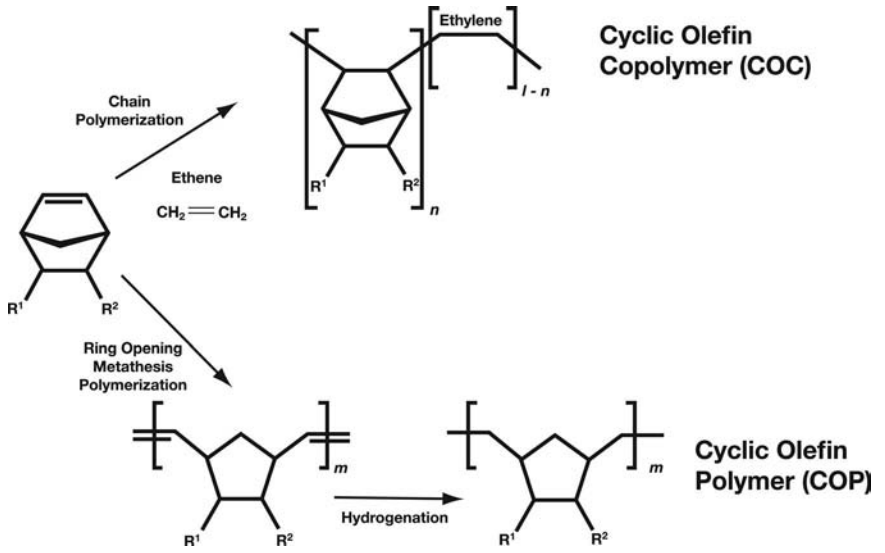

Figure 2 Process of polymerization in the development of cyclic olefin polymers/cyclic olefin copolymers. *Source*: Reproduced from Ref. 2.

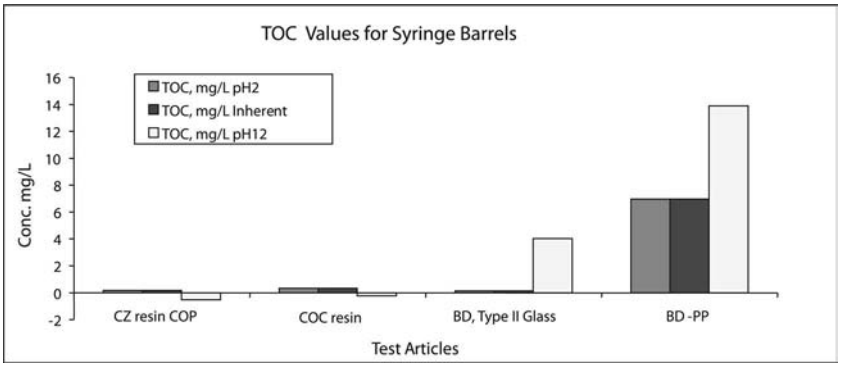

Figure 3 (*See color insert*) Comparison of total organic carbon as an extractable from syringe barrels. *Source*: Reproduced from Ref. 6.

the resultant manufactured components, as well as the parenteral products that are delivered to the end user. The polymers have good melt flow properties that readily lend themselves to plastics processing, for example, molding and thermal forming. The polymers exhibit a high impact and break resistance, and they form an excellent moisture barrier (2–5). Additionally, they possess good chemical resistance to acids, bases and alcohols. These polymers are sterilizable by autoclave, ethylene oxide and radiation sterilization processes. As with most plastics in comparison with glass, the number of potential compounds that may be an extractable or leachable is higher for plastic than for glass because the number of components in the formulation is higher. These compounds are organic, whereas glass potential extractables are inorganic. Plastic vendors can provide a list of potential extractables developed with suitable extracting solutions. A decision may then be made on which potential extractables should be studied as leachables during stability testing. Preliminary studies have shown that, when compared with other materials that are used for parenteral applications, COP and COC exhibit very low extractables (Fig. 3). When studied for total organic carbon (TOC) extracted from syringe barrels at various pH levels, the

Table 1 Features of Cyclic Olefins for Parenteral Drug Delivery

Key benefits	Drawbacks
Glass-like transparency	Gas and moisture barrier properties are less than glass but better than other plastics
Sterilizable (via autoclave, radiation and ethylene oxide)	Sensitivity to scratches
High break resistance	Short-term discoloration due to radiation
Excellent moisture barrier	
Biocompatible (inert, low binding, and ion extractables)	
Design flexibility and excellent dimensional tolerances	
Good chemical resistance	

data shows very low extractable for COP (CZ) and COC compared with PP and glass (6,7). On the basis of this data and other information available, COP and COC are considered to be ideal plastic packaging containers for SVP. There are some drawbacks, however. Understanding these drawbacks will be important in the selection of cyclic olefins as a packaging system (Table 1). These plastic containers cannot match the barrier properties of glass to oxygen and moisture ingress, although they are much superior to other plastics, including PP, polystyrene and polycarbonate (PC). For oxygen-sensitive compounds, this may be a concern. Suitable secondary packaging can prevent moisture loss or oxidation, with the addition of a moisture absorbent or oxygen scavenger material.

High-density polyethylene (HDPE): The polymer is based on a simple repeating carbon/hydrogen molecule that branches out during polymerization to form a polymer with a high degree of regularity. This regularity creates the formation of crystal lattice structures. Polyethylene (PE) is recognized as having a high degree of crystallinity. During polymerization, the amount of branching that occurs during the process will determine the overall density and crystallinity of the resulting PE. As a result of their relatively high degree of crystallinity as compared with lower-density PEs, HDPEs have greater tensile strength and stiffness and have a higher melting point than the low-density polyethylene (LDPE) resins. Another important property is excellent chemical resistance, a characteristic of all polyethylene grades. HDPE is typically used for low-to-medium barrier medications, such as bottles, closures and in some cases, secondary packaging of parenterals and blister packs for solid dosage forms. The material is characterized by strong impact resistance, chemical resistance, drug compatibility for oral dosage forms and temperature tolerance. Both HDPE and LDPE are used to form containers by blow-fill-seal technology, primarily for ophthalmic and nasal/respiratory drugs, but also have been used for both SVP and LVP products.

Polypropylene (PP): PP is the leading plastic employed in containers, disposable syringes, PFS and closures. PP is a linear, high crystalline polymer, made of carbon and hydrogen in a very orderly fashion. The regularity of its structure imparts the high degree of crystallinity found in most commercially available PP. Within the crystal array, the methyl groups impart stiffness to the polymer, making it different from its close relative, polyethylene. PP exhibits a high tensile strength, which is the ability to withstand forces tending to pull apart or distort the material, and is more rigid than HDPE. High tensile strength, in conjunction with a high melting point of 165°C, is particularly important for packaging drugs. Consequently, the material has the ability to withstand higher temperatures of autoclave sterilization for a limited number of cycles. PP is also resistant to chemical attack from organic solvents and strong acids and bases at room temperature. Because of the level of crystallinity present, it is not possible to achieve the optical clarity found with cyclic olefins: the crystal lattice sites tend to refract light, which imparts haze. The resin generates significant demand for the manufacture of blow molded bottles, pouches, laminates and plastic containers. Because of its improved moisture resistance and effective chemical resistance, PP is

typically used in disposable containers or delivery systems. It may have poor impact resistance at lower temperatures and increased extractables, and its translucency limits its role in the storage of parenteral drug and biological products for long duration.

Polyethylene terephthalate (PET): PET is a high-quality thermoplastic polyester that offers good barrier protection, chemical resistance and processing properties. It is typically used in packaging drugs that may require barrier protection as in blister packs and blow molded containers. It is cost competitive with HDPE and PVC and is used in development of bottles and blister sheeting. PET is polyester that is a condensed polymer prepared from ethylene glycol (EG) and either terephthalic acid (TPA) or the dimethyl ester of terephthalic acid (DMT). The EG monomer is prepared using ethane as feedstock and the TPA is manufactured using paraxylene as feedstock. TPA can then be purified by reaction with methanol to form the DMT. PET can exist in an amorphous state, an oriented and partially crystalline state and a highly crystalline state. Because of its low glass transition temperature, PET cannot tolerate autoclave sterilization. The material does hold up well to gamma radiation, making it the preferred method for sterilization. Ethylene oxide sterilization is also acceptable with PET resins. PET film may potentially be used as a coextruded layer of LVP bags (replacing use of PVC resins).

Polycarbonate (PC): PC is known for its mechanical properties and higher clarity with poor barrier properties. PC-based polymers are aliphatic molecules and are synthesized in various forms. These aliphatic PCs become extremely soft in the 40°C to 60°C temperature range. Bisphenol A PC is extremely stable and virtually nondegradable under physiological conditions. PC can be processed readily, possesses high mechanical strength and is very shatter resistant. PCs are used extensively as bottles and containers for parenteral applications. PC resin contains repeating aromatic rings in its main chain structure. The material is a polyester of carbonic acid and is generally produced using an interfacial reaction between dihydric or polyhydric phenols and a suitable carbonate precursor such as dichlorocarbonate. Currently most PCs are produced with a reaction between bisphenol A and carbonyl chloride in an interfacial process. Other polyhydric phenols are sometimes used to form copolymers for special end uses. The material is well suited for the injection molding process. PC shows excellent creep resistance over a broad temperature range, enabling its use in applications previously open only to thermoset materials. There are, however, some areas where PC resins are inferior. PC materials have limited chemical and scratch resistance and a very high water transmission rate when compared with other plastics. The resin also has a tendency to yellow with light exposure and with exposure to radiation sterilization.

Polyvinyl chloride (PVC): Less popular in parenteral packaging, PVC is prepared by polymerizing a gas, vinyl chloride or monochloroethylene, in the presence of organic peroxides or inorganic persulfates as initiators. The length of the molecular chain and the structure of the side chains are altered by the temperature, pressure and the nature of the initiator. PVC's growth in pharmaceutical packaging is much slower compared with its peers because of environmental concerns. This includes the formation of dioxin when PVC is incinerated. Additionally, di(2-ethylhexyl) phthalate (DEHP) plasticizers are used in the production of many PVC materials. These types of phthalates, which are known to leach out of PVC containers, may have potential health risks. Growth has slowed in this area, which probably reflects preferences for better performing and safer plastics.

Multilayer plastics: Plastic bags commonly used for LVP generally consist of between three and five layers of plastic film consisting of two or more different resins. Similarly, plastic film used for blister packaging of tablets is also multilayered. The purpose is to produce a plastic film that combines the best properties of each film including good clarity, excellent flexibility and durability, which also is a strong barrier to water vapor transmission.

Plastics fabrication: There are many processes used to convert plastic resins from pellets into desired shapes or configurations. This is a brief description of the plastic molding

processes. All plastic processes are similar in the use of three basic elements to convert the resin from a pellet to its processed shape.

1. *Heat*: excites the molecular structure to allow free movement of molecules
2. *Pressure*: forms the free-flowing polymer into a desired shape
3. *Time*: allows the transfer of heat into the plastic followed by time for removal of heat (cooling)

Extrusion of plastics: The process of extrusion involves melting a plastic and forcing it through a die under pressure to form a desired shape. There are several types of extrusion, depending on the die arrangement used to form the plastic. The three most widely used for parenteral packaging are flat-sheet extrusion, profile-tubing and blown-film extrusion. Flat-sheet extrusion is a versatile process, with the capability to produce sheet stock over a wide range of thicknesses from a wide range of resins. The process may also be used to produce coextruded sheeting where two or more different resins are brought together in the die manifold from two or more extruders. Flat-sheet extrusions can be used for blister packaging and form, fill and seal packaging. Clear grades of plastic that have a high degree of stiffness are generally preferred for extrusion processing. Another application for this process is the production of LVP containers.

Injection molding: Injection molding is a process used to convert resin from a melt into a molded shape using a mold pattern to form the part. Injection-molded products are replacing materials such as glass, metals and paper in many areas of parenteral drug packaging. The development of newer plastic resins, combined with improvements in the injection molding process, is setting the stage for these changes. For example, materials such as CZ resin have been used to develop larger containers such as the 1-L bottle by injection molding. Many of these newer resins are used for drug delivery systems that are replacing products traditionally made from glass. In this process, plastic resin is melted using the extrusion process and is injected into a mold where the resin is cooled enough to be removed in a solid state. Like the other plastic processes, heat, pressure and time are used in each of the steps to produce a molded product. Injection-molded items are finding many uses in parenteral drug packaging. The injection molding process is also used to produce components such as IV spikes and IV administration sets.

Blow molding: The blow molding process has grown rapidly over the past three decades. The two types of blow molding in use are extrusion blow molding and injection blow molding. A uniform tube of heated resin with one end closed is formed during the extrusion blow molding process and is moved into a mold where the two ends are pinched off, and the material is blown outward into the shape of the mold. The injection blow molding process is similar in concept except that is a two-step process. A preform is molded using a first-stage mold and the principles of injection molding. The form is then transferred into a second mold, and blown outward using pressurized air to form the container. Containers produced for health care applications, such as tablet bottles, are made primarily using the injection blow mold process. With small containers, this process is more cost effective than extrusion blow molding because it is capable of handling a large row of preforms at one time. Extrusion blow molding lends itself to larger containers where it becomes more economical and practical to eliminate the preform step. The blow molding process enhances the physical, chemical and barrier properties of certain materials, for example, PET, because it creates a high level of bi-axial orientation of the polymer. CZ, Zeonex and Topas resins also use the blow molding process to manufacture vials.

VIAL SYSTEMS

Market considerations: A vial is a SVP container with a stopper and a seal, intended to package liquid or a dry powder formulation for either single or multiple doses. Glass vials, typically made of type I glass, are most commonly used as vials for parenteral

applications. Recently there is increased interest in the use of newer plastics, particularly the cyclic olefins, as parenteral vials as they provide clarity and inert surfaces for biopharmaceutical and biological applications. When combined with plastic's inherent break-resistant attribute and the need for biologics to be stored and transported at lower temperatures, the future of cyclic olefin based plastics appears bright. Cyclic olefin polymers (COP) and copolymers (COC) are considered to be an ideal plastic for vial systems because they have glass-like clarity and suitable physicochemical properties and the ability to be sterilized.

The vendors in this area may be divided into those that manufacture the COP and COC resins such as the Zeon Corporation and Topas Advanced Polymers and companies that convert the resin into parenteral containers such as Schott Forma Vitrum that offers a range of sizes of both syringes and vials made out of COC under the brand name Schott TopPac®. Daikyo Seiko, Ltd. of Japan has used a proprietary COP resin to produce a range of sizes of conical, flat-bottom vials and larger screw-top containers, under the brand name of Crystal Zenith. West Pharmaceutical Services, Inc. (West) partners with Daikyo to codevelop, market and sell sterile and nonsterile CZ vials. As a result of the anticipated growth, the suppliers of resins and products have made significant investments to their supply chain to maintain continuity of supply. Rexam offers a new generation of multilayered plastic vials called MLx that are being used as a container with improved barrier properties. The COC vials produced by Aseptic Technologies represent a newer approach to vial handling and filling called the Crystal® technology, licensed from Medical Instill Technologies (Table 2). The vials and stoppers are molded and assembled immediately under clean conditions and gamma sterilized. Filling is achieved by piercing the thermoplastic closure and then immediately resealing the puncture with a laser. COP and COC vials have been tested and used to replace glass in various pharmaceutical parenteral applications. This is because glass contains free alkali oxides and traces of metals and, at higher pH conditions, can undergo delamination, thus affecting the stability of the drug product (8,9). Proteins and peptides can be adsorbed on a glass surface and can either be denatured or become unavailable for treatment (10,11). Glass particles can promote protein particulate formation, and glass is also more likely to break under processing, storage or transportation of biopharmaceutical products, especially at lower temperatures. In these areas and more, plastic vials have made clear in-roads in the pharmaceutical drug delivery market.

Protein and peptide adsorption: Numerous studies have addressed the adsorption of proteins to packaging containers. This interaction of proteins and peptides with the surfaces of storage containers can result in their loss and destabilization (12–14). Although the amount bound is typically low, this problem can be acute at low protein concentration where a substantial portion of what is usually assumed to be solution-state protein may actually be adsorbed to the container walls. Although protein

Table 2 COP/COC Packaging Systems for Parenteral Delivery

Company	Trade-name/type of cyclic olefin	Delivery system/sizes
Amcor/Alcan Packaging	COC	Vials 2 mL and 5 mL
Aseptic Technologies/Rexam	Crystal®/COC	Closed vials[a] 1–50 mL
Becton Dickinson	Sterifill®/Crystal Clear Polymer	PFS[b] 5–50 mL
Daikyo/West	Daikyo Crystal Zenith®/COP	PFS[b] 0.5–100 mL Vials[a] 0.5–1000 mL
Gerresheimer/Taisei Kako	Clearject®/COP	PFS[b] 1–20 mL
Rexam	MLx/COC, COP	Multilayer vials & bottles
Schott	Schott TopPac®/COC	PFS[b] 0.5–50 mL Vials 2–100 mL

[a]Presterilized vials and containers available
[b]Presterilized formats available
Abbreviations: COP, cyclic olefin polymer; COC, cyclic olefin copolymer; PFS, prefillable syringe.

binding is protein and formulation dependent, studies have shown a trend toward less protein adsorption to cyclic olefin containers. Burke et al. (15) compared glass vials with plastic vials made of polyester, PP and nylon for protein binding. Although no clear conclusion could be drawn on the binding characteristics of these primary packaging materials, it was observed that the degree of binding was highly protein-dependent. Qadry et al. (11) showed less protein binding to plastic CZ vials compared with type I glass, suggesting that the CZ vial is a potential candidate for an alternative material to the glass vial because of low affinity of proteins to bind to its surface. Eu et al. (16) compared the level of adsorption between glass and CZ vials and showed that a model protein preferentially adsorbed to glass vials compared with CZ vials. The authors used gold nano-particle staining techniques for a visual comparison of protein adsorbed to vial surfaces, but this technique does not permit quantitation of the amount of protein adsorbed to the surface. Waxman et al. (17) developed methods to quantitate protein adsorption on vial surfaces. One method uses the protein stain colloidal coomassie, which binds to protein adsorbed to vial surfaces and can be eluted and quantitated spectrophotometrically; the other method involves hydrolyzing the protein adsorbed *in situ* and quantitating the peptides released fluorometrically after reaction with fluorescamine. These approaches allow testing over a much broader range of protein concentrations without the use of radiolabeling. Using these methods, the authors confirmed that binding occurs rapidly and the amount of protein adsorbed per SVP vial is typically in microgram quantities. Protein adsorption to CZ vials was found to be independent of ionic strength, likely because of its hydrophobicity; in contrast adsorption to glass vials was inhibited with increasing ionic strength, indicating the effect of electrostatic interaction with glass containers. In our opinion, protein adsorption is clearly protein dependent, and testing needs to include glass and plastic containers with elastomer influence, before optimizing the drug formulation and container closure system.

Storage and transport at low temperatures: In the area of cell therapy, stem cell research holds significant promise for development of innovative therapies for many unmet or partially met disease treatments. As products enter clinical development stages, there is need for clean, clear, biocompatible, low extractables containers. The ideal vial-based system should be a suitable package to store and transport cell therapy products at lower temperatures; it should be suitable for commercial filling and meet pharmaceutical quality requirements. PP is a plastic resin that has been used for decades for various packaging applications including bottles, pouches, prefilled syringes, tubes and containers. Plastic resins have made minimal headway in the area of parenteral vials because of various quality attributes. A study investigated the use of CZ plastic vials for storing and shipping cell therapy products at low temperature ($-85^{\circ}C$) or cryopreserved ($-196^{\circ}C$) for six months using 0.5, 5.0 and 30 mL volume vials (18). Vials were tested for durability and integrity of a filled vial using a 1-m drop test, and for the ability to maintain viability and functionality of stem cells over the time of storage. No evidence of external damage was found on vial surfaces in the 1-m drop test. Post-thaw viability using dye exclusion assay was $>95\%$ and stored cells exhibited rapid recovery two hours post-thaw. Cultures were $\sim 70\%$ confluent within five to seven days, consistent with nonfrozen controls and indicative of functional recovery. CZ vials were durable and allowed for preservation and maintenance of cell viability and functionality, showing that these vials offer significant benefits to storing and transporting biological and biopharmaceutical products for storage, clinical and commercial applications.

Lyophilization and reconstitution: Cyclic olefin based plastics COC and CZ vials have been extensively studied for packaging lyophilized products. Freeze-drying in a plastic vial brings added advantages, especially when cytotoxic and biohazard products need to be packaged. Crystal technology, developed by Aseptic Technologies, applies the closed-vial technology for lyophilization (19) and for liquid fills. After filling closed vials using a piercing needle, a small disposable device called the penetrator reopens the orifice and, when the lyophilization chamber shelves move, the penetrator is

pushed down, releasing the water vapor. Lyophilization of mannitol and arginine was studied in Daikyo's CZ vials and compared with molded glass and tubing glass vials. The crystallinity of mannitol in CZ vials was either greater or comparable to glass vials. There was thermal homogeneity within the CZ vial during the lyophilization cycle, providing more uniformity within the cake (20). Despite the fact that COC and CZ plastics provide a high moisture vapor barrier, it is always recommended that a secondary packaging barrier such as an aluminum pouch or a blister pouch with aluminum lidding and very low water vapor thermoformable film be used to assure adequate shelf-life protection for lyophilized products. For liquid fills in COC or COP, additional barriers are not necessary because of the low moisture vapor transmission rate of cyclic olefins.

Many drug candidates are marketed in lyophilized form to maintain shelf-life stability and require reconstitution prior to administration. Some of these products, including treatments for hemophilia, multiple sclerosis and autoimmune diseases, may be administered in a home environment. Traditional reconstitution requires the use of multiple vials and needles, which can prove to be complicated for patients or untrained personnel, and may increase chances for needle stick injuries. In recent years, there has been an increasing use of safer and more convenient reconstitution devices made out of plastics. These provide simple methods to reconstitute products without the use of needles and may also improve the effectiveness of the reconstitution process and compliance with the dosing regimen. There are several types of reconstitution systems designed to connect the drug container (typically a vial) to a diluent container (either a vial or a prefilled syringe). Plastic reconstitution devices are sterile, nonpyrogenic, biocompatible and fully supported by appropriate regulatory filings (21). They are designed for short-term contact with the drug product, and can be manufactured from a variety of medical grade plastic materials, such as PC and polyolefins, with the precise material selected on the basis of functional requirements. An example of a plastic reconstitution device, a vial adapter, is shown in Figure 4. For most vial adapters, and other components where a plastic spike is required, PC is used as it provides the appropriate balance of rigidity and sharpness to optimize spiking performance and attachment to the vial. Other materials, such as HDPE, can be used for components within the system where a stopcock system is required. These component devices are packaged in a rigid blister, often made from PET, to maintain sterility and to enable ease of handling and protection of the device during use. Plastic vial adapters can provide safe, easy to use and cost-effective diluent transfer to a lyophilized drug vial. The adapter snaps to the neck of the standard vial after the plastic button has been removed. A plastic spike pierces the stopper; needles are not

Figure 4 Vial adapter. *Source*: From Medimop Medical Projects Ltd.

used. Plastic vial-to-vial transfer systems also offer a similar level of simplicity and cost-effectiveness through a double-spike adapter that connects to the top of each vial (lyophilized drug and diluent). This is an ideal solution for connecting vials of different sizes. These advanced plastic reconstitution systems offer several benefits, including ease of use by patients and caregivers; protection against drug spray-back and accidental needle stick injuries; needleless reconstitution and transfer. They may also help drug manufacturers reduce the amount of overfill in the drug vial (22).

Process considerations: Glass vials are washed, depyrogenated and sterilized by heat before they are filled. Plastic containers cannot be heated to high temperatures for depyrogenation, therefore alternative methods are used. Plastic molding and packaging in environmentally controlled clean rooms usually produce products that have very low bioburden and low particulate level. Nonsterilized vials undergo water-for-injection rinses for depyrogenation, followed by sterilization using autoclave, radiation (gamma or e-beam) or ethylene oxide. All handling operations are designed to avoid scratching the vials' outer surfaces, as plastics have a tendency to scratch. To minimize scratching, care is usually taken not to stack vials too tightly in processing. During autoclave sterilization processing, hazing of the plastic walls is known to occur. This is where moisture gets trapped during processing and may take a few days to diffuse out, but the clarity and integrity of the vial is not compromised. Vial spacing during the autoclave sterilization process may help mitigate this effect. For vials in a ready-to-use format, vendors offer sterile vials and containers. Sterile vials or containers are nonpyrogenic and have a very low particulate level, and could be used to store and transport drug products as early as first-in-human studies. Most commercial filling companies can accommodate filling of COP and COC plastic vials if care is taken to accommodate the characteristics of plastic vials. During filling of plastic vials, guide rails and vial handling change parts should be covered with a material that will limit scratching of the vials. The speed of the filling line may also need to be adjusted to accommodate filling of the lighter plastic vials.

PREFILLABLE SYRINGE SYSTEMS

Market considerations: In the current global market, PFS comprise more than 2.0 billion syringes per year in development and use. The origin of the prefilled syringes' rise as the preferred container was an extremely successful market introduction of syringes for heparins by Sanofi and Rhone Poulene-Rorer (Sanofi-Aventis) in Europe in the early 1980s. The PFS market has now exploded because of several factors: the growth of biopharmaceuticals; the need to eliminate overfills; precision of delivery volume; convenience of delivery, cost-effectiveness; elimination of dosage errors or a combination of these factors (23–26). Glass continues to dominate the PFS markets with a significant market share; however, plastic PFS are beginning to make advances, especially where glass has been unsuitable as a delivery system. PFS have been in use as larger volume containers for x-ray contrast media or medical devices such as hyaluronic acid derivatives (23). In the last decade, however, pharmaceutical drug products have been approved for use with prefillable plastic syringes, including a new chemical entity for oncology and a peptide drug product for the treatment of osteoporosis (Table 3).

Table 3 Global Regulatory Approvals of Drug Products in Cyclic Olefin Polymers/Cyclic Olefin Copolymers

Therapeutic area	Plastic packaging	Approvals
Anemia	Cyclic Olefin	Japan
Osteoporosis/Oncology	CZ vials	United States, Europe, Japan
Antifungal	CZ vial	Japan
Osteoporosis	CZ syringe	Japan
Radiology	CZ syringe	Japan
WFI product (for thrombolytic drug)	TopPac® syringes	Europe

Abbreviation: CZ, Daikyo Crystal Zenith®.

Although not reaching the adoption level of glass syringes, plastics syringe systems continue to gain strong acceptance from pharmaceutical manufacturers because of recent improvements in design, composition and manufacture. Plastic syringes were historically made out of PP, however, recent developments in the area of thermo-elastic polymers, such as cyclic olefins, have made substantial headway in the use of plastics as a PFS system. COP is as clear as glass, has low extractables, is less reactive and has better barrier properties compared with PP. Multiple vendors offer different sizes of syringes in sterile nested configuration or as nonsterile bulk syringes. Cyclic olefin plastic barrels are formed by injection molding under clean conditions and assembled in similar conditions, primarily to maintain a high level of cleanliness. Plastic syringes are sterilized either by autoclave, radiation (gamma or electron beam) or by ethylene oxide, but not by dry heat, and are offered as assembled sterile syringes that are ready for filling. The molding process also provides a greater degree of flexibility to include design features such as a plastic finger grip that can be combined with a back stop to prevent the piston being pulled out of the barrel.

To meet the need for lubricity and sealability, syringe manufacturers use silicone to coat the glass barrels and elastomer components. Silicone facilitates ease of movement of pistons in filling and stoppering equipment, and allows pistons to glide smoothly on activation of syringes. Silicone, however, can interact with drug formulation components (27,28). Recent developments to minimize free silicone include baking silicone at high heat onto the glass barrels, thereby minimizing the amount of free silicone that can interact with drug product. Advances in elastomer closure technologies have produced closures that do not require siliconization because of a special polymer lamination applied to the outer surface of the piston, thereby offering a silicone oil–free PFS system such as the Daikyo CZ syringe system. The syringe system includes a plastic COP barrel, nozzle cap and piston laminated with a fluoro-polymer lamination, Flurotec®, and requires no silicone for consistent functionality. Flurotec is a lamination technology using copolymer film of polyethylene tetrafluoro-ethylene (PTFE) or ethylene tetrafluoroethylene (ETFE). Helvoet (Omniflex® 3G) pistons also have a fluoro-polymer coating, however, these typically are coated with a sprayed-on polyvinylidene fluoride (PVDF) and will need siliconization for use with glass or plastic barrels. Use of these coated stoppers provides lubricity for machinability and reduces piston clumping in feeder bowls. Additional benefits, depending on the coating used, include a decrease in particle generation and a reduction of extractables from the elastomer (27,29).

Improving protein stability: Growth in the pharmaceutical industry is expected to be driven by biotechnology products and vaccines. This will be associated with significant challenges in the formulation development of proteins such as monoclonal antibodies, as they are typically administered in high doses. High-concentration proteins have a propensity to interact with each other and with the packaging components and cause protein instability, especially when the volume of delivery is approximately 1 mL. Challenges with glass PFS typically encompass breakage, presence of particulates, glass reactivity to the drug product and potential leachables including silicone, tungsten and adhesive. A plastic PFS offers options to solve such challenges. A plastic PFS can eliminate silicone, tungsten and adhesive, depending on the quality attributes of the entire prefillable system. For instance, the CZ insert needle system uses no silicone for syringe functionality, no tungsten (commonly used during the glass syringe forming process) and no adhesive (commonly used to hold the staked needle in place).

There are reports that the detachment of silicone oil in water-filled syringes is possible (30) and can result in particulate matter and clouding phenomenon. Silicone oil interaction has been suspected as being responsible for aggregation in protein pharmaceuticals. Several publications in the 1980s have discussed this issue, especially with regard to the aggregation of insulin in disposable siliconized plastic syringes (31–33). Surfactants such as polysorbates have been used extensively to prevent/inhibit protein surface adsorption and aggregation under various processing conditions (34,35). One consequence with using polysorbates in protein preparations

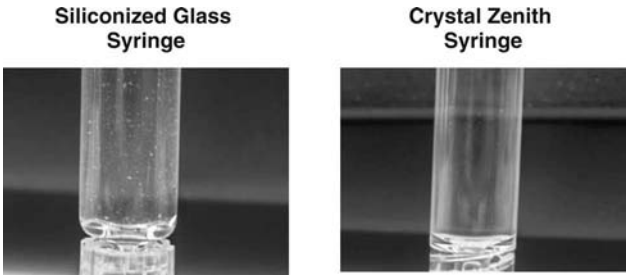

Figure 5 Aggregates in siliconized syringes and silicone-free syringes.

is their potentially adverse effect on protein stability, including the oxidative damage of the residual peroxides in Tweens, which are generated during processing or storage (36). This can pose a serious problem affecting the shelf-life of products. Polysorbates and their concentration should be selected carefully. In addition, the choice of a suitable container will help mitigate significant risks of protein aggregation caused by silicone oil. The propensity of proteins to aggregate when silicone oil is present in formulation was further investigated by Esfandairy et al. (37). Silicone oil–induced aggregation of proteins was studied on silicone oil–free plastic syringe systems and siliconized glass PFS systems. The study included model proteins at low concentrations of 0.35 mg/mL to as high as 25 mg/mL. Although no unambiguous generalization was drawn at lower concentration, there was a clear effect at protein concentrations as high as 25 mg/mL. Effects on protein aggregation with silicone oil were observed during air shipment of samples, caused by effects of agitation and vibration. The study showed that the extent of aggregation in silicone oil–free CZ syringes was less compared with siliconized glass syringes under the conditions examined (Fig. 5). The study recommended that the susceptibility of therapeutic proteins to silicone oil–induced aggregation be investigated on a suitable container closure system before finalizing stabilized formulations and container selection.

