Handbook of Experimental Pharmacology 199

Fiona Cunningham
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Peter Lees
Editors

Comparative and Veterinary Pharmacology

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Preface

The human–animal bond has evolved and diversified down the ages. Dogs, cats and even horses, have long fulfilled the role of faithful companion and indeed, as exemplified by the introduction of seeing and hearing dogs, there may be a critical level of co-dependency between the species. In the twenty-first century, the animal types that are kept as pets in many parts of the world are extensive ranging from reptiles through rodents to ruminants and beyond. As would be predicted by the nature of the relationship, the approach to treatment of a companion animal is often closely aligned to that which would have been offered to their owner. However, an increasing awareness of welfare issues, such as the recognition that animals experience pain and the proven benefits of disease prevention in intensive farming units, together with the growth in zoos and wildlife parks, has increased the likelihood of food producing and non-domesticated animals receiving medicinal products during their life-time.

Although many of the individual drugs or classes of drugs administered to animals are the same as, or derived from, those given to man, the safe and effective use of drugs in animals often cannot be achieved by simply transposing knowledge of drug action on, or behaviour in, the body from one species to another. The impact of the anatomical, physiological and pathophysiological variability that spans the animal kingdom can often profoundly alter drug response. Thus the discipline of veterinary pharmacology, which has grown up alongside and developed from basic and medical pharmacology, has drawn from and built upon, but has sometimes had to markedly adapt data obtained from drug use for the prevention and treatment of disease in man. In compiling this volume, Springer Verlag has provided us with the opportunity to collaborate with world experts in this field in order to bring together a series of succinct overviews in some key areas of veterinary pharmacology and therapeutics. Those topics addressed in the first part of the volume (Chaps. 1–7) illustrate both the commonality and differences between drug pharmacodynamics and pharmacokinetics in animals and man, looking also to the future benefits that introduction of new technologies may bring. Those in the latter part (Chaps. 8–12) demonstrate the potential impact of drug use in animals on man and the

environment, as well as presenting the many benefits to man brought about by the genetic modification of animals.

We hope that you will enjoy reading this volume as much as we, the editors, have enjoyed our role in its creation.

October 2009

Fiona Cunningham
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Part II The Interface of Veterinary Pharmacology and Man

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Part I
Topics in Veterinary Pharmacology

Introduction

Fiona Cunningham, Jonathan Elliott, and Peter Lees

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1 From Materia Medica to Veterinary Pharmacology and Therapeutics

The origins of veterinary pharmacology and therapeutics are the same as those of the equivalent human disciplines, lying in the administration of and responses to plants and extracts of plants containing pharmacologically active compounds. The history of Materia Medica, and then the emergence of pharmacology and therapeutics in humans have been extensively described. Appelgren (2009) has provided a recent summary of both the human and parallel veterinary developments. He describes the early records contained: (a) in Egyptian papyri (1800–1200 BC), the contents of which became known only from 1822 when the Rosetta stone was translated and; (b) in the writings of the Greeks (notably Hippocrates, 430 BC) and later Galen (94 AD). Hippocrates' and Galen's prescriptions dominated European medicine for many centuries, through the medieval periods, until superseded in the Age of Enlightenment. As Appelgren points out, we can certainly conclude that

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the same "drugs" were used in animals and man up to and beyond the Age of Enlightenment. There was, however, at this time an expression of concern relating to the use of drugs therapeutically in animals on the basis of human experience. As voiced by the Swedish botanist and doctor Carolus Linneaus, "human medicines are used for animals without knowledge if they work, which is devastating barbarism". At that time much of the progress in veterinary medicine was made in France, and Linneaus sent Peter Hernquist to France to learn the scientific principles underlying veterinary medicine. In 1791, Charles Vial de St. Bel left the Lyon school to found the first veterinary teaching establishment in the English speaking world, the Royal Veterinary College in London, later to become a constituent College of the University of London.

A key development in the emergence of the science of pharmacology, from the older discipline of Materia Medica, was the progress in organic analytical and synthetic chemistry in the early to mid nineteenth century. One example will suffice to illustrate this historical development, through to the twenty-first century. The therapeutic properties of the leaves and bark of the willow tree had been described in the first century AD by Dioscorides in his pharmacopoeia. Several centuries prior to the birth of Christ, Aristotle had similarly used extracts of the willow to ease the pain of childbirth in humans. The benefits of the willow might have remained as a small historical footnote had the Reverend Edward Stone of Chipping Norton, UK not revived interest in his Philosophical Transactions to the Royal Society.

In the first half of the nineteenth century, chemists isolated from the willow a glycoside, saligenin, one component of which was shown in 1830 to be salicyl alcohol. Recognising this as the active principle of saligenin, chemists converted this to salicylic acid and then to its sodium salt. Finlay Dun (1895) described the therapeutic value of sodium salicylate in the horse and dog, for its analgesic action in joint diseases. We now know that the pathology of degenerative joint disease in these species shares many common features with that occurring in the ageing human population and that the natural wear and tear process is accelerated by extreme activity or sub-optimal conformation.

In 1898, Felix Hoffmann of the Bayer Pharmaceutical Company described the use of the acetyl ester of salicylic acid (aspirin) in his arthritic father and the next phases through to the twenty-first century led to the introduction of successive agents of the non-steroidal anti-inflammatory drug (NSAID) class. However, it was not until 1971 that Vane (1971) discovered the principal mechanism of action of NSAIDs to be inhibition of cyclo-oxygenase (COX), an enzyme which converts the substrate arachidonic acid to a range of locally acting autacoids, described under the collective term eicosanoids. Eicosanoids possess a wide range of properties that include the generation de novo of compounds such as prostaglandins (PG) E_2 and I_2, which exert crucial roles as mediators of acute inflammation, notably in the phenomenon of hyperalgesia, through both local and central actions. It was not until 1991 that the discovery of two COX isoforms, COX-1 and COX-2, led to the concept that the former was involved primarily in a range of protective roles, while the latter was involved mainly in generating inflammatory mediators.

In terms of pharmacodynamics, the veterinary history of NSAIDs has followed human developments, so that the latest phases have included the introduction into veterinary therapeutics of the dual inhibitor class of COX and 5-lipoxygenase (e.g. tepoxalin) and the preferential/selective class of inhibitors of the COX-2 isoform, the COXibs. Veterinary medicine now has five drugs of this class, either in use or currently under review by regulatory bodies, cimicoxib, deracoxib, firocoxib, mavacoxib, and robenacoxib. While the study of the pharmacodynamic properties of novel NSAIDs has followed, in qualitative terms, the developments in human pharmacology and therapeutics, it may be noted that quantitatively significant differences exist between humans and other animals, and between animal species. Thus, there are species differences in potencies for inhibition of the COX-1 and COX-2 isoforms and in COX-1:COX-2 potency ratios. An example is carprofen, which has been shown to be COX-2 preferential in the dog and cat, COX non-selective in the horse, and COX-1 selective in man (Warner et al. 1999; Lees et al. 2004).

It may also be noted that species differences in some pharmacokinetic properties of NSAIDs have been found, in general, to be minor, but for other properties the differences have been marked. Thus, most NSAIDs are highly protein bound and have small distribution volumes (Vdarea and Vdss). On the other hand, differences between species in clearance and elimination half-life are the rule rather than the exception. This is exemplified by phenylbutazone for which elimination half-life values in hours have been determined: 96 (man), 60 (cow), 18 (sheep), 16 (goat), 13 (camel), 5 (horse and dog), 3 (rat), and 2 (donkey) (Lees et al. 2004). Likewise, the pharmacokinetics of aspirin, vary markedly between species. In all species, the drug is rapidly deacetylated to salicylate (the half-life of aspirin in the horse, for example, is 9 min) and the elimination half-life of salicylate in hours varies significantly between species: 22–45 (cat), 8.6 (dog), 5.9 (pig), 3.0 (man), 1.0 (horse), and 0.5 (cow) (Lees 2009). The cat illustrates another aspect of species variability in pharmacokinetics, in that elimination of salicylate is zero order, so that the half-life increases with dose, in consequence of saturation of the elimination pathways.

The pharmacodynamic consequences of COX-2 inhibition are beginning to emerge, with adverse events being reported from the large clinical trials conducted using COXibs. The increased prevalence of acute cardiovascular events in human patients on chronic COXib treatment was unexpected and may be explained by the physiological importance of COX-2 in the endothelium. As the use of COXibs becomes more widespread in dogs, cats and horses, it seems likely that pharmacodynamic species differences, possibly affecting the gastrointestinal and renal systems, will emerge in veterinary pharmacology.

The science of pharmacogenomics is now firmly established as a sub-branch of pharmacology, with many reported differences in the pharmacokinetic properties of various drug classes between differing human racial groups. In veterinary medicine, pharmacogenomics is much less well established but, if clear differences commonly exist between racial groups in humans, differences can likewise be expected between differing breeds of, for example, dogs. The literature evidence is very limited, but clear differences have been shown to occur between mongrel dogs and

beagle dogs for the anti-epileptic drugs phenobarbitone and the NSAID naproxen, the beagle breed having the shorter elimination half-life.

In addition to the well established pharmacokinetic inter-species differences and the, although still largely un-researched, likelihood of inter-breed differences, there is the equal likelihood of intra-breed differences. Again, there are very limited available data, but the study of Paulson et al. (1999) on the COX-2 inhibitor celecoxib clearly showed that dogs of the Beagle breed could be classified into PM or EM groups, "poor" and "extensive" metabolisers, respectively. With the dog genome having now been sequenced and the high degree of relatedness that is recognised within individuals of a given breed because of genetic bottlenecks, it is likely that, in the future, the dog may be an excellent model to determine the genetic basis for particular pharmacological phenotypes that are shared between human and veterinary medicine.

The next phase in gaining increased knowledge of the pharmacology of NSAIDs in species of veterinary interest (as yet still very incomplete) is the recognition that animals in a clinical population, comprising usually many differing breeds and including also animals of varying weight and in various states of health, will almost always have a greater range of values of pharmacokinetic parameters, such as clearance and elimination half-life, which (in part) determine effective dosage schedules. In addition, in clinical populations, the means (as well as range) of values may vary from those determined in healthy animals in pre-clinical studies. This has been shown for two drugs of the COXib class, mavacoxib and robenacoxib, in dogs clinically affected with the condition of osteoarthritis. In both cases, clearance was slower and terminal half-life longer than the values obtained in pre-clinical studies in healthy animals (Cox et al. 2009; Giraudel et al. 2009; Lees 2009).

In veterinary medicine, the transition from materia medica to veterinary pharmacology occurred slowly from the early to mid twentieth century. The discoveries and introduction into veterinary therapeutics of sulphonamides, benzylpenicillin, and then the streptomycins as antimicrobial drugs, of NSAIDs such as phenylbutazone, of sedatives such as acepromazine, of volatile anaesthetics such as halothane, and of injectable anaesthetics of the barbiturate class laid the early foundations of veterinary pharmacology and therapeutics in the period 1930–1960. Nevertheless, at the time of appointment of one of the editors (PL) to the staff of the Royal Veterinary College in 1964, there remained in widespread use older drugs. These included phenothiazine (as an anthelmintic), carbon tetrachloride (as a treatment for liver fluke infestation), chloral hydrate (as a sedative), chloroform (as an anaesthetic), a range of digitalis glycosides (for the control of congestive heart failure), and organomercurials (as diuretics), all of questionable efficacy and/or low safety in clinical use.

Within the 50 year period from 1960 to 2010, veterinary pharmacology and therapeutics have been transformed. This has occurred first through major advances in understanding disease (both infectious and non-infectious) mechanisms at molecular, cellular, organ and whole animal levels and secondly (and in consequence) through the introduction of drugs with increasingly selective actions. In parallel, the identification of drug action at receptor and enzyme levels has led to

improved targeting of therapeutic agents. The veterinary pharmacologist has taken advantage of new knowledge in basic science that leads to drug discovery. Often drugs have been developed for human clinical use before they have been investigated in veterinary clinical patients. Safety and efficacy cannot be assumed to apply across species (e.g. alpha2-adrenoceptor agonist drugs lack sedative efficacy in pigs; ibuprofen has a very narrow therapeutic index in dogs) and careful study in each individual species is required. Likewise, some drugs discarded at an early stage of development for human medicine, prove to be highly efficacious and safe in some veterinary clinical patients (e.g. milrinone in treating dilated cardiomyopathy in dogs). The beneficial result has been the introduction of many novel drugs with increased efficacy and reduced toxicity for human and veterinary use. Some 95% plus of drugs now in widespread clinical use were undiscovered in 1960.

2 Aims of This Volume and Rationale for Inclusion of the Chapters

As the short introduction above illustrates, the discipline of veterinary pharmacology has evolved alongside that of human pharmacology. While the two may, on the face of it, be regarded as essentially similar, there is no doubt that species and breed differences in pharmacokinetics and pharmacodynamics can have a significant impact on the approach to, and outcome of, drug use in animals. It is also the case that between species comparative studies can of themselves inform knowledge of the properties and actions of drugs that are to be used in animals and man. Thus, the principal aims of this volume are twofold. The first aim is to illustrate those aspects of veterinary pharmacology that are unique and the second is to demonstrate the alignment between, as well as the impact on human health of, the use of drugs in animal and human populations.

In the first chapter of this text, Toutain and colleagues review a very large subject, namely the differences between species in pharmacokinetic and pharmacodynamic properties of drugs. Differences between species in drug action have been defined, for example, for COX-1 and COX-2 inhibitors (*vide supra*), but the literature is not extensive in this area and in the absence of data it is commonly assumed (no doubt often incorrectly) that they do not exist or are insignificant.

From the very extensive literature on inter-species variability in drug pharmacokinetics, on the other hand, it is clear that there are both qualitative (inability of dogs to acetylate, cats to glucuronidate drugs, etc.) and quantitative (markedly differing and commonly unpredictable differences in pharmacokinetic parameters, notably clearance and half-life) differences between species. The required dose of any systemically acting drug, for a given pharmacological or therapeutic response, is determined by two pharmacokinetic properties, clearance (Cl) and bioavailability (F) and one pharmacodynamic parameter, potency (usually expressed as 50% of maximum attainable response, EC_{50}):

$$ED_{50} = \frac{Cl \times EC_{50}}{F},$$

where ED_{50} is the dose providing 50% of the maximum response.

As the veterinary clinician has to deal with seven major (horse, dog, cat, pig, cow, chicken and sheep) and many more minor species (including several fish species), it is clear, from the likely inter-species variability in pharmacokinetics and the possible differences in pharmacodynamics, that doses must be set on an individual species basis. Similar considerations apply within a given species, in relation to possible inter- and intra-breed differences. Also reviewed in this chapter are factors such as individual animal versus group/herd treatment, the former being the norm for companion animal species, such as horse, dog, and cat, and the latter being common in farm animal, fowl, and fish therapeutics. This subject is further reviewed in the contribution by Benchaoui. The origins of inter-species, inter-breed, and inter-animal differences are explored in detail. An interesting example, very clearly distinguishing some animal species from humans is coprophagia, a practice which can lead to "a second dose" of drugs. Special considerations apply also to drug action and disposition in poultry and fish species, in consequence of anatomical and physiological differences from mammals, as well as species specific disease conditions. It is predictable that future advances in pharmacogenetics and pharmacogenomics, together with the results of population pharmacokinetic and pharmacodynamic studies, will lead to increased knowledge of mechanisms causing inter-species differences and thereby facilitate the design of more rational dosage regimens.

Mosher and Court extend the basic concepts outlined in the contribution of Toutain and colleagues. Comparative and veterinary studies of pharmacogenomics are providing a novel basis for explaining inter-species, inter-breed and even intra-breed differences in both pharmacokinetic and pharmacodynamic properties of drugs. While still at an early stage of development, animal pharmacogenomics has as its ultimate goal the design of dosage schedules that are optimal for sub-groups (breeds, young vs. old animals, etc.) or even provide individualised dosing regimens. Thus, P4502D15 and P4501AZ polymorphisms in beagle dogs explain the identification of sub-groups classified as poor (PM) or extensive (EM) metabolisers. The well-defined slower metabolism of thiobarbiturates in Greyhounds compared to mixed-breed dogs is consistent with a lack, in Greyhounds (and related breeds), of one or more P450 isoforms. It is likely that this is due to lower expression of CYP2B11.

Important pharmacogenomic variations have also been described for the transporter enzymes, *P*-glycoprotein (*P*-gp) in dogs and mice. The importance of *P*-gp was discovered when knock-out mice lacking MDR-1 gene were treated for a parasitic infestation with ivermectin. These mice died showing neurological signs, whereas the wild type controls showed no adverse signs. A veterinary pharmacologist read the report of this study and wondered whether a polymorphism in the gene explained the sensitivity of Collie types of dog to therapeutic doses of ivermectin used to treat mange mites in the dog (Mealey et al. 2002). This proved to be the case. Pharmacogenomic variation in dogs of *P*-gp, an efflux transporter

located in intestine, liver, kidney, and brain, involving loss of function, leads to increased brain penetration of certain drugs of which ivermectin is the most studied.

Brayden and colleagues review the many aspects of drug delivery either from sites of administration to the circulation or from the circulation to sites of action. A wide range of species specific devices and product formulation approaches to drug delivery are now used in veterinary medicine. These are designed to deliver therapeutic agents at either constant or variable rates for pre-determined times to suit a wide range of species and disease conditions and to meet the needs of both individual animals and herds. This chapter compares and contrasts the technologies and formulations used in animals with those used in humans. The greatest differences are between farm animal and fish species and humans. Thus, in farm animal medicine, where repeated administration of drugs to grazing animals throughout the season is impractical, much use is made of slow release technologies. A single administration can provide relatively constant drug levels over weeks or longer and is equivalent to constant intravenous infusion. Other devices have been designed to provide pulsatile release of anthelmintics such as oxfendazole, the time between the pulses corresponding to just less than the pre-patent period of the nematodes ensuring ingested eggs develop into susceptible stages but are eliminated before they produce eggs which further contaminate the pasture.

In veterinary therapeutics, there are many examples of topical formulations designed to deliver drugs, e.g. insecticides locally for external parasite infection control as well as for systemic absorption through the application of skin patches (e.g. the opioid analgesic fentanyl) or the use of "pour on" products which deliver drugs, e.g. anthelmintics transdermally. An interesting complication of this route is the self-licking and licking of companions leading inadvertently to drug delivery orally as well as transdermally, with the unintended consequence of increased inter-animal variability in dosage received.

Brayden and colleagues also address the rapidly increasing interest in the role of endogenous drug transporters in regulating drug delivery. Expression of ABC transporters confers multi-drug resistance in tumour cells and the resistance of bacteria to antimicrobial agents. Other roles, for example, as efflux transporters in enterocytes and blood–brain barrier endothelial cells, are being actively researched, as is their potential as a cause for drug interactions.

Benchaoui reviews the economic and welfare requirements for, and means of, implementing population medicine in the veterinary care of both livestock and companion animals. The aim of population medicine is the control, and ideally eradication, of infectious and parasitic diseases, in order to ensure the welfare, health, and productivity of livestock through the implementation of whole herd strategies. While control is commonly achieved through the use of vaccines, there remains a major role for the use of chemotherapeutic agents, particularly anthelmintics and antimicrobial drugs, for the treatment of worm and bacterial diseases, respectively.

Population medicine is now a huge discipline and this chapter therefore focuses on selected examples. A major area of concern is the gastrointestinal and liver parasitisms of grazing cattle and sheep, requiring strategically timed anthelmintic medication, a subject also considered in the immediately preceding chapter. An

interesting aspect of increasing importance is the growth of organic farming, wherein the use of anthelmintic drugs is generally restricted. Some cases of poor management on organic farms have resulted in avoidable clinical parasitism in sheep. Other parasitisms in dogs and cats require differing therapeutic approaches.