Various methods are used to siliconize syringes, including stationary nozzles and diving nozzles. Recent studies have shown that (16) silicone oil distribution is often nonuniform, leaving certain areas of the syringe surface without any silicone oil. The low or inconsistent silicone oil coating can have a significant impact on the piston travel/glide forces, especially in the use of autoinjectors. In 2006, lots of Neulasta® delivered by an autoinjector containing a glass PFS were recalled in a number of European countries because of problems with slow or incomplete delivery of the drug (38). Areas of nonuniformity cause travel forces to increase, causing failure or incomplete injection. In addition, there has been significant attention to tungsten as a leachable present in glass PFS. These reports discuss tungsten-based particulate matter leaching and interacting with the protein drug product (39). Tungsten pins are typically used to keep the fluid path open at the nozzle end of the syringe at around 1200°C during the glass syringe forming process. Upon cooling, a needle is staked-in with adhesive, to make a glass PFS with a staked needle. The residual tungsten had migrated into the drug product and caused the protein to form protein-tungsten aggregates. Although this appears to be protein specific, it is important to test for protein-tungsten interaction at an early stage of drug development. In another case, a residue was observed in a PFS during the manufacturer's inspection. Upon investigation, the material was identified as poly (metaxylylene adipamide), a component of the glass fiber pin use by the syringe developer during the needle assembly and curing process (40). Such concerns may be mitigated with the use of COP/COC syringes. Silicone oil–free CZ syringes have been shown to have consistent travel forces over time and temperature. The dimensional tolerance of plastic syringes and consistency of syringe functionality will provide a predictable operation of a drug product–filled autoinjector. CZ syringe systems have no tungsten as a leachable because the needle is insert-molded, avoiding the need for tungsten pins and adhesive, which

are typically used with glass staked-needle syringes. The manufacturer has developed a PFS system intended for biopharmaceutical drug delivery that is free of silicone oil, tungsten and adhesive (41).

Process considerations: In the current market environment, presterilized, ready-to-fill syringes are increasingly more prevalent. PFS are now available in sterile and ready-to-used formats. As glass PFS are already being filled using tubs, a switch to PFS in a similar tub and nest configuration has been achieved using the same filling machines, with minor modification and change parts to accommodate plastics. Most commercial filling companies can accommodate plastic syringes. The control of dimensional tolerances of plastic syringes far exceeds that of glass syringes and, because they are less prone to breakage and shattering, plastic prefilled syringes are generally easy substitutions for glass PFS on modern filling/processing equipment. There are, however, some physical differences between glass and plastic that should be considered before running plastic PFS on a filling/processing line designed for glass PFS. Plastic syringes are prone to scratches and cosmetic defects from contact with metal surfaces in processing equipment and the weight of plastic PFS is less than their glass equivalents. Scratching may create an unacceptable level of cosmetic defects. Lighter weight syringes can cause problems when gravity is responsible for syringes settling into place in processing equipment. The issues of weight and scratching often manifest themselves when metal centering devices are used to hold and center PFS during filling and stoppering processes. These problems can be overcome by reengineering some parts of filling and processing equipment or by running equipment at slower speeds. It is expected that, as the use of plastic PFS becomes more prevalent, manufacturers of filling/processing equipment will design equipment that performs equally well with both glass and plastic PFS.

There are various processes for filling and stoppering PFS. These include filling and stoppering using vent-tube, or vacuum fill or/and stopper placement. Vent-tube is more commonly used for uncoated or partially coated pistons intended for glass and plastic PFS. For coated pistons, vacuum placement works well as the procedure uses differential pressure rather than force to eliminate wrinkling of the lamination. Vacuum placement is particularly important for laminated pistons, especially in CZ syringe systems, which use a piston that is coated on the drug product contact and syringe barrel contact surfaces. The piston provides lubricity for efficient piston release and consistency of travel forces for a silicone oil–free system. An option offered at Hyaluron Contract Manufacturing, Burlington, Massachusetts, for filling PFS, BUBBLE-FREE FILLING®, uses online vacuum filling and online vacuum stoppering (42). The primary advantage is the reduction of the air bubbles that exist between the product and the stopper in traditionally filled syringes. This may help mitigate concerns regarding oxidation of the product.

LARGE-VOLUME PARENTERALS

LVP refer to sterile diluents, electrolytes, irrigating fluids, blood derivatives, nutritional preparations and premixed injectable drugs administered in quantities of over 100 mL. LVP are packaged in semi-rigid plastic containers, flexible minibags and, to a lesser extent, glass containers. Three major global manufacturers of LVP include Baxter, B. Braun and Hospira. The sterile formulation of LVP necessitates the use of containers with good barrier properties and sizes of semi-rigid plastic IV containers range from 250 mL for biologicals and nutritionals up to 4 L for standard diluents (such as sodium chloride and dextrose).

Minibags are used for administering lower-volume parenteral admixtures, and most premixed IV solutions are packaged in specially designed minibags. IV minibags usually contain 50- or 100-mL volumes of solution and are made of PETG, PP and various polyethylene-based coextrusion. These containers provide a sterile format consisting of a drug mixed with an appropriate diluent solution. Premixed minibags eliminate the need for independent admixture preparation and provide significant time, labor saving and waste

reduction advantages. Most major parenteral drugs are now available in this format, including drugs for antibiotic, analgesic, anticonvulsant, cardiovascular, psychotherapeutic and respiratory preparations. Some solutions packaged in the container must be stored frozen and thawed no more than 24 hours prior to use.

Historically, PVC was the leading material employed in the production of LVP configurations. However, this trend has changed because of potentially adverse patient reactions to a plasticizer used to stabilize the resin. Known as DEHP, the plasticizer has been linked to infertility and hormonal imbalances in laboratory animals. Regulatory authorities have recommended that all medical products based on PVC and DEHP be either adapted to alternative materials or include a label warning about the plasticizer. In response, the producers of IV solutions have adopted newer plastics for their containers. B. Braun Medical eliminated the used of PVC in IV packaging. The company's Excel® and PAB® IV containers now include specialized PP materials. Newer, higher-grade plastics, such as PETG copolyester, are being used for minibag applications to keep solutions stable, including Baxter's and Hospira's products. Baxter International recently introduced Buminate® human albumin solution in a Galaxy® minibag that is composed of proprietary, high-barrier plastic film. The new Galaxy package can provide a shelf-life of two years and eliminates the need for preparing admixtures in hospital pharmacies. Hospira's ADD-Vantage® system is a specially designed diluent container that connects to a vial. Once the vial is affixed to the container, the active drug blends with the diluent and creates the finished IV solution. The ADD-Vantage system allows the IV solution to be mixed directly at the site of administration. Another innovative IV minibag system is the Duplex® Drug Delivery System developed by B. Braun. Duplex is a dual-compartment flexible plastic IV bag that stores unit dosages of drug powder and diluent separately in the same container. The health care professional squeezes the bag to break the quick-release seal, mixing the drug and diluent just prior to administration. Designed to simplify the intravenous delivery of antibiotics, the Duplex container reduces product waste, eliminates the use of vials from the preparatory process, and is equipped with a standard linear bar code to reduce dosage errors and track inventory.

X-ray contrast media is also packaged in a range of volumes from 50 to 500 mL in both plastic and glass containers, with the 500 mL containers labeled as pharmacy bulk packages. PP prefilled syringes and prefilled PP cartridges designed to fit a specific range of power injectors for computed tomography are available (43). Another design of a prefilled cartridge called REDIFLOW™ is available in a clear plastic to fit a second range of power injectors for computed tomography as well as PP bottles (44).

PLASTICS AS DISPOSABLE SYSTEMS FOR BIOPROCESSING

Market considerations: Plastic packaging systems for LVP drugs are facing increasing scrutiny. All packaging systems, stainless steel or plastic, need to provide and meet the same requirement for protection, compatibility and safety as those used for SVP (45). This section addresses the use of plastics as disposable bags in packaging large-volume drug substances or drug products in bioprocess development and fill/finish operations (46,47).

According to the report released by the Tufts Center for the Study of Drug Development, Outlook 2009 (48), there are more than 200 new monoclonal antibodies in development worldwide, and the FDA has approved 22 monoclonal antibodies. To support development of these biologics, the biopharmaceutical manufacturing industry is rapidly adapting to disposable systems. Single-use bioprocess systems referred to as disposables have become common in the industry. Disposable systems have gained increased acceptance for manufacturing-scale storage and processing of recombinant proteins and monoclonal antibodies in liquid and frozen forms (49,50). This is driven primarily by the key benefits plastic disposable containers offer over stainless steel containers. These include reduced capital expenses (stainless steel vessels, cleaning and sterilization validations), minimizing cross contamination, flexibility in manufacturing and easier scale up (51,52).

Disposable technology employs a multitude of plastics to customize processing and may include bags, filters and tubing. Plastic materials that make up the critical

Table 4 Commonly Used Plastics in Disposable Systems

Disposable bags	Polyethylene, ethylvinyl acetate, PVDF
Filters	PTFE, polypropylene, PVDF
Tubing	Silicone, PTFE, PVDF

Abbreviations: PVDF, polyvinylidene fluoride; PTFE, polyethylene tetrafluoroethylene.

components of a disposable system include filters (e.g., Millipore, Sartorius, Pall, GE Healthcare), tubing (e.g., Amesil, Saint Gobain), and disposable bags (e.g., Hyclone, Stedim, TCTech, Pall). Disposable bags are larger volume containers that are used for large volumes of drug substances or products and have the greatest dwell time of product exposure. These bags are used in upstream and downstream bioprocessing and in fill/finish operations, examples include media preparation, bioreactor, storage and transportation. Multilayer bags are typically used and are intended to maintain product integrity. These bags provide gas and moisture barrier properties, functionality after sterilization, durability and biocompatibility (Table 4). Very few materials possess a balance of properties in one layer and PVDF film may be the best solution (47). The outer layer of a multilayer bag provides durability, and many materials are used with varying thickness. These materials are made up of nylon, polyesters, ethylvinyl acetate (EVA) and polyethylenes. As a sandwich layer, ethyvinyl alcohol (EVOH) is commonly used. EVOH has extremely low gas permeation and excellent barrier characteristics. Because it has a propensity to absorb moisture and lose its barrier property, it is sandwiched in a multilayer bag. LDPE is commonly used as the drug contact layer because of its good chemical compatibility profile. While EVA films are typically considered superior as the product contact layer, there are limitations to large-scale manufacturing of EVA film, and, consequently, LDPE becomes a good alternative, especially with three-dimensional bags such as those used in disposable mixing applications.

Many factors are usually considered during the design phase when choosing a disposable bag. Two important questions to be addressed are: Is the plastic polymer safe and is it compatible with the solution it is in contact with? Several facets related to the qualification and selection of a disposable container must be considered to address these questions. This includes a validation package from the vendor with information related to the materials of construction, sterility, USP plastic class VI data, extractables, heavy metals, particulates, pyrogens and cytotoxicity testing from the vendor. This information—in combination with knowledge of the drug substance or drug product processing that may include processing volumes, chemical stability, compatibility, number of campaigns, formulation components, processing conditions such as temperature, pressure and, most importantly, extractable and leachable considerations—can provide insights into the choice of disposable bag for bioprocessing. The primary considerations should include:

Chemical resistance study: Chemical compatibility studies should be conducted to evaluate the choice of a single-use container prior to its selection. The tests can include weight loss, clarity, visual inspection, drop test, tensile strength, thickness of the film and testing using various solvent systems including buffers, organic solvents or other components that may be intended for drug product development. For most aqueous formulations, the plastics (e.g., LDPE, HDPE, PP, etc.) have an acceptable compatibility profile. However, organic solvent usage may cause incompatibility issues. Emerging disposable systems bags such as PVDF, which has a chemical compatibility profile similar to Teflon®, may offer options for accommodating formulations based on organic solvents.

Protein adsorption: Single-use systems are increasingly prevalent in downstream processing, final formulation development and in fill/finish of protein solutions. These systems gained acceptance for storage and processing at manufacturing scale of recombinant proteins and monoclonal antibodies in liquid or frozen forms. The

container-protein interactions may include protein adsorption onto the plastic container surfaces. The major driving forces influencing adsorption of protein are hydrophobic and electrostatic interactions. These interactions are responsible for nonspecific protein binding on a variety of surfaces. Interaction factors between plastic surface and protein could be affected by the physical nature of the surface (material surface or any coating), product formulation (pH, ionic strength, surfactant, etc.), storage conditions (temperature and contact time) and the concentration and conformational properties of the protein. Studies have shown a low binding level of model proteins on plastic polymeric surfaces compared with borosilicate glass surfaces. It is important to evaluate plastics using specific protein binding assays under various processing conditions, using large surface-to-volume ratios to determine their acceptability (53).

Extractables and leachables: The release of compounds from the plastic may affect product quality such as plasticizers, stabilizers or solvents. Regulations mandate that the equipment and materials used in the manufacture of pharmaceuticals should not alter the safety, efficacy and potency of the final drug product. An evaluation of potential extractables is required for plastic disposable bags to ensure compliance. Extractables are substances that can be extracted from a plastic using solvent or extraction conditions that are expected to be more aggressive than the processing conditions intended. Leachables are substances that could be present in the finished product because of interactions between plastics and the drug product during the products shelf-life. The suppliers of the plastic bags or components should provide a full and complete potential extractables list which could be used to evaluate product suitability with the plastic disposable bag (54).

Sterile barrier integrity: Maintaining integrity of a disposable device is critical to protect the product from microbial contamination. When plastic bags or components are provided as sterile, the integrity of these products must be demonstrated. Container closure validation can be performed to reduce the risk of compromise. These tests may include helium leak testing, pressure testing, dye ingress or microbial ingress challenges. Guidance documents from the FDA and European Medicines Agency (EMEA) can help to define the level of validation and qualification necessary for the safety of the single-use systems. These include the FDA's guidance document issued in May 1999, "Container-Closure Systems for Packaging Human Drugs and Biologics" (45) and EMEA's guidelines on plastic primary packaging materials (55).

QUALITY AND REGULATORY CONSIDERATIONS

There are numerous plastic containers that have been used for parenteral applications, including drug products in cyclic olefin containers that have been approved for marketing in the United States, Europe and Japan (Table 3). Guidance documents from FDA and EMEA help define requirements and the level of validation and qualification needed. This guidance has been universal to encompass all plastic containers for SVP or LVP, including vials, PFS or flexible bags. The FDA document "Container Closure Systems for Packaging Human Drugs and Biologics" provides the fundamental guidance on container closure systems, including plastic materials (45). The United States has a drug master file system (DMF) in which companies provide confidential information on the manufacturing and the composition of the plastic in a type III packaging material DMF and is incorporated into a letter of authorization for referencing the DMF upon FDA review. Canada has a similar DMF system, except that packaging materials are listed in a type II DMF. In Europe, the EMEA limits the information contained in a DMF to drug substances; therefore, the drug manufacturer will usually provide the required information on the packaging system. Guidelines for plastic containers can be found in the newly revised EMEA's Guideline on Plastic Primary Packaging (55). Both Ph.Eur. and USP have chapters referencing plastic materials and plastic packaging. Ph.Eur. section 3.1 has detailed chapters on various plastics including "polyolefins," and Ph.Eur. 3.2 specifically focuses on plastic containers (56,57). USP combines guidelines for plastic containers and plastic materials in chapter <661> (58). With respect to biocompatibility, both in vitro and in vivo

biological reactivity needs to be performed on plastic containers (59,60). The quality-conscious Japanese market has seen the plastic market grow significantly for SVP. Mitigation or elimination of particulates or defects, safety, break resistance and clarity are clearly the drivers for using plastics in Japan. Key JP guidance is described under General Tests Processes and Apparatus, 7.02 Test Methods for Plastic Containers and General Information 17, Plastic Containers for Pharmaceutical Products (61).

SUMMARY

Application of plastics for parenteral delivery is expected to grow in years to come. Although PP material is more commonly used because of its availability and cost-effectiveness, there has been a recent surge in the use of superior plastics, the cyclic olefins, for parenteral delivery. The features of cyclic olefins are seen very favorable when packaging SVPs, highlighted by properties such as break resistance, glass-like transparency, better barrier properties compared with other plastics and its biocompatibility. However these features need to be balanced with the needs of a drug product, especially in the areas of oxygen or moisture sensitivities, where secondary packaging may help reduce such risks. Plastics are also favored because of their moldability and tight dimensional tolerance and can lead to newer design integrations. Examples include front finger grips, larger flanges and back stops for syringes. This capability is especially important because the home health care market is a growing segment. Many drug products are produced with the intention of being used in a home setting. Material flexibility also allows the same resin to be used in an assortment of designs, from vials through PFS systems, without substantial chemistry differences. Recently cyclic olefin syringes have become available in sterile assembled formats for ease of filling, similar to that of glass syringe packaging, making it easier for drug manufacturers to switch to plastics. Similarly sterile and nonsterile plastic vials and containers are also available.

Plastic container systems can also play a significant role in influencing the stability of a drug product. For example, they are used with drug products that would otherwise delaminate glass or with water-for-injection products to maintain pH. Recent advances in plastic PFS systems include developments in silicone-oil free and tungsten-free syringe systems that can help mitigate or eliminate any potential interaction of leachables from a packaging system. Formulators and package engineers now have more options to evaluate and optimize drug formulation with suitable packaging components at early stages of drug development. Protecting the drug product in a package that does not break or crack is a substantial benefit, especially with biological products that need low temperature storage and transport. Plastic vials are now considered in these areas. In addition, availability of plastic cartridges and plastic dual chambered syringe systems for liquid-liquid or lyophilized powder-liquid systems clearly illustrates the ability of vendors to offer such designs for various drug delivery applications. For large-volume packaging and processing of bulk drug products, plastic disposable bags are being considered. Clearly plastic disposable bags offer many benefits over stainless steel containers in downstream bioprocessing, including fill/finish operations; however, due diligence is a must for the right choice of plastic for the product. Plastics will increasingly be utilized throughout the entire total supply chain of pharmaceuticals and provide opportunities for total life cycle containment of pharmaceutical products. These opportunities can allow for the lowest total cost of ownership to be provided with plastic packaging materials.

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13 Elastomeric closures for parenterals

Renaud Janssen

SUMMARY

The present chapter in this review work intends to give insight into elastomeric closures that are used for parenterals. The single most important reason why elastomeric materials are used for closures for parenterals is that the elasticity of such materials allows for preservation of the sterility of the packaged drug, by ensuring a tight seal between the closure and the container, and by ensuring adequate resealing of the closure after penetration with a needle or with a spike in cases where this is applicable.

Of course sealing and resealing are not the only features that characterize elastomeric materials. Elastomeric closures also have benefits in that they are able to give a property profile that is an ideal combination of physical, chemical, functional and biological performance, combined with microbiological and particulate cleanliness.

This chapter is an endeavor to give the reader insight into this complex system of properties and requirements.

THE MANUFACTURING PROCESS FOR ELASTOMERIC CLOSURES

The text below describes the operation of a typical modern elastomeric closure manufacturing plant. In any such plant, irrespective of the name of the company, the major steps in pharmaceutical rubber stopper manufacturing will consist of weighing according to a recipe, mixing, preforming, molding, die-trimming, washing, drying and packing (Fig. 1).

Raw Materials

The basis for the manufacturing of rubber closures is a so-called rubber compound. It is composed of a number of raw materials.

Raw materials are quarantined upon receipt and there is a system in place for testing of raw materials for identity and purity according to specific procedures and specifications.

Upon acceptance by the control laboratory, raw materials are released for production and a raw material lot number is assigned. All relevant data are stored in a computerized raw material lot file. There are provisions in the manufacturer's quality system to protect against inadvertent use of nonreleased raw material lots.

Mixing and Preforming

Individual rubber compound batches are composed by combining the required amounts of each rubber ingredient in accordance with a formulation sheet ("recipe"). The ingredient's weight accuracy and lot numbers are stored in the compound batch file. Each weighed quantity is duly identified.

Weighing of the ingredients and composition of the individual compound batches take place in specially equipped rooms, designed for cleanliness and logical material flow. Large-volume ingredients such as fillers may be stored in silos in which case they are automatically weighed and delivered directly to the mixer, thus largely reducing the potential for dust and contributing to cleanliness of the manufacturing environment.

The compound ingredients are mixed in a Banbury type mixer. A Banbury type of mixer consists of an extremely robust mechanical chamber in which the rubber ingredients are mixed by the action of cooled cylindrical rolls that rotate into each other. Prior to introducing the ingredients into the mixer, their identity is verified.

The mixing process is highly automated and entirely computer controlled, as it functions according to a predetermined "mixing recipe." The mixer parameters that are important for the quality and the properties of the mixed material typically are constantly monitored and recorded.

At the end of the mixing cycle, the rubber compound batch is transferred onto an open mill where it is cooled and further homogenized. Next the rubber compound batches are

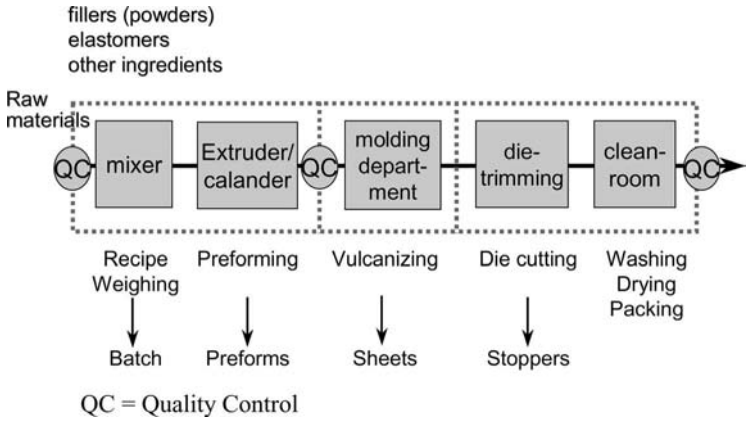


Figure 1 Elastomeric closure manufacturing process.

shaped into "preforms" with the size and weight required for molding in a particular mold. The preforming operation may have different forms. It may consist of passing the mixed and milled rubber through an extruder and cutting the extrudate into bricks of a well-defined form and weight. Alternatively it may consist of a calandering operation where the rubber coming from the calendar is cut into slabs that again have a well-defined shape and weight. At the stage of mixing or preforming typically a sample of each compound batch is checked for correct vulcanization properties by means of a rheometer test. Furthermore, a sample is sent to the laboratory for testing of physical and chemical properties. All data are stored in the compound batch file and are fully traceable.

Molding

Both injection and compression technologies may be used for molding rubber closures. The choice depends on the technical requirements and characteristics of the products.

The rubber preforms are heated under high pressure in multicavity molds. During this process the rubber vulcanizes. In the vulcanization process, by the use of cross-linking agents that are contained in the rubber compound, chemical bonds are formed between individual polymer molecules that form the elastomeric base of the rubber. It is only at the stage of molding that the rubber turns from a plastic into an elastic material, and that it acquires its required shape in the form of a vial stopper, of a plunger for a cartridge or a prefilled syringe, or of any other geometrical form that is intended to shape the rubber in.

The products leave the molds in the form of "sheets," each carrying many closures. The operators performing the molding operation typically examine the quality of the molded sheets at this stage, which marks the first quality check of the elastomeric components.

The use of modern, proprietary compression and injection molding technology, combined with proprietary mold construction technology, results in rubber closures with narrow tolerances and stable nominal dimensions.

Die-Trimming

The sheets with the products are then die-trimmed to result in individualized stoppers. This operation may take place in the immediate vicinity of the molding press or in a separate area that is designed for higher cleanliness. Die-trimming of elastomeric closures requires a trimming agent, which is typically a silicone emulsion, that is then removed by rinsing the freshly die-trimmed stoppers or, in case this is not present, in the next manufacturing step, which is washing.

Washing Process for Elastomeric Closures

The die-trimmed closures are transferred to the washing and posttreatment area. At present time rubber closures for parenteral applications are always washed, regardless of the closure manufacturer.

Washing of rubber closures typically is combined with siliconization. Siliconization of rubber closures is necessary to overcome the stickiness that is inherent to typical rubber formulations that are used for parenteral stoppers. Washing is performed to improve the state of microbiological and particulate cleanliness of the stoppers. Washing and siliconization may take place in washing equipment of various types. Very often, rotating drum type equipment is used for washing, siliconization and drying. However, the state of the art practice is that closures are washed in a pass-through machine. Loading of the closures takes place at the "dirty" side of the machine, while unloading is foreseen at the "clean" side in a room with a controlled state of cleanliness.

Various procedures exist for washing of parenteral closures. Every stopper manufacturer has its own process. More on stopper washing and siliconization can be found in a later paragraph of this chapter. At any rate washing is followed by drying with air of controlled cleanliness.

Packaging

After drying, the rubber closures are immediately packed in clean polyethylene (PE) bags, and sent out of the washing area into the packaging area where the bags are put into cardboard or plastic boxes. The plastic bags and the boxes are labeled with identification data such as product and compound code, lot number, packaging date and information on the final treatment.

In case the closures are manufactured "ready for sterilization" or "ready to use," packing takes place in dedicated functional ready-for-sterilization (RfS) or ready-to-use (RtU) bags. RfS and RtU bags are overwrapped with protective plastic bags before putting them into the cardboard or plastic boxes.

Classification of Manufacturing Environment and Environmental Controls

The manufacturing of rubber still to a large extent is an industrial process, especially in the first steps of mixing and to a lesser extent in molding. Throughout the manufacturing process it is usual that the closure manufacturer progressively implements measures to work in cleaner areas and to protect the products or intermediates from contact with the environment, including the manufacturing personnel.

In practice this comes down to implementing systematic cleaning programs in all areas, sound gowning procedures for operators, for their supervisory personnel and for plant visitors, and appropriate measures to protect products from environmental contamination. In the initial manufacturing steps of mixing, molding, and die-trimming it is not common that a closure manufacturer will classify the manufacturing areas. Exceptions to this are for new plants that are built from scratch. For washing and packaging areas, though, it is common that these areas are classified.

Classification may be done in various ways. Whereas in the past it was most common to speak of class 100 or class 1000 or class 10,000 or . . . in terms of the U.S. Federal Standard 209, today classification is mostly done in terms of International Standardization Organisation (ISO) 14644-1, "Cleanrooms and associated controlled environments—Part 1: Classification of air cleanliness" and/or in terms of the FDA Guidance for Industry, "Sterile Drug Products Produced by Aseptic Processing—Current Good Manufacturing Practice" or the EU Guidelines to Good Manufacturing Practice, Annex 1, "Manufacture of Sterile Medicinal Products" Grade A/B/C/D classification. It may be noteworthy to verify whether a manufacturer claims a classification for his manufacturing areas "at rest" or "in operation."

Classification of manufacturing areas needs to go hand in hand with the implementation of a monitoring system to demonstrate not only initial compliance but also continuous compliance. This system to demonstrate continuous compliance is then based on a sound rationale for measuring nonviable and viable airborne particulates, complemented by measurements of surface microbiological cleanliness, and in the highest degree of sophistication also of contamination of personnel gowning. Since in the final washing of closures for parenteral application, modern standards require that water of defined purity such as purified water and water for injection is used, also monitoring of the compliance of the various water types will need to be part of the manufacturer's total monitoring system.

PARENTERAL CLOSURE TYPES AND DESIGNS

The present part of this chapter gives an overview of the most important and common types and designs of closures that are used as *primary* packaging in parenteral applications. No attempt is made to review components that are used as secondary packaging such as aluminum or aluminum/plastic crimp caps. Since some closure *designs* may be proprietary to the closure manufacturer or end-user, it is impossible to put together an exhaustive listing here. This will not preclude though that the overview below is as complete as possible pertaining to closure *types*.

Stoppers for Vials and Bottles

Closures for Serum Vials

These closures are the rubber stoppers that are used for closing glass or plastic vials or bottles stemming from liquid or dry powder fills (Fig. 2).

These closures consist of a flange having a larger diameter and a plug part having a smaller diameter. The plug part fits into the vial neck while the flange part rests on the rim of the vial.

Closures in this category are usually subdivided by their size. These subdivisions include 13-mm stoppers and 20-mm stoppers for small-volume parenterals (SVP), and 28-, 29-, and 32-mm stoppers for large-volume parenterals (LVP). These sizes do not correspond with any diameter of the closure itself, however they indicate the largest diameter of the vial neck. For example, a 20-mm stopper is used for closing a vial with 20 mm as the outer diameter of the vial neck, while the flange diameter of the stopper typically is between 18.8 and 19.1 mm.

Stoppers in this category have two further features.

1. On the top of the flange there is an antistick marking. Rubber always has a tendency to stick, especially the type of rubbers that most parenteral stoppers are made of. The purpose of the antistick marking is to prevent the two large flat flange surfaces of two different stoppers from sticking together during storage of the stoppers, during steam sterilization and during filling operations at the pharmaceutical company. A well-studied design of antistick markings greatly helps in preventing clumping of stoppers in all these stages.

   The antistick markings also often delineate the target area of the stopper, that is, the area that is intended to be pierced with a needle or a spike.
2. The presence or absence of a constriction just underneath the flange. This constriction is called "blowback." Its role is to fit into a corresponding protruding part of the inner rim of the vial mouth so as to prevent the stopper after placement from rising and popping out of the vial neck. In this respect one also speaks of a "no-pop" feature or a no-pop stopper.

Significant dimensions of this type of stopper to consider are as follows:

1. Flange diameter: obviously this diameter has to be compatible with the outer diameter of the vial neck.
2. Plug diameter: obviously it has to adequately match the inner diameter of the vial neck and in forthcoming case its blowback.

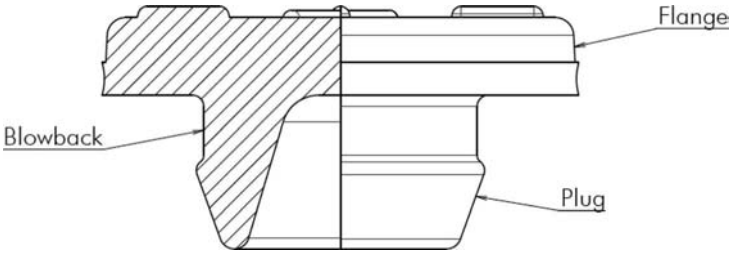


Figure 2 Section of a serum stopper with blowback.

3. Flange thickness: this dimension may be of primary importance for machineability of the stoppers on filling lines. Flange thickness should be well controlled by the stopper manufacturer.
4. Total stopper height: depending on the filling line this dimension can play an important role in stopper machineability.
5. Penetration thickness (the thickness of the stopper in the penetration area): this thickness is one of the contributing factors in determining the coring, the resealing and the penetration behavior of the stopper. Additionally, this thickness after capping of the vial determines the permeability to gases of the stopper/vial/cap combination. Given a certain rubber material, higher penetration thicknesses can lead to higher resistance to permeation of air and moisture into the vial and thus into the drug.

All these dimensions are expressed as nominal values and respective tolerances. Both are partly normalized in ISO standards such as ISO 8362-2 (closures for injection vials) and ISO 8536-2 (closures for infusion bottles).

For design purposes it is necessary to understand that tolerances of rubber parts cannot be as tight as for plastic parts. Dimensions on rubber parts as per ISO 3302-1 can be subdivided into dimensions that are determined by the rubber mold and dimensions that are determined by the rubber molding process. The former ones are tighter than the process related dimensional tolerances, however they are still larger in comparison with what is usual for plastics. With respect to serum stoppers, diameters are mold related, while dimensions such as flange thickness, penetration thickness and total height are process related.

A frequently asked question is where the effective seal between stopper and vial takes place, or which matching surfaces of stopper and vial are responsible for container/closure integrity.

For capped vials, or where under the influence of a crimp cap the underside of the stopper flange exerts a force on the top of the rim of the vial neck, it is this interface (underside flange / top of vial neck) that constitutes the seal. The permanent seal thus is not formed by the sidewall of the stopper plug pressing into the inner diameter of the vial neck. Such a seal can only be effective until the moment the vial is crimped. More on this can be found in the separate chapter of this book on container-closure integrity.

Freeze-Drying Closures

Obviously these closures are not used in powder or liquid fills but in lyophilization, or freeze-dry, applications. In the lyophilization process, the drug in its liquid state is filled into the vials. The freeze-drying closure is put on the vial in a halfway down position, so that there is a vent opening between the inside of the vial and the area around the vial. Through this opening, sublimation of the liquid takes place under the influence of underpressure in the lyophilization chamber and heat that is transmitted by the plates of the freeze-dryer. At the end of the lyophilization cycle the stoppers then are fully pressed down into the vials by the shelves of the freeze-dryer (Figs. 3 and 4).

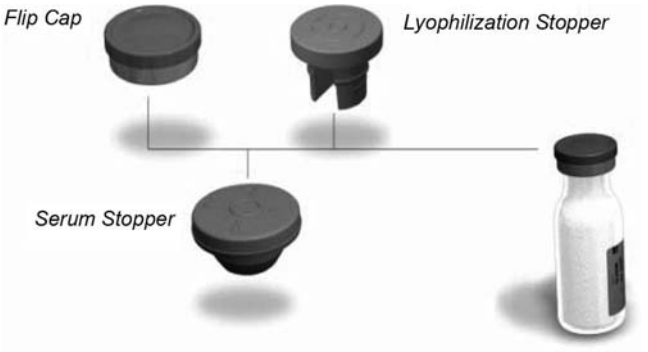


Figure 3 Various closures for serum and for freeze-drying vials.

Figure 4 Lyophilization vials and their stoppers. The vial on the right hand side has a stopper in its halfway down position before freeze-drying; the vial on the left has its stopper fully pressed down after freeze-drying. In front of the vials are stoppers showing their lyophilization opening.

Lyophilization stoppers need to be stable in the halfway down position, to allow for proper mass transfer (sublimation!), and to prevent falling off the vials during the transport between the liquid fill station and the lyophilization chamber.

The dimensions of the closure plug, including diameter and height of the zone underneath the flange to the vent opening, must provide enough surface area to contact the vial in such a way that seal integrity is not jeopardized, from the time between unloading of the freeze-drying vials from the lyophilization chamber to the moment of crimping the vials. In practice several hours may develop between these two time points. However, if the closure dimensions are too large, then interference during initial insertion and during full insertion of the lyophilization closure may pose a problem.

Antistick markings in general are designed as part of the closure to prevent sticking/mating of stoppers during bulk transportation and within feeding lines. Another primary function of these markings with respect to lyophilization closures is to prevent closure adhesion to lyophilizer shelves upon full insertion of the stoppers. If stoppers at this stage adhere to the shelves, vials containing the freeze-dried product remain stuck to the shelves when they retract after pushing down the stoppers. This leads to undesired problems when the freeze-dryer is unloaded and to unacceptable product loss (Fig. 5).

In view of the moisture sensitivity of many freeze-dried drugs, it is clear that for lyophilization closures, penetration thickness and good control of it is of even higher importance than for serum closures.

Like serum stoppers, freeze-drying stoppers can be subdivided by their size. Most commonly found are 13- and 20-mm stoppers. Standards on freeze-drying closure design can be retrieved and ISO 8536-6 (infusion stoppers for freeze-drying) and ISO 8362-6 (infusion stoppers). Notwithstanding these standards, the market offers freeze-drying closures in a broad variety of designs, especially with respect to the design of the plug part. Each of these designs ("igloo design," "two-leg design," "three-leg design," etc.) has specific benefits in areas such as stopper stability, behavior upon reconstitution of the vial contents, and ease of withdrawal of the reconstituted from the vial (Fig. 6).

Components for Prefillable Syringes and for Cartridges

More and more drugs are packaged in prefillable syringes or cartridges, in addition to or instead of a vial presentation. Prefillable syringes are claimed to have distinct advantages over vials, including ease of use, dose accuracy and minimization of product loss in the emptied packaging.

The market offers many different presentations of prefillable syringes and it is impossible to list them all here. They consist of a series of components of various natures, but at a minimum have a barrel in glass or plastic, plus (at least) two different elastomeric sealing components.

Figure 5 On the left, a picture of a pilot scale lyophilization chamber. Vials are placed on the shelves. The shelves can move so that they can bring the stoppers from their halfway down into their fully pressed down position. On the right, a picture of a shelf with vials after unsuccessful insertion of the stoppers. Stoppers got stuck to the shelf that pressed them down!

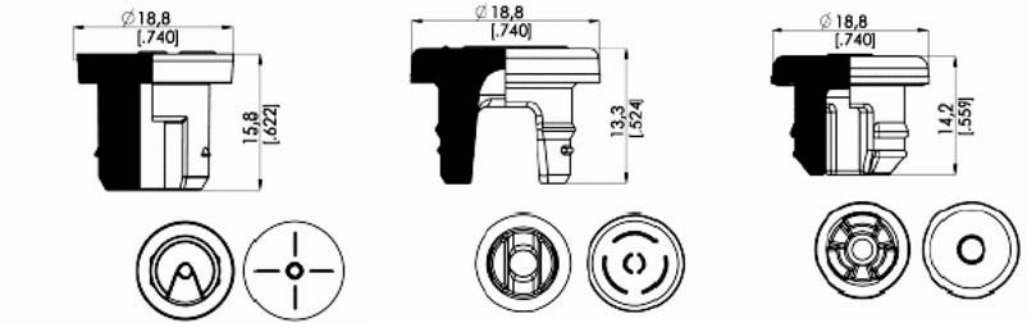


Figure 6 20-mm lyophilization stoppers in various product designs.

- An "internal" component that makes a seal on the internal diameter of the barrel. This component most commonly is called a "rubber plunger," sometimes also a "plunger stopper." After filling of the syringe this plunger is in long-term "intimate" contact with the drug, just as the cavity of the stopper plug is in case of a vial application. During the drug shelf life the plunger must maintain an adequate seal on the inner side of the barrel. However, at the time of administration of the drug to the patient, the plunger also must exhibit efficient gliding behavior in the barrel to adequately transfer the syringe contents into the patient.
- An "external" component that makes a seal between the inside of the syringe and the outer world. Basically the syringe is delivered with either a needle already being present ("staked needle") or with a prevision to place a needle at the time of administration. In the first case the needle will be protected by a rubber needle shield, also called "cover" or "sheath." The tip of the preassembled needle will stick into rubber at the interior of the needle shield, while the opening of the needle shield forms a seal on the tip of the syringe.
- In syringes without staked needle, the latter function is taken over by another rubber component, called "tip cap." The inside of the tip cap takes care of forming a seal on the tip of the syringe.

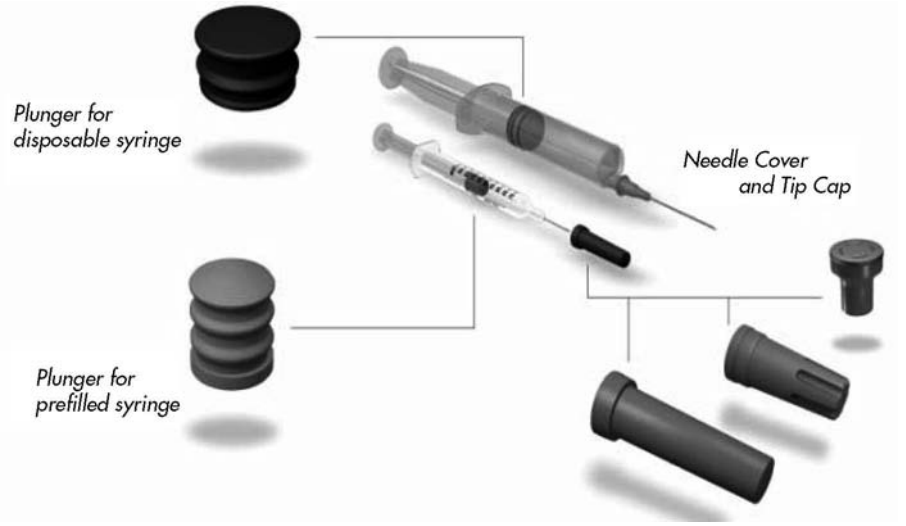


Figure 7 Elastomeric components for prefillable and for disposable syringes. Plungers on the left, needle shield and tip caps on the right.

- Even if the contact area between the syringe contents and the external rubber component may not be claimed to be zero, it is clear that this contact is less "intimate" in comparison with the contact the elastomeric plunger has (Fig. 7).

Whereas needle shields and tip caps in the past were found as components made purely out of rubber, today's tendency is to put these items into a plastic cover and, assembled in this way, to mount them onto the syringe barrel. In this case the market speaks of "rigid needle shields" and "rigid tip caps." Rigid needle shields and rigid tip caps offer or can be designed to offer enhanced product features, including tamper evidence for the syringe and extra protection against needle-stick at the time of drug administration.

Plungers for prefillable syringes are standardized by ISO 11040-5. At the time of writing there is no standard for elastomeric needle covers or tip caps.

Another prominent tendency at this time is to package drugs in cartridges. These cartridges may be intended to be used in self-administration devices, like insulin pens or growth hormone pens, or may be intended for administration by medical staff. The most well-known example in this class is a cartridge with a dental anesthetic. Like prefillable syringes, cartridges are equipped with rubber plungers. However, the second sealing element most frequently consists of a rubber disk being assembled in an aluminum cap. The cap with assembled elastomeric liner is crimped onto the front end of the cartridge. In this case, two rubber components (plunger and disk) are in long-term contact with the drug. At the time of administration the disk is perforated by a double-ended needle, one end making contact with the cartridge contents and the other end being the patient end (Fig. 8).

Typical for nondental applications, such as insulin and growth hormone cartridges, is that the cartridge contains multiple drug dosages. After administration of each dose, the rubber disk must adequately reseal so as to preserve drug sterility, and at every next dose the plunger must again smoothly move over a small distance.

Information on standardization of plungers for dental cartridges and plungers for pen systems can be found in ISO 11040-2 and ISO 13926-2, respectively.

Components for Disposable Syringes

Apart from prefillable syringes and cartridges a very large amount of rubber plungers, sometimes also called "gaskets," are used in disposable syringes that are used to administer parenteral products to patients.

Figure 8 Dental cartridge components.

A similarity between a disposable and a prefillable syringe is that in both cases the plunger must be able to move smoothly, with a well-controlled force to start the movement and with a well-controlled force to sustain the movement as long as this is needed. A very important difference between the plungers in prefillable and in disposable syringes however is the contact time with the drug. For a prefillable syringe this time is expressed in years, whereas for a disposable syringe plunger it will be minutes or hours. This difference has a large impact on the type of material that the plunger is made of. A prefillable syringe plunger will be designed to ensure adequate gliding behavior as well as to aim for low levels of material that could be extracted from the rubber into the drug product as a leachable, while disposable syringe plungers will be designed primarily to ensure acceptable administration behavior.

Plungers for disposable syringes are standardized to some extent by ISO 7886-1.

Other Components

There are many other elastomeric components used in parenteral products, other than the ones listed so far. Among the products that are in long-term contact with parenteral drugs it is worth mentioning here parts that are used in special systems such as dual chamber syringes or vials with two compartments. In the category of short-term contact products certainly components for injection ports on flexible bags and parts used in blow-fill-seal applications take a large part.

RUBBER COMPOUNDS FOR APPLICATION IN CLOSURES FOR PARENTERALS

This part of the chapter contains information on the composition of elastomeric closures for parenterals and explains which rubber compounds are suitable in the various applications.

General Outline

The main characteristic of an elastomeric material is its elasticity. Elasticity is introduced by cross-linking the polymer chains of the elastomer base of the material by using cross-linking agents. This cross-linking process, also called "vulcanization" or "curing," uses curing agents that make chemical bonds between polymer molecules. The vulcanization takes place under the influence of temperature and pressure in a heated mold. During the vulcanization the rubber will adopt the shape of the cavities of the mold in which it is being cured. In this sense one speaks of "thermoset" rubbers.

Before vulcanization, the elastomer behaves in a plastic way, as mechanical deformation will result in a permanent deformation. By cross-linking, the elastomer turns into a rubber. After vulcanization the resulting rubber material behaves in an elastic way, and as such after imposing and taking away a mechanical deformation the material will regain its original shape.

The total set of materials that are used in rubber compounds can be listed as follows:

- *The elastomer*: It is the polymer base of the compound. A rubber compound may either use one single elastomer or a blend of different elastomers. The type of polymer(s) will heavily influence a number of characteristics of the resulting rubber.

- *The cure system*: It consists of a defined set of chemicals that take care of the cross-linking reaction. This set not only comprises the actual cross-linking agent that makes the chemical bonds, but also other chemicals that activate or accelerate the cross-linking reaction.

  There are many types of cross-linking agents of which sulfur for sure historically is the best known. Other types are phenol-formaldehyde resins, peroxides and amines. A well-known activation system is zinc stearate or zinc oxyde in combination with stearic acid. The zinc ion therefore may be readily found in aqueous extracts of quite a number of rubber materials.

  Special caution shall be given to the use of accelerators in rubber compounds for parenteral applications. In fact, these accelerators typically are organic molecules like thiurams, sulfenamides and thiazoles that are relatively easily extractable and some of which, like 2-mercaptobenzothiazole, are directly linked to health hazards, while others may give rise to the formation of hazardous reaction products as nitrosamines. Modern, unconventional curing systems for parenteral rubber compounds therefore will avoid the use of such accelerators.
- *The filler*: It attributes mechanical strength to the rubber compound. In modern parenteral applications the fillers that are used most commonly are inorganic mineral materials like aluminum silicate (clay) and magnesium silicate (talc). Carbon black, which is commonly used in other rubber applications, is avoided for use as filler for parenteral applications. This is due to the potential link with polynuclear aromatics (PNAs) that may pose a health hazard.
- *The pigment*: It attributes a color to the compound. In parenteral applications most components are gray, red, or black. The gray color is obtained by incorporating titanium oxide (white) and minor amounts of well-defined carbon blacks. The red color comes from the use of red iron oxide. Pigments for rubbers for parenteral application preferentially are not of organic nature, again because they may be extractable.
- *Other rubber ingredients*: In this class are various materials that either influence the physical properties of the rubber, like plasticizers, or the physicochemical stability of the rubber compound, like antioxidants and antiozonants, or the surface state of molded products, like migrating plasticizers or waxes. Modern parenteral rubber formulations will use these ingredients only if really needed and at any rate their extractability will be a design factor in the development of the compound.

Halobutyl Compounds

For parenteral applications, the most widely used compounds for long-term contact applications (vial stoppers and plungers for prefillable syringes and cartridges) are pure halobutyl compounds or are blended compounds where the halobutyl polymer is the main elastomer.

There are three major reasons for this. First, halobutyl elastomers allow for the lowest possible gas permeability of polymers that are available worldwide on an industrial scale. For sure in parenteral applications, where oxygen and moisture permeability are an issue, this is of the highest importance. Also, even if it cannot be linked one to one, low gas permeabilities are linked to lower absorption characteristics, especially with respect to preservatives that are present in parenteral formulations, and with lower leaching characteristics into the drug.

Secondly, halobutyl compounds allow using the cleanest curing systems. Accordingly, vulcanization can be obtained using the smallest possible set of curing agents with low extractable potential.

Thirdly, halobutyl elastomers, thanks to their low level of unsaturation, have extremely good ageing characteristics. This allows working with the lowest possible antioxydant levels, thus again preventing extractable and leachable issues, and still achieving a shelf life of multiple years.

Traditional halobutyl elastomers are obtained by polymerization of isobutylene and isoprene, followed by chlorination or bromination of the resulting copolymer. In the mid-1990s

an even more stable brominated copolymer of isobutylene and para-methylstyrene was brought to the market. This new elastomer at present time is used in a small number of parenteral rubber compounds only.

It is to be noted that nonhalogenated copolymer of isobutylene and isoprene, named butyl elastomer, equally may be in use for parenteral applications. Little or no new rubber compounds based on butyl elastomer are however offered to the market anymore.

A frequently asked question is whether bromo- or chlorobutyl is to be preferred. The answer is that principally bromobutyl cross-linking can still be achieved in a "cleaner" way, however the difference with chlorobutyl cross-linking is not of practical relevance. In fact, the use of bromobutyl or chlorobutyl compounds can be linked to a historical or even geographical background. Furthermore, it is very often forgotten that it is not so much the elastomer that is responsible for the chemical cleanliness of a parenteral rubber compound, but rather the rest of the compound recipe!

Poly-isoprene Compounds

Whereas halobutyl compounds stand for impermeability, chemical cleanliness and high stability, it is difficult to achieve with these materials the levels of elasticity that are required in some parenteral applications. Notorious in this respect are multipuncture applications, as encountered for instance with stoppers on insulin vials or with rubber seals on cartridges containing insulin or growth hormone. If the number of penetrations with a needle is tens of times—design specifications sometimes are over 100 times—it is not possible to ensure proper functionality in the sense of adequate resealing and of absence of coring with a pure halobutyl compound. For these applications historically natural rubber compounds or blends of halobutyl and natural rubber or laminates of these two materials were used. Since the last decade of the 20th century however, natural rubber has been largely phased out for use in pharmaceutical and medical rubber since, justifiably or not, it is associated with the risk of "latex allergy." Synthetic poly-isoprene has replaced natural rubber in most applications.

While mechanically superior to halobutyl compounds, poly-isoprene compounds fall short in other areas that make halobutyls so performant for pharmaceutical applications. Oxygen and water vapor permeability of poly-isoprene compounds are one to two orders of magnitude larger than for halobutyl materials. Poly-isoprene compounds also require more complex cure systems, which often means less pure and / or higher concentrated cross-linking agents. Residuals of the cure system in a number of cases may migrate to the surface of poly-isoprene components ("blooming"). Ageing characteristics of poly-isoprene compounds need to be improved by incorporating higher levels of antioxidants and in forthcoming case by including antiozonants.

In a number of applications components made of distinct layers of a halobutyl compound and of a poly-isoprene compound are able to bring a solution that offers the best of both worlds. This type of solution can be applied in the case of seals on insulin cartridges, where the rubber disk may be a laminate consisting of halobutyl material facing the drug and with a poly-isoprene side not in contact with the drug, however ensuring perfect resealability upon multiple puncturing. Unfortunately, such a laminate solution is not industrially feasible for vial stoppers.

Other Compounds

Whereas most parenteral applications call for low permeability compounds, some do just the opposite. The most important example is that of an elastomeric needle shield for a prefillable syringe. In a lot of cases these needle shields are preassembled on the cleaned and siliconized barrels of prefillable syringes with staked-in needles, packaged in gas permeable tubs and then subjected to ethylene oxide sterilization. Since the open end of the needle shield forms a hermetic seal on the hub of the syringe, the ethylene oxide must be able to permeate through the wall of the rubber shield to have its sterilizing effect on the needle that is covered by it. The needle cover thus must have a high instead of a low gas permeability. Rubber compounds used for these needle covers, and partly also for tip caps for prefillable syringes, therefore are made of poly-isoprene compounds, or alternatively of a compound based on a styrene-butadiene elastomer [styrene-butadiene rubber (SBR)]. The latter also displays a suitable gas permeability for this application.

The use of compounds other than halobutyl, poly-isoprene and SBR on the parenteral scene is for the most part restricted to niche applications. Examples are nitrile rubber for use in combination with mineral oil based drug formulations, which is often seen in veterinary applications, and silicone rubber in ophthalmic applications.

COATED CLOSURES

The compounds for elastomeric components for long-term contact with parenterals are designed to have no or the smallest possible level of interaction with the drug. For most applications, halobutyl formulations are able to achieve this goal. However, in a number of cases requirements are so high that halobutyl compounds are not adequate. Worth mentioning in this respect are biotech drugs that are used in very small quantities per dose and where no absorption by the vial stopper is allowed. Another example is cephalosporins, which in contact with halobutyl stoppers always tend to develop a measurable level of turbidity that in a number of cases is not deemed to be acceptable.

For such applications, solutions are offered to the market in the form of coated vial stoppers and coated syringe plungers. The two products that have established an accepted market position utilize fluoropolymer coatings, at least in the contact zone with the drug. Depending on the manufacturer of these coated components, the coating may have a different level of fluorination, but always will be high. Also, in all cases the coating will exhibit barrier behavior between the rubber component and the drug. This means that leaching of materials from the stopper into the drug and from the drug into the stopper is further suppressed. This in combination with the inert nature of the fluoropolymers that are used leads to better stopper/drug compatibility.

It is important to point here to the fact that the barrier function of coatings is not absolute. While extractables and leachables will be reduced, this will not be to a level of zero. The level of extraction will in part be dependent on which extractable is involved, as to whether the barrier function of the coating will be stronger or weaker. Where fluoropolymer coatings are not barriers is against water vapor. Fluoropolymer coatings thus are not suitable for preventing uptake of moisture during steam sterilization.

A difference between the two types of coated closures in the market, apart from the identity of the fluoropolymers, is the area in which the barrier coating is applied and how it is applied.

The first type starts from a fluoropolymer film that in a special type of molding process is applied to the closure in the contact area with the drug only (the largest part of the plug for a vial stopper). Other parts of the stoppers, including the topside, sidewall and underside of the flange and the part of the plug immediately underneath the flange, are left uncoated. This allows for achieving compatibility improvement with the drug with a thicker film of fluoropolymer material. The top part of the flange of these stoppers still needs some sort of siliconization to avoid stopper clumping during transport and machining. Equally it is debatable whether the entire drug contact area is coated or not.

The second type of coated closures uses fluoropolymer that is deposited on the closures in a proprietary type of spray coating process. The coating in this case is thinner, however still clearly exhibits a barrier function. This process enables coating of the entire closure, not only in the drug contact zone but also in all other areas. Since the coating is nontacky in itself, these closures do not require any surface siliconization, which in applications where the drug is sensitive to silicone of course is of highest value. Also coating of the sidewall of the flange is of help in prevention of formation of particulates during machining of the stoppers in feeding bowls and in chutes.

Fluoropolymer coated closures are available as vial stoppers and more recently also as plungers for prefillable syringes. Coated vial stoppers may require minor adaptations to the settings of filling machine but for the rest do not require too much attention in terms of machineability. This is different for coated plungers, especially when they are strongly mechanically stressed when they are inserted into the barrels of the syringe. At this stage the coating may start to exhibit wrinkling which worst case may lead to marginal sealing behavior on the inner diameter of the syringe barrel. Precautions to prevent this are indicated, either by using a suitable filling technique or by using adapted machine parts.

Coated closures mostly are encountered in high value applications, like biotech drugs, or for silicone sensitive drugs like some proteins. Since these closures require the use of costly fluoropolymers plus the use of extra process steps to apply the coating, the cost of coated closures is considerably higher than for uncoated closures. In spite of their superior product properties this high cost precludes their more widespread use, especially in cases where the cost of the component is not negligible compared with the cost of the drug.

PROPERTIES OF PHARMACEUTICAL RUBBER AND OF CLOSURES

This part of the chapter gives an overview of the most important properties that are or can be of interest for closures for parenteral application. The overview lists both properties of the elastomeric material itself and properties of components made thereof.

Physical Properties

Hardness

Hardness is the physical property of a rubber that is most apparent to the user since manipulating the closure or penetrating it with a needle gives an idea of its hardness. The hardness of a rubber is determined by a number of factors. The most important ones are the ratio of filler to elastomer and the presence or absence of a plasticizer. For a given compound system hardness will increase with increasing the amount of filler relative to the elastomer. Hardness of closures for parenteral applications is usually in three ranges: soft, hard and intermediate. The softest formulations can be found in applications where resealing is of critical importance, such as in injection points for flexible bags. These formulations tend to have no or only a low amount of filler. Most vial stoppers on the other hand are in an intermediate range. Softer stoppers, in as far as they do not contain a plasticizer, are made of formulations with relatively little filler, while in harder stoppers the ratio of filler to elastomer is higher. The hardest formulations for parenteral applications will be found in syringe plungers. The background for that is that gliding forces for harder formulations are more favorable than for softer ones.

There are numerous scales in which hardness of materials is expressed. Hardness for rubber formulations for parenteral closures though is expressed in Shore A. Values that are encountered in practice are in a range of grossly between 30 and 55, with exceptionally numbers up to 65° to 70° Shore A.

Hardness of rubber formulations is measured according to standardized methods on test buttons of standardized dimensions. ISO 7619-1, "Rubber, vulcanized or thermoplastic—Determination of indentation hardness—Part 1: Durometer method (Shore hardness)" is such a method. As the title already indicates the hardness of a rubber is determined by measuring the indentation depth of a standardized "pin" into the test button. There is often confusion about the fact that the value that results in this way cannot be reproduced by measuring on the rubber product (stopper or plunger) itself. Values measured on closures therefore will often be out of the hardness range that the closure manufacturer specifies on their data sheets.

Ash Percentage

Ash percentage measures the portion of noncombustible material in a rubber compound. This comes down to measuring the portion of material of inorganic nature to material of organic nature in the rubber material. Inorganic materials in rubber compounds for parenterals are primarily fillers, and to a lesser extent the pigment and potentially a portion of the cross-linking system. Materials of organic nature in rubber compounds are of course the elastomer, and also potentially a plasticizer. Since the primary inorganic and organic constituents are filler and elastomer, respectively, and since hardness is primarily determined by the ratio of these two, it is not surprising that hardness and ash percentage are linked to each other. Basically they yield the same information about the rubber formulation. Hardness though is less laborious and less cumbersome to measure in comparison with ash percentage. A standardized method to measure ash in rubber is ISO 247, "Rubber—Determination of ash."

Compression Set

Rubber is used for parenteral closures because of its elasticity, or its ability to return to its original form after being mechanically compressed. Yet, rubber is not perfectly elastic. This means amongst others that if a mechanical compression is being exerted for a long time on an elastomeric component, that it will not 100% return to its original form again. The difference between the original and the final form is called "permanent deformation." There is a standardized test (ISO 815) that measures permanent deformation of rubber under standardized conditions. It expresses the permanent deformation of a test part as a percentage of the deformation that the part was subjected to. This percentage is called "compression set."

The higher compression set of a rubber is, the higher thus is its permanent deformation under influence of a mechanical load. Expressed differently, the higher is the tendency of the rubber to adapt to the shape of its environment. Translating this into practical terms for prefillable syringe plungers that are compressed for a long time into a barrel, it means that the outer diameter of plungers made from a rubber with a high compression set tends to adapt to the inner diameter of the barrel. Of course, this is not desired or at least must be under control, since the plunger is expected to yield over time a high enough force on the inside of the barrel to guarantee seal integrity before and at the time of activation of the syringe.

Parenteral applications thus call for elastomeric materials with low enough compression sets. When measured according to ISO 815 (24 hours at 70°C) compression sets for rubbers for parenteral applications will be found to be in a large range between 10% and 50%. Depending on the application this may or may not be acceptable. A typical compression set for a halobutyl compound is in the range of 15% to 40%.

It is worth mentioning here that γ irradiation has a significant impact on the permanent deformation of rubber. This means that when rubber is subjected to the simultaneous action of mechanical compression and of γ irradiation its permanent deformation will be larger than when subjected to compression alone. The difference between the two, which is also function of the irradiation dose, can, depending on the rubber, range from significant to very significant. There are rubber formulations that have an acceptable compression set but an unacceptable "irradiation set," which means that under the combined action of compression and irradiation their permanent deformation is too large to still guarantee functionality. This aspect must be taken into consideration when making selections like that of an elastomeric part for a syringe that is irradiated with the plunger being assembled.

Gas Permeability

It has been pointed out in paragraph 3 of this chapter that gas permeability is a property of major importance for elastomeric closures used for parenterals. The majority of parenteral applications call for low permeability of the rubber closure (vial stoppers and prefillable syringe plungers), however as explained in a previous part of this chapter some applications require just the opposite (needle shields and tip caps for prefillable syringes).

The two extremes of permeability in the parenteral area are formed by halobutyl rubber (low permeability) on one hand and poly-isoprene or natural rubber (high permeability) on the other hand. In between are rubbers like SBR. Relative oxygen permeabilities at 40°C for different rubber compounds as cited by literature and confirmed by own measurements are approximately 1 for halobutyls to about 10 for SBR to 20 to 30 for poly-isoprenes. Similar relative rankings apply for moisture vapor permeability measured at the same temperature. Gas permeability of a rubber primarily depends on the type of polymer, but also on other factors as the type and degree of filler. Among external factors that influence gas permeability certainly temperature needs to be mentioned, with higher temperatures causing higher gas permeabilities.

The ISO standard to measure gas permeability is ISO 2782. For pharmaceutical rubber it is however more common to refer to ASTM standards ASTM D3985 (oxygen) and ASTM F1249 (water vapor).

It is worth mentioning here that recently instruments have been introduced into the market to nondestructively measure moisture or oxygen in the headspace of individual vials. The technique is based on laser absorption spectroscopy.

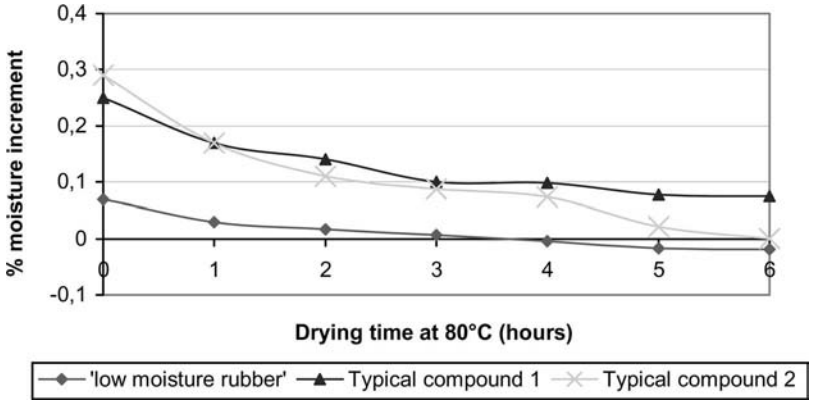


Figure 9 Moisture uptake and release of three different rubber formulations. The curves show moisture uptake at initial steam sterilization (30 minutes at 121°C) and subsequent release at drying at 80°C.

Moisture Absorption/Desorption

Water vapor permeability of the rubber compound influences the amount of water that over time will permeate through the rubber closure into a vial with medicinal product. Another factor that influences the amount of water that will permeate through the rubber closure is the amount of water that is withheld in the stopper itself at the moment it is placed on the vial. This moisture over time will partly end up in the drug product. Whereas for aqueous solutions this will not be of an issue, it can be for moisture sensitive products that are filled as powder or are freeze-dried. The lower the amount of active pharmaceutical product contained in the vial is, the more critical the situation can get. Therefore, in cases where moisture sensitivity of the drug formulation is an issue it is indicated to monitor the moisture content of the elastomeric closure at the moment of filling.

The moisture content of halobutyl stoppers in the state as they are supplied to pharmaceutical companies typically is in the range of 0.3% to 1%. It must be stressed though that by steam sterilizing the stoppers, as is usual for aseptic filling, a significant amount of extra water is "pumped" into the closures. This extra moisture needs to be dried to a level that is compatible with the moisture sensitivity of the drug application. Recently "dry" halobutyl compounds have been offered to the market, or compounds that take up significantly less water during steam sterilization while maintaining the typical drying behavior of halobutyl materials. These dry compounds target specifically lyophilization applications. Figure 9 depicts the moisture absorption/desorption behavior of such a dry compound in comparison with two "traditional" halobutyl formulations. The time point $t = 0$ represents the percentage weight increase of the stoppers as noted during a steam sterilization of 30 minutes at 121°C. The other time points represent the drying behavior at 80°C as found during laboratory drying. It should be noted that since the stoppers before autoclaving also contain moisture, negative values for the drying part of the curve are possible.

A standardized method for measuring moisture of elastomeric closures can be found in ISO 8362-5, "Injection containers for injectables and accessories—Part 5: Freeze-drying closures for injection vials." The principle of the method that is outlined there is a coulometric Karl-Fisher titration of the moisture that is dried off from a part of the stopper. The advantage of this method obviously is that it specifically measures moisture. Simple weight change methods to measure moisture absorption/desorption of elastomeric closures are also frequently used.

Absorption of Preservatives

Many drug formulations are stabilized by the use of preservatives like parabens, m-cresol, or benzalkonium chloride. These preservatives are added in low concentrations, however they have a tendency of being absorbed by rubber, thus loosing their effect in the drug solution. Depending on the combination of type and concentration of preservative and type of rubber

compound this absorption can be more or less pronounced. Development of new rubber compounds and development of drug formulations must take this absorption into account.