The two other examples discussed in this chapter relate to bacterial diseases, one of adult cattle and one of calves and young pigs. The pathogens causing intramammary infections in adult dairy cattle are many, but they are primarily bacteria, thus requiring antimicrobial drug treatment while animals are lactating and prophylaxis for prevention at "drying off". In both circumstances, drugs or drug combinations providing a broad spectrum of activity are required, but in the lactating animals products with a short duration of action are infused into the udder, commonly twice daily for 1–3 days, while in the "dry period" the formulations are designed to maintain antimicrobial levels over several weeks.

The second example of bacterial infection leading to major welfare and production loss issues are the pneumonic conditions which affect calves and piglets. Causative pathogens include viruses and bacterial species, including *M. haemolytica*, *P. multocida*, and mycoplasma species. Infections are commonly mixed. As there are no effective antiviral drugs, therapy is directed against the bacterial and mycoplasma species which either induce disease or are secondary opportunistic pathogens. Drugs of several classes are used and much research has been directed to optimising pharmacokinetic properties through the use of drugs which accumulate in lungs in high concentrations. Several antimicrobial drug classes possess anti-inflammatory and/or immunomodulating properties and there is debate on the extent to which the host effects of, for example, macrolide drugs contribute to therapeutic success.

A common concern arising from the use of both anthelmintic and antimicrobial drugs is the emergence of resistance. In the case of antimicrobial drugs, this concern relates not only to the loss of efficacy in treating animal diseases, but also the possibility of spread of resistance from animals to man, and indeed, from humans to animals also. This topic is also dealt with in greater detail in the chapter of Martinez and Silley.

Several chapters in this text review the impact on the pharmacokinetic and pharmacodynamic properties of drugs arising from species differences. These differences have been addressed through experimental studies in the major veterinary species. However, extending the studies to exotic, wildlife, and zoo species is, in many instances, impractical and economically not feasible. On the other hand, the approach of "trial and error" to selecting drugs and predicting pharmacokinetic and pharmacodynamic properties and hence dosage schedules for such species is scientifically unsound. Hunter reviews the anatomical and physiological differences between species, in relation to their likely impact on the pharmacokinetics and pharmacodynamics of drugs and describes the approaches that have been made to prediction of species variability. The simplest but almost invariably flawed approach is to base dose on body weight, irrespective of body size; this assumes a linear increase in dose with increasing body weight. Allometric scaling is a superior alternative, based on a log–log relationship, first applied in the 1930s to relate metabolic functions to body size.

The chapter by Hunter illustrates the "successes" and "failures" in extrapolating clearance data (a) between mammalian and avian species, (b) between avian species and (c) between various "large" animal species. Drugs which have been found generally to be good candidates for allometric scaling include carbenicillin, diazepam and prednisolone. However, this list is outnumbered by drugs such as paracetamol, fentanyl and xylazine which have been shown to be poor candidates.

Livingston reviews current thinking on mechanisms of pain and its relief in a wide range of animal species, including the human animal. While there are differences of detail, the neuronal pathways, transmitters and receptor types are broadly similar in all mammals and probably also in all vertebrates. However, a crucial difference between man and other animals is the ability of the adult human in most circumstances to describe through verbal communication their perception of pain, including its type, intensity and duration. Therefore, both the evaluation of pain and its relief through the actions of analgesic drugs is assessed in non-human animals differently, by behavioural responses. The problems in studying pain intensity and drug-based alleviation of pain relate not only to the absence of verbal communication, but also to the fact that differing pain stimuli often produce different responses within a species. Moreover, for a similar stimulus, the behaviours vary markedly between species.

In assessing pain, it is necessary to recognise that humans and their close relatives vocalise and display marked escape behaviour and enlist peer support with the objective of avoiding or ameliorating pain. Vocalisation is also characteristic of dogs, whereas animals living in large groups and possibly subject to the attention of predators may react in a less overt manner and this has been regarded as an important element in their survival strategies. In circumstances such as these, should an animal display overt signs of pain, its ability to escape would be reduced. Therefore, the behavioural responses used to assess pain and analgesia differ between the monkey and the sheep and so on.

Many of the pain behaviours used in experimental and clinical pain therefore involve subjective and species dependent assessments, made using semi-quantitative indices of severity (e.g. numerical rating scales) or continuous scales (e.g. visual analogue scales). However, there is increasing use of more objective indices, for example, for assessment of joint pain through the use of force plate analyses.

Riviere offers informed speculation on the manner and extent to which new technologies could transform veterinary therapeutics over the next 30 or so years. All futuristic predictions necessarily comprise a risk exercise, but Prof Riviere is well placed to undertake this task through his own background in technology transfer. Whether at all and to what extent the predictions are realised will depend on several interacting factors, which themselves contain unknowns and uncertainties, relating to societal demands and trends as well as economic issues. To what extent will global financial crises stifle development? Will the global warming consequences of emission of methane and the high conversion cost of generating calorific protein in the cow spell its total or partial demise? These considerations notwithstanding, this chapter reviews the nature of, and potential for, application to

veterinary pharmacology and therapeutics of six areas of endeavour, emphasising the potential for interaction between them:

(a) Further advances in computer technology
(b) Microfluidics
(c) Nanotechnology
(d) High-throughput screening
(e) Increased control and targeting of drug delivery
(f) Increased knowledge of pharmacogenomics

The last two of these fields are further discussed in the chapter by Brayden and colleagues and the developments in pharmacogenomics are also addressed in the contribution of Mosher and Court.

The first transforming factor, computer technology, will certainly play a prominent role in veterinary pharmacology and therapeutics through, for example, even more sophisticated programmes of population pharmacokinetics and pharmacokinetic–pharmacodynamic modelling of data, and similar parallel developments in veterinary toxicology can be anticipated. There will also be further advances in integrating genomic and proteomic data with physiologically based pharmacokinetic models. These models will lead towards development of dosing schedules on individual herd and sub-group bases, as also discussed by Toutain and colleagues.

Microfluidic devices have arisen through computer processor miniaturisation and advances in microscale engineering; these devices allow complete analytical platforms to be contained on the size of a postage stamp. The prospect is for linking these devices to implantable, feed-back controlled, drug delivery devices. Further, these devices may be powered by endogenous ionic substances to energise the internal batteries. The selection of antimicrobial drugs might be facilitated by devices which identify specific genetic determinants of resistance.

Nanotechnology utilises manufactured materials which are less than 100 nm across one dimension and possess unique physical properties. The potential is for the use of nanomaterials as drug carriers targeted to selected organs/tissues, reducing dose and increasing drug safety. The therapy of cancers is the area of greatest promise. Beyond that, they may be used to create artificial ribosomes and even wholly manufactured cells. The cautionary note is that the toxicology of nanomaterials remains to be fully defined.

Wells reviews the use of genetically modified animals in research and the benefits deriving from increasing knowledge of genomes in domestic animal species. Genetic manipulations have been (and will increasingly be) used to investigate specific gene functions, to provide models of human diseases, to treat inherited and spontaneous diseases, and to increase resistance to disease. This chapter traces the history of genetic modifications in pharmacological research, considers recent advances such as the development of induced pluripotent stem cells for increasing the efficiency of producing gene targeted domestic animals, and reviews future prospects. While genetically modified mice have been crucial in understanding gene function, especially in relation to human diseases, there have been (on a lesser scale) transgenic farm animal developments to provide resistance

from disease and to generate nutritionally enhanced food, as well as for facilitating xenografting.

A vast number of murine models of human disease have been developed, which have been invaluable for testing novel therapeutic agents. Knock-out mice have also played important roles in furthering understanding of a wide range of physiological and pathological mechanisms. Nevertheless, mouse models are not without limitations and large animal models, or naturally occurring diseases in veterinary clinical patients, may, in some circumstance, provide more appropriate alternatives.

From the perspective of future advances in veterinary therapeutics, the development of sequencing the genomes of several major veterinary species may be noted. For example, the horse genome is now well mapped and includes approximately 1.5 million single nucleotide polymorphisms from a range of breeds. There are many examples of future prospects for gene therapy for diseases of companion and farm animals. In the field of inherited and acquired diseases, domestic animals are destined to increase in importance for testing genetic-based therapies. They may also themselves be the target for such therapies. Of the domestic species, the dog provides the most useful models of human disease and also provides a source of spontaneous diseases which can be used to assess gene therapies.

Transgenic rabbits, goats and sheep have been developed to synthesise potentially high value biopharmaceutical products. To date, success in this field of "pharming" has been limited. However, one product, recombinant human antithrombin III, has received regulatory approval. A future growth area is likely to be the introduction of transgenic cattle producing humanised polyclonal antibodies for the treatment of pathogens that mutate rapidly.

The development of resistance of bacteria and other microbes to the actions of antimicrobial drugs progresses inexorably and causes considerable concern. As Martinez and Silley indicate, "the global impact of a shrinking therapeutic arsenal has precipitated numerous efforts to track the emergence and prevalence of resistance".

In veterinary medicine, those responsible for the development, licensing, and therapeutic use of antimicrobial drugs have to consider not only the problems relating to ineffective treatment of microbial-based infectious diseases in animals but also the possibly overstated concern arising from the transfer of resistance from animals (or animal food products) to man. Martinez and Silley review all aspects of resistance with emphasis on underlying mechanisms, monitoring programmes, and the impact of clinical use on its emergence and, in particular, with the pharmacokinetic concepts of mutation selection window and the integration of pharmacokinetic and pharmacodynamic data to provide indices, such as Cmax:MIC and AUC:MIC ratios and T>MIC, which are widely used as a rational basis for selecting doses designed to minimise opportunities for the emergence of resistance. The mechanisms of resistance emergence at the molecular level are many but all involve an alteration to proteins synthesised by bacterial cells. The definition of resistance is no simple matter; bacteriologists, pharmacologists, epidemiologists, and clinicians all have their views. There is the added consideration of distinguishing between resistance and tolerance. In this chapter the key consideration has been to consider the innumerable factors which may contribute to resistance and the importance of

selecting dosages which minimise its emergence. The role and importance of biofilms and quorum sensing signalling systems between bacterial cells are also reviewed, as is the role in eradicating bacteria of the immunomodulatory and anti-inflammatory actions on the host exhibited by some classes of antimicrobials.

The therapeutic use of drugs in food producing animal species may lead to residues of drugs and their metabolites. Drug residues in edible fluids and tissues of food-producing animals have become an issue of major public health concern and are now consequently a major consideration in the licensing of drug products for veterinary use. Drug residues comprise a major link between veterinary therapeutics and the health of humans. Reeves outlines the framework for undertaking the food safety risk analysis. This involves four components: hazard identification, hazard characterisation, exposure assessment, and risk characterisation. Dietary risk is defined by the relationship, risk=hazard $\times$ exposure, where risk is the probability of harm for the consumer, hazard is the chemical residue (drug and/or metabolite) in edible tissue and exposure is the dietary exposure of the residue.

As yet, there is no harmonisation at international level governing how residues of drugs and their metabolites should be regulated. However, the guidelines of many jurisdictions work on the principle of maximum residue limits (MRLs) which are set on the basis of a no observable effect level (NOEL), usually defined in laboratory animal chronic toxicology studies, but also involving other possible data sources. This is then translated, using an appropriate safety factor, into an amount of drug plus metabolite residue, which can be consumed daily by humans over a lifetime without causing appreciable risk to human health, the Acceptable Daily Intake (ADI). Some regulatory authorities distinguish between the risks associated with short-term acute toxicity and consumption over a lifetime, enshrined in concepts such as acute reference dose (ARfD). The exposure assessment is on the basis of consumption daily of an assumed diet.

Following risk assessment, regulatory authorities undertake risk management procedures, the essential element of which is setting a withdrawal time, which is the time between last administration of product and the time when an animal can be safely slaughtered for food. Compliance with the withdrawal period is required to provide assurance on safety and there are therefore in place residue surveillance programmes. As well as ensuring consumer safety, drug residues legislation and testing is an important facilitating element in international trade of animal derived foodstuffs. There are several future challenges in relation to residues. They include the need for international harmonisation and close liaison between producers of drug products, regulators and surveillance assessors.

Boxall discusses the release of drugs and their metabolites into the environment and the impact this may have on wildlife, insects, etc., arising from the use of veterinary medicines (both legally and non-legally). The classical example of chemical impact on, and persistence in, the environment is DDT, not a veterinary medicine but a pesticide which, nevertheless, was shown to have a devastating impact on many species of wildlife (notably birds). Companies marketing veterinary medicines are required by regulatory authorities to undertake not only edible tissue residue studies but also environmental risk assessments. This is required to

provide assurance that the impact of veterinary medicinal products and their metabolites on aquatic and terrestrial organisms (including man) is absent, negligible, or at the very least acceptable.

Entry into the environment may arise in several ways; for example, during the manufacturing process, but more commonly from excreta discharged into the environment following livestock treatment and use in aquaculture. Following entry into the environment, the fate of drugs {and their metabolites} should be established in several ways, namely the initial sorption into soil, and then the subsequent persistence in and transport within soil systems. The latter requires data to establish leaching to ground water, run off, and drainflow. Other aspects of fate of excreted drugs and their metabolites include the presence in surface waters and the uptake by biota.

There is finally the question of impact of discharged veterinary residues on the environment, including the possibility of risks to human health. Human exposure can potentially occur through crop consumption or through contaminated ground and surface waters. Estimates of exposure through these indirect routes (as opposed to the direct exposure through consumption of food from treated animals) have, to date, given reassurance on risks to human health.

The environmental impact on non-human organisms is established in studies required by regulatory authorities, and effects are generally classified as chronic or subtle. This chapter cites three classic examples of why vigilance is required in monitoring and assessing the environmental impact of veterinary residues: avermectins and terrestrial and aquatic vertebrates; antimicrobial drugs and soil dwelling microorganisms; and diclofenac and the fate of vultures. The veterinary (but generally non-licenced) use of the NSAID, diclofenac, to treat cattle in India and Pakistan provides an especially dramatic example of the potential hazards to human health. Subsequent vulture feeding on contaminated carcasses led to large scale deaths of the birds through renal failure and visceral gout, thus leading to a decline in the populations. Carcasses are also consumed by feral dogs and as the vulture population has declined the feral dog population has risen. It may be noted that feral dogs are a main source of rabies in India. An estimate has been given of thousands of human deaths through this sequence of events.

Doping as an important aspect of abuse of drugs is of major concern in both human and veterinary sports medicine. It involves the illegitimate use of drugs or any compound with pharmacological activity with a view to altering (enhancing or diminishing) performance. Anti-doping policy is designed to prevent such use of such substances. Several veterinary species are involved in competitive sports and the use of drugs to modify performance is therefore a concern, primarily in all equine competitive sports and dog racing but also, perhaps less obviously, in camel racing and even in bull fighting in some countries. Toutain deals with pharmacological, performance and control issues, relating especially to the horse. There is the key issue of medication versus doping control, between which a clear distinction must be made, as medication is given for the benefit of the animal and withholding drugs required for therapeutic reasons might be unacceptable on welfare grounds.

Once the use of drugs in doping has been clearly identified, there is in place a sophisticated set of procedures and principles for decision taking, which is now operative, at least in relation to the horse. The detection and quantitation of illegal substances is based on sensitive methods of analysis (usually GC-MS or LC-MS) of biological fluids, such as urine and plasma, the results of which are linked to pharmacological considerations of drug potency. The key question is, is it possible to select a breakpoint concentration of active drug which is low enough to guarantee that, despite its detection and quantitation at low concentrations, pharmacological actions will not be exerted to a degree that would alter performance? This is the scientifically acceptable alternative to what has been called "the zero tolerance rule". This new approach attempts to define for each drug an irrelevant urine or plasma concentration, on the basis of pharmacokinetic/pharmacodynamic principles which allows for the detection of therapeutically useful drugs in low concentration. Nevertheless, the zero tolerance rule still applies to compounds which do not have any defined therapeutic uses in the horse. For therapeutic agents, guidance (based on pharmacokinetic principles) is offered to stakeholders on the duration of detection time in plasma or urine following administration of a clinically recommended dose. This duration comprises a withdrawal time before competing. For some drugs and some jurisdictions, there are permitted levels of drugs which do not have to meet the requirement of irrelevant plasma or urine concentrations.

Currently, the major challenges in horse doping control relate to the scientific basis for limiting the illegal use of recombinant biological substances, such as erythropoietin and growth hormone, with potentially long-lasting effects, while parent compounds are not detectable for more than several days. Innovative bioanalytical approaches, using molecular tools, are now being developed to address these issues. The application of proteomic and metabolomic techniques will be used, among others, to address current and future challenges.

The main matrix currently in use for analysis is urine. Actually, plasma is a more robust matrix but is generally not used for practical reasons. The use of faeces and hair (mane or tail) have also been proposed; the latter is of interest as it provides a stable bioenvironment, in which drugs have been identified and quantified years after their administration. In pigeon racing, faeces (actually a mixture of urine and faeces) are the matrix used. Finally, it is to be hoped that increasing levels of international harmonisation will be reached on veterinary doping issues. There are current differences between jurisdictions.

In summary, this volume contains a series of chapters that can each stand alone as a state of the art review but which also, together, serve to illustrate current knowledge of key topics in the speciality of veterinary pharmacology and how the discipline links to, and interfaces with, that of human pharmacology.

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Species Differences in Pharmacokinetics and Pharmacodynamics

Pierre-Louis Toutain, Aude Ferran, and Alain Bousquet-Mélou

Contents

Abstract Veterinary medicine faces the unique challenge of having to treat many types of domestic animal species, including mammals, birds, and fishes. Moreover, these species have evolved into genetically unique breeds having certain distinguishable characteristics developed by artificial selection. The main challenge for veterinarians is not to select a drug but to determine, for the selected agent, a rational dosing regimen because the dosage regimen for a drug in a given species may depend on its anatomy, biochemistry, physiology, and behaviour as well as on the nature and causes of the condition requiring treatment. Both between- and within-species differences in drug response can be explained either

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by variations in drug pharmacokinetics (PK) or drug pharmacodynamics (PD), the magnitude of which varies from drug to drug. This chapter highlights selected aspects of species differences in PK and PD and considers underlying physiological and patho-physiological mechanisms in the main domestic species. Particular attention was paid to aspects of animal behaviour (food behaviour, social behavior, etc.) as a determinant of interspecies differences in PK or/and PD. Modalities of drug administration are many and result not only from anatomical, physiological and/or behavioural differences across species but also from management options. The latter is the case for collective/group treatment of food-producing animals, frequently dosed by the oral route at a herd or flock level. After drug administration, the main causes of observed inter-species differences arise from species differences in the handling of drugs (absorption, distribution, metabolism, and elimination). Such differences are most common and of greatest magnitude when functions which are phylogenetically divergent between species, such as digestive functions (ruminant vs. non-ruminant, carnivore vs. herbivore, etc.), are involved in drug absorption. Interspecies differences also exist in drug action but these are generally more limited, except when a particular targeted function has evolved, as is the case for reproductive physiology (mammals vs. birds vs. fishes; annual vs. seasonal reproductive cycle in mammals; etc.). In contrast, for antimicrobial and antiparasitic drugs, interspecies differences are more limited and rather reflect those of the pathogens than of the host. Interspecies difference in drug metabolism is a major factor accounting for species differences in PK and also in PD (production or not of active metabolites). Recent and future advances in molecular biology and pharmacogenetics will enable a more comprehensive view of interspecies differences and also between breeds with existing polymorphism. Finally, the main message of this review is that differences between species are not only numerous but also often unpredictable so that no generalisations are possible, even though for several drugs allometric approaches do allow some valuable interspecies extrapolations. Instead, each drug must be investigated on a species-by-species basis to guarantee its effective and safe use, thus ensuring the well-being of animals and safeguarding of the environment and human consumption of animal products.

Keywords Pharmacokinetics · Pharmacodynamics · Species variation · Drug administration · Drug disposition

1 Introduction

Veterinary medicine faces the unique challenge of having to treat many types of animals, including livestock, companion animals, working animals, sports animals, laboratory animals, and some invertebrates such as honeybees. Most are domesticated species, but exotic animal species are also kept as pets (reptiles, amphibians, birds) and may therefore require treatment. In veterinary medicine, the main

challenge is not to select a drug but rather to determine, for the selected agent, a rational dosing regimen (involving dose rate, inter-dosing interval, duration of treatment and modalities of administration), because the dosage regimen for a drug in a given species may depend on its anatomy, biochemistry, physiology, and behaviour, as well as on the nature and causes of the condition requiring treatment. Hence, major biological differences impacting on dosage exist between animal species. In addition, within some species there may be considerable differences within and between breeds in pharmacokinetic (PK) and pharmacodynamic (PD) profiles; veterinary pharmacogenetics is a new branch of veterinary science which aims to identify genetic variations (polymorphisms) as the origin of differences in the drug response of individuals within a given species. These between- and within-species differences in drug response are largely explained by variations in drug PK and PD, the magnitude of which varies from drug to drug.

This chapter highlights selected aspects of species differences in PK and PD and that considers underlying physiological and patho-physiological mechanisms in the main domestic species for which a useful review has been published (Baggot and Brown 1998). A text book (Baggot 2001) entitled "*The physiological basis of veterinary clinical pharmacology*" provides numerous examples of interspecies differences in drug disposition and effects together with their physiological basis. Questions relating to zoological pharmacology have recently been reviewed (Hunter 2009) and will not be covered in this chapter.

2 Diversity of Species and Breeds of Interest for Veterinary Medicine

There are more than 40 domestic livestock species. The World Watch List for Domestic Animal Diversity [WWL-DAD:3; (FAO 2000)] issued by FAO provides inventories of the species and breeds of the domestic animals used for food production. Of this number, 13 species contribute to most of the world's food and agricultural production and are of veterinary interest. The evolutionary inter-relationships and population size of these domestic mammalian and avian species are summarised in Fig. 1. Some species are classified as major and others as minor species by the regulatory agencies in Europe and the US (Table 1). It should be stressed that some species classified as minor by Western country scientists, such as buffaloes and goats, have a worldwide population large enough to confer a status of "major" species on such animals. For example, there are about 150 million buffaloes and 800 million goats in the world. The physiological characteristics of these different species reflect adaptations that have evolved over the last millennia, not only to promote their survival in local environments but also as a consequence of their usage by man. This explains why these 13 predominant species have evolved into genetically unique breeds. A breed is defined as a group of animals having common ancestry and certain distinguishable characteristics developed by artificial selection and maintained by controlled propagation (Fleischer et al. 2008).

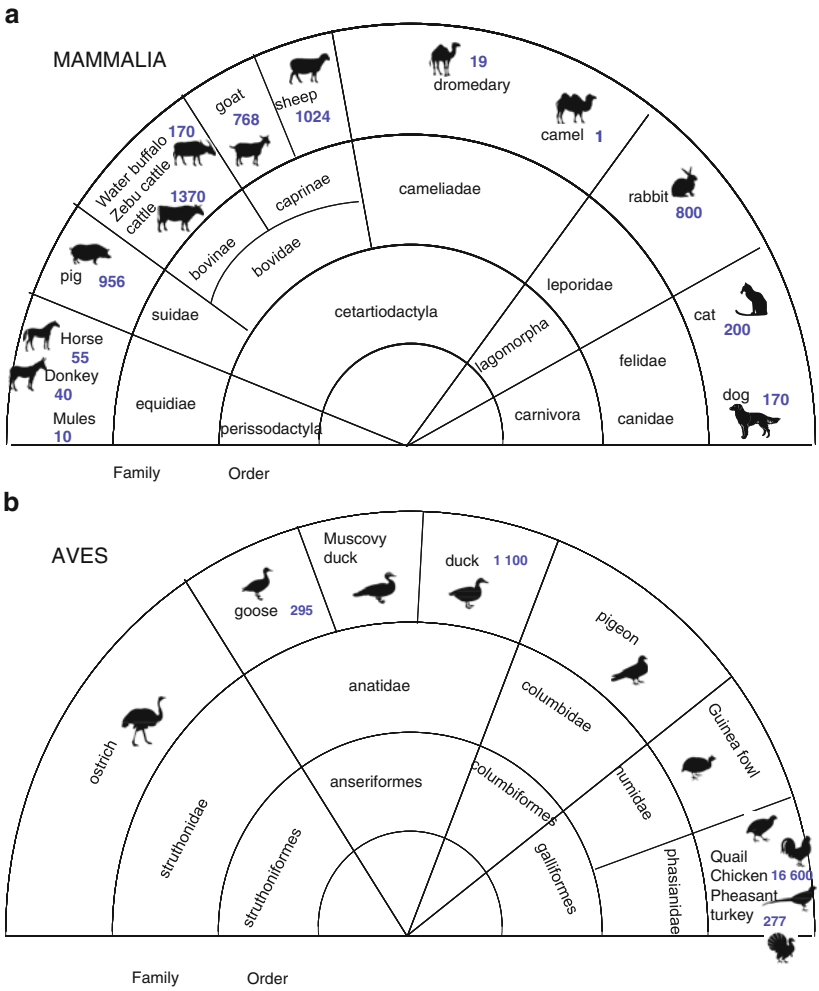

Fig. 1 (**a**, **b**): Species of veterinary interest with the worldwide size ($\times 10^6$)

For domestic species, about 400 of the 6,000 identified breeds are of economic relevance for meat, milk, and egg production.

Dogs were the first animal species to be domesticated and subsequently they have been selectively bred for thousands of years. This has resulted in a wide variety of more than 400 breeds worldwide with differing anatomical, physiological, and behavioural traits. Consequently, it can be inappropriate to generalise PK and PD drug properties to "the dog". For example, certain dog breeds carry a mutation in the ATP-binding cassette (ABC) gene ABCB1, encoding for the efflux transporter P-glycoprotein (PgP). This results in impairment in the barrier function of the blood–brain barrier with the result that these animals are more sensitive to

Table 1 Major and minor species (EMEA/CVMP 2003) in the EU and the USA

Major food-producing species for MRLs	Cattle (dairy, meat animals)
	Sheep (meat animals)
	Pigs
	Chicken (including laying hens)
	Turkey (USA but not EU)
	Salmonidae
Major non-food-producing animals	Cats
	Dogs
Minor food-producing species for MRLs	Other ruminants (bovidae including caprinae and their milk, deer, reindeer)
	Sheep (dairy)
	Other avian species and their eggs
	Other fish species
	Other mammalian species (horse, rabbit, dromedary)
	Honey bees
Minor non-food-producing species (distinguished from wildlife, exotic species in the USA)	All other species used as pets

certain antiparasitic agents, such as the ivermectins, and to morphine derivates, such as loperamide. The dog is the first species for which genotyping was performed to select (or to avoid) drugs that are substrates of PgP (Mealey 2009). There is much less genetic diversity in cats, because they have been domesticated more recently and they were not selected for purposes other than to be a "home" animal.

Recent selection processes have accelerated the development of diversity within the most productive breeds, leading to the creation of distinct strains primarily in high input systems such as for poultry and also for laboratory animals (dogs, rodents). Strains are an artificially created variety of descendants from a common ancestor that have been developed with the aim of improving some special morphological or behavioural characteristics, or enhanced performance. This is not without consequences for the PK and PD properties of drugs. For example, it has been shown that the plasma clearance of celecoxib, a selective cyclooxygenase (COX)-2 inhibitor, can be 2.5-fold higher in some strains of beagle dogs than in others (Paulson et al. 1999). This is of major concern because beagle dogs are usually selected for regulatory purposes as the model breed in pre-clinical trials to determine the initial drug dosage regimen in dogs.

The multiplicity of species of veterinary interest with their large interspecies differences in PK and PD prompted a generation of pharmacologists to attempt to establish some universal "law" or, more modestly, to develop some modelling tools to extrapolate PK and even PD parameters between species. Allometry is one of these systematic approaches; it is the study across species of the influence of body size on the numerical values of PK or PD parameters. Allometry may be useful in drug development to provide a first estimate of a dosage regimen but it is only usefully predictive if the differences between animal species are of a quantitative rather than a qualitative nature. Most drugs are not scalable across multiple species (Riviere et al. 1997) and there can be no replacement for experimental PK data. The same

conclusion holds for birds (Hunter et al. 2008). For a review including applications to veterinary species, see Mahmood (2005) and Hunter's Chapter of this book.

Physiologically based pharmacokinetic modelling (PBPK) is another approach to the extrapolation of data from one species (usually a major species) to another (usually a minor species). A key element in this type of model is to make explicit the biological system-specific properties i.e. anatomical, physiological, and biochemical factors determining the disposition of the drug in question. Then, transposition of the values of parameters (as, for example, blood flow, organ weights, tissue/serum partition, or *in vitro* estimated metabolic rate) from the known species to a second species enables a simulation to be carried out of what might be the drug disposition profile in the second species. Another application of PBPK to food animals is prediction of the rate of depletion of residues from edible tissues (Craigmill 2003).

3 Origin of Interspecies Differences in Modalities of Drug Administration

Differences in the modalities of drug administration across species result from anatomical, physiological, and/or behavioural differences. It may also depend on animal and management husbandry procedures, as in the case for food-producing animals, for which treatments are often collective. In cattle, the udder has a single large teat canal for each quarter leading to a single large teat cistern and this enables the entire mammary quarter to be treated conveniently by intramammary infusion. In horses, intramammary infusion is also possible but it should be considered that each teat has two streak canals leading to two separated lobulo-alveolar tissue systems with no communication between the two halves of the udder. In dogs, the nipples have several fine openings (7–16) preventing local drug administration.

Intramuscular administration is a popular modality of drug administration in veterinary medicine and apparently no major differences exist across species in their skeletal muscular systems, so that no major differences are expected between species for the bioavailability of intramuscularly injected drugs. In poultry, however, the site of administration should be carefully selected (generally the pectoral muscle is chosen), because the bioavailability of a drug injected into the leg can be low or even null due to a first-pass effect in the kidney. This is because the kidney in birds is of a reptilian type with a renal portal system draining the lower regions of the body. Reptiles should also be injected in the anterior portion of the body when a drug is eliminated by the kidneys (Hunter 2009). For subcutaneous administration, it is reasonable to assume considerable similarity between species regarding the local tolerance of formulations. However, it should be noted that cats are specifically prone to the development of localised fibrogranulomatous reactions to injectable vaccine products, producing vaccine-induced sarcomas as a result of malignant transformation of the fibroblastic cells associated with the prolonged inflammatory reaction (Séguin 2002).

Oral administration is the most natural route of drug administration and there are many examples of interspecies differences in the modalities of oral administration that are linked to some aspects of feeding behaviour. There are also many examples illustrating that a rational drug formulation can take advantage of some species–specific factor. This is the case for some oral formulations developed for dogs and cats. Pets are often difficult to medicate due to owner inability or reluctance to administer injectable formulations, and for some chronic treatments (for example, cardiovascular therapy, alternate day corticotherapy) only the oral route is a realistic option. Administration of oral preparations such as tablets or capsules to dogs or cats by inserting a tablet directly in the mouth requires owner skill; in a survey of 95 dog owners, it was reported that only 44% achieved 100% compliance in administering an oral antibiotic treatment for 10 days (Maddison 1999). Thus, with increased use of chronic medications in dogs and cats, there was a need for highly palatable solid oral dosage forms which would be voluntarily accepted either from a feeding bowl or from the outstretched hand of the pet owner (Thombre 2004). This became feasible due to specific carnivore behaviours regarding attitude to new food.

During evolution, food behaviour adaptation allowed animals to select carefully their food through a variety of sensorial and inherited or learned behavioural mechanisms (Bradshaw 2006). Neophobia and neophilia are two contrasting feeding strategies. Neophobia is the fear and rejection of new food. In contrast, neophilia is the interest and preference for any new food providing an opportunity to eat new food in case of food shortage. Both are important to the survival of wild species. Neophilia is often found in dogs, especially in such breeds as Labrador retrievers and Cavalier King Charles spaniels. This opportunistic feeding behaviour enabled appetent formulations to be developed and now in the veterinary market many appetent formulations exist for non-steroidal anti-inflammatory drugs (NSAIDs), angiotensin converting enzyme (ACE) inhibitors, etc. for companion animal therapy. There are however important differences between the dog and the cat regarding taste. The dog is rather insensitive to salt, whilst the cat is insensitive to sugar (Bradshaw 2006). It is known that cats prefer fish and dogs prefer beef, pork, and lamb to chicken, liver, and horsemeat. These findings are utilised when formulating palatable oral tablets by adding food-based products or flavour ingredients.

In contrast, neophobia protects animals against toxic substances. Neophobia is typically observed in non-vomiting species, such as rodents and horses and this makes it difficult to administer any drug requiring voluntary ingestion. Therefore, gavage or naso-gastric intubation may be required. Alternatively, the drug must be hidden in bait as, for example, in rodenticide substances. In horses, drugs are traditionally mixed with bran in some appetent mash to facilitate voluntary ingestion, because placing the drug as a powder directly on to food is generally not appropriate in this species, as the horse displays sniffing behaviour towards any odorous foreign substances.

A unique feature of veterinary medicine is the case of collective treatments. Veterinary drugs may be administered to food-producing animals (pigs, poultry, cattle, sheep, etc.) either individually or, more often, at a herd or flock level. For

the latter purpose, the oral route is chosen because only this oral route enables large numbers of animals (sometime several thousands) to be treated conveniently and cheaply at the same time. For antibiotic use in livestock, the objective is commonly to limit the progression of contagious disease in the overall population, rather than to treat a single subject as for companion animals. Thus, the oral administration of drugs in drinking water (e.g. in poultry) or as medicated feed (e.g. for pigs) ensures that all animals are treated with minimum of labour. Another advantage of the oral route is the absence of stress that may occur with individual treatments that require first catching and then restraining and injecting animals individually. In addition, it is important for food producing animals to avoid both tissue damage and the presence of local residues, as is often the case for drugs administered individually by a parenteral route, especially for the so-called long-acting/depot formulations. Typical of collective treatments are antimicrobial and antiparasitic drug classes, such as coccidiostats.

In veterinary medicine, there are two differing modalities for collective antibiotic administration: (a) prevention in a non-infected setting; this is a prophylaxis at the herd level when a risk factor, such as weaning in piglets or transport in calves, is present; and (b) treatment triggered when illness is actually recognised in a usually small proportion (often 10%) of the animals; this is metaphylaxis, also called prevention in the infected setting. These mass medications have been criticised on the grounds that they inevitably increase antibiotic consumption more than selective/individual curative treatments do and as such they favour the emergence of antibiotic resistance. However, it should be noted that these treatments are initiated in animals when they still have a low pathogen inoculum size at the infection site, a situation in which the selection pressure of antibiotic is minimal or absent (Ferran et al. 2009). The main difficulty with these collective treatments is to guarantee an equal or similar drug exposure in all treated animals. Population PK seeks to measure the inter-individual variability that in turn reflects variability in feeding behaviour in a competitive environment. Figure 2 illustrates the high inter-individual dispersion of doxycycline exposure in a sample of 215 pigs under field conditions (del Castillo 2006). This is of concern for the emergence of antimicrobial resistance, because inter-animal variability in the level of drug exposure is a major risk factor. This arises because under-exposure of the target pathogen in only a few animals within a flock or herd may lead to the establishment in these animals of a less susceptible sub-population of the pathogen that subsequently may transmit resistance genes horizontally to the other members of the group (Lees et al. 2006).

4 Origin of Interspecies Differences in Drug Disposition and Drug Action

The causes of interspecies differences in drug disposition or PK are numerous and reflect species differences in physiological processes involved in the handling of drugs (absorption, distribution, metabolism, and elimination, ADME). This is the

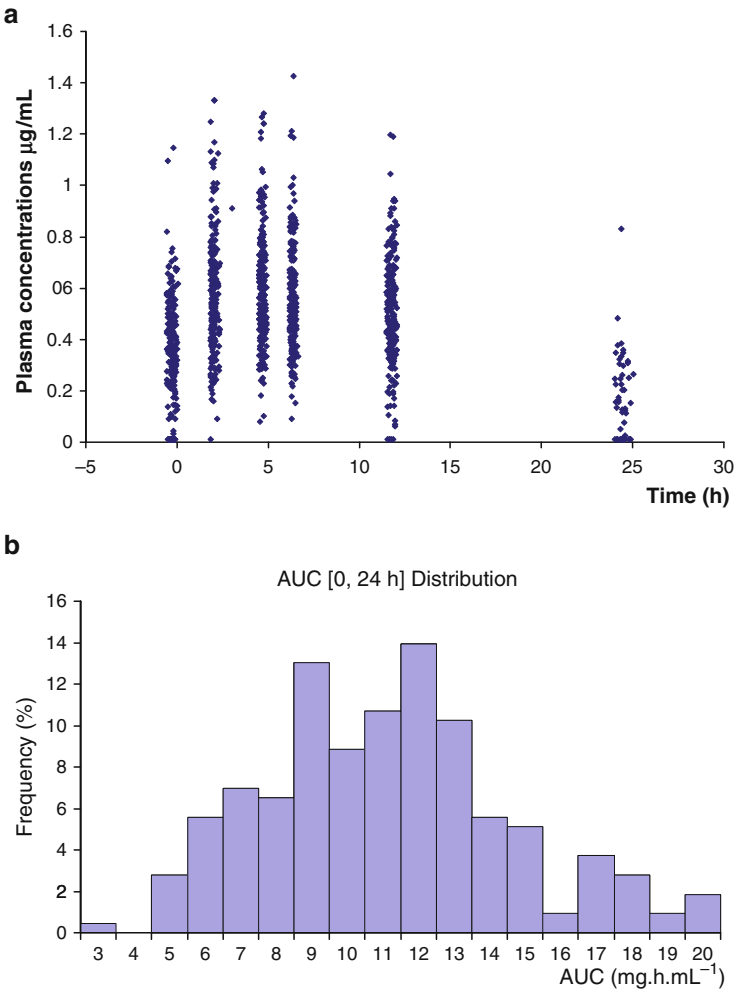

Fig. 2 (**a**): Plasma concentration of doxycycline in 215 pigs under field conditions. Doxycycline was administered by the oral route as a metaphylactic treatment: the first dose (5 mg/kg, nominal dose) was given at 18–19 h (evening dose) and the second dose (5 mg/kg, nominal dose) was administered at 8–9 h (morning dose) on the following day. Blood samples were obtained approximately 30 min before, and approximately 1.8, 4.5, 6.7, and 11.5 h after the second administration. For 25% of the pigs, a final blood sample was obtained 24 h after the second administration. Visual inspection of the raw data reveals the large variability of plasma doxycycline concentrations. (**b**): Histogram of the area under the concentration-time curve (AUC from 0 to 24 h) for the 215 pigs. The range of exposure is approximately 4–5 (from Lees et al. 2006)

main cause of observed inter-species differences and the veterinary literature is regularly enriched with new examples. Species differences in drug action and effect, i.e. of PD, reflect differences in target functions (anatomy, physiology, pathology) and/or target receptors including those of parasites and bacteria.