Swelling

Many drug formulations are aqueous solutions. Water to a certain extent is absorbed by the rubber closure. Where water is in contact with the closure it may cause a local discoloration, for example, a dark gray stopper may discolor to a lighter gray. This discoloration is not of any functional concern and can be reversed by drying the stopper.

In contrast with water, other drug diluents may display a higher amount of absorption into the rubber closures. They cause a clearly measurable increase in the weight and, in forthcoming cases, in the dimensions of rubber closures. In this case one speaks of "swelling" of the stopper. Swelling usually is expressed as a percentage of weight gain of the stopper.

Oils are known to make rubber swell. For example, vegetable oils over one month will typically cause a 3% to 4% weight increase in halobutyl stoppers. Usually this will not hinder the functionality of the closure. Mineral oils on the other hand will cause a much higher swelling in halobutyl stoppers and therefore are incompatible with them. In such cases either the use of special rubber formulations (nitrile rubber) or of coated closures is indicated.

Apart from the physical effect of swelling, the diluent that penetrates the closure and is absorbed there also may dissolve rubber chemicals and act as carrier for leachables into the drug solution.

Chemical Properties

Extractables According to Pharmacopeial Methods

As set out earlier, rubber compounds are composed of different materials that have been vulcanized through a curing step at elevated temperature. In contact with a drug solution some of these materials, their impurities, their reaction products or their thermal breakdown products may be extracted from the rubber closure.

A common way to make an assessment of extractables from pharmaceutical rubber is to prepare an extract of the rubber under well-defined model conditions and then, by using primarily wet chemistry methods, to measure for extractables. Such methods can be found in all major pharmacopeia, specifically in U.S. Pharmacopeia (USP) <381>, "Elastomeric Closures for Injections," in European Pharmacopeia (Pharm. Eur.) 3.2.9, "Rubber closures for containers for aqueous parenteral preparations, for powders and freeze-dried powders" and in Japanese Pharmacopeia (Pharm. Jap.) 7.03, "Rubber Closures for Aqueous Infusions." Also ISO 8871-1, "Elastomeric parts for parenterals and for devices for pharmaceutical use—Part 1: Extractables in aqueous autoclavates" is such a method.

The methods for measuring extractables in USP <381> as from 2009 on are extremely close to the methods in Pharm. Eur. 3.2.9 and in ISO 8871-1.

All three aforementioned methods use water as a model solvent and extract rubber by autoclaving it for 30 minutes at 121°C in a ratio of 1 cm^2 of rubber surface area exposed per 2 mL of water. In the aqueous extract that is obtained in this way, a number of determinations are done, including measurement of acidic or alkaline substances, measurement of reducing substances, assessment of the UV absorbance spectrum of the extract, and measurement of volatile sulfides and of zinc (both are common rubber chemicals). The results of the testing have to be within certain "type I" limits or within more loosely set "type II" limits as "fallback position." The idea behind this is that rubber for parenteral applications should be as clean as possible and thus meet the type I requirements. However for rubber articles where the mechanical requirements are so high that they cannot be met by using the cleanest cross-linking systems, the less strict type II limits allow these compounds still to qualify as "pharmacopeia compliant" or "ISO compliant."

In view of the fact that the ratio of surface area of rubber per volumetric unit of water is constant, the results for chemical testing of USP <381>, Pharm. Eur. 3.2.9 and ISO 8871-1 are independent of the size of rubber product that is extracted. Pharm. Jap. 7.03 is different. It also uses water as model solvent, however it extracts rubber in a fixed ratio of 1 g of rubber per

10 mL of water. As a consequence, for smaller rubber parts that are lighter in weight, relatively more surface area will be exposed to the extraction medium. Therefore for such small parts it is relatively more difficult to comply with Pharm. Jap. 7.03. Also the list of tests in Pharm. Jap. 7.03 is quite different from the other pharmacopeia and there is only one single set of limits.

Extractables and Leachables

No doubt the most discussed topic in the area of elastomeric closures for parenterals in the last decade has been the subject of extractables and leachables.

It has become clear that whereas pharmacopeial extractable methods are able to discriminate between cleaner type I formulations and less clean type II rubber compounds, they are not appropriate to distinguish between rubber formulations that have a general low extractable profile and compounds that are especially developed to release as little as possible to drug formulations. Also pharmaceutical companies and health authorities definitely want to know more about the specific identity of species that are released by packaging materials so that appropriate toxicological assessments can be performed.

Pharmacopeial extraction methods, with the exception of the determination of zinc, are not able to offer this. Therefore, more and more they are considered as a base level of extractable documentation that must be supplemented with more and more specific information. At the time of writing there are no standardized methods yet that describe how such additional extractable data can be obtained. However, initiatives such as the Product Quality Research Institute (PQRI) Working Group on Extractables and Leachables are underway. These initiatives no doubt over time will generate standardized methods for determining extractables under model conditions in model solvents and most likely will introduce concepts of threshold values below which extractables are accepted as safe, and above which toxicological assessments will be needed. What is then still left is the task to describe and ideally standardize the way to assess compounds from packaging materials that end up in real drug products, not in model solvents, in other words: how to assess leachables, not extractables.

A far more elaborate discussion about extractables and leachables is offered in a separate chapter in volume 3 of this reference work.

Functional Properties

Container/Closure Seal Integrity

The ultimate function of a parenteral closure is that it is able to guarantee integrity of the seal that it is forming with the container on which it is placed. Only in this way it is assured that sterility of the vial contents is preserved and that label claim specifications are met. USP <1>, "Injections," in this respect states that "containers are closed or sealed in such a manner as to prevent contamination or loss of contents." For a stopper sitting on a vial, the seal, after capping of the vial, is formed between the underside of the flange of the stopper and the top part of the vial neck. For a plunger for a prefilled syringe the seal is formed between the ribs of the elastomeric plunger and the inside surface of the glass or plastic barrel. For prefilled syringe needle covers and tip caps the seal of the elastomeric part with the cone of the syringe barrel must exhibit integrity.

USP's general chapter <1207>, "Sterile Product Packaging—Integrity Evaluation" discusses the maintenance of microbiological integrity of sterile product packaging over the life cycle of the medicinal product. Integrity testing should take place during three phases: product package development phase, routine manufacturing phase and marketed product stability phase.

Closure/vial seal integrity testing methods fall into two classes: microbiological methods and physical methods. Microbial methods include liquid immersion challenge tests and airborne microbial challenge tests. Under the physical methods there is a whole array including generally accepted dye ingression methods, gas leak methods, vacuum or pressure decay or retention methods, and relatively simple weight loss/weight gain methods.

Since closure/vial seal integrity is so intimately linked to microbial integrity and preservation of sterility, one would expect that standardized microbiological challenge test methods would have developed and could be found in the major pharmacopoeia and in international standards. This however is not the case. In none of the pharmacopeia are any microbial ingression test methods described in concrete wording, while in existing ISO standards all closure/vial seal integrity testing methods to date are physical methods, notably dye ingression methods.

At this place no extensive overview of closure/vial seal integrity methods will be given. An extensive discussion of the topic is given in a separate chapter of this volume. Also PDA's technical report no. 27, "Pharmaceutical Package Integrity," 1998, is a very useful review document.

Coring

Functional test methods for elastomeric closures that are well described in pharmacopeia are coring, penetration and resealing after puncturing. A description of test methods for closures intended to be pierced with a hypodermic needle is available in Pharm. Eur. 3.2.9, as well as in USP <381>. The test methods are the same as in ISO 8871-5, "Elastomeric parts for parenterals and for devices for pharmaceutical use—Part 5: Functional requirements and testing."

Coring, sometimes also termed "fragmentation," is the phenomenon whereby upon puncturing a stopper, small parts of the closure are dislodged by piercing or by abrasion. These small particles risk eventually being injected into patients. The latter of course is undesired.

Looking at vial closures for SVP and hypodermic needles, coring test methods consist of piercing a fixed number of closures a fixed number of times and collecting on a filter the particles that are formed by these penetrations. The number of particles that is visible with the naked eye must not be larger than a certain limit value.

Factors that influence the result of the coring test for SVP closures are multiple. A perhaps still nonexhaustive list is the following:

- *Physical properties of the closure*: Most important in this respect are the closure's hardness and tear strength which are both linked to the closure composition. In general softer closures tend to be less prone to coring. So are closures made from elastomeric formulations with high tear strength. The link between these properties and coring results however is not unique, as there are formulations with more elevated hardness that still are acceptable in terms of coring behavior.
- *Penetration thickness of the closure*: All other things remaining the same, higher piercing thicknesses increase coring tendency.
- *Single versus multiple piercing*: Clearly multiple piercing of the same closure increases the risk for coring. For closures that are intended to be pierced a high number of times, using special rubber compound formulations may be indicated.
- *Irradiation sterilization of the closure*: With quite many elastomeric closure formulations an increase in coring is seen after γ irradiation. The increase is higher with higher irradiation dose. However, typical doses of 25 kGy for various closure formulations are enough to cause coring results to go out of compliance with compendial limits. Use of specially developed compound formulations is indicated in these cases.
- *Quality and size of the needle*: Especially the finishing of the tip and of the sharp edges of the canula and the surface state of the needle are important. Dull needle tips and sharp edges that have a rough finish increase coring. The outer surface of the needle should have an adequate finish, meaning a surface that is not too rough and that is adequately siliconized, not to cause abrasion when penetrating the stopper. Thicker needles tend to yield higher coring results.
- *Surface state of the closure*: Also the surface state of the closure must be sufficiently lubricious. This can be achieved by adequately siliconizing the closure, or, in case of totally coated closures, by taking care that the coating displays enough lubricity.

- *The way the closure is pierced*: When piercing the closure out of its target area, or when penetrating it with the canula nonperpendicular to the closure surface, or when penetrating it with too high speed, the risk of coring increases.

Of course also for LVP coring is an issue. In case the LVP is contained in a glass bottle or in a Blow-Fill-Seal package ("bottelpack") the elastomeric closure will be pierced with a spike of considerably larger outside diameter than a hypodermic needle. Spikes of this type, unlike hypodermic needles, are made out of plastic. The same list of factors influencing coring as for SVPs is valid. Coring of LVP closures that are penetrated with a plastic spike is not described in any pharmacopeia. Test methods can be found in standards ISO 8536-2, "Infusion equipment for medical use—Part 2: Closures for infusion bottles" and ISO 15759, "Medical infusion equipment—Plastics caps with inserted elastomeric liner for containers manufactured by the blow-fill-seal (BFS) process." These test methods use steel spikes with specified dimensions.

Penetration Force

Elastomeric closures for parenterals must have an adequate penetration force, or a force high enough to feel some resistance upon puncturing but more importantly not too high. With respect to factors influencing penetration force again the same list as above can be used, although single/multiple piercing is not relevant for this property. Penetration force testing for SVP and LVP closures is described in the same pharmacopeial paragraphs and the same ISO standards as for coring.

Typical penetration forces for SVP elastomeric closures are between 2 and 3 N.

Resealing

Resealing of an elastomeric closure concerns its ability to perfectly reseal after being punctured and after withdrawal of the needle (or in forthcoming case the spike). Resealing must be guaranteed to preserve sterility of the vial contents before the next penetration of the closure. It is clear that resealing is only relevant for closures that are intended to be pierced more than once. Resealing of elastomeric vial stoppers for SVP's again is described in the same pharmacopeia and standards as where coring and penetration force are described. The type of test method that is found in standards always is a physical dye ingress method. A number of penetrations equal to 10 is assumed. In practice, for some drug products the number of penetrations can still be higher. In the development stage of such products this must be taken into account. SVP stoppers that are crimped on vials are pierced 10 times. Thereafter the vials + stoppers are put in a dye bath where they are subjected to an underpressure for a certain time. After atmospheric pressure has been restored it is observed that no dye has ingressed through the stopper area where the multiple piercing took place.

Applications where the number of penetrations definitely is higher than 10 are cartridges, an example of which is those that contain insulin or human growth hormone. Such cartridges are intended to be used in pen systems for self-administration by the patient. They consist of a glass barrel that is sealed at one end by a rubber plunger and at the other end is crimped with an aluminum cap containing an elastomeric liner of thickness 1.5 to 2 mm typically. At every activation of the pen system a new double-ended needle is to be used. One end of the needle penetrates the rubber liner, the other end penetrates the patient's skin. The number of activations for such pen cartridges may go up to 50 or more times. In the development stage of such products a safety factor concerning number of penetrations is taken into account, as even if the cartridge is developed to contain 50 doses, testing of resealing during system development will take place at two to even three times this number of penetrations. A perfect reseal of the elastomeric liner is difficult to realize. Substantial improvement can be achieved by using a laminate liner, or a liner that consists of two layers of nonidentical elastomeric formulations. The layer that is not in contact with the drug is made of a formulation that is specially developed with a view to multiple piercing and perfect reseal while the layer in contact with the drug is made of a cleaner rubber formulation. In practice the layer that promotes resealing (and at the same time also improves the coring behavior of the

seal) will be a poly-isoprene formulation, while the contact layer with the drug will be a halobutyl formulation.

Spike Retention Force

LVP closures are pierced with a spike. This spike is part of an infusion set that makes the connection between the contents of the LVP package and the patient. The spike will be sitting in the closure for the entire duration of the administration of the LVP to the patient. Since the LVP package itself during administration will be hung up, the spike will be remaining in a hanging position in the closure for potentially several hours. During this time the closure should exert sufficient force on the spike, so that it does not slip out of its position, also not when the patient is transported between different locations in the hospital. This force is called retention force.

Retention force testing may take place in two ways, a static way and a dynamic way. In the static testing mode a well-known weight is attached to the spike for a well-known time. During this time the spike shall not slip out of the closure, nor shall any leakage of liquid be observed in the seal area between the spike and the closure. In the dynamic testing mode the force needed to pull the spike out of the closure is measured on a force testing machine.

Methods for testing spike retention can be found in ISO 8536-2 and in ISO 15759, both of which were previously mentioned in the paragraph "coring."

Gliding Behavior

Vial stoppers take care of closure/vial seal integrity during the shelf life of the medicinal product and play their functional role when at the time of administration they are pierced with a needle or a spike. Syringe plungers partly have a different functionality. Clearly they assure closure/vial seal integrity, but obviously they are not pierced. Instead at the time of administration to the patient of the drug in the syringe they must be able to assure a smooth gliding in the syringe barrel.

When looking at the gliding behavior of syringe plungers one makes distinction between the force that is needed to make the plunger start moving and the force that is needed to sustain movement of the plunger. The former is typically called "activation force" or "break-loose force," while for the latter the names "gliding force" or "extrusion force" or "propagation force" are used.

A typical force curve for the gliding of a plunger in a prefilled syringe is given below. The curve displays the force that is needed to move the plunger as a function of the distance that the plunger travels into the syringe barrel. From this curve it follows that it needs a certain build-up of force to start the movement of the plunger. Thereafter the force to keep the plunger moving decreases. Gliding forces thus are typically lower than break-loose forces. Break-loose forces must be low enough to guarantee smooth activation of the syringe. Gliding forces equally must be at an acceptably low level. Moreover gliding forces must be continuous, or without increases and decreases. Should the movement be "interrupted," then one speaks of shattering of the syringe. Shattering obviously for the comfort of the patient must be avoided (Fig. 10).

There are many factors that have an impact on gliding behavior of plungers in a syringe. One variable for sure is the design of the plunger. Forces are higher the more surface area of the rubber part is in contact with the inside of the barrel. The number of sealing ribs of the plunger and the way they are dimensioned thus play a role. Next there are the physical properties of the plunger. Harder plunger materials tend to yield lower gliding forces. Also the barrel material has an impact. Glass and plastic barrels of the same dimensions will give rise to different gliding behavior of the same plungers. Furthermore there is the surface state of the elastomeric plunger and of the inside of the barrel. This surface apart from exceptional cases is always siliconized. The degree and way of siliconization of the plunger, the degree and way of siliconization of the inside of the barrel and the homogeneity of siliconization of the inside of the barrel over the total path length of the plunger strongly influence break-loose and gliding forces. More sophisticated application methods that guarantee better homogeneity of silicone distribution in barrels as well as methods to verify this distribution recently have emerged.

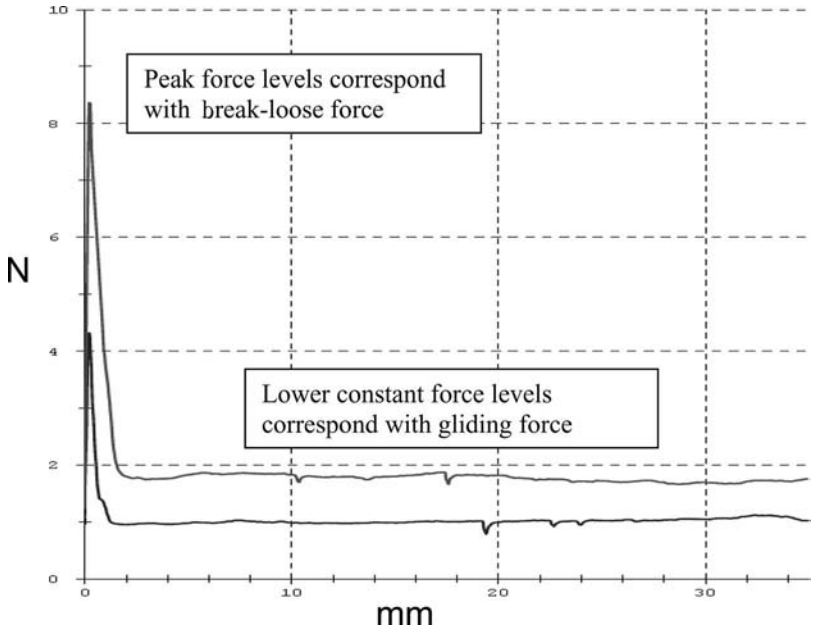


Figure 10 Gliding curves of two different plungers in the same type of barrel. The curves display gliding force as a function of the pathway of the plunger. At the left hand side, peaks correspond with break-loose (or activation force). The lower part of the curves corresponds with the gliding force for the two different plungers.

Biological Properties

In this paragraph the biological properties of materials for elastomeric closures are discussed. Discussion of the state of biological cleanliness of elastomeric closures themselves in terms of presence/absence of endotoxins and colony-forming units will take place in the next chapter.

The leading reference about biological properties of elastomeric closure materials is USP. USP <1031>, "The Biocompatibility of Materials Used in Drug Containers," spends a separate paragraph on elastomeric closures. There it is stipulated that the biocompatibility of an elastomeric material is evaluated according to a two-stage testing protocol specified in section "Biological Test Procedures" of USP <381>. Unlike plastics thus no class I-VI designations are assigned to elastomeric materials.

USP <381>, "Elastomeric Closures for Injections" in turn refers to USP <87>, "Biological Reactivity Tests, In Vitro" as the first-stage test to be performed. The tests in USP <87> are designed to measure the response of mammalian cells to specific extracts prepared from the closure material. If the requirements of USP <87> are met, then no further testing is required. If however the elastomeric material does not meet the requirements of the first-stage testing as per USP <87>, then it may still qualify as a biocompatible material by passing the "more forgiving" second-stage testing as per USP <88>, "Biological Reactivity Tests, In Vivo." USP <88> tests are designed to measure the response of animals to the injection of specific extracts prepared from the elastomeric material under test. Unlike the situation with chemical properties of elastomeric closures no class or type distinction is made between elastomeric materials that meet the requirements of first-stage testing and those that qualify as biocompatible meeting the second-stage requirements only.

USP <87> lists three possible test methods: the agar diffusion test, the direct contact test, and the elution test. In practice however it is always the Elution Test that is carried out.

USP <88> equally lists three possible test methods: the systemic injection test, the intracutaneous test, and the implantation test. Since the latter is not of relevance to elastomeric closures only the first two are carried out in practice.

Not meeting the requirements of USP <87> but still passing USP <88> is typical for elastomeric materials that use certain rubber chemicals, notably accelerators, that have a cytotoxic effect on mammalian cells as per the test conditions of the "Elution Test" in USP <87>.

The relevant ISO standard on biological material properties of elastomeric closures is ISO 8871-4, "Elastomeric parts for parenterals and for devices for pharmaceutical use—Part 4: Biological requirements and test methods." In essence however this is a copy of what is described in USP. At some places ISO 8871-4 refers to the ISO 10993 series of standards, "Biological evaluation of medical devices." Also this reference however does not preclude that ISO 8871-4 and USP come to the same result regarding biological properties of elastomeric closure materials.

Compatibility Behavior

The term compatibility behavior in the case of an elastomeric closure refers to its capability to preserve identity, strength, purity and stability of the drug product that it is in contact with. A closure that is compatible thus will not interact with the dosage form in such a way as to cause unacceptable changes in the quality of either the dosage form or the closure itself, an example of which would be by an unacceptable degree of swelling.

FDA's 1999 Guidance for Industry, "Container Closure Systems for Packaging Human Drugs and Biologics—Chemistry, Manufacturing and Controls Documentation" is the most prominent document that further discusses the subject of compatibility of primary packaging components including elastomeric closures with pharmaceutical dosage forms. This document, amongst others, lists examples of interactions, such as "loss of potency due to absorption or adsorption of the active drug substance, or degradation of the active drug substance induced by a chemical entity leached from a packaging component; reduction in the concentration of an excipient due to absorption, adsorption or leachable-induced degradation; precipitation; changes in drug product pH; discoloration of either the dosage form or the packaging component; or increase in brittleness of the packaging component."

Investigating compatibility of the elastomeric closure with the dosage form is the responsibility of the pharmaceutical company that is qualifying the closure. Changes noted during pre or postapproval stability studies thus shall be adequately addressed.

Ageing Behavior

The ageing behavior of an elastomeric closure refers to the evolution of the property profile of that closure over time. Closures that are affected by ageing will show a deterioration of some of their properties over time. By adequate studies it must be assured that this deterioration is not in conflict with the shelf life of the dosage form that uses that particular closure.

When ageing has an effect on an elastomeric closure, then that will most likely be seen in either the surface properties or the functional properties of the closure.

In terms of surface properties various effects are possible. One of those effects is that over time ingredients of the rubber migrate to the surface and form a layer there that is different in composition compared with the bulk of the article. The phenomenon is also known as "blooming." Blooming ingredients typically are low molecular weight ingredients like accelerators, oils and waxes, and fatty acids and their salts, like zinc stearate. Blooming will have an effect on the chemical properties of the closure. Blooming clearly can only occur with rubber formulations that contain certain rubber ingredients. Avoidance of these ingredients is indicated. If this is not possible, then only storage under well-controlled conditions can help to suppress surface migration.

Another ageing effect is the change of the skin of the elastomeric closure as a result of the attack of oxygen or of ozone. Particularly ozone attack is able to induce cracks at the surface of some rubber formulations. Those cracks however may penetrate further into the body of the elastomeric part, especially in components that are mechanically stressed when they are in use. Cases have been reported of ozone cracks in tip caps for prefilled syringes that resulted in splits of the entire sidewall of the tip cap. Consequently the integrity of the seal of the cap on the tip of the syringe barrel was at stake. Ageing as a result of oxygen or ozone attack is typical for particular elastomeric formulations based on natural rubber, poly-isoprene rubber and SBR that have not been adequately formulated, or those that do not contain enough antioxydant and antiozonant of the correct type. With halobutyl formulations in general there is no issue with neither oxidation nor ozone attack.

Still another ageing effect involving the surface of the elastomeric component has to do with surface siliconization. Surface siliconization of elastomeric parts is necessary to prevent clumping of the parts during storage and transport before use and to enable processing of the parts on filling or assembly lines. Surface silicone however, depending on the type of silicone and on the type of the rubber formulation, over time can be absorbed by the closure. Hereby the silicone becomes inactive at the surface. Stickiness, clumping and in the worst of cases deformation of the parts will develop. Absorption of silicone can be countered by choosing higher molecular weight silicones or by choosing silicones that are able to crosslink and so increase in molecular weight. Silicone absorption will take place earlier in rubber formulations with high permeability such as poly-isoprene. Again, in halobutyl formulations, depending on the molecular weight of the silicone, adsorption will not be or at least will be less of an issue.

Finally, also functional properties of elastomeric closures may be affected by ageing. Particularly coring, sealing and resealing behavior are to be mentioned in this respect. Again, in halobutyls, worsening of these properties over time at most is a slow process. Yet it is indicated to check as closures before they are assembled on vials may already have some age—practice shows that this can go up to two to three years—and to this the shelf life of the pharmaceutical product still has to be added.

At present there is no standard that is dedicated to ageing of pharmaceutical rubber parts. General guidance is given by ISO 2230, "Rubber products—Guidelines for storage." For halobutyl products, at least when stored under appropriate conditions of light and temperature, an indicative shelf life of seven years is given. For poly-isoprene articles this is less. Indicative shelf lives for such articles are three to five years.

Machineability

Machineability of elastomeric closures refers to the processes at pharmaceutical or at medical device companies that are used to bring closures into their final position on vials or in syringes or cartridges. Therefore machines will be used that are designed to have a certain capacity. Such machines typically involve feeding bowls in which the elastomeric parts, mostly after sterilization, are brought in, then feeding lines or chutes that bring the closure in the vicinity of the vial or syringe and next a pick-up and positioning mechanism that assembles individual closures onto or into individual vials or syringes.

A first prerequisite is that elastomeric parts do not clump when they are brought into a feeding bowl. Clumping is very typical for halobutyl components. Clumping behavior can largely be prevented by giving an appropriate surface state to the closures. For nonpolymer coated closures this means that the surface of the closure must be designed so as to maximally prevent sticking of individual parts by including antisticking dots or bars, that the surface of the closure has an adequate roughness that is "copied" from the roughness of the mold out of which it is produced, and that the closures have an adequate degree of surface siliconization. Furthermore care shall be taken so that closures are put into their transport packaging when they are at or close to room temperature, that they are not packed too tightly and that their shelf life for storage is taken into consideration.

Feeding behavior of closures in feeder bowls and chutes mostly is a matter of adequate surface states and of adequate dimensioning of closures and machine parts, however minute details in design may have an unexpected impact here.

Insertion behavior of stoppers into vials and of plungers into syringes or cartridges also primarily is a matter of assuring the dimensions and the surface state of the closures, vials, syringes and machine parts are well adapted to each other and are well controlled.

CLOSURE WASHING AND SILICONIZATION

Elastomeric closures for parenterals are manufactured under industrial circumstances with still a lot of manual operator intervention and using industrially available materials. Closure manufacturers spend a great deal of effort to improve the cleanliness of their plants and to tighten their procedures and quality systems so as to guarantee the quality and the cleanliness of their products. Yet, unlike with plastic products, it is not possible to collect at the end of the molding and die-trimming process the resulting products and to pack them without first subjecting them to a washing process.

There are several reasons for this.

1. Before washing, the products are not in a controlled state of cleanliness. After molding most closures are die-trimmed. Silicone in some form is used as a die-cutting agent that prevents the trimming die from getting dull. This silicone, together with the whole manufacturing history of the closures that precedes die-trimming, brings the closures in an undefined state of particulate and microbiological cleanliness. Washing of the closures is necessary to bring the closures within clear specifications, therefore to bring them in a certifiable state of cleanliness, both from the point of view of microbiological and of particulate cleanliness.
2. Closures have not been subjected to a depyrogenation process as required by regulations. FDA's 2004 "Guidance for Industry—Sterile Drug Products Produced by Aseptic Processing—Current Good Manufacturing Practice" states that "containers and closures shall be rendered sterile and, for parenteral drug products, nonpyrogenic." Nonpyrogenicity is obtained by subjecting the closures to well-defined washing, rinsing and drying processes. More and more this washing is delegated to the closure manufacturer who therefore needs to develop validated washing programs.
3. If not siliconized, closures will clump and machineability cannot be guaranteed. As indicated in the previous chapter uncoated closures need siliconization in order not to develop clumping during storage and to be machineable on filling or assembling lines. Closure siliconization typically is combined with the final washing and drying at the closure manufacturer.

Washing Procedures for Elastomeric Closures

The washing of elastomeric closures can be performed in different types of washing machines. Most often encountered are machines of the rotating drum type and, alternatively, machines that are based on an "overflow" principle. The former ones consist of a rotating drum with a perforated wall through which contamination can be removed. It is necessary for the machine to supply water of different types and the necessary auxiliaries, including silicone in forthcoming case. The drum can be partitioned or not, as it can consist of a number of smaller segments that each contain a smaller number of products. Washing and drying either take place in the same machine, or the washer is combined with the necessary dryers, equally of the rotating drum type. In overflow machines the flow of water is from the bottom of the machine through the stopper bed to the overflow. The closures are in a kind of fluidized bed state and contamination is continuously removed via the overflow. In some machines of both types apart from washing and drying also steam sterilization of the closures can be performed.

Washing programs for elastomeric closures vary from company to company, irrespective of whether it concerns a pharmaceutical company that still washes the closures or the closure manufacturer. A typical washing and drying program of elastomeric closures consists of the following steps:

- A washing step with water of a specified grade plus a detergent.
- A number of rinsing steps with water of specified grades. One of the rinsing steps may be combined with siliconization of the closures.
- A drying step with hot filtered air.

As to the types of water used for the washing of elastomeric closures it is worth pointing to two documents. The first of these documents is the 2004 FDA Guidance for Industry that was cited already earlier. This guidance mentions that "at minimum the initial rinses for the washing process should employ at least Purified Water, USP, of minimal endotoxin content, followed by final rinse(s) with WFI (water for injection) for parenteral products." The second document is the European Agency for the Evaluation of Medicinal Products (EMA)'s 2002 "Note for Guidance on Quality of Water for Pharmaceutical Use." For closures that are used for sterile parenterals this document equally speaks of purified water for initial rinses and water for injection for the final rinse. The major closure manufacturers therefore have invested in

water plants and control systems for these plants so that they are able to guarantee the quality of the water that is used in the various stages of closure washing. What they have also invested in is the installation of clean rooms in which the washing and final packing of closures is performed and in developing monitoring schemes to demonstrate that these rooms are in compliance with standards for biological and particulate cleanliness.

Microbiological Cleanliness

The microbiological state of cleanliness of elastomeric closures relates to the presence or absence of microbiological contamination at their surface. This contamination may be present either in the form of bioburden that can be expressed as colony-forming units, and/or as endotoxins, expressed as endotoxin units.

Bioburden

In the majority of cases closure manufacturers do not sell their product sterile (or even "sterilized"). Alternatively, they sell their products with a defined state of high microbiological cleanliness, or low bioburden levels. This is particularly the case when closures are not rewashed at the pharmaceutical company itself. Closures in case of aseptic manufacturing at the pharmaceutical company then are rendered sterile prior to filling, mostly by steam sterilization.

Bioburden on elastomeric closures can be determined with a method as described in ISO 8871-4, "Elastomeric parts for parenterals and for devices for pharmaceutical use—Part 4: Biological requirements and test methods." Such method consists of an "extraction" or "rinsing" phase where bioburden is transferred from the stopper surface to the extracting liquid, followed by determination of the number of colony-forming units in the rinsing liquid. The latter typically is done by filtration on a filter with a suitable growth medium and incubation of the filter. From the result the number of colony-forming units per square centimeter of stopper surface area or per stopper then can be calculated. Methods for bioburden determination on elastomeric closures need to be validated.

Endotoxins

In case of elastomeric closures the absence of bacterial endotoxins is taken as a synonym for the absence of pyrogenic components. As with bioburden closure manufacturers will sell their product with a defined state of endotoxin cleanliness. Determination of endotoxins equally is described in ISO 8871-4. Methods are similar to bioburden determination methods in that they consist of an extraction or rinsing step, followed by a determination step. Current practice is that most often determination is performed using an instrumental LAL method, or alternatively the LAL gel clot method. Also methods for endotoxin determination on elastomeric closures need to be validated.