In contrast with interspecies differences in PK, examples of PD interspecies differences are less numerous and not so well documented. One such example is that of mydriatic drugs, with the parasympatholytic agent atropine being mydriatic in mammals but not in birds. In birds, the iris and ciliary muscles are composed mainly of striated muscle fibres with an associated nicotinic cholinergic neuromuscular junction (Glasser et al. 1995; Pilar et al. 1987). These striated muscle fibres facilitate a rapid pupillary and accommodation response and this is likely to be an adaptation to the visual requirement of flight. In birds, atropine, a muscarinic receptor antagonist has no mydriatic effect, whereas neuromuscular blockers such as d-tubocurarine dilate pupils. In mammals, the iris is composed of smooth muscle, and muscarinic agonists act on receptors of the M3 subtype to cause pupil dilatation.

For drugs affecting reproduction, very large interspecies differences exist and these are easily explained by basic mechanisms of reproductive physiology. For example, the annual pattern of the reproductive cycle is seasonal in small ruminants, but not in cattle. It was established that seasonality is under photoperiodic control through the secretion of melatonin by the pineal gland during the dark phase of the nycthemere. Thus, melatonin was rationally developed as a drug for use in sheep and goats to hasten the onset of the breeding season and to increase prolificacy. The estrus cycle is qualitatively similar in cattle, sheep, goats, and horses but its overall duration and the relative duration of the luteal and follicular phases have led to specific recommendations regarding the rational use of different hormones used to control the estrus cycle and also to the design and development of controlled release vaginal and non-vaginal systems such as subcutaneous implant containing GnRH or ear implants releasing steroids (for a review see Rathbone and Witchey-Lakshmanan 2000).

Prostaglandin analogues are used to induce farrowing (parturition) within a time limit of 3 days, because in the pig, progesterone of ovarian origin (corpus luteum) is necessary to maintain a pregnancy until term. This is not the case in sheep where progesterone of placental origin becomes sufficient to maintain pregnancy after a delay of about 2 months. This explains why luteolytic substances that suppress progesterone production by the corpus luteum are abortive in sows throughout pregnancy but not beyond 55 days in sheep.

In ruminant species, cortisol of foetal origin plays a pivotal role in the initiation of parturition in the last days of pregnancy and synthetic fluorinated corticoids (dexamethasone, flumethasone, betamethasone) are also able to trigger parturition within the 2 last weeks of pregnancy in cows providing that the foetus is alive, while the same fluorinated corticosteroids are not effective in horses, pigs, and dogs. The size of the prostate gland is species-specific, and in domestic species it is only in dogs that the prostate attains the same degree of compactness as in man; in other species including bulls, sheep, goats and pigs the prostate is rudimentary. In addition, the prostate (even rudimentary) atrophies in the absence of circulating testosterone. These differences explain why prostatitis is an infectious condition that often affects entire male dogs that is difficult to treat because of a prostate barrier due to the tight junctions

between the epithelial cells, while this condition does not exist in stallions or in bulls.

Xylazine, an alpha2-adrenergic agonist, used widely in several species as a sedative, is also a reliable emetic, particularly in cats, in which it stimulates the chemoreceptor trigger zone (CTZ) in the medulla oblongata, while it does not induce emesis in species such as ruminants and horses lacking the vomiting reflex.

In the preceding examples, the interspecies differences were easily explained by anatomical, biochemical, histological, or physiological differences. However, this is seldom the case when species are compared for functions involving the central nervous system, for example with some behavioural responses and the response to pain. The International Association for the Study of Pain (ISAP) defines pain in man as "*an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage*". This definition is not directly transposable to animals due to its cognitive dimension. Therefore, pharmacologists prefer the use of the word "nociception" to describe what they observe, as for any other somatosensation. There is evidence of nociception across all domestic species including fish. However, what is particularly difficult to appreciate let alone quantify, when evaluating the benefit of analgesic therapy in animals, is the level of suffering across species, because suffering implies a mental dimension and there are no means of quantifying levels of suffering.

Observations of animal behaviour as a basis for assessing pain can be very misleading, especially when using some resilient behaviour. Resilience is a passive adaptative strategy to cope with adversity including pain. For example, the ass is a resilient species that expresses its pain only at a high threshold, while the opposite applies to horses (Ashley et al. 2005). This creates problems for the assessment of presence and level of pain and the efficacy of analgesics in asses; conditions such as colic can proceed to advanced irreversible stages before they are detected. Resilience may also be observed in dogs in some circumstances; Hansen (2003) reported that, until recently, dogs in his intensive care unit did not receive analgesics after major surgery, in part because they did not meet the expectations of their caregivers for pain behaviour. Some strains of beagles have been deliberately selected and/or trained to express resilient and passive behaviour as a desirable trait in investigations concerning toxicology assessment but these unresponsive dogs are inappropriate for testing analgesic drugs (Toutain, unpublished observations). There is experimental evidence in both mice and man that responsiveness to pain may be modulated by social status and the social environment and also that empathy exists in animals; it has been reported that expression of pain in a test subject may be influenced by the presence or absence of a familiar conspecific animal, rendering the assessment of analgesic efficacy complicated (Gioiosa et al. 2009; Langford et al. 2006). For fish, the situation is even less clear and there is no consolidated indicator of pain, so that efficacy of analgesia associated with general anaesthesia is often judged by the ability to handle fish without difficulty. For a review on the difficulties of assessing pain, see Anil et al. (2002). Animal pain, including anthropomorphic considerations, is reviewed in the chapter, "Pain and Analgesia in Domestic Animal Species" of this text.

5 Origin of Interspecies Differences in Dosage Regimens

For each drug in each species an efficacious and safe dosage regimen (dose level, interval of administration, and dosing duration) should be determined. Dosage regimens may vary markedly between species, even when doses are expressed by kg of body weight. For xylazine, an alpha-2 agonist used as a sedative, the effective dose is tenfold lower in cattle than in horses, despite similar PK profiles (Garcia-Villar et al. 1981). For morphine, the effective dose is ten-fold lower in cats than in dogs; for aspirin, dosage is approximately 40 times higher in cattle than in cats, while for suxamethonium (succinylcholine), a depolarising neuromuscular blocker, the dose in cattle is some 40 times lower than in cats. From these examples, it seems that no generalisation can be established between interspecies variability and certain physiological traits, such as herbivorous vs. carnivorous species or monogastric species vs. ruminants. Rather, it should be realised that a dosage regimen is a PK/PD hybrid variable with two major components: a PK and a PD component (1):

$$\mathrm{ED} = \frac{\mathrm{Clearance} \times \mathrm{EC}}{\mathrm{Bioavailability}}, \tag{1}$$

where *ED* is an efficacious dose, *Clearance* is the plasma (total) clearance, *Bioavailability* is the extent of the systemic bioavailability (for extravascular routes of drug administration) and *EC* is the efficacious plasma concentration. Clearance is a pharmacokinetic parameter expressing the overall capacity of the body to eliminate the drug. Bioavailability is a PK variable expressing the percentage of drug that actually reaches the systemic circulation after administration by an extravascular route and *EC* is the PD parameter expressing drug potency (Toutain 2009). Thus, interspecies variability may have either a PK (as for aspirin or suxamethonium), and/or a PD (as for xylazine) origin. It should be understood that the 3 factors determining a dose are themselves hybrid parameters and recognising their biological determinants helps to identify the different anatomical, physiological, and/or behavioural sources of interspecies differences. For example, plasma clearance is the sum of different clearances (2):

$$\mathrm{Clearance}_{\mathrm{total}} = \mathrm{CL}_{\mathrm{hm}} + \mathrm{CL}_{\mathrm{nhm}} + \mathrm{CL}_{\mathrm{b}} + \mathrm{CL}_{\mathrm{r}} + \mathrm{CL}_{\mathrm{other}}, \tag{2}$$

where the total clearance is the sum of the hepatic ($\mathrm{CL}_{\mathrm{hm}}$) and non-hepatic metabolic clearance ($\mathrm{CL}_{\mathrm{nhm}}$), the biliary clearance (Cl_{b}) and the renal clearance (CL_{r}), with other clearances ($\mathrm{CL}_{\mathrm{other}}$) generally being negligible. Thus, the interspecies variability in plasma clearance reflects differences in the relative importance of the several potential pathways of drug elimination (liver and non-liver metabolism vs. biliary elimination vs. renal elimination) and the capacity for any given mode of elimination.

The molecular weight (MW) of a compound is a key factor determining the extent of biliary vs. non-biliary excretion. Experiments in rats with compounds

covering a wide range of MW (150 to > 700) demonstrated an increase in the proportion of compounds excreted in the bile vs. urine as the MW increased [see Calabrese (1983) for a review]. Urinary excretion accounts for almost all the elimination of compounds having a MW < 250, whereas it becomes negligible for compounds with MW > 600–800. The same phenomenon has been reported in other species but with some differences in ranges of MW. Substances with low molecular weights (MW < 300) are primarily eliminated by renal clearance (glomerular filtration) in most species; substances (drug, metabolites, conjugates) with MW > 600 are typically eliminated in the bile by active carrier-mediated transport. For substances having MWs between 300 and 600–800, the preferential route of elimination may show large species differences and it is for this MW range that the likelihood of interspecies differences is the highest, with so-called poor biliary excreters (rabbits, ginea-pigs, man), good biliary excreters (rats, chickens, dogs) and intermediate species (cats, sheep) having been classified. These species differences arise from the fact that the value of the threshold MW for appreciable biliary excretion varies from species to species. It should be stressed that the definition of poor or good biliary excreters is not related to the rate of bile flow, which is very high in rabbits (90 mL/min/kg), a so-called poor biliary excreter, and much lower in dogs (4–10 mL/min/kg), a so-called good bile excreter.

For liver metabolism, the hepatic clearance is given either by (3) or (4) for substances having a low or a high extraction, respectively:

$$\mathrm{CL}_{\mathrm{hm}} = fu \times Cl_{\mathrm{int}} = fu \times \frac{V_{\max}}{K_{\mathrm{m}}}, \tag{3}$$

$$\mathrm{CL}_{\mathrm{hm}} = \dot{Q}h. \tag{4}$$

In (3), the elimination by the liver of drugs having a low extraction ratio has only two determinants: the extent of binding to circulating plasma proteins as expressed by *fu*, the unbound fraction (from 0 to 1) and Clint, the intrinsic clearance which is determined by the ratio of the maximal metabolic capacity ($V_{\max}$) and K_{m}, the parameter that expresses the affinity of a substance for the metabolic enzymes. $V_{\max}$ is related to the nature of the enzymes (e.g. different families of P450 cytochromes) and interspecies differences in hepatic metabolism can be explained in terms of $V_{\max}$. For drugs with a high extraction ratio (4), the clearance is equal to the hepatic blood flow (first pass effect) and for these drugs interspecies differences reflect differences in hepatic blood flow (that are *a priori* known) and not of drug specificities. Therefore, it is for drugs having a low extraction ratio that the most important interspecies variability is expected, because the P450 cytochromes (i.e. $V_{\max}$) can vary considerably from species to species (*vide infra*).

Even in closely related species, major differences may exist, for example between horses (*Equus caballus*) and donkeys (*Equus asinus*). Thus, for phenylbutazone (PBZ), the plasma clearance is about 10 times higher in donkeys than in horses. Therefore, to achieve the same PBZ exposure in the two species, the dose

should be 10 times higher in donkeys. Similarly, PK data indicate that plasma clearance is higher in the donkey than in the horse for several antibiotics, including benzylpenicillin, ampicillin, amoxicillin, and oxytetracycline, but not aminoglycosides (amikacin, gentamicin). Hence, no generalisation is possible and the PK of each drug must be specifically investigated in the donkey i.e. the donkey should be recognised and treated as a species in its own right [see review by Lizarraga et al. (2004)].

In (1), bioavailability is likely to be the factor accounting for the greatest interspecies difference, at least for the oral route of administration. Defining the physiological characteristics among domestic species may help in understanding and even predicting and managing the interspecies variability in drug bioavailability, which includes not only anatomical and physiological factors but also behavioural traits such as feeding pattern. However, extrapolation between species is hazardous when veterinarians wish to apply data from man to dogs or cats. The mean bioavailabilities in humans and dogs were compared for a series of 43 drugs with different physicochemical and pharmacological properties and with a range of the mean F values between 1.5 and 100%. The overall correlation was relatively poor ($r = 0.51$), indicating that data derived in dogs may be inapplicable to man (Chiou et al. 2000) and *vice versa*.

6 Pour-on Formulations: Dermal or Oral Route of Administration?

The behavioural origin of interspecies differences and species-specific issues are often overlooked in veterinary medicine, especially when drugs are investigated in an experimental setting in which the natural behaviour is often altered, controlled or even deliberately suppressed for convenience. This is the case for pour-on formulations used in cattle. Pour-on administration is the topical application of a drug on the skin in a liquid formulation; it is a very popular mode of administration for endectocides (ivermectin, doramectin, eprinomectin, moxidectin). Topical administration is very convenient, without risk of tissue damage and without persistent residues at the site of administration, in contrast to products containing the same drugs administered by subcutaneous or intramuscular injection. However, pour-on formulations are not and cannot be a purely topical route of administration in cattle, under all normal husbandry conditions. In a natural environment, cattle lick themselves (allolicking) and lick other cattle (heterolicking). It was shown that either expression or prevention of this physiological behaviour has a marked influence on systemic bioavailability of ivermectin in cattle: most of the ivermectin poured on skin was actually absorbed by the digestive tract (Laffont et al. 2001) and pour-on formulations, under these conditions, were predominantly an oral rather than a topical formulation. This explains why the systemic availability of the pour-on drug formulation is both highly variable and unpredictable (Gayrard et al. 1999). Moreover, allo-grooming might result in cross-contamination of untreated cattle

(Bousquet-Mélou et al. 2004) and thereby give rise to undesirable sub-therapeutic concentrations in both treated and untreated animals. A further consequence is the occurrence of unexpected drug residues in edible tissues of untreated cattle and in underexposure of treated animals, which may be a factor in the development of drug resistance. It must also be stressed that the relatively poor and erratic bioavailability of pour-on formulations has led to an increase in the dose of ivermectin, doramectin, and moxidectin by a factor of 2.5 compared with subcutaneous administration, thereby contributing to an unavoidable increase in the environmental burden for the parent drug. Environmental issues are addressed in the chapter, "Veterinary Medicines and the Environment" of this text.

The influence of licking or analogous behaviour such as grooming in cats deserves attention for topically applied drugs and may explain unexpected interspecies differences. For example, it was shown for selamectin, a semi-synthetic avermectin agent primarily used to kill adult fleas and ticks, that the absolute bioavailability was only 4.4% in dogs vs. 74% in cats. In addition, the peak plasma concentration was 63 times higher in cats than in dogs, suggesting that a major fraction of the dose was ingested during grooming (Sarasola et al. 2002). In goats, a non-licking species, the absolute bioavailability of ivermectin after a pour-on formulation is likely to be very low (about 4–8%), as estimated approximately from published IV AUC (Gokbulut et al. 2008; Gonzalez et al. 2006) and pour-on AUC (Scott et al. 1990) data. The cause of the low systemic availability of pour-on formulations in goats remains unclear but the lack of licking behaviour, as seen in cattle, may be a partial explanation.

7 Consequence of Coprophagia on Drug Disposition and Responses

Coprophagia is another individual and social behaviour phenomenon specific to veterinary medicine. It comprises the consumption of faeces by animals. Many domestic species such as horses, pigs, and dogs either regularly or occasionally practise coprophagia (Soave and Brand 1991). In contrast, some species including goats (at least in an unconfined grazing system) will almost always reject any plants contaminated with the scent of their own species' urine or faeces. Coprophagy may lead to drug transfer between animals (allocoprophagy) or drug recycling when an animal re-ingests its own faeces (autocoprophagy) or its own manure. The faeces of treated foals may contain extremely high concentrations of antibiotics especially for drugs with poor oral bioavailability. If such contaminated faeces are ingested by an adult horse, ingested concentrations of antimicrobial drugs may suffice to disrupt the normal adult colonic microflora, which are exquisitely sensitive to some antibiotics.

In Sweden, it was shown that mares practising coprophagia with their foal faeces, while the foals were receiving erythromycin for the treatment of *Rodococcus equi*, had a high incidence of colitis (Baverud et al. 1998). Adult horses may eat manure.

Lees et al. (1986), Norgren et al. (2000) and Wennerlund et al. (2000) reported that untreated horses that were housed for several days in boxes previously allocated to horses treated with flunixin or naproxen also eliminated the drugs. This suggests some cross contamination via the bedding. Possible contamination by ingesting straw contaminated by urine was also well-documented for meclofenamic acid (Popot et al. 2007). Therefore, it was concluded that spurious urinary drug rebound may lead to some positive doping tests, despite observance of the recommended withholding time.

In dogs, coprophagia is frequent and, under experimental circumstances, treated vs. non-treated dogs should be separated to avoid cross contamination. Food companies produce feed additives that can be added to the animal's food to prevent coprophagia by making faeces unpalatable. Rabbits produce two types of faeces: soft faeces, also named caecotroph, that are rich in proteins, vitamins etc. and regular hard faeces. By re-ingesting caecotroph directly from the anus, the rabbit has a second opportunity to benefit from valuable nutrient substances. Such physiological recycling, that typically occurs twice each day, should be considered when treating rabbits because any drug/metabolites eliminated/produced in the digestive tract will be partially recycled and possibly reabsorbed in the small intestine. This has been described for chloramphenicol. For this drug, a plasma concentration rebound was observed 24 h after an IV administration (Guillot et al. 1988).

The crop of broiler chickens has been implicated as a major source of *Salmonella* contamination probably due to coprophagy (Corrier et al. 1999) and it was shown that a feed withdrawal that increases coprophagy also increased contamination of the crop by food-born zoonotic pathogens such as *Salmonella* and *Campylobacter* species.

8 Interspecies Differences in Drug Disposition in Relation to Digestive Tract Physiology

After a drug administered by the oral route has been swallowed, it passes to the stomach from the esophagus. Esophageal transit normally takes a few seconds but the esophagus shows large interspecies histological differences with practical consequences for therapeutics. Esophageal muscle is striated in fish but smooth in birds, whilst mammals show considerable species variation in the presence of these two types of muscle. Both layers of muscle are striated throughout the length of the esophagus in dogs, sheep and cattle, whereas in cats the esophagus contains smooth muscle over approximately the terminal 8% of its length and 16% for its circular muscle. This explains why cats are prone to retain foreign bodies in the distal part of the esophagus, including drug tablets. This may be problematic if highly acidic medications are retained that may cause severe irritation. The more extensive section of smooth muscle is seen in the horse. From a PD perspective, striated esophageal muscles are paralysed by curare but unresponsive to drugs acting on the sympathetic/parasympathetic (autonomic) nervous system, while the inverse is true

for esophageal smooth muscle. In addition, the oesophagus relaxes under the influence of oxytocic substances and this complicates prediction of the overall response of the esophagus in different domestic species. Horses are prone to choke, a condition in which the esophagus is blocked in its distal segment where the muscles are smooth, so that oxytocin has been recommended in cases of choking to relax the distal portion of esophagus (Wooldridge et al. 2002).

Major differences exist between monogastric species in the gross anatomy and structure of the stomach. An overview of interspecies differences in gastrointestinal physiology with special emphasis on monogastric species has recently been provided by Martinez et al. (2002) and a review on companion animals and dosage forms has been given by Sutton (2004). In monogastric species, the stomach plays an important role in the disintegration and dissolution of drug formulations and gastric emptying, with the pylorus acting as a sieving gate leading into the duodenum. This is the most important physiological factor controlling the rate of access of a drug to its site of absorption in the proximal part of the gut.

In ruminants, the reticulo-rumen (RR) has a profound influence on the fate of all drugs due to its large capacity (225L in adult cattle), which leads to dilution and affects the residence time of orally administered drugs. In addition, the microflora of the rumen can inactivate drugs by metabolic or chemical reactions.

The rumen wall is keratinised and not well designed to absorb drugs except for some weak acids such as sulfonamides and salicylic acid (Baggot 1977). Conversely, the RR fluid, being both very large and acidic (from pH 5.5 to 6.5), can be a trapping compartment for circulating weak bases and thus influence their systemic disposition through the classical Henderson–Hasselbalch mechanism. As in monogastric species, the main site of drug absorption in ruminants is the proximal part of the gut requiring that a drug transits from the rumen through the omasum and abomasum and the pylorus. Between the reticulo-rumen and the omasum, the reticulo-omasal orifice (ROO) has a sieving function that can be viewed as the "pylorus" of the reticulo-rumen. It allows only the passage of small and dense particles and of solution. When a drug is released from its pharmaceutical form it may be either in solution in the liquid phase of the ruminal content or be bound to the ruminal contents such as cellulose. When the drug is in solution, the transit of the ruminal liquid phase becomes the limiting factor with a relatively slow turnover rate in the range of 6–15 h. This explains why drugs having a very short half-life by the IV route, such as salicylic acid (1 h), may nevertheless give sustained plasma concentrations in ruminants when administered by the oral route, because it is the rate of transit to the duodenum that controls the overall rate of the systemic availability (flip-flop phenomenon). The improved bioavailability of several benzimidazole anthelmintics, such as fenbendazole, oxfendazole, and albendazole, when directly deposited in the rumen is probably due to slow transit from the reticulo-rumen to the abomasum allowing a prolonged duration for drug absorption in the more distal part of the digestive tract because there is evidence of saturability of benzimidazole absorption (Sangster et al. 1991). If a drug is strongly bound to cellulose, the transit to the distal part of the gut will be associated with that of small

particles that require cellulose breakdown and the delay will be longer as the turnover time for the solid phase is approximately 50–60 h.

A vast reticulo-rumen functionally isolated from the most distal part of the digestive tract by the ROO offers the unique therapeutic opportunity to administer a large depot of drug using a delivery device to provide prolonged and sustained release of appropriate amounts of drugs (mainly anthelmintics), trace elements, anti-foaming agents, etc. Such drug delivery systems are designed to remain lodged in the reticulo-rumen for several days, weeks, or even months. Such an approach is not possible in monogastric species, due to the gastric emptying that occurs within the 12 h following administration. In ruminants, regurgitation of the device is prevented by an appropriate geometric design, for example, expanding plastic wings. Thereafter, the device should reside permanently in the reticulum or the cranial sac of the rumen to release drug close to the ROO and in the fluid phase of the RR contents and not in the distal part of the rumen, where there is the possibility of being entrapped in the fibrous raft that floats above the ventral ruminal fluid. The density of the device should suffice to avoid floating in the ruminal liquids. A density of 2.25–3.5 g/cm^3 is required in grazing animals, but a lower density (1.8 g/cm^3) may be sufficient in animals fed a cereal-based food. For a review on ruminal drug delivery systems, see Vandamme and Ellis (2004) and also the chapter, "Drug Delivery Systems in Domestic Animal Species" of this text.

The forestomach precedes the true secretory stomach or abomasum. In the newborn ruminant, the reticulo-rumen is not developed. From a digestive physiology point of view, the calf is essentially a monogastric animal and the RR should be bypassed when the animal is fed with milk. This is possibly due to the presence of a reticular groove. The reticular groove is formed by muscular folds, able to form a closed channel that extends from the cardia to the abomasum. Reticular groove closure occurs reflexively in pre-ruminant animals in response to different stimuli including suckling, which allows ingesta (milk with drug in solution) to bypass the rumen to gain access directly to the abomasum.

In adults, the closure reflex of the reticular groove no longer operates but it may be facilitated by some practises like yarding in small ruminants. Yarding consists of holding sheep in a paddock with little or no food for 12–24 h. Yarding (but not withdrawal of water) for 24 h before drenching has been shown to stimulate RR bypass in approximately 35% of a group of 9-month-old lambs (Sargison et al. 1999). Yarding (12 $\pm$ 24 h before drenching) has been proposed as a method to improve the efficacy of anthelmintics (Prichard and Hennessy 1981).

The horse and rabbit are hindgut fermenters and the caecum and colon are major sites of microbial digestion of feed for these species. This makes horses and rabbits particularly prone to antimicrobial drug-induced enterocolitis, secondary to disruption of their normal microflora leading to an overgrowth of pathogenic microorganisms like *Clostridium* ssp. In the rabbit, *C. spiriforme* has been implicated as the primary causative agent producing iota toxin and causing enterotoxaemia and death. It should be stressed that the delay between the end of antibiotic administration and these catastrophic events may be up to 10 days, making it sometimes difficult to identify the origin of an enterotoxaemia. For this reason, antibiotics administered

by the oral route and having a poor bioavailability and also extensively excreted in the bile (such as oxytetracycline) or by enterocyte efflux (doxycycline) after parenteral administration should not be used or should be used with caution in horses and rabbits. Lincomycin and clindamycin in horses and penicillins (amoxicillin, ampicillin) and some cephalosporins (ceftiofur) in rabbits, irrespective of route of administration are well-recognised to be associated with enterocolitis and should be avoided in these hindgut fermenters. Trimethoprim-sulfonamides and macrolides, such as erythromycin and spiramycin, have also been reported to disrupt horse gut flora. It is interesting to note that this disruption may be more marked in some parts of the world than others, as is the case for doxycycline. This drug is considered to be dangerous for horses in Europe but safer in the USA, possibly a consequence of regional differences in gut flora.

In horses, a large fraction of the oral dose of administered drugs of the NSAID class, including phenylbutazone, flunixin, and meclofenamic acid may be adsorbed onto the cellulose and conveyed to the caecum and proximal colon in bound and non-absorble form, where cellulose digestion takes place releasing the adsorbed drug. This explains the profound influence of the diet and the schedule of drug administration in relation to feeding on the bioavailability (rate and extent of absorption) (Maitho et al. 1986; Lees et al. 1988). This also is a likely explanation of the local side-effects of NSAIDs on the digestive tract in horses; erosions may occur in the distal part (colon) rather than, or as well as, in the stomach as in other monogastric species.

9 Species Variation in Drug Metabolism

Biotransformation is a major factor accounting for species differences in the disposition of drug. The group of P450 cytochromes (CYP450) represents a large superfamily of oxygenases. The CYP450 enzymes are considered to be the most important metabolising enzymes for xenobiotics. In veterinary medicine, one of the main motivations to study drug metabolism across different animal species is the need to identify and describe the depletion of residues in the tissues of farm animals intended for human consumption (see chapters, “Pharmacogenomics in Domestic Animal Species” and “Drug Delivery Systems in Domestic Animal Species” of this text). Hence, knowledge of biotransformation in cattle has been used to predict the biotransformation (and tissue distribution) in sheep, which is classified in most countries of Europe as a minor species. It seems that the inter-species differences are so great that a prediction of biotransformation in sheep from cattle data is not valid (Watkins et al. 1987). The same conclusion was drawn for avian species, making a cross-species comparison with poultry virtually impossible (Cortright and Craigmill 2006).

Another objective of studies of comparative metabolism is to explain major interspecies differences in drug effects including safety. In clinical practise, fatal intoxication may occur in horses exposed to monensin, an ionophoric coccidiostat

used in poultry. One likely reason for their high susceptibility in horses is their relative inability to demethylate compounds that are not CYP2D substrates. Comparative investigations with microsomes from various animal species, including horses, pigs, broiler chickens, cattle and rats, showed that the horse had the lowest catalytic ability to demethylate (and hence detoxify) monensin (Nebbia et al. 2001).

Comparative metabolism is also relevant when identifying animal models for humans that have an appropriate similarity, as required for regulatory toxicology. With the aim of establishing the best animal model for human CYP450-related research Bogaards et al. (2000), undertook a comprehensive comparative investigation of the enzyme activities and kinetic parameters of nine prototypical substrates for individual CYP450 activities in liver microsomes derived from mice, rats, rabbits, dogs (beagle), micropigs (Yucatan), monkeys (*Macaca fascicularis*) and man. The overall conclusion of this comparison was that none of the investigated species matched all the typical CYP450 activities as described in humans. This may also be true within a species when some breeds are selected to fit laboratory conditions, as in the case of minipigs (Göttingen minipig and the Yucatan micropig) that have a higher total CYP450 activity than farm breeds of pig (Sakuma et al. 2004; Vaclavikova et al. 2004).

In veterinary medicine, inter-species quantitative differences in phase I and qualitative differences in Phase II metabolism have been known for several decades. Examples are the poor capacity of the cat to carry out some glucuronidations, the deficiency of dogs for acetylation reactions and the low level of sulphate conjugation in pigs (Baggot 1977).

A common assertion in veterinary pharmacology is that in general drugs have lower clearance in carnivores than in herbivores with omnivores having an intermediary position. Herbivores are also reported to be well-endowed with oxidative enzyme systems such as those of the cytochrome P450 group, providing rapid drug clearance for drug elimination by hepatic metabolism. An historical example is salicylic acid, a ubiquitous plant stress compound, found in high concentrations in some forage like lucerne. The terminal half-life of salicylic acid varies considerably between species between less than one hour in herbivores (cattle, horses), from 3 to 6 h in omnivores (man, pigs) and up to 9 h in dogs (Lees 2009). In cats the half-life is even longer (22–48 h) due to a deficit in glucurono-conjugation. Several other examples may be cited from the literature, illustrating a faster clearance of drugs in herbivores but more recent functional and genetic studies have shown that there are no common patterns distinguishing polygastric ruminants from the monogastric species like pigs, and even amongst ruminants there are no obvious links between cattle and sheep. However some differences exist, with goats having generally a more active metabolism than either sheep or cattle. This is linked to their respective feeding behaviour; goats are natural browsers that can stand on their hind legs or even climb trees. They preferably eat leaves, shrubs, flowers and fruits, thus choosing the most nutritious available food but also the portions of plants containing many toxic alkaloids that need to be metabolised by a hepatic first pass effect. In contrast, cattle are a non-selective bulk feeder that grazes non-selective grass generally low in terms of alkaloid content.

Advances in molecular biology and pharmacogenetics has enabled more comprehensive data to be obtained in the face of more protean situations, due to the multiplicity of species, the multiplicity of breeds within each species with possible polymorphism and the lack of catalytic specificity of cytochrome P450 isoforms. For a recent comprehensive review, see Fink-Gremmels (2008).

The CYP450 system is complex and CYP members are subdivided into families and sub-families. The naming of CYP enzymes should now follow the system based on DNA sequencing; therefore the naming based on catalytic activity or substrate specificity should be avoided once the gene has been cloned (Cribb 2003). Practically, there are 3 CYP families involved in drug metabolism: CYP1, CYP2 and CYP3 families. The naming system consists of adding an Arabic number for a family (requiring 40% identity of amino acid sequence as CYP1, CYP2 or CYP3). For a sub-family (requiring 40–80% identity) a letter is added (A, B, C. . .), e.g. CYP1A, CYP1B. Finally, an additional Arabic number is added to identify individual members of a sub-family (as an isoform) like for CYP1A2 or CYP3A4 etc. It should be stressed that a completely named CYP enzyme can only be found in one species because a given amino acid sequence can only derive from one species. For example CYP3A4 can only be found in humans while the ortholog (comparable) enzyme in the dog is CYP3A12. This naming system based on sequences homology offers the advantage of being unequivocal but it may render difficult the presentation of interspecies variability in drug metabolism. Indeed, even if CYP families share general sequence properties, there exist considerable differences within a given family in terms of substrate specificity and of enzyme regulation (induction, inhibition). Thus, it is possible to identify two very similar (ortholog) CYP enzymes in two different domestic species (in term of amino-acid sequence) but having very different substrate (biotransformation) specificities. Conversely, a given drug (substrate) may be metabolised *in vivo* by two or more unrelated (different families) CYP enzymes leading to a multiplicity of substrate/enzyme combinations. This makes species extrapolation of CYP450 specificity very difficult. For example, a comparison of the cDNA homologies showed a particularly high homology between human and pig CYP3A enzymes. However, the response to chemical inducers varied: rifampicin is an inducer of the nuclear factor PXR in both human and pig hepatocytes, whereas dexamethasone, which is also an inducer in man failed to induce testosterone 6β-hydroxylation and midazolam-4-hydroxylation in pig hepatocytes (Monshouwer et al. 1998; Lu and Li 2001).

An additional complication is that some CYPs are subject to considerable intraspecies variability as a result of both environmental and genetic factors. Polymorphism within a given species is a challenge to defining general guidelines for a given species. A single amino-acid change resulting from a single nucleotide change can markedly modify the functional properties of the enzyme (expression and substrate specificity). Although it is expected that these types of polymorphisms may exist in all animal species, they have not yet been investigated in detail. Polymorphic differences exist in certain breeds of dogs. For example, beagles have been shown to have a significantly greater propofol hydroxylase activity than greyhounds (Court et al. 1999), due to a high CYP2B11 activity. Greyhounds also had a much slower

metabolism of antipyrine than beagles, resulting in a significantly longer half-life (greyhound 1.09 h, beagle 0.55 h) (KuKanich et al. 2007). Even within one breed, such as the beagle, different sub-populations exist. This is illustrated by celecoxib, where approximately 45% of the animals were extensive metabolisers of celecoxib, while the remaining 55% of the tested population metabolised the drug only slowly and incompletely (Paulson et al. 1999). Whether or not these differences are related to polymorphisms in CYP2D15 is unknown.

It is for phase II metabolism that the main qualitative interspecies differences have been reported and explained by genetic factors, namely deficiencies in glucuronidation in felidae and acetylation in canidae. Glucuronidation represents one of the major phase II reactions in the metabolism of drugs. Among food-producing animals, rabbits, pigs, and horses show the maximal glucuronidating capacity towards phenolic substrates, while broilers and cattle display a relatively low conjugation rate.

Cats are known to be very sensitive to phenolic compounds as well as some drugs such as acetaminophen (paracetamol). It was historically observed that this sensitivity was attributable to a low glucuronidation capacity, since cats are hardly able to glucuronate simple phenols like phenol itself and simple aromatic acids such as benzoic acid. However, cats are able to conjugate diphenyl acetic acid. Recently Court et al. (1999) carried out a genetic analysis and found that cats (and other felines) have a very low glucuronidation capacity due to a mutation in the UDPT (uridine-diphosphate-glucuronosyl transferase) 1A6 gene, resulting in the expression of a pseudoenzyme i.e. a non-functional protein.

Interspecies variability in acetylation has been known for many years. *N*-acetyl-transferases are widely distributed among animal species and are active in metabolising sulfonamides. Two families of *N*-acetyl-transferases (NAT) have been recognised and called NAT1 and NAT2. Rabbits and pigs have high acetylating capacity, while chickens and horses are poor acetylators. Dogs and other canids fail to express functional NAT-1 and NAT-2, which are essential for the excretion of sulfonamides while in cats, only NAT1 is expressed. In man and rabbit, NAT activity is subjected to genetic polymorphism resulting in "low" and "high" acetylator phenotypes. For further discussion, see chapter, "Pharmacogenomics in Domestic Animal Species" of this text.

10 Kidney Function and Urinary pH

Kidney function is well-conserved across mammalian species with no major anatomical and physiological differences except concentrating ability related to the development of the renal medulla. Concentrating ability is low in pigs (urine to plasma concentration ratio of 3:1) and relatively high in cats (urine to plasma concentration ratio of 10:1) accounting for the fact that the cat is a species prone to form calcium oxalate urinary calculi. The consequences of urine dilution on antibiotic efficacy to treat cystitis are unknown. Urinary pH differences are evident between species. Urine pH is determined mainly by the composition of the diet, with alkaline urine generally produced in herbivorous species and acidic urinary pH

typical in carnivores. In horses, the variability between animals may be very large (from pH 5 to 9) and urinary pH displays a bimodal distribution in horse populations (Houston et al. 1985).

Urinary pH may be relevant to overall drug elimination via renal clearance and is a critical consideration for the actual presence of a drug or its metabolites in urine. This is a direct and major interest for doping control as well as for the treatment of some lower urinary tract infections. Salicylic acid (SA) is extensively conjugated to glucuronic acid in herbivorous species, forming a hydrophilic glucuronoconjugate with a low pKa (approximately 2.5) and a high renal clearance. In the alkaline urine of herbivores, the SA conjugate is wholly in the ionised form and therefore cannot be re-absorbed by the nephron. This leads to the rapid elimination of SA or of any derived glucuronide in herbivores. Urinary pH may also be relevant for drugs with a low renal clearance, as in the case of phenylbutazone (PBZ) in the horse. In horses, renal PBZ clearance is less than 10% of total clearance (Authié et al. 2009) but the actual urinary level of PBZ in post-race urine is strongly correlated with the pH, with higher concentrations found in alkaline urine than in more acidic urine (Houston et al. 1985). See chapter, "Veterinary Medicines and Competition Animals" of this text for further discussion.

11 Specificity in Drug Administration and Disposition in Poultry

Variability amongst bird species is as large as that observed between mammals and each species must be treated as a species in its own right. Marked variability has been noted for the disposition of three NSAIDs (sodium salicylate (SA), meloxicam and flunixin) in 5 bird species (ostrich, pigeon, duck, turkey, and chicken). Ostriches had the fastest elimination rate for all 3 NSAIDs. In chickens, flunixin had a half-life (5.5 h) that was 10 times longer than the other bird species (0.17–0.6 h). On the other hand, for SA the hierarchy was different with a shorter half-life in the chicken (3.13 h) than in pigeon (14.93 h) and duck (5.41 h). Therefore, no general principles allow extrapolation from one avian species to another and the rational design of dosage schedules must be based on data generated separately in each.

Another consideration in poultry medication is that drugs are most often administered collectively at the flock level and by the oral route (90% of all treatments). To achieve an effective collective treatment in poultry, special attention must be paid to poultry feeding and drinking behaviours and to the species characteristics of digestive tract physiology. For a comprehensive review on drug administration to poultry, see Vermeulen et al. (2002).

Drinking water is the preferred mode of administration for drugs, especially for antibiotics, because diseased birds usually tend to stop eating but will usually continue to drink. To achieve an effective dose the drug concentration in the

drinking water should take account of the species specific daily water consumption. In addition, within a given species, many biological (body weight, age, gender), environmental (lighting period, environmental temperature) and managerial factors (flock size, composition of the diet) can influence individual animal water intake. For example, the external temperature may influence drug exposure as water consumption is increased by approximately 7% for every 1°C above 21°C. Moreover, birds do not drink in dark periods so the light period may be manipulated to increase drug exposure especially for drugs having a short half-life.

The alternative to the drinking water is the administration of a drug through the food *via* pre-mix formulations. In contrast to water that is offered *ad libitum*, food may be given and is ingested in a restricted way and competition exists between birds. Therefore, the pecking order that influences food intake will modulate drug exposure and lead unavoidably to differences between individuals. Parenteral administration is seldom used in poultry but is frequent in pet birds.