Particulate Cleanliness

Elastomeric closures like vial stoppers and prefilled syringe plungers are part of a packaging system for injectables. Injectables are subjected to requirements on the presence/absence of particulate matter, including USP <788>, "Particulate Matter in Injections." Elastomeric closures thus are linked, be it indirectly, to the particulate cleanliness of parenteral products.

Particulate cleanliness of elastomeric closures can be approached from various sides. As explained, rubbers are composed of various raw materials that are mixed. If mixing is not perfectly homogeneous this may lead to imperfect dispersion of ingredients like fillers or pigments. This may be visible by a trained eye or under magnification as small particulates of ingredients like filler particles that are different in color from the rest of the stopper. These particles however are still firmly embedded in the rubber matrix and they will not be dislodged from this matrix. Thus they will never compromise particulate cleanliness of the parenteral product.

For particulate contamination that is present at the stopper surface in loose form this is different. These particles effectively may be transferred from the closure into the medicinal product without particular effort. Particulate contamination on elastomeric closures may still have the same material identity as the closure itself, may be part of the ingredients of that closure formulation (endogeneous particles), or may be contamination from the manufacturing

environment that either has not been removed by washing or that is the effect of or a recontamination after washing (exogeneous particles).

USP <788> refers to microscopic methods and to light obscuration methods for the determination of particulate contamination in injections. For the determination of the particulate state of cleanliness of stoppers methods of the same types are standardized in ISO 8871-3, "Elastomeric parts for parenterals and for devices for pharmaceutical use—Part 3: Determination of released-particle count." The methods consist again of two steps. In the first step the particulate contamination is transferred from the stopper surface into an extraction or rinsing liquid and in a second step the contamination that is transferred is sized and counted. For subvisible particulates a light obscuration technique is used. Particles typically are sized in classes 2 to 10 μm, 10 to 25 μm, and >25 μm. For visible particulate contamination particles are collected on a membrane filter where they are sized and counted, either by an operator or by a microscope that is connected to a suitable software system for sizing and counting of particles. Visible particles are typically sized in classes 25 to 50 μm, 50 to 100 μm, and >100 μm.

At present time there are no limit values for subvisible or visible particulate contamination of elastomeric closures, neither in any pharmacopeia, nor in the aforementioned ISO 8871-3. Limit values may be present in quality agreements between manufacturer and customer, but this is on a voluntary basis. The same holds for biological cleanliness of closures.

In case limit values for particulate cleanliness are agreed on, it must be assured that determinations at the closure manufacturer and at the user yield sufficiently comparable results. Although it seems logical that a determination method yields a result with a certain precision and accuracy, intralaboratory repeatability and interlaboratory comparability of particulate cleanliness determinations on elastomeric closures is known to be poor in comparison with other analytical methods.

Closure Siliconization

The purpose of closure siliconization has been explained before. Siliconization of closures usually is part of the final washing of the parts. In one of the rinsing steps silicone is added to the rinsing water. Closures pick up some of the silicone. The water that at the same time is picked up is removed in the drying step of the washing/drying program.

There are various types of silicone that are used for closure siliconization and there are various ways to introduce these silicones into the closure washing machine. Silicone (polydimethylsiloxane) may be introduced as pure silicone or as a silicone emulsion that makes uses of an emulsifier to hold the silicone in an emulsion. The former method is preferred since the emulsifier is not removed by drying. This means that it stays on the closure and, in case of renewed contact with an aqueous medium, as often is the case with a drug product in a vial or a syringe, it will bring the silicone in emulsion again. This emulsified silicone is detectable as subvisible particulate matter. Silicone thus acts as an important source of particulate matter in parenteral products. Also in case no emulsifier is used it deserves attention to bind the silicone as well as possible to the rubber surface. A way to achieve this is to use silicone of higher viscosity, or of higher molecular weight. The longer polydimethylsiloxane chains have lower mobility and attach better to the stopper surface. An alternative way to immobilize silicone at the closure surface is to use a crosslinkable silicone. Such silicone typically is not added in the washing stage of the stoppers but in an earlier stage when the stoppers have not yet been die-trimmed from the sheets in which they are molded. Crosslinkable silicone may be sprayed on the sheets that subsequently are subjected to a silicone curing reaction.

Silicones used for siliconization of elastomeric closures are subjected to the requirements of the USP chapter "Dimethicon" and to Pharm. Eur. 3.8.1, "Silicone used as a lubricant." The viscosity ranges of silicone in these two documents do not perfectly match. The lower limit for Dimethicon is 350 cSt (centistokes) while the lower limit as per Pharm. Eur. is 1000 cSt.

Validation of Stopper Washing

FDA's 2004 Guidance for Industry "Sterile Drug Products Produced by Aseptic Processing—cGMP" mentions that "containers and closures should be rendered sterile and, for parenteral drug products, nonpyrogenic" and that "the validation study for such a process should be adequate to demonstrate its ability to render materials sterile and nonpyrogenic." For

pharmaceutical companies who wash elastomeric closures themselves and then sterilize them, this implies that they develop validation programs for closure washing and sterilization. At many occasions however it is closure manufacturers who perform the last washing of elastomeric closures. In this situation, closures are not rewashed by the pharmaceutical end-users, and only the sterilization is taken care of by them. This practice implies that the depyrogenation process of the closures is delegated to the closure manufacturer who consequently must avail of a validation package for their washing program. The core of such validation studies is inspired by the statement in the Guidance that "the adequacy of the depyrogenation process can be assessed by spiking containers and closures with known quantities of endotoxin, followed by measuring endotoxin content after depyrogenation.... Validation study data should demonstrate that the process reduces the endotoxin content by at least 99.9% (3 logs)." The closure manufacturers will therefore have to develop rationales for the closures to be included in their studies so as to bracket the relevant product portfolio and for which (worst case) conditions are going to be adopted in validation experiments. Not all closures are equally easy to wash. It is accepted in the industry that the ease with which endotoxin can be removed from closures is related to the ease with which the washing and rinsing water have access to the concave parts ("cavities") of the closures. Endotoxin spiking thus for validation purposes shall be done at these parts of the stoppers. For larger stoppers with shallow cavities it will prove to be easier to demonstrate a log 3 endotoxin reduction than for smaller closures with deeper cavities.

Validation of closure washing, apart from the essential part of endotoxin reduction, will also contain validation data about the microbiological cleanliness of the parts after the depyrogenation process. Other properties such as reduction of particulate burden by washing, particulate cleanliness of washed and dried parts, siliconization and presence/absence of washing detergent may form part of washing validation, also when not required by the aforementioned Guidance.

STERILIZATION OF PARENTERAL CLOSURES

Sterilization of parenteral closures may take different forms. The contact area of the parenteral closure with the drug product must be sterile at the time of use. This is achieved by either terminal sterilization of the packaged drug or by aseptic filling where all packaging materials are sterilized prior to filling. In case of plungers for disposable syringes sterilization takes place on the assembled and packaged syringe.

Steam Sterilization

The most common method to sterilize closures for parenteral applications is by steam sterilization, either prior to aseptic filling or by terminal sterilization whereby the packaging components are already assembled. The most typical sterilization temperature that is used for sterilization of elastomeric closures is 121°C, the most typical length of the cycle is 30 minutes. Only in seldom cases higher steam sterilization temperatures such as 134°C are used. For some applications such as blow-fill-seal packages lower temperatures of 106°C or 110°C are applied. Of course every sterilization process of packaging components shall be validated.

As mentioned before steam sterilization puts a considerable amount of moisture into elastomeric closures. Therefore closures after steam sterilization shall be dried again using appropriate procedures that take into account the sensitivity of the drug product to residual moisture in the closure. Closures for lyophilization applications therefore often will be dried to lower residual moisture than closures for liquid fills. Typical drying temperatures for elastomeric closures range from 80°C to 110°C. In a number of cases drying times of only one hour are applied, in other cases drying cycles of up to sixteen hours are qualified.

Other than the moisture uptake, steam sterilization of elastomeric closures, followed by drying, will not affect their functional properties. This still holds when the cycle is applied more than one time on the closures, albeit that this shall not be encouraged and that for multiple sterilizations a check on closure functionality may be indicated, depending on the exact use of the closure in question.

Notes

1. Whereas drying at temperatures of 80°C to 110°C will not affect elastomeric closure functionality, the same does not hold for substantially higher dry heat temperatures. Depending on the elastomeric formulation in question dry heat treatments where closures are exposed to temperatures of approximately 150°C or higher for longer times (15, 30, ... min) are to be avoided. Dry heat sterilization of elastomeric closures is to be totally advised again.
2. It is worth mentioning here that steam sterilization obviously has a sterilizing effect on elastomeric closures, however it cannot serve as depyrogenation process.

Sterilization by Irradiation

Of increasing importance is the use of irradiation sterilization for elastomeric closures. In such cases the pharmaceutical user will choose to be supplied with closures that have been washed by the closure manufacturer and that then have been subjected to a γ irradiation treatment at a sterilization contractor (see also later under "Packaging Ready to Use").

Sterilization by β irradiation of elastomeric closures is not excluded, however γ irradiation because of its much higher penetration capability is preferred. γ Sterilization of elastomeric closures can take place on entire pallets with closures packed in cartons, but more often is carried out with a more limited number of cartons, typically six or eight, being put together in sterilization "totes." One of the advantages of tote sterilization is that the dose distribution over the different cartons with closures will be more homogeneous, as the ratio of maximum to minimum dose achieved over the entire tote is smaller than the same ratio in an irradiated pallet. Since in case of irradiation sterilization the objective is to reach a validated minimum dose, the maximum dose in the case of tote sterilization therefore will be smaller compared with the case of pallet sterilization.

This is of significant importance, since unlike steam sterilization, γ irradiation is more likely to have an effect on the functional properties of the closures. γ Irradiation may have different effects in elastomeric closures. Depending on the formulation of some rubbers, additional cross-linking may take place. In others just the opposite occurs, or the rubber is de-crosslinked to a certain extent. Because of these effects in some rubbers loss of elasticity is found, resulting in a certain "hardening," "stiffening," increase in coring rate and, worst case, inadequate resealing behavior. In other cases closures after γ irradiation exhibit increased tackiness. All of these effects are more pronounced with increasing γ dose. For every individual application it shall therefore be investigated whether the applied irradiation dose does not affect the closure performance up to a level that it is no longer compatible with the requirements of the application. Especially attention has to be given to multidose applications where the closure by the nature of the application is penetrated multiple times. If there is an effect of γ irradiation on the functional properties of elastomeric closures, it will be noticed immediately after the irradiation, unlike with plastics where the effect may be delayed and become apparent only longer time after irradiation.

The most encountered γ dose applied for elastomeric closure sterilization in the past was 25 kGy. As a result of the publication of ISO 11137 on radiation sterilization of health care products newer applications use lower doses that are friendlier to elastomeric components. Of course such lower doses must be demonstrated to be efficient, therefore capable of guaranteeing a certain sterility assurance level. Information and instructions on how to achieve this are given in the same standard.

The effect of γ irradiation is most prominent with respect to the mechanical and functional properties of elastomeric closures. The effect on chemical properties is less evident. On the level of pharmacopeial compliance no effects will be noticed that would turn a compliant elastomeric formulation into a noncompliant one. On a more detailed level of extractables effects are not excluded, certainly not at higher irradiation doses.

Ethylene Oxyde Sterilization

Ethylene oxyde sterilization is very commonly used for the sterilization of disposable medical devices. In the area of elastomeric components for parenteral closures the most important case

is the sterilization of disposable syringes that very often contain an elastomeric rubber plunger. Sterilization is achieved by the action of ethylene oxyde gas on the biocontamination that is present on the plunger surface. To make this action possible the syringes will be packed in gas permeable packing that allows the gas to enter into the syringe. It is well known that ethylene oxyde sterilization leaves chemical residues in the form of residual ethylene oxyde and of ethylene chlorohydrine. Suitable aeration times that allow these residues to decrease below certain levels that are considered as safe must be established.

Apart from disposable devices ethylene oxyde sterilization is very common in one other application in the parenteral field, namely in the sterilization of assemblies of needle covers and tip caps on the barrels of prefillable syringes. One way to come to a presentation of a drug in a prefilled syringe is that pharmaceutical companies purchase syringe barrels with needle covers of tip caps already assembled on them at syringe system manufacturers. The system manufacturer performs the assembly of needle covers on syringes with a staked needle or of tip caps on syringes without needles. The assemblies are then put into tubs that carry a gas permeable plastic film. The tubs next are subjected to ethylene oxyde sterilization. In the case of needle covers the ethylene oxyde has to permeate through the wall of the needle cover to reach the needle surface where the ethylene oxyde has its sterilizing effect. Also these processes of course include suitable aeration or "degassing" cycles. The sterilized barrels may then be directly aseptically filled by the pharmaceutical company and subsequently stoppered with elastomeric plungers that are sterilized prior to aseptic filling.

PACKAGING FOR ELASTOMERIC CLOSURES

The last step in the manufacturing of elastomeric closures is a packaging step. The packaging for closures may just be a transport packaging or may have enhanced features.

Nonfunctional Packaging

In case of nonfunctional packaging the closures are put in single or multiple bags and the bags then are placed into cartons or some type of bulk packaging. Other than just the containment of the closures the bags also take care of preserving their state of particulate cleanliness. Bags of this type are simple polyethylene bags that themselves of course should not shed particles or fibers.

The pharmaceutical user will unpack the closures from the bags and, in case of aseptic filling, transfer them to containers that are compatible with their own sterilization process. These may be containers that are placed in an autoclave. Alternatively the pharmaceutical user may decide to rewash the closures.

Functional Packaging

In case of functional packaging the bags that contain the closures have an additional function at the time of sterilization of the closures. In case of steam sterilization one speaks of packaging "ready for sterilization," in case of irradiation sterilization the term RtU packaging is used.

Packaging Ready for Sterilization

The function of "RfS" bags is that the same bags are used to contain the closures during transport and during steam sterilization. In this case the pharmaceutical user will unpack the RfS bags with the closures from their protective wrapping and transfer them directly into his autoclave for steam sterilization. No rewashing of closures is undertaken.

RfS bags thus must have the following properties:

- They must resist autoclave conditions. RfS bags that currently are in the market resist to temperatures up to 125°C. They are compatible with steam sterilization at 121°C, but not at 134°C. Above 125°C they start to weaken and eventually melt.
- They must be permeable to gases. They must allow air to be evacuated during the vacuum phase at the beginning of the steam sterilization process. Then they must permit steam to enter into the bag to have its sterilizing action. During the drying phase at the end of the autoclave cycle they must allow water vapor to be evaporated.

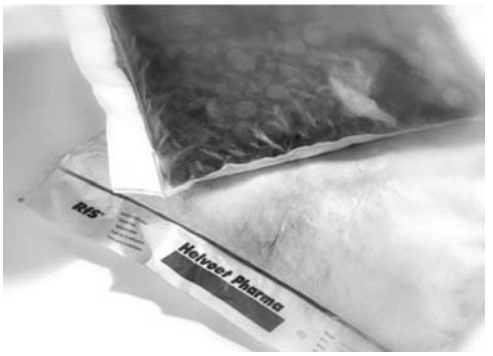


Figure 11 Picture of a ready-for-sterilization bag. The bag on the bottom has its Tyvek side up; the bag on top has its non-Tyvek side up.

- They must be impermeable to microbiological contamination. At the end of the sterilization cycle the closures in the bags are sterile. The bag must be able to guarantee that no microbiological recontamination takes place.

The market offers many types of RfS bags. The ones that are used for steam sterilization of elastomeric closures are composed of two layers of polyethylene in different physical form that are welded together. The welding must be very solid since the weight of the closures in the bag is considerable. One layer of the RfS bag consists of a nonwoven form of polyethylene that is known in the market as "Tyvek." Tyvek has the unique property of being permeable to gases, but not to microbial contamination. The second layer of the bag consists of a regular form of polyethylene that has high enough temperature resistance. This layer is not permeable to gases, nor to microbiological contamination.

It is clear that RfS bags need to have a defined level of particulate cleanliness (Fig. 11).

Packaging Ready to Use

RtU bags are suitable for γ irradiation of elastomeric closures. The closure manufacturer will after washing and drying pack the closures in the RtU bags and provide these bags with protective overwrapping in the form of one or more regular polyethylene bags. From there the closures are transported to an irradiator contractor who performs the γ sterilization of the closures. The pharmaceutical user who is the last in the chain will take off the protective wrapping from the RtU bags and transfer the closures directly to the filling lines in their sterile area. No rewashing nor sterilization of closures is undertaken. As such, RtU bags must be impermeable to microbial contamination.

RtU bags may be made of different types of polymers. Polyethylene can be sufficient since γ irradiation does not have a destructive effect on it. Other types of bags are however possible.

Rapid Transfer Port Packaging

A special case of functional packaging that is gaining more and more attention is rapid transfer port (RTP) packaging. Such packaging is designed to be easily connectable to dedicated ports on isolators or "restricted access barrier systems" (RABS). RTP packaging for elastomeric closures exists in both irradiation sterilization and in steam sterilization compatible forms. RTP bags will always have a "collar" integrated into them. This collar is the mobile part of a two-component system of which the port on the isolator or RABS is the fixed part. When the collar is docked onto the port a system is created that allows aseptic transfer of the sterilized components contained in the RTP packaging into the isolator or RABS (Fig. 12).

Packaging Validation

Validation of the packaging of elastomeric closures in particular is of relevance for functional packaging. At some point in their life cycle such packaging will contain sterile products. The validation of functional packaging comes down to yielding evidence that this packaging is

Figure 12 Picture of two different rapid transfer port bags. The collars are intended to be docked onto a restricted access barrier system or isolator port.

"tight and strong," both before and after sterilization. Microbial tightness of the packaging is important because ingress of microbiological contamination must be avoided before sterilization and of course recontamination after sterilization must be avoided at all times. Apart from choosing the correct materials for construction of the bag, assuring bag tightness can be obtained by developing suitable sealing processes after packing of the closures. The heat-sealing process for the bag shall be capable of generating a seal that is tight before sterilization and that is not affected by the steam sterilization or γ irradiation process. Demonstration of tightness of the seal can be done using microbiological methods or physical methods as a dye ingress method. Equally the sealing process shall generate a seal that is sufficiently strong to resist the weight of the closures, the stress during the steam sterilization process and the handling that inevitably is associated with the bags. Demonstration of the strength of the seals can be given by measuring tear strength of the seals. In the case of γ irradiation it shall equally be demonstrated that there is no effect of time after irradiation on seal strength. Validation of RTP packaging involves demonstration of tightness and strength of yet another seal, namely that of the collar on the bag material. Other points in validation of functional packaging may relate to particulate cleanliness of the bags and in case of γ irradiation to yielding data about discoloration of the bags after irradiation.

QUALITY CONTROL AND QUALITY ASSURANCE IN ELASTOMERIC CLOSURE MANUFACTURING

In-Process Control

Many controls can and will be executed during the manufacturing of elastomeric closures. They range for instance from checking weight on preforms to in-process monitoring of component height, to a visual check of the trimming edge of freshly trimmed stoppers. It is up to the closure manufacturer to determine which particular controls are deemed to be significant and should consequently be performed and documented.

The present paragraph does not intend to discuss further the aforementioned types of controls. Instead a further discussion will be made on controls that generally are formally carried out and documented by qualified people from a quality department.

Included in the category of in-process-controls are tests that serve to confirm the identity of the material that is being processed. Particularly after mixing or preforming, the manufacturer wants to confirm by testing that the material displays all the intended identity characteristics. This is possible by taking samples of the mixed or preformed material and by verifying physical and chemical properties on appropriate test plates made from it.

Physical properties may include a selection or the totality of the following tests:

- Specific gravity
- Ash percentage
- Hardness
- Aspect (assessment of color and homogeneity)
- Rheometry

It is to be noted that the aforementioned tests include only properties that can be affected by the weighing, mixing and preforming operations and do not relate to pure material properties such as gas permeability.

Chemical properties may include a selection or the totality of tests performed according to a standardized method such as USP <381>, Pharm. Eur. 3.2.9 or ISO 8871-1.

None of the aforementioned determinations is capable of confirming on its own the identity of the rubber material. However, every determination leaves its fingerprint and by combining the results of all tests the identity of a rubber compound can undisputedly be confirmed.

In addition to confirming the identity of the material, by carrying out these tests, data are generated that may be used for compiling the Certificate of Analysis or Certificate of Conformity of the product batches that result from the material.

Finished Product Inspection

The term finished product inspection describes the activities that are carried out on closures at the end of the manufacturing process. The tests at this stage comprise a selection, or if applicable the totality, of the following tests:

- Visual inspection of a sample of the inspected batch for the presence of *cosmetic defects*. Included in the category of cosmetic defects are only those defects that constitute a cosmetic failure and that will not influence the functional performance of the part. Cosmetic defects may be further subdivided into critical, major or minor, usually on the basis of their size. At any rate, if such subdivision is made an appropriate definition of the different classes needs to be made.
- Visual inspection of a sample of the inspected batch for the presence of *functional defects*. Functional defects are those defects that with a certain likelihood could lead to inadequate functional performance of the part. They may also be subdivided into critical, major and minor. Again, definitions of "critical," "major," and "minor" need to be established, whereby it is logical that critical defects must not be present in the sample.
- Check on a sample of the inspected batch for *dimensional compliance* with the product drawing. A distinction can be made here between product dimensions that are affected by the manufacturing operations of the part or those that are not affected by the manufacturing process. A typical example of the former class is the total height of a part; a typical example of the latter class is the depth of a product cavity that is determined by the mold dimensions only, and not by the molding operation. Finished product inspection will at least check a dimension that is affected by the manufacturing process, typically total height or flange thickness.
- Check on *functional performance*. In the case of a stopper, such tests can consist of determining coring, self-sealing, and penetration characteristics. Product specific testing may also be introduced under this heading, such as the determination of the holding force of needle shields on prefilled syringe barrels.
- Check on *surface siliconization* (for siliconized parts). This check may be carried out using a chemical analytical technique or may just consist of an assessment based on comparison with parts of known siliconization degree.
- Check on *particulate cleanliness*. Such a check includes the determination of visible and/or subvisible particulate cleanliness on a sample of the batch.
- Check on *microbiological cleanliness*. This check entails the determination of the bioburden and/or endotoxin load on a sample of the batch.
- *Chemical testing*. The manufacturer may decide to document chemical cleanliness of the material on finished product and not in-process. For coated parts, incorporating chemical cleanliness testing as part of finished product inspection testing is most logical.

Finished product inspection levels are usually taken from standards such as ISO 2859-1, "Sampling procedures for inspection by attributes—Part 1: Sampling schemes indexed by acceptance quality limit (AQL) for lot-by-lot inspection," for which the still much cited Military

Standard MIL-STD-105E has served as a basis. Both standards use the concept of "AQL" or "acceptable quality limit." The basis for the acceptance of a product batch is the occurrence of an acceptable number of defects in a statistical sample of the batch, whereas the rejection of a product batch is based on the occurrence of a number of defects that exceeds the acceptable limit. Sampling schemes, sample sizes, number of accepted defects, etc., are regulated by the standard.

Every user of elastomeric closures of course is permitted to make his own listing of defects to which he attributes acceptability or nonacceptability. A potentially useful, although not in all aspects up-to-date, reference that may be helpful in this respect is the "Defect Evaluation List for Rubber Parts," edited by Editio Cantor in Germany. This list has been compiled by a consortium of major German pharmaceutical companies that are active in parenterals.

Quality Systems

It is typical for elastomeric closure manufacturers to maintain a Quality System as per ISO 9001, "Quality management systems—Requirements." This system will usually cover their manufacturing, testing, sales and R&D activities. Apart from the normative aspects of ISO 9001, the Quality System will contain elements of current Good Manufacturing Practice (cGMP) that are typical for the pharmaceutical industry and that many times go beyond the scope of ISO 9001. Until recently every manufacturer at its own discretion included those elements that he thought were pertinent. An emphasis thereby typically was on traceability and on disposition status (released/rejected/quarantined) of raw materials, in-process materials and finished materials. A more comprehensive guideline in this respect has been offered by ISO 15378, "Primary packaging materials for medicinal products—Particular requirements for the application of ISO 9001:2000, with reference to Good Manufacturing Practice (GMP)." Certification against this relatively new standard is finding acceptance with elastomeric closure manufacturers.

STANDARDS FOR ELASTOMERIC CLOSURES FOR PARENTERALS

There are many standards that relate to elastomeric closures for parenteral use. In some cases this relation is very explicit as in pharmacopeia and ISO standards, however in some cases as FDA Guidances the relation can be less explicit. In this paragraph only a discussion of pharmacopeial sections related to elastomeric closure testing is given, as well as a listing of the most relevant ISO standards.

Pharmacopeia

There are three major pharmacopeia that impose requirements on elastomeric closures for parenterals: USP, Pharm. Eur., and Pharm. Jap. The relevant sections are USP <381>, Pharm. Eur. 3.2.9 and Pharm. Jap. 7.03. The types of tests that are contained are as listed in the table below.

	Chemical (extractables)	Functional	Biological
USP <381>	Yes As from May 1, 2009 on aqueous extract only and large degree of alignment with Pharm. Eur.	Yes As from May 1, 2009 on fully harmonized with Pharm. Eur.	Yes, through reference to USP <87> and USP <88>
Pharm. Eur. 3.2.9	Yes	Yes	No
Japanese Pharmacopeia **7.03**	Yes No harmonization with USP and Pharm. Eur.	No	Yes (hemolysis and pyrogens)

Abbreviations: USP, U.S. Pharmacopeia; Pharm. Eur., European Pharmacopeia.

ISO Standards

- ISO 247: Rubber—Determination of ash
- ISO 2230: Rubber products—Guidelines for storage
- ISO 2859-1**:** Sampling procedures for inspection by attributes—Part 1: Sampling schemes indexed by acceptance quality limit (AQL) for lot-by-lot inspection

- ISO 7619-1: Rubber, vulcanized or thermoplastic—Determination of indentation hardness—Part 1: Durometer method (Shore hardness)
- ISO 8362-2: Injection containers for injectables and accessories—Part 2: Closures for injection vials
- ISO 8362-5: Injection containers for injectables and accessories—Part 5: Freeze drying closures for injection vials
- ISO 8536-2: Infusion equipment for medical use—Part 2: Closures for infusion bottles
- ISO 8536-6: Infusion equipment for medical use—Part 6: Freeze drying closures for infusion bottles
- ISO 8871-1: Elastomeric parts for parenterals and for devices for pharmaceutical use—Part 1: Extractables in aqueous autoclavates
- ISO 8871-2: Elastomeric parts for parenterals and for devices for pharmaceutical use—Part 2: Identification and characterization
- ISO 8871-3: Elastomeric parts for parenterals and for devices for pharmaceutical use—Part 3: Determination of released-particle count
- ISO 8871-4: Elastomeric parts for parenterals and for devices for pharmaceutical use—Part 4: Biological requirements and test methods
- ISO 8871-5: Elastomeric parts for parenterals and for devices for pharmaceutical use—Part 5: Functional requirements and testing
- ISO 9001: Quality management systems—Requirements
- ISO 11040-2: Prefilled syringes—Part 2: Plungers and discs for dental local anaesthetic cartridges
- ISO 11040-5: Prefilled syringes—Part 5: Plungers for injectables
- ISO 11137: Sterilization of health care products—Radiation (3 parts)
- ISO 11608: Pen-injectors for medical use (3 parts)
- ISO 13926-2: Pen systems—Part 2: Plungers and discs for pen-injectors for medical use
- ISO 14644-1: Cleanrooms and associated controlled environments—Part 1: Classification of air cleanliness
- ISO 15378: Primary packaging materials for medicinal products—Particular requirements for the application of ISO 9001:2000, with reference to Good Manufacturing Practice (GMP)
- ISO 15759: Medical infusion equipment—Plastics caps with inserted elastomeric liner for containers manufactured by the blow-fill-seal (BFS) process

ACKNOWLEDGMENT

Thanks, Lisa, for reviewing this text!

14 Parenteral product container closure integrity testing

Dana Morton Guazzo

INTRODUCTION

The definition of container closure integrity is simply, the ability of a package to adequately contain its contents by preventing content loss or contamination. This basic description is clear and straightforward. But the concept of container closure integrity is surprisingly complicated given the variety and complexity of parenteral product dosage forms and their packaging.

The demands placed on parenteral product packaging often exceed the requirements of other dosage form containers. Clearly, all pharmaceutical product package systems must prevent content leakage or spillage. But for some parenteral product packages, product loss includes vacuum loss or escape of inert gases or solvent vapors. All pharmaceutical packages must prevent contamination from environmental dirt or debris. However, parenteral product packages must also preclude microorganism contamination. And for some parenteral products, contamination may include unwanted chemicals, even moisture, originating from the outside environment or leaching from the package components themselves.

Another complicating factor of parenteral container closure integrity is the multiplicity of parenteral package designs. For instance, many products are contained in vial package systems. A typical vial package is comprised of a glass or plastic vial or bottle stoppered with a viscoelastic closure compressed against the vial mouth and held in place via a crimped aluminum cap. Prefilled syringes and cartridges, made of either glass or plastic, are becoming increasingly popular. Such systems include a closure or plunger that must adequately contain and protect the contents but must still glide smoothly along the barrel wall at time of drug delivery. The delivery port for cartridges and syringes consists of either an adhesively bonded needle covered with an elastomeric shield, or a luer tip protected with an elastomeric or plastic closure. Flame-sealed glass ampoules were once very common, but are infrequently used for today's new products. On the other hand, plastic blow-fill-seal (BFS) ampoules often package nebulizer solution preparations. Ophthalmic solution products are primarily contained in plastic bottles with uniquely designed plastic caps for easy product use. The closure mechanisms of such bottle/cap systems often include screw-threaded closures and plug- or compression-fitted components. Larger volume intravenous infusion solutions are typically packaged in plastic bags with elastomeric ports for spike access, held together via heat seals and/or ultrasonic welds.

Taking one step back, many parenteral product formulations, and even active ingredients, must be aseptically stored prior to filling into the final product package system. Such bulk storage systems must meet critical package integrity criteria. To make matters even more challenging, finished product, bulk formulation and active substance package systems vary extensively in design and materials of construction.

Given the diversity of packages, products, and integrity requirements, it is no surprise that a universally acceptable container closure integrity test method is nonexistent. Even selecting one appropriate method for any given product package system can be daunting. Much discussion and research over the last three decades has focused on identifying and validating suitable parenteral product container closure integrity test methods for some of the more common packages. Microbial challenge tests continue to be used, although a growing number of approaches for leak testing packages by physicochemical methods are available. When validating a physicochemical container closure integrity method, debate continues on the need for a comparison study against a more traditional microorganism challenge test, how to perform such a comparison, and what should be the acceptance criterion.

Fortunately, consensus on how to evaluate the integrity of at least some parenteral product packages appears to be evolving. This chapter will attempt to introduce container

closure integrity concepts as they relate to some of the more widely used parenteral product packages, and to share new directions in finished product parenteral package integrity verification.

PACKAGE SEAL CHARACTERIZATION AND OPTIMIZATION

Package closure is effected either by physically mating package components or by chemically bonding them together. To ensure adequate container closure integrity, package design and development should include both theoretical and practical closure characterization and optimization studies. A clear understanding of critical component dimensions, materials of construction, and design enables the establishment of appropriate component purchasing specifications and quality controls. Package integrity studies during later development stages should also incorporate packages assembled according to actual or simulated manufacturing operation conditions. Containers assembled by hand or using laboratory scale equipment may not perform comparably to those assembled on automated, high speed manufacturing lines.

Mechanically Fitted Seals

Mechanically fitted components rely on precise dimensional fit, adequate compression, and/or tortuous paths for seal integrity. Therefore, component dimensions and tolerances should ensure the worse case "loosest" fit will still preclude leakage gaps, while the worse case "tightest" fit will permit successful, damage-free package assembly. Checking component dimensional specifications and tolerances provides a theoretical analysis of worse case component fit. However, package assembly line trials performed under anticipated manufacturing conditions play an important role in package integrity validation.