Birds have no teeth (no chewing is possible) but a beak that is often trimmed at hatching and re-trimmed later to prevent behavioural problems (feather pecking). It was shown that for all diets including medicated feed, birds with short upper beaks consumed significantly less than birds with long upper beaks. In many bird species (scavenging birds, etc.), the cervical esophagus is expanded to form a crop allowing the storage of food before digestion occurs. Drugs are not absorbed in the crop, which has a keratinised epithelium. The pH of the crop is about 6 and some drugs ingested in solution in drinking water can precipitate in the crop, resulting in delayed transit and poor absorption, as in the case of tetracyclines. The presence of a *lactobacillus* flora in the crop can inactivate antimicrobial drugs of the macrolide group. Drinking water (and drug in solution) passes directly through the crop, but solid or pasty feed (possibly containing medication) may reside for a long time in the crop with an emptying time in broilers ranging between 3 and 20 h. This is the case when direct administration of food into the crop is carried out at the fattening stage for fattened duck or goose. The food passes from the crop to the stomach. The stomach consists of two parts: the proventriculus that is the glandular portion of the stomach secreting acidic digestive juices, followed by the muscular gizzard that contains gravel (or grit) which works together with the muscles to grind up food. Most drugs used in poultry (antibiotics, coccidiostats, etc.) are weak organic bases and are not absorbed by the proventriculus. The gizzard is a powerful triturating apparatus (replacing the teeth), and in birds any solid dosage forms are rapidly disintegrated to release active ingredients. Most drugs ingested as a solution pass rapidly along the crop and the two stomach chambers to arrive within a few minutes in the intestine. The alkaline pancreatic juice neutralises the acidic contents leaving the gizzard and absorption in birds occurs, as in mammals, in the duodenum and upper jejunum. This rapid transit to the small intestine and the limited development of the distal part of the digestive tract (related to adaptation to flight) explain the very rapid overall transit time of about 5–6 h in broilers of those drugs that are not entrapped in the crop with food.

Force feeding in ducks or geese produces a liver that is six to ten times its ordinary size. The storage of fat in the liver produces steatosis of the liver cells. This

is a metabolic adaptation observed in wild migrating birds and fish (e.g. cod), in which hepatic steatosis occurs naturally as a consequence of energy storage before migration. The process of hepatic steatosis is facilitated in these oviparous species, because the liver is the major site of *de novo* lipogenesis, which is not the case in mammals. Foie gras represents a quasi-pure form of acquired and reversible hepatic steatosis of nutritional origin. The tissue is not diseased, as degenerative events such as necrosis or cirrhosis never occur, so that the metabolism of drugs is minimally affected in fattened duck and goose.

Glomerular filtration is not constant in birds, for example in the face of varying perfusion pressure, and this can have an impact on drug PK. Avian species appear to be more susceptible to renal ischaemia and tissue damage caused by NSAIDs than to the gastro-intestinal tract side-effects. Recently, a decline in the vulture population in India and Pakistan was observed (Oaks et al. 2004) and a direct correlation was shown between renal residues of diclofenac (a NSAID) and renal failure, because in these countries the primary food source for vultures is dead domestic livestock and it was hypothesised that the most probable source of diclofenac exposure was the consumption by vultures of treated livestock.

12 Species Variability in Drug Administration and Disposition in Fish

The main farmed fish in Europe are salmon and rainbow trout. Establishing dosing schedules for drugs in fish must take into account the major differences in anatomy from mammalian species as well as the husbandry circumstances. Several routes of administration are possible. The commonest method and the only one practicable for treating farmed fish (e.g. salmon and trout) is in medicated feed as for anti-parasitic drugs like praziquantel for removal of tapeworms. This requires spontaneous ingestion. As appetite in fish is severely depressed in cases of infection, antibiotherapy should be prophylactic. Gastric emptying in trout and salmon is a very slow process compared with mammals. Drug absorption occurs in the stomach and weak bases are generally well-absorbed, by the classical Henderson–Hasselbalch mechanism. Following absorption, a drug undergoes a possible hepatic first-pass effect, due to a liver portal system as in mammals, and the extent of bioavailability depends on the extent of hepatic catabolism.

Tetracycline has a low bioavailability in fish ($< 10\%$), due to binding with sea-water-borne divalent cations such as Mg^{2+} and Ca^{2+}. It is noteworthy that non-bioavailable tetracyclines contaminate the environment. For example, it has been shown that residues of oxytetracycline in marine sediments were very stable over months. See chapter, "Veterinary Medicines and the Environment" of this text for further discussion. Generally, drug bioavailability is lower in fish than in mammals: for example, 55% for enrofloxacin but only 2% for sarofloxacin (Martinsen and Horsberg 1995).

Biliary elimination and enterohepatic recycling of drugs occur in fish. Topical treatments are available and fish may be placed in baths for treatment in water. Drug examples are dichlorvos and trichlorfon, two organophosphates that are used to treat sea lice infestations. The main challenge of this modality of treatment is the pollution of the environment and the possible impact on non-target species, as discussed in chapter, "Veterinary Medicines and the Environment" of this text. The main route of absorption for drugs directly administered in water is via the gills so that water properties including pH and composition influence drug absorption, as only unionised fractions are absorbed. When oxygen tension decreases, the fish passes more water over its gills for oxygen capture and this can indirectly increase the exposure of the fish to drugs in solution in the water. A large fraction of drug absorbed by the gills is initially transported to the kidneys and can undergo a renal first-pass effect. Moreover, it is noteworthy that enzyme induction at the kidney level in fish exposed to foreign compounds is observed before corresponding hepatic induction occurs.

The fish kidney is similar to the mammalian kidney but with a renal portal system; blood from the portal vein bathes the tubules and exposes them to a much higher fraction of the cardiac output than in mammals. In addition, xenobiotics can be presented directly to the tubules via the caudal vein. This explains why injections into muscle should be made (as in poultry and reptiles) in the cranial segment of the fish.

In fish, drugs may accumulate in fat depots and subsequent removal of feed will promote mobilisation of lipid reservoirs with redistribution throughout the body. Drug metabolism is qualitatively similar in fish and mammals for phase I and phase II processes but the kidney may be the main site of drug metabolism in fish. Fish are heterotherm animals and water temperature has a major influence on the rate of drug metabolism, so that the values of PK parameters are variable rather than fixed; they are temperature-dependent variables. However, acclimatisation is possible and the same rate of drug metabolism may occur at 5°C and 25°C, providing the fish are at their respective acclimatisation temperature. The temperature dependency of drug PK is an important consideration for drug residues. The elimination half-life of antimicrobial drugs increases significantly as the temperature decreases, a fall in temperature from 20 to 10.8°C being associated with an increase of up to 100% in elimination half-life. Ideally, drug dose should be adjusted according to water temperature, but in clinical practise the dose is normally fixed. Therefore, for farmed trout and salmon, the withdrawal times, based on temperature dependent residue levels, are determined in *degree days* (°C × days). Degree-days are calculated by multiplying the mean daily water temperatures by the total number of days measured.. Thus, 160° days represents a withdrawal period of 16 days at 10°C or of 10 days at 16°C. It should be stressed that drug activity can also be temperature dependent; for example when tested against *Aeromonas salmonidae*, the MICs for several quinolones were 2–3-fold higher at 4°C than at 15°C (Martinsen et al. 1992). For an old but still valuable review, see Ingebrigtsen (1991) and for a more recent review see Shao (2001). An exhaustive and searchable data base on drug residues and PK parameters in aquatic species is available online (Reimschuessel et al. 2005).

13 Conclusions

The main conclusion from this review is that differences between species are numerous and often unpredictable in terms of both drug PK and drug PD. The ass is not a rustic horse; the horse is not a large rat; the sheep is not a small cow; "dog" does not exist as a single, simple entity and the concept of poultry or of non-salmonide fishes as simple entities are not applicable in veterinary pharmacology. No generalisations are possible. Rather, each drug must be investigated on a species-by-species basis to guarantee the effective and safe use of drugs, thus ensuring the wellbeing of animals and safeguarding also the environment and human consumption of animal products.

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Comparative and Veterinary Pharmacogenomics

Carrie M. Mosher and Michael H. Court

Contents

Abstract Pharmacogenomics is the study of the impact of genetic variation on drug effects, with the ultimate goal of achieving "personalised medicine". Since the completion of the Human Genome Project, great strides have been made towards the goal of personalised dosing of drugs in people, as exemplified by the development of gene-guided dosing of the anticoagulant drug, warfarin. Although the pharmacogenomics of domestic animals is still at an early stage of development, there is great potential for advances in the coming years as the direct result of complete genome sequences currently being derived for many of the species of significance to veterinary and comparative medicine. This sequence information is being used to discover sequence variants in candidate genes associated with altered drug response, as well as to develop whole genome high density single nucleotide polymorphism arrays for genotype–phenotype linkage analysis. This review summarises the current state of veterinary pharmacogenomics research, including drug

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response variability phenotypes with either known genetic aetiology or strong circumstantial evidence for genetic involvement. Polymorphisms and rarer gene variants affecting drug disposition (pharmacokinetics) and drug effect (pharmacodynamics) are discussed. In addition to providing the veterinary clinician with useful information for the practise of therapeutics, it is envisaged that the increasing knowledge base will also provide a resource for individuals involved in veterinary and comparative biomedical research.

Keywords Cytochrome P450 · Drug metabolism · Malignant hyperthermia · *N*-acetyltransferase · *P*-glycoprotein · Pharmacogenomics · Thiopurine methyltransferase · UDP-glucuronosyltransferase

1 Introduction

Pharmacogenomics is the study of the impact of genetic variation on drug pharmacokinetics and pharmacodynamics, and is concerned with understanding interactions between drugs and the entire set of genes in an organism (i.e. the genome) (Court 2007). Pharmacogenomics is a relatively new discipline that arose less than 15 years ago from the field of pharmacogenetics (a term first coined in 1956 by Carson et al. (1956)), which typically addresses a single, or relatively few, genes. The two terms are often used interchangeably, and for the purposes of this discussion, pharmacogenomics will be used inclusively. As pointed out in a recent review article (Court 2007), over the last 15 years, there has been almost exponential growth in the volume of published pharmacogenomics research. The initial driving force behind this growth was likely the Human Genome Project, initiated in 1990 and culminating in 2003 with the publication of the complete human genome sequence (www.ornl.gov/sci/techresources/Human_Genome/home.shtml). A direct outcome of the Human Genome Project was the International Hapmap Project (2003–2008), which recently completed mapping the common patterns of genetic variation in four different human populations (www.hapmap.org). Currently, the 1000 Genomes Project (2008-onwards) proposes to provide a high resolution map of human genetic variation through sequencing the entire genomes of approximately 1,200 individuals from around the world (www.1000genomes.org).

Unfortunately, research into the pharmacogenomics of non-human species has lagged significantly behind that of human research. However, the recent release of genome sequences for a number of domestic animal species of direct relevance to veterinary clinical medicine and comparative animal research now provides a scaffold on which veterinary pharmacogenomics research can be constructed, hopefully at a more rapid pace. At the time of this writing (April, 2009), complete genome sequences have been deposited in the US National Center for Biotechnology Information (NCBI) Genbank database for the human and mouse, while draft sequence assemblies (in various stages of completeness) are available for a further

29 mammalian species, including dog, cat, rat, rabbit, horse, cow, sheep, and pig (see http://www.ncbi.nlm.nih.gov/genomes/static/gpstat.html). This list will likely continue to grow at a more rapid pace given the recent availability of "massively parallel sequencing" technologies that enable large-scale "shotgun sequencing" of entire genomes on a single chip-based platform within a relatively short period of time (hours for small genomes to weeks for mammalian genomes) (Wheeler et al. 2008). Finally, sequence variation in many of these newly derived genomes is being catalogued to generate single nucleotide polymorphism (SNP) maps that span the entire animal genome. High density SNP chips are now available through various commercial sources (such as Illumina and Affymetrix) for species including dog, horse, sheep and pig, thereby providing a powerful tool for the identification of genes linked to disease and animal production characteristics, as well as those associated with altered drug response.

In this chapter, we outline the current status of comparative and veterinary pharmacogenomics, focusing on drug response phenotypes in companion animals, farm animals and laboratory animal species that have either an established genetic aetiology or, at least, reasonable circumstantial evidence for a significant contribution of genetic variation to phenotype. While it is apparent that the majority of genetic variants identified to date affect drug absorption and disposition (pharmacokinetics), it is likely that variants directly altering drug effect (pharmacodynamics) will continue to be discovered, as the molecular mechanisms underlying drug action continue to be elucidated. In addition to providing the veterinary clinician with useful information for the practise of therapeutics, it is envisaged that this will also provide a resource for individuals involved in veterinary and comparative biomedical research.

2 Drug Disposition

2.1 Oxidative Enzymes

2.1.1 Cytochrome P450 2D15 in Dogs

In humans, cytochrome P450 (CYP) 2D6 is responsible for the metabolism of a large number of drugs. It is also polymorphic, with about 10% of White people classified as "poor metabolisers" (PM) while the remaining 90% are considered "extensive metabolisers" (EM). In dogs, CYP2D15 is presumed to be the orthologous (equivalent) enzyme to human CYP2D6. Like human CYP2D6, canine CYP2D15 phenotypic variation has been reported. In a landmark study of the pharmacokinetics of celecoxib in a colony of 242 Beagle laboratory dogs (from the USA), there was a clear bimodal distribution of drug clearance with 45% having an EM and 53% having a PM phenotype (2% were uncertain) (Paulson et al. 1999). There was also a similar distribution of each phenotype within each sex. Evaluation

of in vitro celecoxib hydroxylase activity in liver microsomal preparations from representative EM and PM phenotyped dogs showed a similar pattern of bimodal phenotypes and concordance between low in vitro celecoxib oxidation and low in vivo celecoxib clearance (Fig. 1). Screening of recombinant canine CYP2B11, CYP2C21, CYP2D15, and CYP3A12 identified CYP2D15 as the main isoform mediating celecoxib hydroxylation, as well as bufuralol oxidation, which is a selective marker for human CYP2D6 activity. Celecoxib metabolism was also potently inhibited by quinidine, a selective inhibitor of human CYP2D6. Taken together, these results indicate that there may be a genetic polymorphism associated with canine CYP2D15 that results in decreased expression or activity of this enzyme. Two amino acid variants of CYP2D15 (CYP2D15*2 – I250F and I307V; CYP2D15*3 – G186S), as well as a putative splice variant that deleted 51 amino acids in exon 3 (CYP2B15del), were identified by sequencing Beagle dog genomic DNA (Paulson et al. 1999) or cDNA derived from dog liver mRNA (Roussel et al. 1998). Recombinant expression of these variants indicated only minimal, if any, effect of the *2 and *3 amino acid variants, and almost complete abolition of activity with the CYP2D15del variant. However, as yet it is not clear whether the presence or amount of the CYP2D15del mRNA variant (relative to full length UGT2B15 mRNA) is associated with decreased CYP2D15 activity in dog liver. Also, there has been no attempt to correlate the presence of any CYP2D15 genetic polymorphism with celecoxib metabolism phenotype. This polymorphism may affect metabolism of other drugs, including propranolol, bufuralol and dextromethorphan, which are known CYP2D15 substrates (Tasaki et al. 1998; Shou et al. 2003).

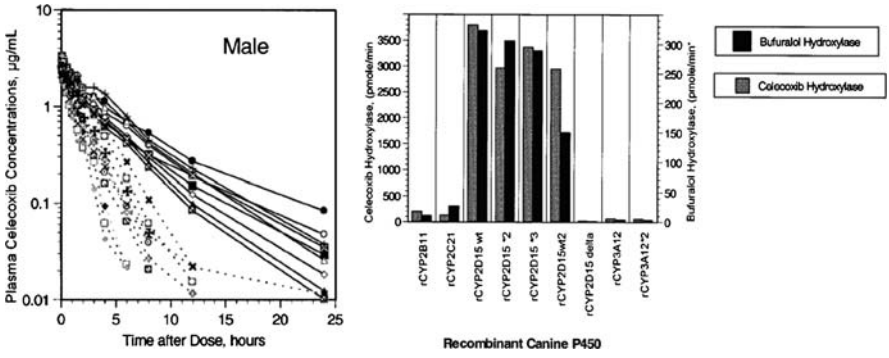

Fig. 1 Polymorphism of celecoxib pharmacokinetics in Beagle dogs (from (Paulson et al. 1999)). Shown in the *left panel* are plasma concentrations of celecoxib following intravenous administration of 5 mg/kg bodyweight of celecoxib to 19 male Beagle dogs. Results with female dogs were similar. *Solid lines* indicate dogs with the PM phenotype, while *dotted lines* indicate dogs with the EM phenotype. The *right panel* shows celecoxib and bufuralol hydroxylase activities for recombinant canine CYP isoforms indicating that both drugs are oxidised by CYP2D15 and that the exon 3 deletion (rCYP2D15delta) greatly decreases activity

2.1.2 Cytochrome P450 2D in Rats

Unlike humans, who have only one functional CYP2D subfamily protein (i.e. CYP2D6), rats have six CYP2D subfamily enzymes, including CYP2D1, 2, 3, 4, 5 and 18. Of these, CYP2D2 may be the most similar to human CYP2D6, based on sequence homology and the ability to metabolise debrisoquine. The Dark Agouti (DA) rat has been used as an animal model of the human CYP2D6 PM phenotype, as this strain of rat metabolises debrisoquine and other CYP2D6 substrates much slower than other rat strains (including Sprague-Dawley and Wistar) (Schulz-Utermoehl et al. 1999). Over 10 years ago, it was determined that low debrisoquine metabolism in DA rats was probably the result of low protein levels of CYP2D2 in the liver (Schulz-Utermoehl et al. 1999). More recent studies have demonstrated lower CYP2D2 mRNA levels (Kawase et al. 2008). However, the molecular genetic cause of this difference is not understood. A polymorphism in diazepam *para*-hydroxylation has also been identified in rats with lowest activity in DA rats compared with Brown Norway, Sprague-Dawley and Wistar strains (Sakai et al. 2009). Polymorphism within the Wistar strain was also observed with both EM and PM phenotypes. A recent study established that this polymorphism is the result of a nucleotide (thymine) insertion into exon 8 of the CYP2D3 gene, resulting in a reading frame shift and enzyme protein truncated prior to the critical heme-binding domain (Sakai et al. 2009).

2.1.3 Cytochrome P450 2C41 in Dogs

To date, two members of the CYP2C subfamily have been studied in dogs, CYP2C21 and CYP2C41. CYP2C21 shares 70% amino acid identity with CYP2C41 and both are similar to the human CYP2C isoforms expressed in liver, including CYP2C8, CYP2C9, and CYP2C19 (Shou et al. 2003). Of seven different canine CYPs tested (including CYP2C21 and CYP2C41), canine CYP2C21 was most active against the human CYP2C9 substrate, diclofenac (Shou et al. 2003). On the other hand, neither of the canine CYP2Cs metabolised the human CYP2C19 substrate *S*-mephenytoin and CYP2C41 possessed only low activity for all substrates evaluated (Shou et al. 2003). Both CYP2C21 and CYP2C41 are expressed in the canine liver; however, while CYP2C21 mRNA was detected in all nine (100%) Beagle livers examined, CYP2C41 mRNA was detected only in one of the nine (11%). A later study confirmed this polymorphism, showing that only 5 of 11 (45%) canine livers expressed CYP2C41 (Graham et al. 2003). The molecular basis for this polymorphism appears to be deletion of the CYP2C41 gene encompassing at least exon 4 to exon 7 (Blaisdell et al. 1998). Genotyping of dogs indicated complete concordance between the lack of expression of CYP2C41 mRNA in the liver and the presence of the CYP2C41 gene deletion. Furthermore, the deletion was identified in mixed-breed dogs, suggesting that it is not specific to Beagles. It is not yet clear what impact this polymorphism will have on canine drug metabolism because high turnover substrates for CYP2C41 have not been identified to date.

2.1.4 Cytochrome P450 1A2 in Dogs

A pre-clinical pharmacokinetic study of a novel cognitive enhancer (AC-3933) in Beagle dogs revealed highly variable drug clearance with a bimodal distribution, such that dogs could be divided into EM and PM groups. In vitro studies confirmed 50- to 100-fold lower intrinsic clearance (V_{max}/K_m) values in liver microsomes from PM compared with EM dogs (Mise et al. 2004b). Furthermore, CYP1A protein could not be detected by immunoblotting in the PM dogs (Mise et al. 2004a; Mise et al. 2004b) (Fig. 2). Two groups independently identified a nonsense mutation in canine CYP1A2 cDNA that introduced a premature stop codon instead of an arginine at position 373 (c.1117 C>T; R373X), thereby eliminating the predicted heme-binding region and resulting in a non-functional enzyme (Mise et al. 2004a; Tenmizu et al. 2004). The genotypic frequency of the homozygous mutant genotype in CYP1A2 was 0.11 (in 149 Beagles) and 0.17 (in 65 Beagles) implying that 11–17% of Beagles would not express CYP1A2 protein (Mise et al. 2004a; Tenmizu et al. 2004). Pre-clinical studies of a novel phosphodiesterase type 4 inhibitor, YM-64227, also metabolised by canine CYP1A2, reported 17 and 27 times higher maximum plasma drug concentrations and bioavailability, respectively for PM dogs compared with EM dogs following oral administration, thus indicating a large effect of CYP1A2 deficiency on first-pass drug metabolism (Tenmizu et al. 2006a, 2006b). More recently, the substrate specificity of canine CYP1A2 was examined and, while there was overlap between human and canine substrates, some typical human CYP1A2 substrates, including caffeine and oestradiol, were not good substrates for dog CYP1A2 (Mise et al. 2008). Nevertheless, as the canine CYP1A2 R373X polymorphism represents a true "null" allele, this polymorphism should provide a useful tool for in vitro and in vivo studies seeking to identify the contribution of canine CYP1A2 to metabolism of drugs in dogs. To

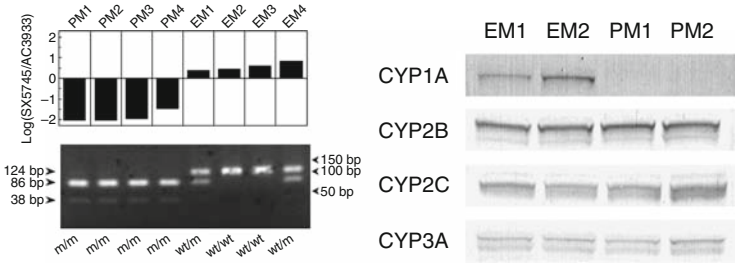

Fig. 2 Canine CYP1A2 protein and activities in dog liver genotyped for the *CYP1A2* c.1117c>t (R373X) polymorphism. *Left panel* (from Mise et al. (2004a)) shows the log ratios of metabolite (SX5745) to parent (AC3933) concentrations in the plasma of four PMs and four EMs Beagle dogs after being administered the putative CYP1A2 substrate, AC3933. Below are the CYP1A2 c.1117c>t genotypes for each animal determined by PCR-RFLP analysis (wt = wild-type C allele; m = mutant T allele). The *right panel* (from Mise et al. (2004b)) shows immunoblots of liver microsomes from EM and PM dogs probed with antibodies that recognise CYP1A, CYP2B, CYP2C, and CYP3A enzymes. PM dogs do not express any detectable CYP1A protein

date, the incidence of this polymorphism in canine breeds other than Beagles has not been reported.

2.1.5 Cytochrome P450 2B11 in Dogs

The pharmacokinetic disposition of some drugs is markedly different in Greyhounds compared with other breeds of dog (Court 1999). Nearly 40 years ago it was recognised that Greyhounds tended to recover relatively slowly from the effects of thiopentone, an ultra-short acting intravenously administered barbiturate used for anaesthetic induction and short duration anaesthesia in dogs (Court 1999). It was initially speculated that the relatively low body fat content typical of all Greyhounds may limit the rate of redistribution of drug from the central compartment to adipose tissue within the peripheral compartment, thereby delaying anaesthetic recovery. However, a series of elegant studies demonstrated that the difference may in large part be the result of slower drug metabolism in Greyhounds (Sams et al. 1985; Robinson et al. 1986; Sams and Muir 1988).

A pharmacokinetic study of thiopentone and thiamylal (a structurally related thiobarbiturate in clinical use at that time) demonstrated that plasma elimination of both drugs following intravenous administration was much slower in Greyhounds ($n = 12$) compared with mixed-breed dogs ($n = 10$) (Sams et al. 1985). Although it was possible to calculate pharmacokinetic parameters for both drugs in the mixed breed dogs, it was not possible to do so for the Greyhounds because plasma concentrations decreased not only more slowly but also in a non-exponential (i.e. non-first-order process) manner from 20 to 480 min after drug administration. Over this same period, drug concentrations in plasma also averaged over 3-fold higher in Greyhounds compared with non-Greyhound breeds for both drugs. Complete anaesthesia recovery (time to standing) was also much slower, by more than 3-fold, in Greyhounds. However, in contrast to the thiobarbiturates, for the ultra-short acting oxybarbiturate, methohexitone, there was a much smaller difference, with plasma clearance being about 45% lower in Greyhounds, while pentobarbitone (a medium acting oxybarbiturate) possessed essentially identical pharmacokinetic parameters for both Greyhound and non-Greyhound breeds. Anaesthesia recovery times were also somewhat slower with methohexitone but indistinguishable for pentobarbitone for Greyhounds compared with mixed breed dogs. The pharmacodynamic sensitivity of Greyhounds to thiobarbiturates is not likely to account for these differences in recovery times, as plasma drug concentrations at recovery were not lower in Greyhounds compared with mixed-breed dogs. These differences in anaesthesia recovery times between Greyhound and mixed breed dogs were confirmed in another study by the same group that also evaluated possible differences in cardiovascular and pulmonary effects (Robinson et al. 1986). Finally, in a third study, thiopentone pharmacokinetics was determined before and after treatment of Greyhounds for 14 days with the liver enzyme inducer, phenobarbitone, and the results compared with a matched control group of Greyhounds. Significant reductions in thiopentone concentrations were observed in the phenobarbitone treated

group but not in the control group. Furthermore, the pharmacokinetics of thiopentone in phenobarbitone-treated animals following a typical exponential decay consistent with first order elimination and half-life and clearance values approximated those previously reported for mixed-breed dogs (Sams and Muir 1988). Given the known importance of cytochrome P450 in determining the elimination of barbiturates in other species, these findings are consistent with a lack in Greyhounds of one or more cytochrome P450 isoforms responsible for thiobarbiturate (but not pentobarbitone) metabolism in dogs.

Elimination of several other drugs is also reported to be significantly slower in Greyhounds compared with other canine breeds. These include propofol (Zoran et al. 1993), antipyrine (KuKanich et al. 2007), ketoconazole (KuKanich and Borum 2008b) and celecoxib (Hunter et al. 2005), all of which are metabolised by cytochrome P450. On the other hand, methadone, a substrate for cytochrome P450 3A, was reported to have a higher clearance in Greyhounds compared with Beagles (KuKanich and Borum 2008a), suggesting that not all cytochrome P450 isoforms are deficient. Amikacin, a polar drug which is cleared primarily unchanged by the kidney, is reported to have a 30% lower plasma clearance in Greyhounds compared with Beagles, although the volume of distribution is also lower in Greyhounds such that elimination half-life is similar between the breeds. Morphine, which is cleared primarily by glucuronidation and also by sulphation, also showed a somewhat lower clearance by about 30% in Greyhounds compared with published values for other dogs, although again the elimination half-life was similar because of a lower volume of distribution (KuKanich and Borum 2008b). Finally, the depolarising muscle relaxant succinylcholine, which is metabolised by plasma pseudocholinesterase, has a similar duration of muscle relaxant effect in Greyhounds compared with mixed-breed dogs (Curtis and Eicker 1991). The same study also demonstrated similar pseudocholinesterase activities in plasma collected from the same dogs regardless of breed.

In vitro studies have explored the molecular basis for slower metabolism of propofol, a non-barbiturate ultra-short acting anaesthetic, in Greyhounds (Court et al. 1999; Hay Kraus et al. 2000). An in vitro assay using dog liver microsomes was developed to measure the rate of cytochrome P450 mediated oxidation of propofol to 4-hydroxyprofol, the major propofol metabolite in dogs (Court et al. 1999). Using liver microsomes from male Greyhound, Beagle, and mixed-breed dogs (five animals each), it was found that the rate of propofol oxidation was about 3 times slower in Greyhound liver compared with Beagle liver, while mixed-breed dog liver activities were intermediate (Fig. 3). Enzyme kinetic studies showed similarly that the difference primarily was not associated with altered enzyme affinity (based on K_m values) but was the result of lower V_{max} values in the Greyhound livers (Hay Kraus et al. 2000). This suggests that Greyhounds may have lower expression of the main cytochrome P450 responsible for propofol hydroxylation in dogs. Further work using P450-specific chemical and antibody inhibitors indicated that this isoform is probably CYP2B11, a major isoform expressed in dog liver. However, as yet it is unclear whether a genetic polymorphism accounts for low CYP2B11 expression, and whether low CYP2B11

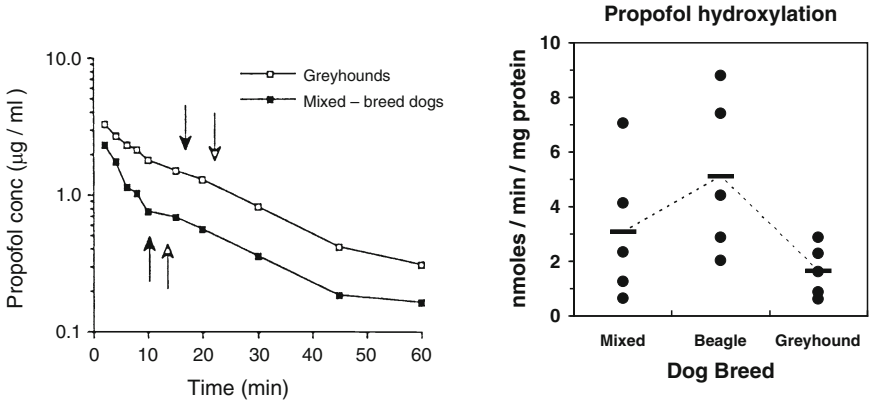

Fig. 3 Propofol clearance from plasma in vivo and propofol hydroxylation by liver in vitro is slower in Greyhounds compared with mixed-breed and Beagle dogs. The *left panel* (from Zoran et al. (1993)) shows differences in mean propofol concentrations between Greyhound ($n = 10$) and mixed breed ($n = 8$) dogs after intravenous administration of 5 mg/kg body weight. The *arrows* indicate time of return of the righting reflex (*filled arrow*) and ability to stand (*open arrow*). The *right panel* (adapted from Court et al. (1999)) shows differences in propofol hydroxylation activity measured by HPLC of in vitro incubations using liver microsomes prepared from male Greyhounds, beagles, and mixed-breed dogs ($n = 5$ each). The points represent data from individual dog livers, while the *horizontal lines* indicate the mean values of each group

expression also accounts for decreased clearance of thiobarbiturates or other drugs in Greyhounds.

2.1.6 Esterases in Horses and Rabbits

Benzylpenicillin is the most frequently used penicillin in equine therapy, and is often given intramuscularly as a procaine salt. However, occasionally horses demonstrate adverse effects following injection of procaine penicillin ranging from sweating, staggering and tachycardia to seizures and death (Olsén et al. 2007). While this has been attributed primarily to an immune-mediated hypersensitivity reaction to the penicillin, procaine also has the potential to cause cardiotoxic and neurotoxic effects (including seizures) as the result of inhibition of voltage-gated sodium channels. Procaine is normally rapidly metabolised by plasma esterase to the nontoxic metabolites *para*-aminobenzoic acid (PABA) and diethylaminoethanol following intramuscular injection and is unlikely to reach toxic concentrations (Tobin et al. 1976). However, in a study of plasma collected from 27 horses including Thoroughbreds and Standardbreds, high variability in plasma procaine esterase activities was observed between individual horses, although the distribution of activities was considered to be uniform (i.e. not bimodal with clear PM and EM phenotypes) (Tobin et al. 1976). The possible molecular genetic basis for this variability in esterase activities in horses has not been explored. A recent Swedish study demonstrated that plasma procaine esterase activities were lower in horses

that had previously experienced an adverse reaction to procaine penicillin, compared with a matched control group of horses (Fig. 4; Olsén et al. 2007). However, the involvement of procaine in these reactions is not certain as the reactions in most horses occurred within minutes of administration, which is too rapid for absorption of procaine from intramuscular sites. In addition, the difference in median esterase activities between reactor and control horses while statistically significant, was relatively small (less than 10% difference) and this may be of limited biological significance. The authors also reported a difference in breed susceptibility with Warmbloods and Thoroughbreds being over-represented out of the 59 identified reactor horses, although this could simply reflect the population of horses being treated with procaine penicillin (Fig. 4).

The ability of rabbit serum to hydrolyse procaine, as well as the anticholinergic drug atropine, varies greatly both between and within rabbit strains (Harrison et al. 2006). New Zealand white (NZW) rabbits were shown to have a bimodal distribution of esterase activities (Stampfli and Quon 1995) with 70% EM and 30% PM phenotypes. There was also high concordance between atropine and procaine esterase activities suggesting that they were metabolised by the same enzyme in rabbits. However, the molecular genetic basis for this variability is not understood.

Paraoxonase is an arylesterase that hydrolyses and inactivates neurotoxic organophosphate metabolites (Richter et al. 2009). Rabbits have high paraoxonase activity compared with other species and have been used as a model for studies of organophosphate (insecticide and nerve gas) toxicity. This enzyme is also polymorphic in both people and rabbits and amino acid variants have been identified that explain low paraoxonase activity in humans (*hPON1* Q192R) and rabbits (*rPON1* P82S, K93E and S1O1G) (Watson et al. 2001).

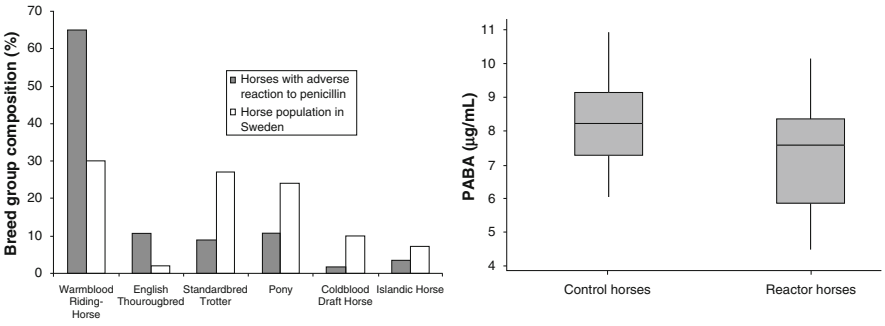

Fig. 4 Adverse reactions to procaine penicillin and serum procaine esterase activity in Swedish horses (from (Olsén et al. 2007)). The *left panel* shows the breed distribution of horses ($n = 59$) with reported adverse reactions to procaine penicillin administration compared with the distribution of horses in Sweden. The *right panel* is a box and whiskers plot (median, interquartile range) that shows the difference in serum procaine esterase activities (formation of *para*-aminobenzoic acid (PABA) from procaine) in blood collected from horses that had adversely reacted to procaine penicillin ($n = 29$) compared with a matched group of control horses ($n = 17$)

2.2 *Conjugative Enzymes*

2.2.1 *N*-acetyltransferase in Mice, Rats, Hamsters, Rabbits and Rhesus Macaques

In humans, slow *N*-acetyltransferase (NAT) metabolism resulting from polymorphisms in the *NAT* genes has been implicated in adverse reactions to amine drugs such as isoniazid and procainamide, as well as susceptibility to develop cancer, particularly bladder cancer (Martell et al. 1991; Sim et al. 2008). Consequently, laboratory animals, including mice, rats, hamsters, rabbits and rhesus macaque with naturally occurring *NAT* polymorphisms have been used to model *NAT* polymorphisms in people to investigate altered risks for drug toxicities and cancer.

Humans have two *NAT* genes, *NAT1* and *NAT2*, encoding NAT1 with *para*-aminosalicylic acid as the prototypical substrate, and NAT2 with isoniazid as the prototypical substrate. Polymorphisms in human *NAT2* are thought to be responsible for adverse reactions to the antituberculosis drug, isoniazid (http://N-acetyltransferasenomenclature.louisville.edu). The number of *NAT* genes varies between species, such that cats only have NAT1 (with very low turnover rates), dogs and musk shrews (*Suncus marinus*) completely lack *NAT* genes (or *N*-acetylation activity), while rodents have three genes designated *Nat1, Nat2,* and *Nat3* (Trepanier et al. 1997; Trepanier et al. 1998; Sim et al. 2008). However, it should be noted that, based on tissue distribution and substrate specificity, murine *Nat1* is most homologous to human *NAT2*, and murine *Nat2* is most homologous to human *NAT1* (Boukouvala et al. 2002). The reason for this somewhat confusing nomenclature is that the polymorphic murine *Nat2* locus was named after the polymorphic human *NAT2* locus and this nomenclature was maintained despite subsequent studies demonstrating different substrate specificities (Sim et al. 2008). The functional significance of murine *Nat3* is unclear, as high turnover substrates for this enzyme have not yet been identified and deletion of the *Nat3* gene in mice does not significantly alter *N*-acetylation of a range of substrates (Sugamori et al. 2007).

Polymorphic sulfadiazine acetylation was first described in rabbits over 45 years ago (Frymoyer and Jacox 1963) and it was subsequently determined that this was the result of a deletion of *NAT2* (Sasaki et al. 1991). Sequencing of the murine *Nat1* and *Nat2* genes in phenotyped fast and slow *N*-acetylator mouse strains identified a N99I amino acid change in the *Nat2* protein that was associated with slow *N*-acetylation (Martell et al. 1991; DeLeon et al. 1995). No polymorphisms were identified in the murine *Nat1* gene. The N99I substitution was found to conformationally modify the Nat2 enzyme with both diminished stability and decreased substrate affinity (Martell et al. 1991; DeLeon et al. 1995). Furthermore, this polymorphism was associated with an increased susceptibility to teratogen-induced cleft lip/palate in the slow acetylators (Boukouvala et al. 2002). In rats, two slow acetylator inbred strains have been identified, including WKY and NSD strains. Both strains have the same two amino acid substitutions (V121I and V266I) that likely account for

the slow metaboliser phenotype. Again, functionally significant variants of the rat *Nat1* gene have not been identified and rat *Nat3* has only a single reported polymorphism (A207S) of uncertain significance (Walraven et al. 2007). Polymorphic *N*-acetylation has also been described in the Syrian hamster and was attributed to a nucleotide substitution, resulting in a premature stop codon at amino acid position 243 (R243Stop) (Ferguson et al. 1996). Finally, a polymorphism (V231I) has been identified in the *NAT2* gene in the rhesus macaque (*Macaca mulatta*) that results in altered *N*-acetylation activity (Fakis et al. 2007). A comprehensive database maintaining *NAT* allelic variants for many non-human mammalian species is available online (http://www.mbg.duth.gr/non-humannatnomenclature/).