The vial/elastomeric closure/aluminum seal parenteral package (vial package) is an excellent example of a mechanically sealed package. The plug dimension of an elastomeric closure for a vial package should be sufficiently narrow to allow easy insertion into the vial neck, and so minimize vial breakage or closure "pop-up." Then again, some compression is necessary if the package must maintain an inert gas or vacuum atmosphere prior to aluminum seal capping. Elastomeric closure design, formulation, lubrication and polymer coatings all influence stopper insertion and closure-plug/vial-neck seal integrity. The vial throat dimension and design (i.e., absence or presence of a locking ring or "blow-back" feature) also significantly impact stoppered vial integrity and machinability. Finally, the aluminum seal height should be long enough to allow proper seal tuck under the stoppered vial flange, but not be so long that assembled packages exhibit inadequate closure flange compression. All these factors make a purely theoretical evaluation of such a package's closure mechanisms nearly impossible. Often vial, closure, and seal components are sourced from multiple suppliers making it difficult to ensure an optimally designed fit given all possible component combinations. Some pharmaceutical firms use computer modeling software to simulate closure compression during vial-neck insertion and seal capping. Certainly, such tools are useful, but the only way to be confident of a package's leak tightness is to integrity test finished containers, representing multiple component lots assembled at manufacturing line operational limits, using appropriately sensitive test methods. Reportedly, a few firms have gone so far as use vials made to worst case dimensions, and closures lubricated to either extreme for such studies.

Another example of a mechanically sealed system is the ophthalmic dropper-tip bottle with a screw-cap closure. Typically, the dropper-tip base snaps into the bottle neck creating a valve seal fitting. The other critical seal occurs where the inner top surface of the torqued cap presses down against the dropper-tip opening. Small shifts from optimum component designs or dimensions at these critical locations can have disastrous results. Plastic resin changes may affect component viscoelasticity which ultimately can also impact package integrity. For example, the screw cap may back off and/or component polymer creep may occur over time, especially upon exposure to temperature swings, shock or vibration. To ensure package integrity, assembled container leak test methods should identify leakage from these critical sealing locations. Supplier specifications and controls should be in place to ensure that molded components are made from approved materials, and that they conform to dimensional tolerance limits and to absence of defects specifications. Ophthalmic package production line

assembly trials prior to product launch can help identify unanticipated problems. For instance, marketed product-package integrity failures have resulted from incomplete insertion of the dropper tip into the bottle neck, insufficient or excessive screw-cap torque force, and gaps at the dropper-tip/torqued-cap sealing interface.

A syringe or cartridge has a mechanically fitted closure (also called a "plunger") positioned inside the syringe/cartridge barrel to prevent content leakage, yet is designed to glide smoothly with minimal resistance at time of drug delivery. The dimensions of the closure and barrel, and the closure's viscoelastic properties determine this mechanical seal's effectiveness. The amount of lubrication on the barrel wall and the closure also impacts closure performance. For this reason, studies to evaluate both syringe leakage and functionality may use components made to simulate tightest and loosest fit, lubricated and sterilized under the most challenging anticipated conditions.

Chemically Bonded Seals

Chemical bonding techniques are used for sealing various pharmaceutical packages. Heat sealing using thermal impulse or conductive heat sealers is one such technique. Examples of packages sealed in this manner include plastic bags for sterile powder storage, and barrier laminate pouches for protecting semi-permeable plastic BFS ampoules. Consistent seal strength and barrier properties rely on proper characterization and control of heat seal layer polymer composition, molecular structure, and laminate thickness. In addition, the heat sealing process critical parameters of heating, cooling, pressure and time should be controlled and monitored within optimized ranges along the entire length of the seal.

Ultrasonic welding is another well-known process used to create polymer-polymer seals for pharmaceutical packages, although other industries use this technique to bond metals to plastics or even metals to metals. Ultrasonic welding is very fast and usually produces welds relatively free of flash making it attractive in clean room settings. A welding tool transmits ultrasonic energy to the part to be bonded, causing mechanical vibration and frictional heat at the sealing interface. Rapid melting and bonding occurs at the connecting surfaces statically pressed together. Effective ultrasonic welding requires that the bonded polymer materials exhibit nearly equivalent melting points. Amorphous thermoplastics weld more efficiently than semicrystalline materials, harder materials with high modulus are also easier to weld. Thus, consistent welding requires proper characterization and control of polymer layers' thickness, composition and molecular structure. Optimization and control of ultrasonic frequency, oscillation amplitude, power level and pressures are vital, as well as the tool design used to direct energy between the welded parts.

Adhesives can also accomplish a chemical bond between package surfaces. For example, UV and visible light curing adhesives effect the bond between stainless steel needles and the tips of glass or plastic syringe barrels. Semi-rigid plastic trays used for many medical devices or drug-device combination kits often incorporate porous barrier lidding materials, such as Tyvek® or low-linting papers, bonded to the tray with a heat-activated adhesive. Well-sealed bonds depend on the adhesive's chemical composition and quality, the adhesive application process, and the curing process, as well as the nature and quality of the bonding surfaces.

Contiguous containers, such as flame-sealed glass ampoules, represent another chemical bonding process. Glass ampoules filled with product are sealed by one of two methods. In the first case, the ampoule's stem is flame-heated at the intended point of closure. As the distal tip is pulled away the stem narrows and closes. The second glass ampoule sealing process involves heating the ampoule's open end until the glass softens and closes under gravity. Ampoule seal integrity and quality is a function of several factors, including glass formulation, ampoule wall thickness, line speed, ampoule rotation speed, ampoule tip "draw" speed (if applicable), and flame heat. Typical glass ampoule defects include cracks, as well as pinholes, channels, and weak, thin-wall areas usually located at the sealed tip.

Plastic BFS ampoules, another type of contiguous container, are created, filled and sealed in one continuous, aseptic manufacturing process. Dosage forms packaged in BFS ampoules include unit-dose sterile solution products, such as nebulizer solutions and intravenous line flushing solutions. Integrity of these packages is a function of the plastic formulation and the forming/sealing parameters of time, pressure and temperature. Defects that can result in

package leakage include pinholes, thin-wall areas, and burrs or other contaminants trapped in the plastic wall.

LEAKAGE THEORY

Leakage occurs when a discontinuity or gap exists in the wall of a package that allows the passage of gas under the action of a pressure or concentration differential existing across the package wall. Leakage differs from permeation, which is the flow of matter through the barrier itself. Both leakage and permeation play vital roles in the study of parenteral product package integrity.

Permeation

Permeation is passage of a fluid into, through and out of a solid barrier having no holes large enough to permit more than a small fraction of the molecules to pass through any one hole. The process always involves diffusion through a solid, and may involve other phenomena such as adsorption, migration, solution, dissociation, and desorption. Permeation rate is a function of the permeant's concentration, its solubility in the barrier material, as well as the molecule's physical ability to migrate through the barrier.

The general equation for permeation is given by equation (1), where Q, the mass flow rate (Pa m^3/sec m^2) is a function of the permeation rate constant (K_P), which is a product of the solubility coefficient (S), and the diffusion coefficient (D). Permeation is directly proportional to A, the area normal to permeation flow (m^2), and ΔP, the partial pressure drop across the flow path (Pa), while inversely proportional to l, the path flow length (m) (1).

$$Q = K_P A(\Delta P/l) = (SD)A(\Delta P/l) \tag{1}$$

Permeation plays a role in package integrity assurance if the package must prevent loss of critical headspace gases or vacuum, restrict loss of product solvents or other permeable ingredients, or limit migration of external gases or vapors into the package. For example, small volume plastic BFS ampoules containing nebulizer solution are generally semi-permeable containers requiring a barrier laminate pouch secondary package to prevent the product from drying out over shelf life. Packages for hygroscopic lyophilized products or aseptically filled powders must limit moisture ingress from the outside environment or even from the package components themselves. Pharmaceutical products subject to oxidative degradation must be contained in packages that limit oxygen permeation. Some lyophilized products in vial packages require a vacuum headspace to help draw diluent into the vial upon reconstitution. Therefore, atmospheric gas permeation leading to loss of vacuum can make product use difficult and may cause end-users to question product quality.

Leakage Flux

Diffusion

Leakage is defined as the movement of molecules by convection plus diffusion through one or more gaps in the package barrier wall. The driving force for gas or liquid convective flow through a leak path is the pressure differential that exists across the barrier. If no pressure differential exists, only the concentration gradient of the leaking molecule existing across the barrier drives molecular flux according to diffusional flow kinetics.

Gas diffusion follows Fick's laws of diffusion (2). Fick's first law defines diffusion assuming a plane of infinitely small thickness [eq. (2)]. The negative sign means that when $\delta C/\delta x$ is positive, flux is in the direction of decreasing x or decreasing concentration.

$$J = -D(\delta C/\delta x)_t \tag{2}$$

where

J = amount of diffusion	$g/m^2 \cdot sec$
D = diffusion constant	$m^2 \cdot sec$
C = diffusant concentration	g/m^3
x = barrier thickness	m
t = time	sec

Fick's second law takes into consideration a barrier of measurable thickness, where the diffusant concentration varies across the barrier thickness and changes continually over time, thus changing the rate of flux.

$$\delta C/\delta t = D(\delta^2 C/\delta x^2) \tag{3}$$

An example of diffusional flux occurs in a parenteral vial package sealed under a nitrogen blanket. In this case, the vial interior contains a higher concentration of nitrogen and a lower concentration of oxygen than exist outside. Thus, nitrogen gas will tend to diffuse out of the vial, while oxygen will tend to leak into the vial. This tendency is especially true for stoppered vials prior to aluminum seal capping. While studies may show a stoppered vial capable of preventing ingress of relatively large air-borne microorganisms, gas molecules will readily diffuse across the tiniest leak paths.

Convection

For the most part, parenteral package integrity is concerned with fully assembled container closure systems, where measurable leakage linked to either dosage form loss or microbial ingress is chiefly convective, with little or no diffusional flow. So for the remaining discussion, unless otherwise specified, the term "leakage" refers to convective flow of gases moving from higher to lower pressure sides of a package boundary, without diffusional flux or permeation components.

Different physical laws relate leakage rate to the differential pressure gradient across the leak, the range of absolute pressure involved, and the nature of the gas moving through the leak. The five main types of pneumatic gas leak flow are turbulent, laminar, molecular, transitional, and choked flow. Approximate gas flow rates for these pneumatic modes are as follows (1):

1.	Turbulent flow	$>10^{-3}$	Pa m^3/sec
2.	Laminar flow	10^{-2}–10^{-7}	Pa m^3/sec
3.	Molecular	$<10^{-6}$	Pa m^3/sec
4.	Transitional	Between molecular and laminar	
5.	Choked	When flow velocity approaches the speed of sound in the gas	

Laminar and turbulent flow are both classes of viscous flow. Because turbulent flow is rarely encountered in leaks, the term viscous flow is sometimes incorrectly used to describe laminar flow. This chapter focuses on leakage ranging from turbulent to molecular flow—the leak rates of greatest concern for most nonporous parenteral packages. Laminar flow occurs when the mean free path length of the gas (λ) is significantly smaller than the leak path's cross-sectional diameter ($\lambda/d < 0.01$). The mean free path length is that at the average pressure within the leaking system. The leak rate (Q) follows Poiseuille's law for laminar flow through a cylindrical tube (1).

$$Q = [(\pi r^4)/(8nl)][P_a(P_1 - P_2)] \tag{4}$$

or

$$Q = [(\pi r^4)/(16nl)][(P_1^2 - P_2^2)] \tag{5}$$

where

Q = gas flow rate	Pa m^3/sec
r = leak path radius	m
l = leak path length	m
n = leaking gas viscosity	Pa sec
P_1 = upstream pressure	Pa
P_2 = downstream pressure	Pa
P_a = average leak path pressure, $\frac{(P_1+P_2)}{2}$	Pa

Molecular flow occurs when the mean free path length of the gas is greater than the cross-sectional diameter of the leak path ($\lambda/d > 1.00$). Molecular flow leak rates are defined according to Knudsen's law for molecular flow through a cylindrical tube, neglecting the end effect, as per equation (6) (2). By comparing equation (6) with equations (4) and (5), it is evident that laminar flow is a function of the leaking gas's viscosity, whereas molecular flow is a function of the gas's molecular mass.

$$Q = (3.342)(r^3/l)(RT/M)^{1/2}(P_1 - P_2) \tag{6}$$

where

Q = gas flow rate	Pa m^3/sec
r = leak path radius	m
l = leak path length	m
M = molecular weight of leaking gas	(kg/mol)
T = absolute temperature	Kelvin
R = gas constant, 8.315	J/(mol K)
P_1 = upstream pressure	Pa
P_2 = downstream pressure	Pa

Transitional flow occurs when the mean free path length is about equal to the leak's cross-sectional diameter ($\lambda/d = 0.01 - 1.00$). The equations for transitional flow can be quite complex. For further discussion on convective flux, refer to *The Nondestructive Testing Handbook* (1).

Practical Application

Package integrity research studies utilize the above equations and concepts in a variety of useful ways. For example, a leak path's nominal width can be calculated by measuring the gas flow rate through the leak (the leak rate), assuming either molecular or laminar gas flow behavior. University of Iowa researchers measured the helium leak rate through various capillary tubes embedded in the walls of glass vials to estimate these artificial defects' diameters (3).

In another example, package leakage through a hypothetical defect can be calculated and compared with actual package leakage, thus confirming the defect's absence or presence. For instance, consider a lyophilized product sealed under vacuum conditions in a stoppered/capped vial. The lower pressure conditions in the vial act to draw air into the package through any gaps present. By knowing the vial headspace volume and the absolute pressure in the package at time of capping, the theoretical vacuum loss over time due to a given-size leak can be modeled using convective flux equations. Actual headspace pressure readings below modeled predictions confirm the vial's integrity. Similarly, Fick's laws of diffusion can predict the rate of oxygen ingress into an inert gas flushed, stoppered vial as a function of a hypothetical leak. Both of these predictive models are explored more fully later in this chapter.

Leakage Units of Measure

Leakage rate is the amount of gas (mass or volume) which passes through a leak path under specific conditions of temperature and pressure. Therefore, leakage rate has dimensions of pressure multiplied by volume, divided by time. Table 1 lists several common leak rate units of

Table 1 Mass Flow Conversion Factors for Common Leak Rate Units

Pascal cubic meter per second	Standard cubic centimeter per second	Mol per second	Millibar liter per second	Torr liter per second
Pa m^3/sec	Std cm^3/sec Alternatively, sccs	mol/sec	mbar L/sec	torr L/sec
1	9.87 ($\simeq$10)	4.4×10^{-4}	1.00×10^{1}	7.50

Source: From Ref. 4.

measure. The international standard SI nomenclature is pascal cubic meter per second (Pa m^3/sec). To express leak rate in mass flow units, rather than volumetric flow units, the results must be converted to standard conditions of 101 kPa (760 torr) and 0°C (32°F). When expressing leakage volumetrically, test pressure and temperature conditions are specified.

PACKAGE LEAKAGE ACCEPTANCE LIMITS

Since leakage is the rate of gas flow through a leak path, it is meaningless to say that a package has zero leakage, or is leak-free without reference to a leak rate specification. This is similar to saying that a pharmaceutical ingredient is pure or a dinner plate is clean. These expressions are only meaningful when compared with some purity or cleanliness standard. In the same way, a leak-free package simply means the package does not leak above some acceptable leakage limit. The key to setting leak rate specifications is to select meaningful limits, while avoiding unreasonable, and costly requirements. Unnecessarily small leak rates limits will result in expensive instrumentation, increased test time, and rejection of otherwise acceptable product.

Setting realistic and useful leak rate specifications for parenteral products requires characterization of the package sealing mechanisms as well as an understanding of finished product dosage form specifications and the package's performance requirements. This enables logical and practical integrity test method selection. For example, all parenteral products must be sterile; therefore, all packages must be able to prevent liquid- and/or air-borne microbial ingress. All parenteral product packages must also contain the product, preventing loss. Thus, for liquid dosage forms the packaging must also prevent liquid leakage. Studies have shown that leaks that allow liquid flow are also at risk of microbial ingress; the larger the leak, the greater the risk. Conversely, when liquid cannot pass through a leak, microbes cannot (5–7). For this reason, leak tests capable of identifying the smallest leak paths able to contain liquid or permit liquid flow may serve to verify a package's microbial integrity. This microbial ingress/liquid leakage relationship, briefly introduced at this point, is a topic explored extensively throughout this chapter.

Some leak tests, such as helium mass spectrometry, provide test results in quantitative gas flow rate terms. Therefore, when using such methods it is important to know how gas leak rates correlate to critical package performance requirements. For example, helium trace gas leak test studies have linked gas flow rates as small as about 10^{-6} Pa m^3/sec to the smallest leaks able to permit liquid leakage plus microbial ingress (8). Leak detection texts define water-tight seals as meeting limits of about 10^{-4} Pa m^3/sec, whereas, relatively large leaks from misassembled, misshapen or damaged packages are most often above 10^{-4} Pa m^3/sec (9).

Gas headspace preservation is a practical package performance requirement linked to leakage acceptance criteria. For instance, if the product requires low oxygen container headspace content, then oxygen permeation plus air leakage must remain below a specified limit. Similarly, hydroscopic product packages must limit moisture ingress. Integrity tests that specifically monitor gas or vapor migration are reasonable options in such cases. For packages sealed under negative pressure, instruments to monitor headspace pressure are preferred.

LEAK TEST METHODS

Many leak test methods exist for testing everything from soft drink cans to vacuum pumps to heart pacemakers. Even within the relatively small world of parenteral packaging, numerous leak test methods apply (10). Rather than provide an exhaustive survey of all potentially useful leak test methods, this chapter will focus on those testing techniques having the broadest application for the most common parenteral packages, namely, vial packages, prefilled syringes, ophthalmic dropper bottles, and plastic or glass ampoules.

Microbial Challenge Methods

A microbial challenge test procedure includes filling containers with either growth-supporting media or product, followed by closed container immersion in a bacterial suspension or exposure to aerosolized bacteria or bacterial spores. Test containers are incubated at conditions that promote microbial growth, and container contents are then inspected for evidence of microbial growth. Positive challenge organism growth is indicative of package leakage.

Currently, no standard microbial challenge test method exists (10). In reality, any one of many possible microbial challenge methods may prove satisfactory as long as it is scientifically sound, given the package type and its protective function, and the product's anticipated exposure to conditions of processing, distribution, and storage. The following discussion explores factors to consider when designing a microbial challenge test.

1. Challenge mode. If a package is able to tolerate liquid immersion, then this approach is generally favored for parenteral package system testing, as it presents the greatest challenge to package seals. Aerosol challenge testing is most appropriate for packages that rely on tortuous paths, or seals not intended to prevent liquid leakage. Aerosolized challenges are frequently used in the food and medical device industries. Static testing, where packages filled with media are simply stored under normal warehouse conditions or in stability storage chambers, affords no definitive bacterial challenge and no significant pressure differential to the seals. If such long term storage of media-filled units is part of an integrity verification program, then some known bacterial challenge to the packages at the end of the storage period is appropriate.
2. Challenge parameters. Liquid immersion challenge tests preferably include vacuum/pressure cycling simulating pressure variations anticipated during product life processing, distribution and storage. These cycles will enhance flow of packaged media into any leak paths present, thus encouraging potential microbial ingress. For this reason, package position during the challenge test should ensure packaged media contact with seal areas. An aerosol challenge test chamber size and design should guarantee uniform distribution of viable aerosolized bacteria or spores around the test packages, considering factors such as chamber temperature and humidity, as well as airflow patterns and speed.
3. Challenge microorganism. Liquid challenge organism size, mobility and viability in the packaged media are important factors for consideration. Bacteria concentration in the challenge media at the initial time point should ensure a high concentration of viable organisms at the test's conclusion (e.g., $\geq 10^5$ CFUs/mL at end of test). Bacteria used in published immersion challenge studies include, but are not limited to *Escherichia coli*, *Serratia marcescens*, *Clostiridium sporogenes*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, and *Brevundimonas diminuta*. When performing aerosol challenge tests, aerosolized microorganism concentration and uniformity are important factors, as well as viability in the packaged media. Reportedly, aerosol challenge testing commonly uses *Bacillus atrophaeus* spores and *Pseudomonas fragi* microbes.
4. Growth promotion media. All challenge tests require test containers filled either with growth-promoting media or product that supports microbial growth. The product formulation itself or a product placebo is preferred as it most closely simulates the product package system. However, this may not be practical if the intention is to validate a variety of products in similar packaging. Verification of the media's growth promotion capability at the completion of the package integrity test is important, especially if the test sample holding time is lengthy.
5. Test package preparation. Two approaches are possible for preparing sterile packages for testing. Either previously sterilized package components are aseptically filled with the growth-promoting vehicle, or media-filled packages are terminally sterilized. If feasible, the sterilization procedures and package assembly processes chosen should mirror those used for the actual product. Otherwise, the test package and seal may differ in some respect from the marketed product package system. For example, vial package capped closures exhibit a certain amount of sealing force on the vial land seal surface. This residual seal force will noticeably decay upon terminal steam sterilization, thus potentially changing the seal quality (11,12). Similarly, plastic bag test samples exposed to gamma irradiation post heat sealing may not represent product bags normally sealed using ethylene oxide sterilized materials.
6. Test package quantity. There is no guarantee of microbial ingress even in the presence of relatively large defects. Microbial ingress is a notoriously probabilistic

phenomenon. For this reason, a valid test requires a relatively large population of test samples and positive controls.

7. Positive and negative controls. All leak test validation protocols, including microbial challenge tests, require positive control or known-leaking packaging in the test package population to demonstrate the test's leak detection ability. Negative controls, or so-called good packages, are also important to establish a baseline of intact package performance. Additional information on positive controls is included under a separate heading.

Microbial challenge tests have been used to verify container closure integrity for decades. However, there are problems with solely relying on this approach. First, microbial challenges, especially immersion tests, do not simulate real life, product bio-exposure conditions. Simply put, package seals are not typically soaked in media highly concentrated with microbes, while differential pressures promote liquid and microbial entrance. Yet, even under these extreme challenge conditions, the highly probabilistic nature of any microbial challenge test makes results difficult to interpret. Leak paths several fold wider than a microorganism will not guarantee microbial ingress, as numerous studies have shown (5,7,8,13). On the other hand, the rare occurrence of microbial grow-through across a package's fitted seam during an exceptionally severe biochallenge may negate the use of an otherwise acceptable container closure system, even though such a challenge does not realistically portray naturally occurring phenomena.

Conversely, inappropriately designed microbial challenge tests can easily make bad packages look good. Short exposure times; minimal or no differential pressure application; small test sample populations; and positive control packages with very large leaks all help samples with questionable seals pass a microbial challenge test, thereby falsely implying package integrity. In some cases, reliance on such tests has kept leery companies from adopting more reliable, physicochemical leak test methods, despite known product package integrity problems.

Suitably designed and executed microbial challenge tests, if used, are of greatest value during package development and early clinical research programs. Microbial challenge tests are one of the few appropriate tests for integrity verification of porous barrier materials and tortuous path closure systems. However, reliance on microbial challenge tests for most package types throughout a product's life cycle has disadvantages. Results are prone to error and the test itself consumes resources of time, space, equipment, and staff, making it much more expensive than cost of materials implies. Microbial challenge tests are not practical, for instance, for routine production lot integrity testing, for forensic investigations of recalled product, or when studying package component and assembly process variables. In addition, unless the product formulation supports microbial growth, the test cannot definitively validate the integrity of the actual product package system. Nevertheless, because parenteral packages must prevent sterility loss, microbial challenge tests will likely remain part of the package leak testing arsenal for some time to come.

Dye and Liquid Tracer Methods

A liquid tracer leak test consists of immersing test packages in a solution of either dye or other chemical tracer, then allowing time for liquid to migrate through any leaks present while pressure and/or vacuum are applied. After the liquid challenge, test packages' contents are checked for liquid leakage as evidenced by visual inspection or other appropriate analytical method. Liquid leak tests are relatively inexpensive, simple to perform and conceptually easy to understand. However, the test is destructive to the package, and results may vary considerably on the basis of several factors.

Test method parameters that promote greater liquid tracer test sensitivity include longer immersion times, increased pressure and vacuum conditions, smaller volumes inside the test package, and lower surface tension challenge liquids. On the other hand, debris in the challenge liquid may clog small leaks, and airlocks in leak paths may prevent liquid ingress. Restraining package part movement (e.g., partially filled syringes), or package expansion (e.g., flexible pouches) during vacuum exposure helps keep package internal pressure constant, thus ensuring consistent leakage driving forces.

The compatibility of the dye or tracer element with the package and its contents should be verified. Dyes may quickly fade or adsorb onto package surfaces shortly after leak testing; therefore, time gaps between testing and inspection or analysis should be limited and specified. Analytical methods for dye or tracer detection require appropriate validation. For the most reliable visual inspection results, qualified inspectors following defined inspection procedures in well-lit, controlled inspection environments are called for. Inspection procedures should dictate lighting intensity and color, inspection angle, background color(s), background luster, inspection pacing, and any comparator negative control package(s) used. Inspector qualification protocols should entail accurate segregation of packages containing trace amounts of dye from negative controls in a randomly mixed, blinded test sample population. A multisite study lead by H. Wolf demonstrated how differences in inspector capabilities and inspection environments play a significant role in interpreting dye ingress test results (14).

Numerous published leak test studies incorporate dye or liquid tracer test methods, some of which are described in section "Test Method Validation" (5,6,13). U.S. compendia (15), EU compendia (16), and ISO international standards (17) all specify methylene blue dye ingress tests for demonstrating punctured closure reseal properties. But before using such closure reseal methods for whole-package integrity testing, test parameters should be optimized and the methods validated using known positive and negative control packages. The importance of this was demonstrated in the previously cited study by Wolf et al., in which 1-mL water-filled syringes with laser-drilled defects in the barrel wall ranging in nominal diameter from 5 to 15 μm were leak tested according to the closure resealability dye ingress tests described in the U.S. and EU compendia and in ISO standards. None of these standard test methods permitted accurate identification of all defective syringes (14).

Vacuum Decay Leak Test Method

A vacuum decay leak test is a whole-package, nondestructive leak test method. Vacuum decay methods relate pressure rise, or vacuum loss, in an evacuated test chamber containing the test package to package leakage. A typical test cycle consists of placing the subject container in a test chamber, then closing the chamber and evacuating it to a predetermined vacuum level. Upon reaching this target vacuum within an allotted time segment, the test system is isolated from the vacuum source, and a short time for system equalization elapses. A defined test time segment follows for monitoring any subsequent pressure rise (vacuum decay) inside the test chamber. Rise in pressure above baseline, or background noise level, signifies package headspace gas leakage, and/or vaporization of product liquid plugging leak path(s). Total test cycle time is normally less than 30 seconds, but may vary with the test system, the product package tested, and the desired sensitivity level.

A package "fails" or "leaks" if any one of several events occurs during the vacuum decay leak test cycle. Failure modes include (*i*) failure to achieve initial target vacuum, indicative of largest leaks, (*ii*) rise in pressure above a defined reference pressure at any time throughout the test cycle, indicative of medium size leaks, or (*iii*) rise in pressure above a defined differential pressure value during the final test time segment, indicative of smallest leaks. Figure 1 illustrates these various failure modes.

The combination of test equipment, package test chamber, and testing cycle is unique to each product package system, and is identified on the basis of the package's contents (liquid or solid, with significant or little gas headspace), and the nature of the package (flexible or rigid, porous or nonporous).

Uniquely designed test chambers snugly enclose the test package, minimizing test chamber deadspace for maximum test sensitivity. Added features may be required to limit package movement or expansion during the test. For example, prefilled syringes require special fixtures to restrict plunger movement. Test chambers for flexible packages, such as bags or pouches, include flexible surfaces that conform to the package and prevent expansion that may stress package seals. Test chambers designed to test trays with porous barrier lidding have a single flexible bladder that masks gas flow through the porous barrier, allowing detection of leaks located around the seal perimeter or through the nonporous tray (19).

Test method reference parameters maximize test method sensitivity for each product package. These parameters include: Time to reach initial target vacuum, equalizing time,

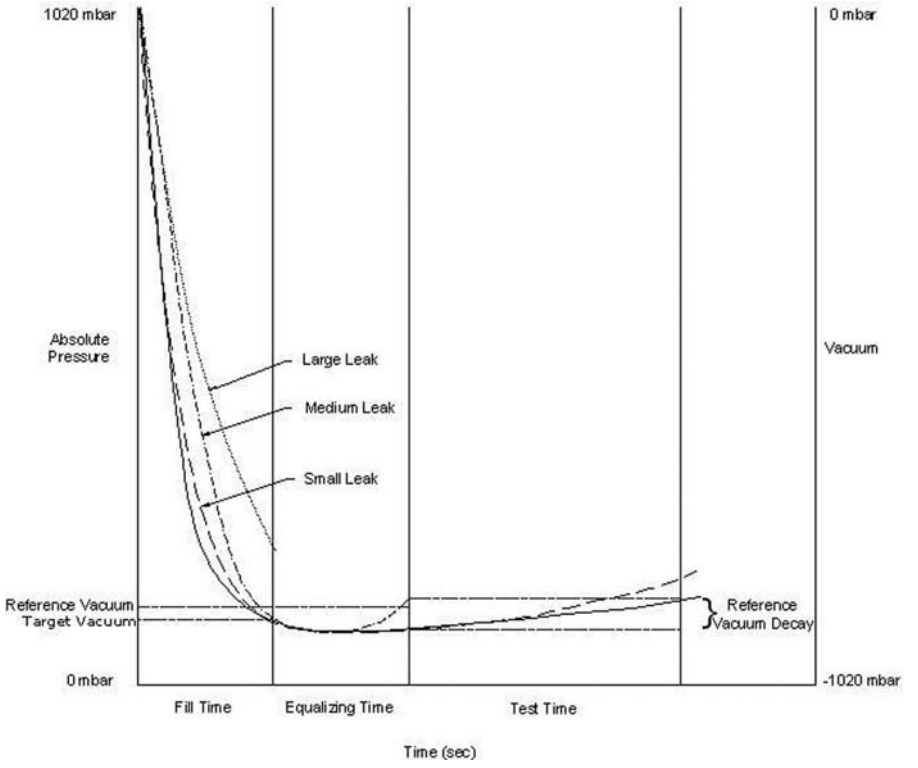


Figure 1 Pressure readings as a function of time during a vacuum decay leak test method for packages with and without leaks, according to ASTM F2338-09 *Standard Test Method for Nondestructive Detection of Leaks in Packages by Vacuum Decay Method*. *Source*: From Ref. 18.

vacuum loss test time, target vacuum level, and pressure loss limits. For instance, leaks plugged by liquid require target vacuum below the liquid's vaporization pressure, so that vaporized liquid yields a measurable rise in pressure. On the other hand, gas leaks are detectable at less severe vacuum settings. Pressure loss limits close to baseline make the test more sensitive, but run the risk of false positive test results. Generally, longer total test cycles improve test sensitivity, especially for gas leaks.

Vacuum decay leak tester designs vary among instrument manufacturers. While most models rely on a single 1000-torr gauge transducer, some instruments use a dual transducer system with either a 1000-torr gauge or absolute transducer coupled with a more sensitive, higher resolution 10-torr gauge transducer. One manufacturer that relies on the single gauge transducer approach also incorporates special software that continually readjusts the no-leak baseline to account for atmospheric pressure changes and no-leak noise variations that can affect test sensitivity. Another manufacturer is able to eliminate atmospheric pressure variation concerns and the need for calculated baseline adjustments by utilizing an absolute pressure transducer as part of their dual transducer test system (19). Automated multistation linear or rotary-style equipment enables 100% on-line testing; semi-automated or manually operated test systems with either single- or multiple-package test stations are useful for testing one or several packages simultaneously. In general, longer tests possible with off-line testers enable smaller leak detection. Thus any given vacuum decay leak test method is not only specific to the product package system, but also to the leak test instrument and its manufacturer.

Test method development and instrument functionality checks often utilize a calibrated airflow meter for artificially introducing leaks into the test chamber containing a negative, no-leak control package. Airflow meters certified by the National Institutes of Standards and Technology (NIST) or other recognized certification bodies are recommended for such

purposes. The smallest rate of airflow that triggers a significantly greater rise in pressure above background noise level is the limit of detection for the leak test. However, use of calibrated airflow standards alone is not sufficient for complete test method development and validation.

For instance, consider a grossly leaking package with very small gas headspace volume. If the time allotted for reaching initial target vacuum is too long, the headspace will be rapidly lost, preventing leak detection during the pressure rise test phase. Whereas, the same test performed using a flowmeter with unlimited gas supply will still yield test phase pressure rise despite the longest chamber evacuation times. In another example, consider a plastic bottle with a pinhole-size leak in the induction seal, beneath the torqued screw-thread cap. A proper test cycle may require additional time to draw out trapped air in the cap's threads, before leakage from the induction seal hole can be observed. This phenomenon would likely be missed if test method development only used a flowmeter for leakage simulation. Further, consider the fact that leaks simulated using a calibrated flowmeter only represent gaseous leakage and not leakage from liquid-plugged leak paths. Generally, liquids clogging leaks quickly volatilize once test pressure falls below the liquid's vaporization pressure. At this point, solvent volatilization causes a rapid rise in test system pressure, which quickly stops or perhaps fluctuates once saturation partial pressure is reached. This difference in leak behavior often requires different testing parameters when checking for gas versus liquid leaks, or some combination of both.

Negative controls used for vacuum decay test method development and validation may consist of actual no-leak packages, or they may be solid material, package-shaped models. However, at some point, tests using larger populations of actual, filled, no-leak packages will ensure the baseline represents all possible package-to-package variations. Actual leaking packages filled with placebo or product are also very useful to verify the test method's ability to find various types of leaks located at various seal locations. Prior to testing actual product packages, cleaning procedures should be in place in anticipation of test equipment contamination from leaking containers.

Two vacuum decay leak test research studies reported in the literature used Wilco AG leak test systems. For both studies test samples consisted of glass vials with micropipettes affixed into the glass vials to simulate leaks. Test package leakage was quantified using helium mass spectrometry, a leak test method previously compared with liquid-borne microbial challenge tests. In the first study, air-filled vials were vacuum decay leak tested (20). The second study evaluated vials filled with various solvents that plugged the leak paths using a so-called LFC pressure rise or vacuum decay approach. This concept required the test pressure to be substantially lower than the vapor pressure of the packaged liquid (21). LFC method test results indicated potentially greater sensitivity when testing liquid-filled vials.

ASTM F2338-09 *Standard Test Method for Nondestructive Detection of Leaks in Packages by Vacuum Decay Method* (22) is a recognized consensus standard by the U.S. Food and Drug Administration (FDA), Center for Devices and Radiological Health (CDRH), effective from March 31, 2006 (22). According to the FDA Consensus Standard Recognition Notice, devices that are affected include any devices that are sterilized and packaged. Packages that may be nondestructively tested by this method include: Rigid and semi-rigid nonlidded trays; trays or cups sealed with porous barrier lidding materials; rigid, nonporous packages; and flexible, nonporous packages.

The ASTM method includes precision and bias (P&B) statements for various types of packages based on round robin studies performed at multiple test sites with multiple instruments. P&B studies have looked at porous lidded plastic trays, unlidded trays and induction-sealed plastic bottles with screw caps. The most recent P&B studies used glass prefilled syringes. Test packages included empty syringes, simulating gas leaks; and water-filled syringes, simulating leaks plugged with liquid (liquid leaks). Laser-drilled holes in the syringes' glass barrel walls ranging from 5 to 15 μm in nominal diameter served as positive control leaks. The leak testers used incorporated an absolute 1000-torr transducer coupled with a 10-torr differential transducer, manufactured by Packaging Technologies & Inspection, LLC of Tuckahoe (New York, U.S.). Two different test cycles were explored; one with a target vacuum of 250 mbar absolute for testing gas leaks only, and another with a target vacuum of about 1 mbar absolute for testing both gas and liquid leaks. Results showed the leak tests reliably identified holes as small as 5 μm in both air-filled and water-filled syringes (23).

In summary, vacuum decay is a rapid, noninvasive and nondestructive leak test method. Depending on the test system, holes as small as 5 μm in a variety of nonporous, rigid packages are reliably detected. Vacuum decay is a practical tool for optimizing package-sealing parameters and for comparatively evaluating various packages and materials. Test methods are suitable as a stability program integrity test or as an in-process check of clinical or commercial manufacturing lots. Larger scale, on-line equipment may be used for 100% production lot testing, although leak test sensitivity is considerably less than for the most sensitive off-line instruments.

Electrical Conductivity Leak Test

Electrical conductivity testing relies on the application of a high frequency electrical current near the test package. Any liquid of greater conductivity than the package material present in or near a leak path located near the detector will trigger a spike in measured conductivity (Fig. 2). Conductivity spikes occur even if leak paths are clogged with dried product—an advantage not shared with other test methods that require an open leak path. This approach for testing liquid-filled packages has the added benefits of being extremely rapid, nondestructive and clean.

Electrical conductivity testing is appropriate for a wide variety of container closure systems, including plastic or glass ampoules, vial packages, prefilled syringes, and liquid-filled pouches. Electrical conductivity is not appropriate for testing flammable liquid products. In addition, only leak paths near detectors are identifiable; therefore, either package surfaces are checked using multiple detectors, or only the areas of greatest risk for leakage are monitored. Package rotation during testing may be required to capture defects around a package's circumference. Test method validation for a given product package requires demonstration of the test's ability to detect leaks at all likely package locations.

The electrical conductivity test, also known as high-voltage leak detection (HVLD), is widely employed for 100% on-line testing of plastic BFS ampoules and glass ampoules. Möll and colleagues described test method development and validation of an electrical conductivity test used for gel-filled low density polyethylene ampoules (24). Positive controls consisted of ampoules with laser-drilled holes positioned at the most likely zones for leaks to occur: the sealing zone at the ampoule bottom, and the top tear-off area. The voltage setting and the sensitivity or "gain" setting were the two parameters optimized to establish a window of operation that finds all defective ampoules and rejects few, if any, good ampoules. Replicate testing of a randomized population of negative and positive control test samples took place over three days. Each day of operation the HVLD test successfully "failed" all 210 positive control ampoules (150: 5–10 μm; 60: 10–20 μm), and "passed" 3830 negative controls. A dye ingress test confirmed the presence of defects in two of three so-called negative controls consistently rejected by HVLD. Therefore, the electrical conductivity test correctly identified all defective units and falsely rejected only one negative control sample.

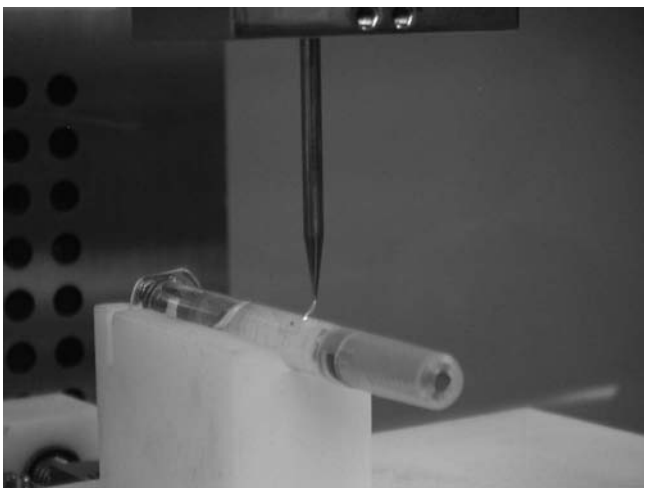


Figure 2 A glass prefilled syringe containing an aqueous liquid being tested using Nikka Densok's electrical conductivity method. Positive electrical current occurred near a laser-drilled hole in the glass barrel wall. *Source*: Courtesy of Nikka Densok, Inc., Lakewood, Colorado, U.S.

Frequency Modulation Spectroscopy

Frequency-modulated spectroscopy (FMS) is a rapid, nondestructive analytical method suitable for monitoring oxygen and water vapor concentrations as well as evacuated pressure levels in the headspace of sterile product containers. Frequency modulation spectroscopy was developed in academic and industrial laboratories in the 1980s and 1990s. Over the last 10 years, the technology has found commercial application in the pharmaceutical industry for leak detection (25), moisture monitoring (26) and oxygen monitoring (27). Systems for rapid nondestructive headspace analysis were first introduced to the pharmaceutical industry in 2000 (28), and are now routinely used in product development, process development and commercial manufacturing.

The key to these test systems are diode laser devices fabricated to emit wavelengths in the red and near-infrared regions of the electromagnetic spectrum where molecules such as oxygen and moisture absorb light. Containers made of glass (amber or colorless) as well as translucent plastics allow the transmission of near IR diode laser light and are compatible with FMS test methods.

The underlying principle of laser absorption spectroscopy is that the amount of light absorbed by a molecule at a particular wavelength is proportional to the gas concentration and the gas pressure. Therefore, FMS technology works by tuning the wavelength of light to match the internal absorption wavelength of a molecule and recovering a signal where the amplitude is linearly proportional to gas density (e.g., headspace oxygen and moisture) and the signal width is linearly proportional to gas pressure (e.g., vacuum level in the headspace of a sealed vial). Figure 3 presents a simple schematic of the FMS technique. Laser passes through the gas headspace region of a sealed package; light is absorbed as a function of gas concentration and pressure; the absorption information is processed using phase sensitive detection techniques; a mixer demodulates the radio frequency signal; the output voltage, proportional to the absorption lineshape, is digitally converted and further analyzed by a microprocessor, yielding final test results.

Examples of demodulated absorption signals for headspace oxygen, moisture and total pressure are shown in Figures 4 to 6. Figure 4 shows how the oxygen concentration in the headspace of a sterile product vial varies linearly with the peak to peak amplitude of the FMS signal. Figure 5 compares frequency modulation signals from vials filled with varying amounts of moisture. The total area is proportional to the moisture partial pressure and concentration. Figure 6 shows how the moisture laser absorption signal measures the total headspace pressure in a sealed container. As described above the moisture absorption signal width is linearly proportional to the total headspace pressure. As the total pressure rises because of a leak, the absorption signal broadens proportionately because of an increase in the collision frequency between moisture molecules and other gases. In general, measurements of higher headspace pressure require higher levels of moisture in the vial headspace.

A variety of diode laser-based system configurations can accommodate process monitoring and control and/or inspection of individual containers for oxygen, moisture or vacuum. Lighthouse Instruments, Inc., of Charlottesville, Virginia provides benchtop systems for laboratory use, as well as at-line, fully automated systems for 100% monitoring, control and

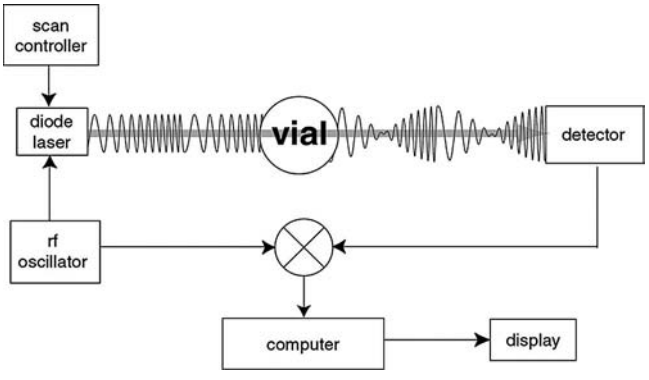


Figure 3 A schematic diagram of the frequency modulation spectroscopy technique. The frequency-modulated diode laser output is converted to an amplitude modulation after passing through a gas sample, which absorbs at a particular wavelength. The amplitude modulation is proportional to gas concentration and can be phase sensitively detected. *Source*: Courtesy of Lighthouse Instruments, Inc., Charlottesville, Virginia, U.S.

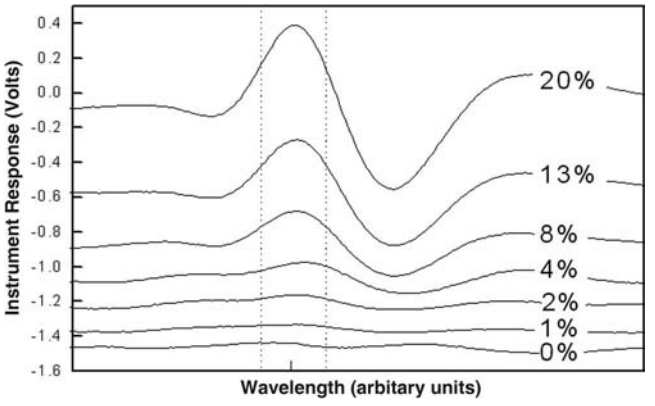


Figure 4 Frequency modulation signals from oxygen absorption. The peak-to-peak amplitude of each spectrum is proportional to oxygen concentration. *Source*: Courtesy of Lighthouse Instruments, Inc., Charlottesville, Virginia, U.S.

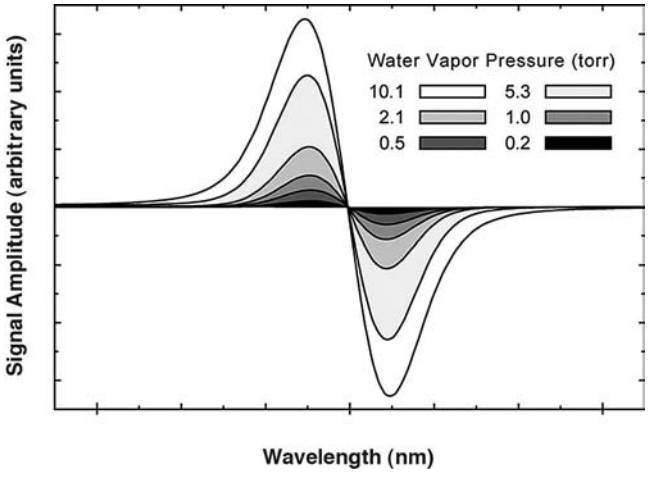


Figure 5 Frequency modulation signals from moisture absorption using 10-mL vials filled with certified amounts of moisture. Since the absorption strength of water vapor is 1000× stronger than oxygen in the near infrared, the total area of the absorption profile can be used to determine water vapor concentration. In these scans, the total area is proportional to the moisture partial pressure and concentration. *Source*: Courtesy of Lighthouse Instruments, Inc., Charlottesville, Virginia, U.S.

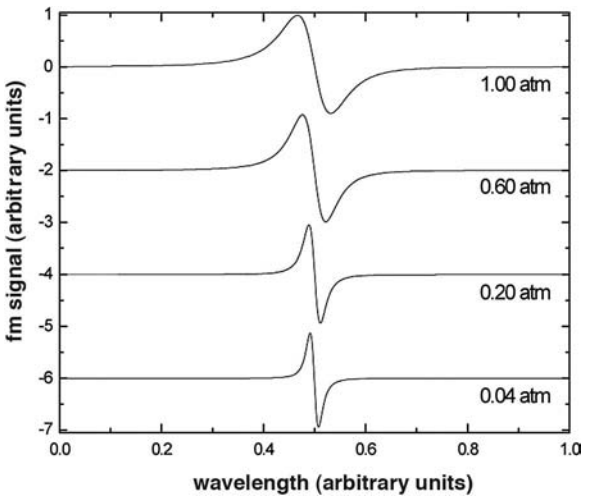


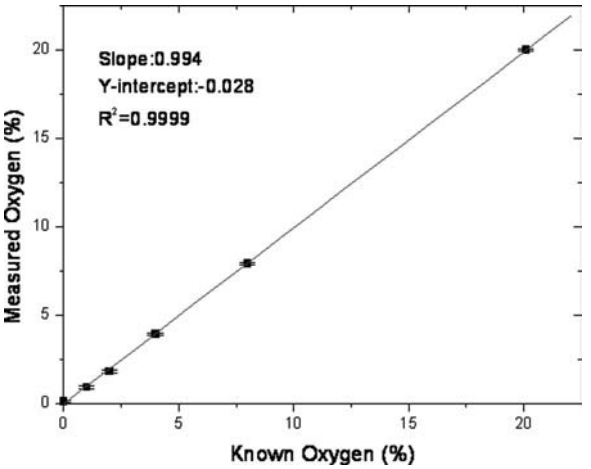
Figure 6 Frequency modulation signals due to pressure broadening of a moisture absorption signal. In principle, any molecule undergoes pressure broadening and can be used for measuring the gas pressure in a sealed container. These scans show how the absorption signal broadens as the total gas pressure increases from full vacuum to an intermediate pressure and finally to atmosphere. *Source*: Courtesy of Lighthouse Instruments, Inc., Charlottesville, Virginia, U.S.

inspection. Typical measurement times can be varied from 0.1 to 1 second corresponding to line speed throughput of 60 to 600 vials per minute. Maximum machine speeds will depend on the details of a particular application. Key parameters that impact maximum speed are container diameter and reject specification. Both faster speeds and smaller diameter packages increase measurement standard deviation.

Test systems are calibrated using NIST traceable standards of known gas concentration or pressure. Standards are constructed from the same containers used to package the pharmaceutical product, so that calibration represents containers identical to the test sample containers. For example, an oxygen-monitoring instrument would utilize standards of known oxygen concentration in containers of the same type and diameter as test sample containers. Datasets of standards measurements versus certified values enable calibration constant or calibration function generation. Subsequent measurements of unknown samples use this calibration information to convert measured absorption signals into meaningful values of headspace gas concentration and/or gas pressure. System measurement performance (method validation) is demonstrated by repeatedly testing a set of gas or pressure standards, evaluating the data following guidance in the U.S. Pharmacopeia, General Information <1225> for accuracy, precision, linearity and limit of detection (29). Figure 7 illustrates system performance data generated from 100 measurements of NIST oxygen concentration standards.

FMS offers invaluable insight for monitoring and controlling aseptic manufacturing processes. Oxygen sensitive products typically require an inert gas headspace, and lyophilized products often require either vacuum or inert gas headspace. Vial package systems, typically used for such products, cannot guarantee maintenance of inert gas or vacuum content post stoppering, prior to capping. Variations in component dimension, elastomer lubrication, gas flushing, stopper insertion, even handling, are only some of the factors that may influence the outcome. Upstream processing controls and monitors give some assurance of success, but a strong likelihood exists that some small percentage of the lot will not meet specifications. Destructive testing for either oxygen content or vacuum level using other off-line test methods is costly in terms of loss of product, and cannot provide timely information to correct a manufacturing deviation. And such test results cannot differentiate between a random glitch in the process versus system-wide failure. In contrast, FMS can be incorporated at-line for 100% automatic headspace content testing. Thus, FMS provides real-time headspace verification, enabling every unit not meeting specifications to be culled.

By testing sealed product some time post packaging, FMS technology can also verify container closure integrity, or absence of leakage. In the case of product sealed with an inert gas overlay, leakage of oxygen into the container will be a function of diffusive flow, driven by



Accuracy	0.2%
Precision	±0.08%
Linearity (R^2)	0.9999
Limit of detection	0.3%

Figure 7 Frequency modulation spectroscopy method linearity for oxygen measurement in a 10-mL vial. *Source*: Courtesy of Lighthouse Instruments, Inc., Charlottesville, Virginia, U.S.

Table 2 Time for Oxygen to Diffuse into a 10-mL Vial Container Through Holes 2 and 5 μm in Nominal Diameter

Predicted rise in package oxygen content		Time to reach predicted oxygen levels	
Partial pressure (atm)	Oxygen concentration (% atm)	5-μm hole (days)	2-μm hole (days)
0	0	0	0
0.005	0.5	<1	4
0.01	1	1	8
0.02	2	3	17
0.04	4	6	36
0.08	8	13	81

Note: Initial oxygen partial pressure is 0 torr. The defect length is assumed to be 0.1 mm. *Source*: Courtesy of Lighthouse Instruments, Inc., Charlottesville, Virginia, U.S.

Table 3 Predicted Vacuum Loss in a Leaking 10-mL Vial, Fully Evacuated Prior to Stoppering and Capping

	Package headspace pressure assuming stated leak size and laminar flow kinetics (torr)	
Time post package closing	5-μm diameter leak	2-μm diameter leak
0 min	0	0
1 min	13	2.4
5 min	63	12
10 min	126	24
60 min	756	144
5 hr	760	720
8 hr	760	760

Note: Laminar flow kinetics were modeled assuming a leak path length of 1.5 mm and air viscosity of 1.8×10^{-7} Pa·sec.

the greater oxygen partial pressure outside the container. Following Fick's laws of diffusion [eqs. (2) and (3)], assuming a 10-mL vial with initial oxygen partial pressure of 0 torr, and a length of 0.1 mm separating the vial headspace and the outside environment, oxygen ingress as a function of time can be predicted (Table 2). The results show that holes $\geq$5 μm will permit oxygen levels to rise above 1% within one day; 2-μm holes will bring about oxygen content greater than 1% after about eight days. Caution is advised, however, when attempting to predict package integrity for longer periods according to diffusion kinetics. Over time, packages are exposed to pressure differentials from changes in altitude or weather, or even by doors opening and closing, all of which drive faster, convective flux leakage, thus complicating such projections.

Consider a second scenario, in which a 10-mL vial containing lyophilized product is stoppered under vacuum. In this case, the differential pressure between the evacuated container and the atmosphere will drive air into the package according to either molecular or laminar flow kinetics, depending on the leak path diameter, the mean free path length of the leaking gas, and the package internal pressure. Table 3 presents the projected vacuum loss that will occur for a 10-mL vial initially stoppered under full vacuum (0 torr), assuming a leak path length (vial wall thickness) of 1.5 mm, and laminar gas flow leakage. Calculations assumed laminar flow [eq. (5)] and air viscosity at 15°C (1.8×10^{-7} Pa sec). Tabulated predictions show that leakage through a hole as small as 2 μm wide is evident within several minutes after package closing; vacuum is completely lost in less than eight hours. Therefore, FMR spectroscopy is reliable and sensitive approach for verifying the integrity of every evacuated container unit both upon package sealing and as a function of stability.

Trace Gas Leak Test Methods

Leak detection by trace gas analysis is the most sensitive leak test method available. Helium is the most common trace gas used for package integrity testing, although hydrogen is also used

(30,31). Detection of helium by mass spectrometry is capable of detecting large leaks of 10^{-2} Pa m^3/sec down to ultrafine leaks as small as 10^{-11} Pa m^3/sec. Helium trace gas testing is most useful for testing leaks in the moderate to ultrafine leak range. Greatest sensitivity is possible using the vacuum mode, in which a helium-flooded sealed package is exposed to vacuum conditions while inside a closed test fixture. Mass spectrometry detects helium drawn into the fixture from the leaking package. Alternatively, the sniffer mode works by scanning the test package's exterior surfaces checking for helium leakage into the atmosphere or into a special scanning fixture. The sniffer mode can pinpoint leakage location, and is especially suited for packages that cannot tolerate test vacuum conditions. ASTM F2391-05 *Standard Test Method for Measuring Package and Seal Integrity Using Helium as the Tracer Gas* describes both vacuum mode and sniffer mode techniques (32). The ASTM method text includes P&B data demonstrating the vacuum mode's ability to differentiate between cold-form aluminum foil blister packages punctured with a needle and covered with aluminum foil laminate tape (leak rate approximately 10^{-8} cc/sec/atm), to those punctured but masked with more permeable Scotch™ tape (leak rate approximately 10^{-6} cc/sec/atm).

There are possible sources of error or method interferences unique to helium mass spectrometry. Background helium present in the testing environment can mask package leaks. Steps to prevent elevated helium levels in the test area include proper ventilation, remote helium cylinder location, and proper sample isolation fixturing. "Virtual" leaks resulting from helium adsorbed onto package surfaces or trapped in seal areas can be mistaken for true leakage. "Washing" surfaces free of helium using an inert gas, or drawing off adsorbed helium by adding a preliminary vacuum cycle to the leak test are sometimes used to avoid virtual leaks. Helium easily permeates through many materials, especially plastics and some elastomers. Thus, helium permeation through the test package should be known to prevent misinterpretation of results. Care should be exercised when large leaks are suspected, as helium can be quickly lost even prior to conducting the test. Finally, sensor calibration using helium reference leaks is required to ensure accurate results.

Research teams lead by Kirsch (3,8,21) and Nguygn (20) used the helium mass spectrometry vacuum mode to measure the leak rates of positive control vials prior to microbial challenge and vacuum decay leak testing. More recently, Miyako and colleagues (33) used helium mass spectrometry for verifying the integrity of a double-bag system used for holding and transporting sterile freeze-dried powder from the bulk manufacturing site to the finished product packaging site. The bulk powder was bagged in a sterilized aluminum laminate bag which was flooded with sterile-filtered helium and subsequently sealed. This inner bag was then placed in a sterile polyethylene bag which was also sealed. The helium leak test was performed by placing the double-bagged package in a vacuum chamber. After target vacuum was reached, the vacuum source was isolated from the chamber and the double-bagged package remained under vacuum for up to one hour, allowing helium leakage to occur. The chamber was then flooded with sterile-filtered nitrogen, and a sniffer probe connected to the test chamber was used to collect a gas sample for helium detection. The helium leak test was able to find pinholes present in both bags between 20 and 500 μm in size. The size of the bag and the location of the sniffer probe inserted into the test fixture influenced leak detection.

Helium leak detection is a very useful tool for container closure integrity evaluation of packages in the research and development stages of a product's life cycle. Because some expertise is required to design and conduct leak tests by helium mass spectrometry, this technology is best performed in a laboratory setting by skilled workers. When properly performed, helium mass spectrometry provides valuable information on the quantitative leak rate of a package, as well as the package's leak location.

INTEGRITY TESTING THROUGH PRODUCT LIFE CYCLE STAGES

Changing Demands Through the Life Cycle

The scope of leak tests performed may change as a product moves through the various life cycle phases of product development, marketed product manufacturing, and marketed product stability (34). Package design and development involving seal characterization and optimization demand the most package integrity support, and may in some cases, require

multiple leak tests for verifying different performance criteria of individual seals. Once the package system and the assembly processes are well defined and controlled, leak tests used to support manufacturing practices may be able to focus on detecting larger leaks resulting from defective components or poor assembly.

For example, highly sensitive and quantititative helium mass spectrometry tests can be quite useful when characterizing a vial package system during package design and development. Helium leak test methods readily detect leaks at or below liquid leakage cut-off specifications. However, helium tracer tests take time to perform, are destructive to the package, may miss larger defects, and require considerable operator expertise, making this approach impractical during routine manufacturing. At the manufacturing stage, more rapid, nondestructive vacuum decay leak tests or electrical conductivity tests may make more sense for identifying leaks resulting from damage or misassembly.

While gas tracer or vacuum decay leak test methods are generically used for many container closure systems, other test methods are more product package specific. For example, electrical conductivity leak detection rapidly detects defects in liquid-filled glass or plastic packages, and is most useful in production environments for testing entire lots. Frequency modulation spectroscopy is ideally suited for testing vial package systems intended to maintain a low-oxygen or low-pressure headspace. This method is very rapid, highly sensitive, and nondestructive making it useful throughout all product life cycle phases, from research through 100% on-line production lot testing.

Integrity as a Function of Product Stability

Regulatory agencies around the world either imply or require product container closure system integrity verification as a function of stability to support new product market applications and to provide on-going postmarket product quality data. The U.S. FDA has issued several Guidances to Industry on this topic, discussed below.

The U.S. FDA Guidance of 1999 regarding container and closure systems for packaging human drugs and biologics (35) indicates the need for all pharmaceutical packaging to be suitable for its intended use. One aspect of suitability is protection—the ability of the container closure system "to provide the dosage form with adequate protection from factors (e.g., temperature, light) that can cause degradation in the quality of that dosage form over its shelf life." Common causes of degradation linked to package integrity cited in this Guidance include loss of solvent, exposure to reactive gases (e.g., oxygen), absorption of water vapor, microbial contamination, and contamination by filth. Package suitability verification provided in any new product submission must therefore include package integrity study results. As stated in the Guidance, "... the ultimate proof of suitability of the container-closure system and the packaging process is established by full shelf life stability studies." And later, "Stability testing of the drug product should be conducted using the container-closure systems provided in the application ... The container-closure system should be monitored for signs of instability. Where appropriate, an evaluation of the packaging system should be included in the stability protocol." Thus, integrity testing as part of stability protocols is strongly encouraged.

The U.S. FDA Guidance for Industry describing sterilization process validation submission documentation directly communicates the need to demonstrate the ability of a container closure system to maintain the integrity of its microbial barrier, and, hence, the sterility of a drug product through its shelf life (36).

More recently, an FDA Guidance for Industry addresses the issue of integrity as part of pre- and postapproval stability protocols for sterile biological products, human and animal drugs, including investigational and bulk drugs (37). As noted, manufacturers of drugs and biologics purporting to be sterile must test each lot or batch prior to release to ensure that the product conforms to sterility requirements. While stability testing must provide evidence on how the quality of a substance or product varies with time and under specific storage conditions. Stability protocols must therefore include a method(s) that supports the continued capability of containers to maintain sterility. Sterility testing satisfies this requirement; however, this newer Guidance acknowledges practical and scientific limitations for the sterility testing approach. Therefore, this Guidance allows the substitution of other integrity tests in stability protocols according to the information and recommendations spelled out.

The FDA Guidance of 2008 does not suggest specific test methods and acceptance criteria, nor does the agency provide comprehensive lists of tests. Instead, good scientific principles are recommended, taking into consideration the container closure system, product formulations, and, where applicable, routes of administration. The Guidance states, "Any validated container and closure system integrity test method should be acceptable provided the method uses analytical detection techniques appropriate to the method and is compatible with the specific product being tested. Innovative methodology is encouraged. Information submitted to the agency should detail what the test method evaluates and how it is applicable to microbial integrity. A test method is adequately validated if it has been proven through scientifically accepted studies to be capable of detecting a breach in container and closure system integrity." The selected integrity test should be "conducted annually and at expiry, or as otherwise required by applicable regulations." Both physicochemical and microbiological challenge methods are mentioned, but the onus for proper test method selection and validation lies with the product manufacturer.

Integrity as a Function of Distribution and Use

A complete package development program should include package integrity tests performed in conjunction with distribution and end-user handling challenges. Ship testing, whether simulated in a laboratory or performed in the field, provides much more meaningful data if packages are integrity tested before and after exposure to the distribution conditions. Otherwise, it becomes difficult to ascribe package damage discovered at the end of a study to the distribution challenge. Therefore, a nondestructive leak test method is best able to detect damaged product both before and after shipping.

Use testing provides valuable insight into the functionality and integrity of packages placed in the hands of the end-user. Studies comparing package use by subjects provided with careful product package usage instructions to those given no direction provide interesting and practical information that can help in final package optimization and product literature preparation. End-user populations should vary in age, sex, education, and skill level as appropriate. This is especially important for products intended for homecare administration, or for use by the elderly or physically impaired.

Production Lot Integrity Testing: 100% Vs. Statistical Process Control

The 2008 revision to Annex 1 of the European Union Good Manufacturing Practices (GMPs) for sterile products states that "Containers closed by fusion, e.g., glass or plastic ampoules should be subject to 100% integrity testing. Samples of other containers should be checked for integrity according to appropriate procedures" (38). Additionally, "Containers sealed under vacuum should be tested for maintenance of that vacuum after an appropriate, pre-determined period." Concerning stoppered vials, "Vials with missing or displaced stoppers should be rejected prior to capping." Another reference to integrity testing in the EU GMPs states: "Filled containers of parenteral products should be inspected individually for extraneous contamination or other defects." Direction is given for human inspection, and "where other methods of inspection are used, the process should be validated and the performance of the equipment checked at intervals."

The 2004 U.S. FDA Sterile Drug Products Aseptic Processing GMPs delineate similar standards (39). Referring to inspection of container closure systems, "Any damaged or defective units should be detected, and removed, during inspection of the final sealed product. Safeguards should be implemented to strictly preclude shipment of product that may lack container-closure integrity and lead to nonsterility. Equipment suitability problems or incoming container or closure deficiencies can cause loss of container-closure system integrity. For example, failure to detect vials fractured by faulty machinery as well as by mishandling of bulk finished stock has led to drug recalls. If damage that is not readily detected leads to loss of container-closure integrity, improved procedures should be rapidly implemented to prevent and detect such defects." Appendix 2 *Blow-Fill-Seal Technology* states the following: "Container closure defects can be a major problem in control of a BFS operation. It is critical that the operation be designed and set-up to uniformly manufacture integral units. As a final measure, the inspection of each unit of a batch should include a reliable, sensitive, final product examination that is capable of identifying defective units (e.g., *leakers*). Significant defects due

to heat or mechanical problems, such as wall thickness, container or closure interface deficiencies, poorly formed closures, or other deviations should be investigated in accordance with §§ 211.100 and 211.192."

USP <1207> *Sterile Product Packaging—Integrity Evaluation* discusses the issue of 100% testing versus sample testing. This general information chapter emphasizes that control of critical production processes is paramount to integrity assurance, regardless of the integrity testing approach used (34).

To summarize, mandates to leak test every product package unit released for market currently exist only for glass and plastic BFS containers. Still, the pharmaceutical manufacturer is responsible if defective, leaking containers of any type enter the marketplace. Component quality and manufacturing process control are keys to ensuring integral packaged product, but experience says that defects still occur even under the best circumstances. For this reason, it is sensible to integrity test every production lot at least on a statistical sampling basis. Upon finding leaking packages, further lot testing and a full investigation to determine and correct the cause of the defect and to eliminate other defective units are called for. As leak test methods become available for rapid and nondestructive detection of leaks in various product package systems, it is logical to expect their implementation will become standard practice.

TEST METHOD SELECTION

Integrity test method selection is based on many factors largely addressed elsewhere in this chapter. The following brief listing summarizes major selection criteria, along with a few examples.

1. Package design and construction. Rigid, nonporous packages best tolerate test methods requiring vacuum or pressure challenge conditions, such as dye ingress tests, vacuum decay tests, or the helium mass spectroscopy vacuum mode test. Flexible packages tested by such methods require special tooling to restrict significant package expansion that may damage seals or negatively influence test method sensitivity. Packages with a porous component, such as a Tyvek lidded tray, can be tested by vacuum decay as long as a test chamber fixture or other means is used to mask the porous lidding material. Packages made of permeable materials, for example, plastics or elastomers, may not accommodate trace gas testing using gases such as helium. Electrical conductivity leak detection is able to find defects in liquid-filled packages if the liquid is more conductive than the package material.
2. Seal type and location. Package seal type and location can influence test method selection. For example, ophthalmic dropper bottles have two main seals: the dropper-tip/bottle-neck valve seal and the dropper-tip/screw-cap seal. Both seals are hidden from view under a screw-thread cap making it impossible to inspect for evidence of liquid leakage at the actual seal locations. Thus, a whole-package test able to detect gas leakage, such as vacuum decay, makes more sense in this case. On the other hand, a translucent plastic bag is easily inspected for evidence of dye migration through heat sealed areas. Electrical conductivity leak detection is an excellent choice when checking physically accessible locations at higher risk for leaks, such as the seal tip end of a plastic BFS ampoule. If a seal relies strictly on a tortuous path or the quality of a porous barrier material, then microbial challenge testing may prove necessary.
3. Critical leakage rate. Seals made to prevent liquid leakage and microbial ingress require less stringent leak rate criteria than seals meant to prevent loss of vacuum or inert gas. When verifying absence of leaks $\geq$5 μm in a nonporous, rigid package to minimize risk of liquid loss and/or microbial ingress, viable options include electrical conductivity, vacuum decay, and liquid tracer tests, assuming appropriate method optimization. Frequency modulation spectroscopy is very appropriate for headspace content verification of clear or translucent packages, both upon initial sealing and over product shelf life. With appropriate fixturing and instrumentation, helium mass spectrometry is able to quantitatively measure package leaks ranging from 10^{-2} down to 10^{-11} Pa m^3/sec. However, such trace gas methods are perhaps most useful when detecting leaks not easily found with other leak test methods, namely, below about 10^{-5} Pa m^3/sec.

4. Product life cycle phase. Tests to prove a package's most critical leakage rate of concern are commonly performed during package design and development phases. Early research may also include a wide variety of tests to satisfy particular study objectives. Once package components and assembly are optimally defined, fewer test methods may be implemented to verify absence of larger, random defects or package misassembly. For example, early development of a vial package for a liquid formulation may incorporate helium mass spectrometry to verify the critical leak rate specification; a dye ingress test as a visual aid for finding package defects; and a vacuum decay test for supporting distribution and stability studies. Later in production, an on-line electrical conductivity test may check for package defects or improper assembly.
5. Regulatory and validation requirements. Region- or country-specific regulatory requirements influence leak test method selection. A parenteral product approval to market application often includes microbial challenge test data, along with sterility tests performed as a function of product stability. However, this trend is changing. A nonmicrobial method may successfully substitute for microbial challenge tests, or replace the sterility test performed through product expiry, if strong scientific rationale and validation data supporting the alternative method are provided. A study correlating the sensitivity of the alternative method to a microbial ingress test is helpful; such comparison may be theoretical or practical. Regardless, it is important to use validated test methods to support a product approval to market application or marketed product lot release. It is not adequate simply to follow an internationally recognized ISO, ASTM or compendial method. (ASTM methods typically include P&B statements based on round robin studies. These data provide a useful starting point for test method development and validation.) Even these methods require validation studies specific to the product package system, the test equipment and the test method parameters. Validation should include verification of method robustness, reliability, accuracy and range of leak sizes detected (sensitivity). Therefore, ease of method validation is also a factor in test method selection.
6. Cost versus benefit. The costs of package integrity test methods range from a few thousand to a several hundred thousand dollars, depending on the test method and its implementation. The least expensive tests include dye, liquid tracer, and microbial challenge tests, and are therefore often preferred. However, these probabilistic tests require the destruction of large test sample populations to generate the most reliable data. Conducting such tests expends resources of time, staff, equipment, and space. Human inspection processes for detecting dye or microbial ingress are especially costly, and results are prone to error. Numerous other challenges face microbial challenge test methods, as discussed in section "Test Method Validation."

   Sometimes a given test method may vary in expense as a function of the equipment manufacturer and the method's manner of application. For example, vacuum decay leak testers come as single-chamber, manually operated test systems costing tens of thousands of dollars, or as multichamber, rotary, 100% on-line systems costing hundreds of thousands of dollars, or more. The single-chamber manual systems are not well-suited for 100% testing of large lots, but they are less costly, easier to validate, and are capable of detecting smaller leaks. Each vacuum decay equipment manufacturer uses a different methodology for detecting leakage pressure rise, which then influences the validation approach and related costs. Which test system and manufacturer is most appropriate depends on many factors, including the product, the pharmaceutical manufacturer's philosophy, the nature and size of the leaks anticipated, and the quality control systems in place for incoming package components and product manufacture. Regardless, some significant investment in integrity test method selection, validation and implementation should be expected.

TEST METHOD VALIDATION

Package integrity test methods should be validated for robustness, reliability, accuracy, and range of leak sizes detected. Quantitative analytical methodology routinely relies on these test method validation concepts. But in the case of parenteral product package physicochemical leak

tests, often some assessment of the method's sensitivity to risk of microbial ingress is presumed, whether on the basis of scientific rationale or on the basis of actual laboratory studies.

Leak Test Sensitivity by Direct Comparison with Microbial Challenge Tests

How physicochemical integrity tests compare with microbial ingress tests is a topic frequently explored in publications from the food, pharmaceutical and medical device industries and academia. Generally, a population of both good and defective package units tested by both microbial ingress and the alternative container closure integrity method provide a direct comparison of the two approaches. The studies summarized below provide interesting insight on how to perform direct comparison studies, and perhaps, whether such comparisons are warranted.

About 20 years ago, the author and a team of researchers compared gas leak rates with liquid and microbial ingress from vial packages (5,40). Vials were made of stainless steel, electropolished to ensure exceptionally smooth sealing surfaces. Disc-shaped closures made of various elastomers, either uncoated or laminated with a variety of fluorocarbon- or propylene-based polymeric materials, were capped onto the metal vials at various seal forces. Test packages were mounted onto a manifold enabling them to be internally pressurized with filtered nitrogen. Package leak rates were determined by pressurizing the manifold-vial test system to target pressure, then monitoring the system's pressure drop over time. Measured gas flow rates ranged from 10^{-3} to 10^{-7} Pa m^3/sec, at 3 pounds per square inch gauge differential pressure test conditions. For the comparative microbial challenge test, each sterilized, manifold-mounted vial was filled with a suspension of *P. aeruginosa* ($\geq 3 \times 10^8$ CFUs/mL). The vial packages were submerged closure-end-down in sterile saline while being internally pressurized via the manifold. Microbial leakage into the saline was determined using a filter plate count method. In like manner, the liquid leakage test was performed by filling the vials with an aqueous solution of copper sulfate, and testing for copper ion presence in distilled water collection fluid by atomic absorption. No packages of gas leak rates less than 10^{-5} Pa m^3/sec demonstrated microbial or liquid tracer leakage. Interestingly, liquid passage occurred for every package exhibiting gas leakage at or above this rate limit, while microbial leakage only occurred sporadically, with the number of colony forming units moving across the seal bearing no relation to the gas flow rate.

In the 1990s, a team led by Lee Kirsch at the University of Iowa correlated helium leak flow rate from glass vial packages to microbial ingress and liquid leakage (8). Positive controls were made by imbedding glass micropipettes of various nominal diameters (0.1–10 μm) into the walls of glass vials. Vial package leakage was quantified by flooding open vials with helium just prior to stoppering and capping, then testing the packages using helium mass spectrometry according to the vacuum mode method. Microbial and liquid leakage through these same leak paths was determined by first filling each vial with sterile saline lactose broth. Broth-filled packages were immersed in a 60°C water bath for one hour, followed by immersion in a 25°C saline lactose broth, spiked with magnesium ion trace element, for another hour to allow the vial content's temperature to equilibrate to 25°C. The purpose of this procedure was to eliminate airlocks in the leak path. Next, the bath was spiked with 10^8 to 10^{10} viable *B. diminuta* and *E. coli* organisms/mL, and the vials continued to be immersed for 24 hours at 35°C. Post 13 days of incubation, vials were inspected for evidence of microbial growth, and vial contents were assayed for presence of magnesium tracer using atomic absorption spectroscopy.

Initially, the University of Iowa researchers only reported microbial ingress data for those test packages confirmed to contain magnesium; units failing to demonstrate a liquid pathway were eliminated from the analysis. Given these criteria, the probability of microbial ingress was near 100% at helium leak rates of about $10^{-1.9}$ std cm^3/sec (sccs), which was equivalent to about an 8-μm nominal diameter leak. An 80% probability of ingress corresponded to a leak rate of about $10^{-2.5}$ sccs (about 5 μm), and a 50% probability of ingress corresponded to a leak rate of about $10^{-3.7}$ sccs (about 0.7 μm). The likelihood of microbial failure at leak rates $\leq 10^{-5}$ sccs was remote; of the 66 test units with leak rates less than $10^{-4.5}$ sccs, only three failed the microbial ingress challenge.

Later, Kirsch used this same body of research to explore the relationship between liquid leakage verified by magnesium tracer and the likelihood of microbial ingress (6). He concluded

that both liquid leakage and microbial ingress are probabilistic occurrences. For any given leak, liquid passage was more likely to occur than microbial ingress. However, even at relatively large gas leak rates greater than 10^{-4} sccs liquid leakage at times failed to occur. Microbial ingress only occurred when liquid leakage was also present, but liquid leakage did not guarantee microbial ingress. Thus, it was concluded that microbial ingress through a leak sized at $<10^{-2}$ sccs requires liquid penetration through the leak path. And liquid leakage likely depends on variables such as liquid surface tension, defect diameter, leak morphology, leak surface conditions, environmental contaminants blocking the leak, and procedural technique.

Burrell et al. compared an ISO dye ingress method with a liquid immersion microbial challenge integrity test using vial packages (13). Positive controls were created by inserting polyimide-coated glass microtubes ranging in internal diameter from 2 to 75 μm through the elastomeric closures of 5-mL vial packages. Vials were challenged with dye solution (1% FD&C Red No. 40% and 0.25% sodium dodecyl sulfate) following procedures described in ISO 8362-2 Annex C (41). Exceptions to the ISO procedure included use of red dye, rather than methylene blue, and analysis by spectrophotometry, rather than by visual inspection. Challenge conditions included package immersion in dye solution for 30 minutes at 22 in Hg (75 kPa) vacuum, followed by rapid vacuum release and 30 minutes of dye immersion at ambient pressure. There was no attempt to eliminate airlocks in the microtubes. The microbial challenge test used positive and negative control packages, filled with saline lactose broth and immersed in an *E. coli* suspension ($\geq 10^8$ CFUs/mL), challenged according to the same ISO procedure. Results showed the dye ingress test and the microbial challenge test were equally sensitive. Dye and microbial ingress occurred in at least half the units with microtubes 10 μm in diameter. No leakage of any kind was detected in packages with smaller defects (2 and 5 μm). All units of microtubes ≥20 μm demonstrated dye leakage and microbial ingress. Therefore, the ISO dye ingress method was equally sensitive to a microbial challenge test performed according to identical challenge conditions.

Keller and team published an interesting study in 2006, further exploring the relationship between critical leak size and package sterility (7). Leaking package models were created using nickel microtubes, 7 mm long, with inner diameters of 2, 5, 7, 10, 20, and 50 μm, each placed through the elastomeric septa of a small glass cell encased in a glass water jacket. Negative controls utilized solid tubes. Sterilized test cells filled with nutrient broth were placed in an aerosol chamber with tube-end down to ensure liquid broth contact with the microtube opening. Motile *P. fragi* microorganisms were aerosolized to establish a concentration of approximately 10^6 CFUs/cm^3 during the 30-minute come-up period; static conditions followed for an additional 5 minutes. Post exposure incubation continued for 72 hours at 25°C. Test cell media turbidity was indicative of microbial growth. Special ports added to each test cell enabled the simulated packages to be exposed to various controlled pressure/vacuum/temperature conditions during the biochallenge. A randomized block design allowed independent measurement of each test variable's influence on test package sterility. Considering all test variables, results showed microbial ingress can occur through microtubes as small as 5 μm in diameter; 2-μm tubes and negative controls showed no growth in any case. Test conditions that promoted broth flow into or through the tubes correlated to higher risk of microbial ingress; the greater likelihood for liquid flow, the greater the sterility loss risk. For instance, static conditions in which no differential pressure was applied only triggered microbial ingress through two of nine tubes sized 50 μm wide. Factors that promote product liquid flow and therefore increase risk of packaged product sterility loss include defect size, liquid product surface tension and the pressures imposed on the package during processing, distribution and storage.

In conclusion, all studies described illustrate the probabilistic nature of microbial ingress through package defects. Microbial challenge tests require carefully designed and conducted procedures using relatively large test sample populations to support convincing conclusions. Numerous studies have attempted to pinpoint the critical leak size that corresponds to risk of product sterility loss. Results vary, with some studies implicating leaks as small as 0.2 μm, while others imply leak paths 10 μm and larger. Regardless, and perhaps most importantly, all research shows that liquid presence in the smallest defects is required for microbes to enter. Therefore, it seems logical that industry should move away from directly correlating

physicochemical leak tests to microbial challenge tests, to examining the leak test method's ability to detect defects capable of liquid passage—a less stochastic and more easily verified parameter.

Leak Test Sensitivity by Indirect Comparison with Microbial Challenge Tests

Literature studies describe indirect means of correlating physicochemical leak tests to risk of microbial ingress. In two publications, vacuum decay leak tests results were compared with helium trace gas detection by mass spectroscopy. Previously, the helium mass spec method had been judged against a microbial ingress test using the same test sample population type; thus establishing an indirect relationship between vacuum decay test results to risk of microbial ingress (20,21).

Another indirect comparison approach, explained under test method "*Frequency Modulation Spectroscopy*," is based entirely on gas leak rate predictions through a theoretical defect into an evacuated vial package. In the example cited, laminar gas flow theory was used to predict the pressure rise in 10-mL vial packages, initially sealed under vacuum, with leaks 2 and 5 μm wide. The text noted that as long as the actual vial package in question maintains an internal pressure at or below leaking package predictions, then no leaks of that equivalent size are present.

The works described in the previous subsection, "Leak Test Sensitivity by Direct Comparison with Microbial Challenge Tests," suggest that the presence of liquid in or moving through a leak path provides a better indication of the risk to package sterility afforded by the defect than a biological challenge test performed under the same test conditions. In fact, without liquid presence, microbial ingress through very small defects less than about 10 μm in nominal diameter appears improbable. With liquid presence or passage, sterility loss risk increases significantly. Therefore, a leak test reliably able to detect liquid passage can be indirectly assumed as good as, or better than, a microbial challenge test performed under the same test conditions.

Leak Test Sensitivity Based on Leak Rate Standards

Leak test method sensitivity may also be determined quantitatively using calibrated reference leak standards. Calibrated physical leaks are designed to deliver gas at a known flow rate. There are many types of standard leaks, falling into two main categories: (*i*) reservoir leaks that contain their own tracer gas supply and (*ii*) nonreservoir leaks that rely on tracer gas addition during testing. Calibrated gas leaks perform by one of two methods. Either the leakage rate depends on the permeation of specified materials by certain gases, or an orifice is present allowing specified gas flow rates under prescribed differential pressure conditions. Some leak test instruments, for example, helium mass spectrometry, incorporate internal reference standards to verify test system functionality.

Other leak test instruments that rely on air movement for leak detection, for example, vacuum decay testers, may utilize a calibrated variable rate flowmeter or a fixed size orifice to artificially introduce leakage into a test chamber during equipment qualification or start-up.

Whenever possible, leak test instrument performance should be challenged using such calibrated standards. The *Nondestructive Testing Handbook*, Volume 1 *Leak Testing* (42) is an excellent resource for precautions and limitations regarding calibrated leak usage. While calibrated leak standards provide valuable instrument functionality and sensitivity information, it is still important to challenge a leak test method using known positive and negative control package samples.

Positive Control Test Samples

Defect Types

Leak test sensitivity verification is not complete without a demonstration of successful leak detection using a randomized population of negative and positive control test samples. A positive control is a known-leaking test package. A common misconception is that a media-filled package used for a growth promotion check in a microbial challenge test is equivalent to a positive control test sample. A growth promotion test only proves that the packaged media can support microbial growth; it does not prove that bacteria would or could actually enter the package. Another false perception is that a calibration standard, such as a calibrated airflow

introduced into a vacuum decay leak test chamber, satisfies the need for a positive control test. Certainly, such a test is important as it correlates equipment response (pressure rise) to a known challenge (airflow rate). However, it does not prove that the method can detect leaks of various sizes or types at various locations on the package.

Simple ways commonly used to create positive control test samples involve inserting microtubes or needles through package walls, placing wires or film between sealing surfaces, or adhering thin metal plates with microholes over package surface openings. These types of defects are inexpensive, simple to create, and give a quick assessment of a leak test's capabilities. Because microtubes, microholes and needles have fixed diameters, test results infer detectable leak path sizes. On the other hand, such positive controls do not truly represent defects most likely to occur in actual product packages. Liquid or microbial migration around or through an item foreign to the package (e.g., needle, film, microhole, or microtube) may be very different from leakage through an actual defect located in or between package components.

A study by Morrical and associates illustrated this very point, by comparing helium leakage and microbial ingress through two types of defects in glass vial packages (43). One defect type consisted of a laser-drilled microhole in a thin metal plate mounted on a holed-stopper, capped on each test vial. Microholes ranged in diameter from 0.5 to 15 μm. The other leak type was a copper wire placed along the sealing surface between the elastomeric closure and the glass vial. Wire thicknesses ranged from 10 to 120 μm. Helium trace gas leakage was detected using mass spectrometry. The microbial challenge test included a suspension of *S. marcescens* ($\geq 10^8$ CFUs/mL). Challenge conditions consisted of one hour at 0.4 bar vacuum followed by one hour at 0.4 bar overpressure. Both test methods showed different leakage behavior for the two positive control types. Helium leak rates through the microholes matched theoretical predictions for gas moving through an orifice, whereas helium flow rates through the wired samples displayed complex, less predictable, gas flow dynamics. Microbial ingress occurred in at least a portion of the samples with microholes $\geq$4 μm (helium leakage rate $\geq 6.1 \times 10^{-3}$ mbar L/sec), while units with holes $\leq$2 μm ($\leq 1.4 \times 10^{-3}$ mbar L/sec) saw no microbial leakage. Microbial challenge results for hand-capped vials with wire defects demonstrated microbial leakage for wire diameters $\geq$20 μm (helium leakage rate $\geq 2.2 \times 10^{-5}$ mbar L/sec).

Whenever possible, positive control test samples should incorporate defects simulating actual leaks likely to occur. For example, typical vial package defects may include glass cracks or breaks (Fig. 8), misaligned or misshapen closures, and poorly crimped seals. Therefore, a laser-drilled hole in a glass vial wall could simulate vial breakage. Including defects positioned above and below the liquid fill level is important if the leak test method's performance is a function of liquid or gas presence in the leak path. Scoring the vial finish might represent another type of glass defect (Fig. 8). Removing slices along a closure's sealing surface, or loosely capping seals can replicate closure and seal defects, respectively. Pouch or bag positive control samples might include pinholes, open seals, channeled or wrinkled seals, weak seals, "burned" seals, and seals with trapped product inclusions. Ophthalmic dropper bottle positive controls could include loose caps, missing or poorly inserted dropper tips, defective tips or caps, and pinholes in the bottle.

With the exception of laser-drilled hole defects, the positive controls described will not necessarily provide information about the exact sizes of detectable leaks, but they will help define detectable leak locations and types. Risks inherent in this approach include the possibility that the leak test would not find all nonhole positive controls, and that the irregularities in defects' shapes or sizes may not permit statistically sound method reliability and sensitivity assessments. Nevertheless, including such positive controls in leak test method feasibility and optimization studies can provide invaluable information on the method's capabilities. Knowing this may give insight into ways of limiting the occurrence of actual defects not readily found by the chosen leak test method.

Defect Sizes

Published studies using microtubes or other artificial means to create leaks have unfortunately resulted in an expectation that all leak test methods need to detect defects as small as 0.2 μm in diameter, otherwise, the test method cannot compare to microbial ingress.

Figure 8 Defects found in glass vials. *Top row*: Line-over defect likely created during vial manufacturing process. *Middle row*: Crack in vial finish likely created during vial manufacturing or distribution to end-user. *Bottom row*: Crack in vial shoulder (*left*) and vial neck (*right*) likely created at the end-user manufacturing site. *Source*: Anonymous upon request.

The first problem with this premise is creating defects 0.2 μm in size. Experience says naturally occurring leaks in packages below a few micrometers wide are extremely rare, if they occur at all. Also, defects are not hole-shaped, but are complex tortuous paths. Even artificial laser-drilled holes through the walls of glass vials or syringes are really a convoluted matrix of capillaries and chambers (Fig. 9). Companies that laser drill holes certify their size by comparing the rate of pressurized gas flow through each hole with flow rates through standard orifices in thin metal plates. Generally, the smallest possible laser-drilled holes through small volume glass or plastic containers range from about 3 to 5 μm in nominal

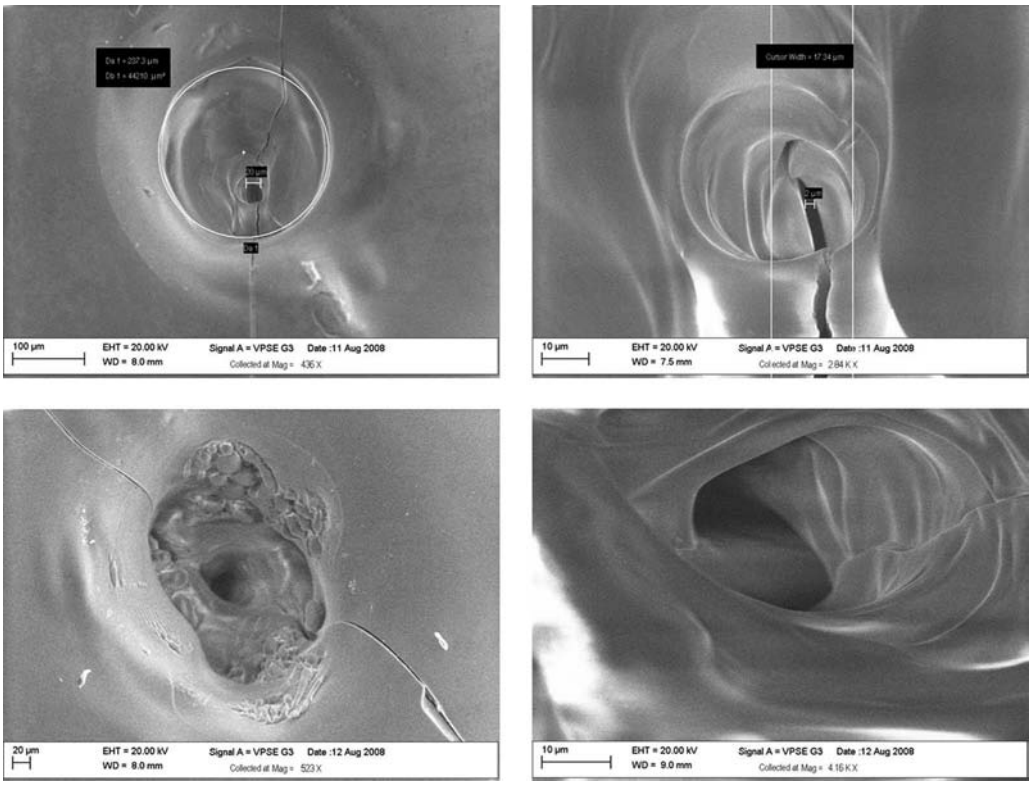


Figure 9 Scanning electron micrographs of laser-drilled holes through the glass barrels of 1-mL prefillable syringes. Each hole was nominally sized by comparing the rate of pressurized airflow passing through each hole with the flow rate through precisely formed, standard holes in thin metal plates. Nominal hole sizes are 10 µm (*top row*) and 15 µm (*bottom row*). *Source*: Reprinted from Amgen, Inc., Thousand Oaks, California, U.S.

diameter; smaller holes are difficult to make and readily clog. The smallest feasible holes through flexible laminates or films may vary from about 2 to 10 µm in diameter depending on the packaging material. Without a way of creating and sustaining holes sized below these practical limits, positive control test samples with smaller defects are not possible.

The other factor complicating this requirement is even typical microbial ingress tests cannot find 0.2-µm defects. Microbial ingress tests by Kirsch et al. (8) only found submicron-sized defects in a very small fraction of samples, under extreme challenge conditions, after meticulous measures to eliminate leak path plugs and airlocks. The risk of microbial ingress rose significantly for defects >1 µm, exceeding 80% probability for defects about 5 µm, and approached 100% probability for 8-µm defects. All defects considered in this analysis where those already confirmed as allowing liquid passage. In the absence of liquid passage, no microbial ingress occurred with any size defect (6). Research by Burrell et al. linked microtube defects ≥10 µm to a significant chance of dye and microbial ingress (13), while Keller's work using aerosolized microorganisms implicated microtube leaks ≥5 µm (7). Morrical detected microbial ingress in a portion of vial packages topped with thin metal plates having microholes ≥4 µm (43).

Therefore, positive control leaks should be as small as reasonably possible, given the type of package, the package dimensions, and the materials of construction. Parenteral product package positive control test units used for checking the lower limit of sensitivity of physicochemical leak test methods generally include defects ≥5 µm in diameter. Positive control sample populations should include larger defects as well as smallest defects, to represent the full range of anticipated leak sizes.

CONCLUSION

Container closure integrity is an easy concept to grasp. Simply put, packages must contain and protect their contents, preventing leakage in or out. However, the many parenteral product types and package integrity requirements make leak test method selection and leakage measurement anything but a simple process. First, leakage is not a straightforward, yes-or-no phenomenon. All package seals have the potential to leak gases to some extent; therefore, an understanding of leakage flux and critical leak rate specifications is necessary. When selecting leak test methods, microbial challenge tests are the traditional choice, despite their cumbersome application and demonstrated lack of reliability and sensitivity. Alternative physicochemical leak test methods are increasingly popular, including dye or liquid tracer methods, vacuum decay leak tests, electrical conductivity tests, frequency modulation spectroscopy, and trace gas detection. Each approach has unique advantages and disadvantages. Often more than one test may be necessary to provide full product support through all product life cycle phases. Any test selected must be appropriately developed, optimized and validated prior to use. Tools necessary for this process include calibrated reference leak standards, and positive and negative control test samples. The technique used to create leaks in positive control packages, and the size of these leaks, are significant factors in leak test sensitivity interpretation. Traditionally, final definition of leak test sensitivity requires some indirect or direct correlation to risk of sterility loss. Debate continues on the best approach to address this expectation, but mounting evidence supports a shift away from microbial ingress direct comparison studies. In summary, the last three decades have seen parenteral product container closure integrity move from a package testing afterthought to a major feature of product quality assessment. This evolution will likely drive the development of more reliable and sensitive package integrity test methods for future parenteral products.

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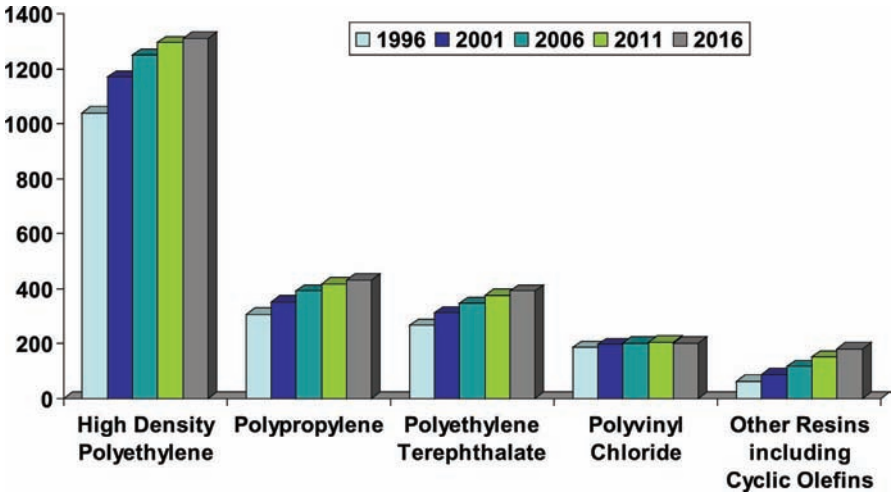

Figure 12.1 World pharmaceutical packaging plastics demand by resin (million pounds) (*see page 306*).

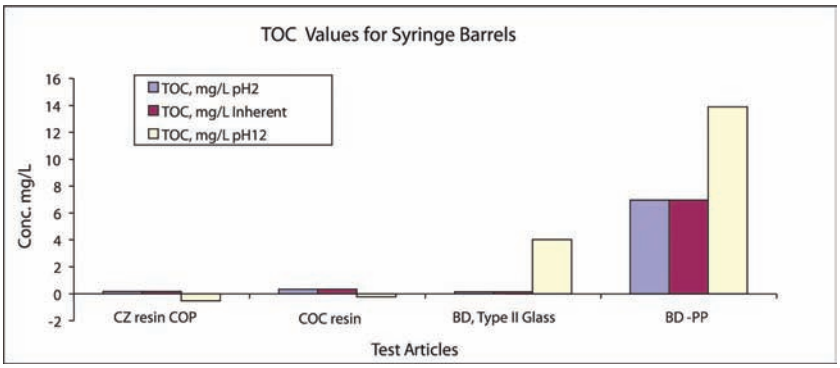

Figure 12.3 Comparison of total organic carbon as an extractable from syringe barrels. *Source*: Reproduced from Ref. 6 (*see page 307*).

Pharmaceutical Dosage Forms: Parenteral Medications

Third Edition

Volume 1: Formulation and Packaging

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ISBN 978-142008643-0

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