2.2.2 UDP-Glucuronosyltransferase 1A in Rats

The Gunn rat is a mutant strain of the Wistar rat, first identified over 60 years ago, that develops a severe unconjugated hyperbilirubinaemia (jaundice) resulting from deficient hepatic bilirubin UDP-glucuronosyltransferase (UGT) activity. It has been used extensively as a model of unconjugated hyperbilirubinaemia in people (particularly Crigler-Najjar syndrome, type 1), as well as providing an invaluable tool for UGT enzymology and molecular genetics (Iyanagi et al. 1989). In all mammalian and avian species evaluated to date, the *UGT1A* gene encodes multiple UGT1A subfamily enzymes through mRNA splicing of different exon 1 regions encoding the N-terminal half of the enzyme to shared exons 2–5 that encodes the C-terminal enzyme half. In the Gunn rat (and in some instances of Crigler-Najjar syndrome in people), there is a single base deletion that introduces an in-frame stop codon in a common region of the *UGT1A* gene (Iyanagi et al. 1989; Roy-Chowdhury et al. 1991). Consequently, all proteins of UGT1A isoforms are truncated, resulting in a severe phenotype that includes deficient glucuronidation of bilirubin (by the UGT1A1 isoform) as well as various drugs (Iyanagi et al. 1989; Roy-Chowdhury et al. 1991). However, recent work suggests that there is a degree of compensatory upregulation of other UGTs (especially UGT2B subfamily isoforms that are encoded by individual genes) and of other drug-metabolising enzymes, including cytochrome P450s, and therefore detoxification is not as severely impaired as would be expected (Dietrich et al. 2001; Haraguchi et al. 2004). Increased adverse effects of various drugs and intoxicants that are glucuronidated have been studied in the Gunn rat. An increase in the covalent binding of the carcinogen benzo[a]pyrene to liver microsomal protein was reported in Gunn rats, presumably because of deficient glucuronide conjugation of activated metabolites generated by cytochrome P450 (Hu and Wells 1992). Similarly, acetaminophen (paracetamol) exerts greater hepatic and renal toxicity in the Gunn rat, associated with a 72% reduction in acetaminophen glucuronidation (de Morais and Wells 1989). Enhanced toxicity of irinotecan, which is commonly used in people to treat colon cancer, has also been observed in Gunn rats compared with Wistar normal rats, associated with decreased glucuronidation and increased levels of the active metabolite SN-38 (Onoue et al. 2008). Most recently, the Gunn rat has served as a useful model for evaluating and

developing gene therapy methods for the correction of monogenetic disorders (Miranda and Bosma 2009).

2.2.3 UDP-Glucuronosyltransferase 2B2 in Rats

Another example of a rat strain deficient in a glucuronidation activity is the low androsterone (LA) Wistar rat. This mutant rat strain was identified initially through demonstration of a masculinised phenotype accompanied by increased levels of the androgen androsterone resulting from LA glucuronidation activity in the liver. LA Wistar rats demonstrate approximately 50-fold lower androsterone glucuronidation by hepatic microsomes, as well as substantially decreased biliary excretion of exogenously administered androsterone (Corser et al. 1987). Subsequent studies identified UGT2B2 as a major isoform glucuronidating androsterone and other endogenous androgenic steroids in rat liver (Haque et al. 1991). It was subsequently demonstrated that UGT2B2 mRNA is not present in the liver, and it was determined that a major portion of the coding region of the gene is deleted (Corser et al. 1987; Homma et al. 1992). Similar to Gunn rats, the LA phenotype demonstrates autosomal recessive inheritance and is present with variable incidence in Wistar rat colonies throughout the world (Matsui and Watanabe 1982; Homma et al. 1992).

2.2.4 Thiopurine Methyltransferase in Cats, Dogs and Mice

The pharmacogenetics of the thiopurine methyltransferase (TPMT) enzyme has been extensively studied in people, as this enzyme metabolises a number of important cancer and immunosuppressant drugs (including 6-mercaptopurine and azathioprine), and genetic variants causing low enzyme activity enhance adverse drug effects such as bone marrow toxicity (Kidd et al. 2004; Marsh and Van Booven 2009). The incidence of TPMT polymorphism is relatively high in humans, with 12% of Whites being heterozygous, and 3% homozygous for alleles that result in low or absent TPMT activity (Salavaggione et al. 2002). In addition to humans, TPMT activity has been identified in the red blood cells of dogs, cats, horses, rats, and mice (White et al. 2000). Highly variable and/or polymorphic activity has been described for mice, cats, and dogs (Fig. 5). In a study of 177 dogs, there was a 9-fold range in TPMT activities, with certain breeds having low activities (such as Giant Schnauzer) while other breeds (such as Alaskan malamute) had relatively high activities (Kidd et al. 2004) suggesting genetic variation as a cause. In another study, the canine TPMT gene was sequenced and a total of nine polymorphisms were identified, including six SNPs and three insertion/deletion variants. Six of these polymorphisms explained 40% of the variance between dogs (Salavaggione et al. 2002). In contrast to dogs and humans, a study of TPMT in cat blood showed relatively low TPMT activities, likely explaining the sensitivity of cats to thiopurine treatment (Foster et al. 2000; White et al. 2000). In addition, 31 polymorphisms

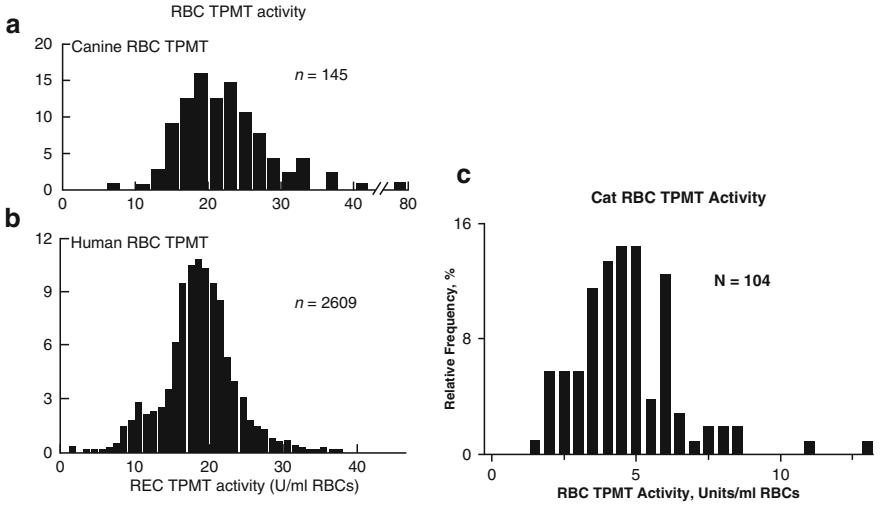

Fig. 5 Population distributions of red blood cell thiopurine methyltransferase (TPMT) activities measured in (**a**) dogs ($n = 145$), (**b**) humans ($n = 2{,}609$) and (**c**) cats ($n = 104$). Dog and human data from (Salavaggione et al. 2002); cat data from (Salavaggione et al. 2004)

were discovered, with 12 of these accounting for 30% of the total variance in TPMT activities representing an approximately 10-fold range (Salavaggione et al. 2004).

Variability in TPMT activity between inbred mouse strains has served as a useful model system for studying TPMT (Hernandez et al. 1990). C57BL/6J and AKR/J have been identified as TPMT slow metaboliser strains, while DBA/2J is a fast metaboliser phenotype (Otterness and Weinshilboum 1987a; Otterness and Weinshilboum 1987b). Breeding studies determined that slow TPMT metabolism is inherited as an autosomal recessive trait. Although it had been initially proposed that the different murine phenotypes were due to variations in the quantity of TPMT protein (analogous to human TPMT variation), inter-strain variations appear to be primarily related to differential mRNA expression (Watters and McLeod 2002). A genetic study of mouse strain crosses identified only two major haplotypes in the mouse *Tpmt* gene and those haplotypes were highly correlated with TPMT activity (Watters et al. 2004). However, no particular variant could be identified that explained the phenotype.

2.3 Transporters

2.3.1 *P*-glycoprotein in Dogs and Mice

Perhaps the best example of clinically important pharmacogenomic variation in dogs involves the drug transporter *P*-glycoprotein (*P*-gp), which is the product of

the *ABCB1* (formerly *MDR1*) gene (See also chapter, "Drug Delivery Systems in Domestic Animal Species" Sect. 4). *P*-gp is an ATP-dependent efflux drug transporter protein, located primarily in the intestine, liver, kidney and brain where it limits systemic and brain uptake of drugs and enhances drug excretion (Mealey et al. 2008). Consequently, loss of *P*-gp function (genetically or through drug–drug interactions) can lead to increased drug levels and enhanced brain penetration of certain drugs. For a number of years, it was recognised that Collies were unusually susceptible to the CNS depressant side effects of ivermectin (Paul et al. 1987). In one study, the lethal dose of ivermectin in Collies was only 1/10th to 1/20th of the lethal dose in Beagle dogs (Pulliam et al. 1985). However, it was later shown that there were no significant differences in plasma pharmacokinetics of ivermectin between sensitive and non-sensitive Collies, suggesting a role for either enhanced CNS sensitivity or enhanced brain penetration of drug in susceptible animals or both (Tranquilli et al. 1989). Subsequently, it was demonstrated that mice with genetic ablation of *Mdr1a* (the mouse homolog of *ABCB1*) were also exquisitely sensitive to the CNS toxic effects of ivermectin (Schinkel et al. 1994), which identified canine *ABCB1* as an appropriate candidate gene for ivermectin sensitivity in Collies. Examination of the *ABCB1* sequences of three ivermectin-sensitive Collies revealed an identical 4-bp deletion that causes a frame-shift mutation resultant premature stop codon and produces a truncated, non-functional protein (Mealey et al. 2001). As probably *P*-gp is important for the disposition of many other drugs in addition to ivermectin, it is likely that dogs with the *ABCB1*-del4 will be susceptible to the adverse effects of a range of other drugs. Indeed, excessive sedation with loperamide (Sartor et al. 2004) and toxicity of vincristine (Mealey et al. 2003) have been observed in dogs with *ABCB1*-del4.

ABCB1-del4 primarily affects herding breed dogs, particularly Collies (Neff et al. 2004; Mealey and Meurs 2008). Seventy-five percent of Collies in the United States, France, and Australia have at least one mutant allele (Hugnet et al. 2004; Neff et al. 2004; Mealey et al. 2005; Mealey and Meurs 2008). As shown in Table 1, other herding breeds with a relatively high prevalence (10% or more carriers) include Australian Shepherd, German Shepherd, and Shetland Sheepdog, and there is also a low prevalence in the Border Collie, Bearded Collie, and Australian Cattle Dog (Mealey et al. 2003; Mealey and Meurs 2008). Interestingly, certain non-herding breeds including the Silken Windhound and Long-haired Whippet have a high prevalence (31–58%) of the mutation, suggesting common ancestry with the Collie dog, perhaps reflecting a previous breeding strategy to achieve and maintain a desirable characteristic. Also noted in Table 1 is that mixed-breed dogs of uncertain ancestry may be carriers (up to 11%), although as the data were obtained from a clinical *ABCB1*–del4 genotyping service, it is likely that the DNA samples were being submitted for testing by owners and veterinarians because of suspicion that the dogs might have herding-breed ancestry. In addition to the clinical implications of the *ABCB1*–del4 mutation, because affected dogs are essentially a naturally occurring large animal (non-rodent) *P*-gp knockout model, they have also been used for research into the role of *P*-gp in the blood–brain and blood–cerebrospinal fluid barriers (Mealey et al. 2008). Mutant dogs are also being

Table 1 Frequency of ABCB1 genotypes (No. of dogs [%]) determined via analysis of DNA in buccal swab samples collected from 5,368 dogs in North America between May 1, 2004, and September 30, 2007

Breed	No. of dogs	Genotype		
		ABCB1 wt/wt	ABCB1 mut/wt	ABCB1 mut/mut
Australian Shepherd	1,421	754 (53)	525 (37)	142 (10)
Border Collie	306	301 (98)	4 (1)	1 (0.003)
Collie	1,424	322 (23)	598 (42)	504 (35)
English Shepherd	28	28 (100)	0 (0)	0 (0)
German Shepherd	166	149 (90)	14 (8)	3 (2)
Herding-breed mix[a]	312	276 (89)	32 (10)	4 (1)
Longhaired Whippet	24	10 (42)	14 (58)	0 (0)
Miniature Australian Shepherd	285	180 (63)	96 (34)	9 (3)
McNab	1	1 (100)	0 (0)	0 (0)
Mixed breed[b]	238	212 (89)	19 (8)	7 (3)
Old English Sheepdog	40	39 (97.5)	1 (2.5)	0 (0)
Shetland Sheepdog	448	395 (88)	47 (11)	6 (1)
Silken Windhound	16	11 (69)	5 (31)	0 (0)
Other purebreeds	659	659 (100)	0 (0)	0 (0)

ABCB1 genotyping was performed according to previously published methods. ABCB1 wt/wt = Dogs with 2 ABCB1 wild-type alleles. ABCB1 mut/wt = Dogs with 1 ABCB1 wild-type allele and 1 ABCB1-1 Δ allele. ABCB1 mut/mut = Dogs with 2 ABCB1-1Δ alleles

[a]Herding breed mix was a dog in which at least 1 parent was known to be a herding breed.

[b]Dogs were not identified specifically as a herding breed mix or a non-herding breed mix; the proportion of the former in this group is not known

From (Mealey and Meurs 2008)

used to define the role of *P*-gp in drug disposition for preclinical drug development studies in dogs (Kitamura et al. 2008).

Shortly following the discovery that *Mdr1a* gene knockout mice were susceptible to ivermectin toxicity, a sub-population of the CF-1 strain of mice was identified that was also susceptible because of a lack of *P*-gp expression (Umbenhauer et al. 1997). Subsequent studies showed that these mice expressed a truncated *Mdr1a* mRNA with deletion of a portion corresponding to exon 23 (Jun et al. 2000; Pippert and Umbenhauer 2001). Interestingly, genomic DNA sequencing identified a large DNA insertion at the exon 23 intron–exon junction with high homology to a murine leukaemia virus. This insertion resulted in aberrant splicing of the mRNA and the loss of exon 23 during RNA processing.

2.3.2 MRP2 in Rats

Multidrug resistance-associated protein 2 (MRP2; also called ABCC2) is an ATP-dependent active transporter of organic anions, such as glutathione, glucuronide, and sulphate conjugates located in the canalicular membrane of hepatocytes as well as polarised kidney and intestinal cells (Wright and Dickinson 2004). TR^- (transport negative) rats are a mutant strain of the Wistar rat that have an autosomal recessive defect in the biliary excretion of many organic anions resulting from

deficient Mrp2 expression (Wright and Dickinson 2004; Leslie et al. 2007). The genetic basis for the defect involves a single base-pair deletion at amino acid 393 that results in a frameshift and premature stop codon and truncated Mrp2 protein (Paulusma et al. 1996). TR^- rats have been used for a number of years as a model of human Dubin–Johnson syndrome, which is characterised by a conjugated hyperbilirubinaemia resulting from decreased ability to excrete conjugated bilirubin from the hepatocyte into the bile (Paulusma et al. 1997). Like the TR^- rat, people with Dubin Johnson syndrome have various genetic defects in the gene encoding human MRP2 (Paulusma et al. 1997; Wada et al. 1998). The TR^- rat has also been useful in identifying drugs and substrates other than bilirubin glucuronide that are substrates of MRP2 including valproic acid, acetaminophen, and gemfibrozil (Xiong et al. 2000; Kim et al. 2003; Wright and Dickinson 2004).

2.4 Other

2.4.1 Albumin in Rats and Dogs

Polymorphism in the binding of drugs to albumin in rats with consequential effects on pharmacokinetics has recently been described (Ito et al. 2007). Specifically, it was observed that D01-4582, a drug candidate under development, had over 6-fold higher plasma clearance values in SD compared with the CD strains of the Sprague-Dawley rat. Studies using isolated hepatocytes from each strain indicated that hepatocellular drug uptake was markedly different when incubated in the presence of plasma. Further studies on plasma protein binding using plasma from six different rat strains identified low-K_d/high affinity rat strains (CD, EHBR and Lewis) and high-K_d/low affinity rat strains (SD, Wistar and Brown Norway), which agreed with the classification based on pharmacokinetic phenotype for CD and SD rats (Ito et al. 2007). Sequencing of the rat albumin gene cDNA identified 11 different polymorphisms. Of these, two amino acid substitution mutations (V238L and T293I) were identified only in the high-K_d strains and were suggested to cause decreased binding affinity of D01-4582 to rat albumin with a resulting higher clearance. Interestingly, the same group recently reported a similar effect of two linked amino acid polymorphisms in the canine albumin gene (A335S and G450E) on the pharmacokinetics of D01-4582 in Beagle dogs (Ito et al. 2009). The prevalence of this allele in 47 Beagle dogs from their colony located in Japan was 40%. As yet, it is not clear what other drugs will be affected by this polymorphism, and whether Beagles in other colonies or other dog breeds carry the allele.

2.4.2 Arylhydrocarbon Receptor in Mice and Rats

The arylhydrocarbon receptor (AhR) mediates the genetic response to polycyclic aromatic hydrocarbons and dioxins, resulting in enhanced expression of a multitude

of genes including those encoding oxidative enzymes (such as CYP1A1) and conjugative enzymes (such as UGT1A6) (Poland et al. 1994). In mice and rats, the *Ah* gene which encodes for AhR has several known alleles that affect its responsiveness to dioxins. In mice, there are four alleles, Ah^{b-1}, Ah^{b-2}, Ah^{b-3}, and Ah^{d}, of which the first three display high ligand affinity and differ only by a few point mutations in the common open reading frame and additional sequence at the carboxyl ends (Poland and Glover 1990). In contrast, the Ah^{d} allele encodes the receptor with the lower binding affinity, attributable to valine at position 375 (Poland et al. 1994). The aromatic hydrocarbon responsiveness is inherited as an autosomal dominant trait and the alleles were named for the mice initially found to carry them (Ah^{b} for C57BL/6 mice, and Ah^{d} for DBA/2 mice). It is now known that AKR, DBA/2 and 129 strains carry the Ah^{d} allele, C57BL/6, C58 and MA/My strains carry the Ah^{b-1} allele, the Ah^{b-2} allele is found in BALB/cBy, A and C3H strains, while the Ah^{b-3} allele is found in *Mus caroli, Mus spretus* and MOLF/Ei (Poland and Glover 1990). In rats, there is at least a 1,000-fold difference in sensitivity to the lethal effects of dioxins between the sensitive Long–Evans (L–E) strain and the resistant Han/Wistar (H/W) strain resulting from a point mutation in the H/W *Ahr* allele that forms an abnormal C-terminus transactivation domain and a smaller AhR protein (Simanainen et al. 2003). In addition to the mutation, L–E rats appear to have higher total hepatic levels of AhR than H/W rats (Pohjanvirta et al. 1999).

3 Drug Effect

3.1 Malignant Hyperthermia in Pigs, Dogs, Cats and Horses

Malignant hyperthermia is a heritable drug hypersensitivity syndrome that classically displays as muscle rigidity, elevated core body temperature, hypercapnia, acidosis, hyperkalaemia, sympathoadrenal activation, and rhabdomyolysis after administration of a triggering agent. This is typically an inhalant anaesthetic and/or succinylcholine. The biochemical basis for the syndrome involves aberrant regulation of calcium release from sarcoplasmic reticulum stores within skeletal muscle. Based upon literature reports, affected species include humans, pigs, dogs, cats, and horses (reviewed in Brunson and Hogan (2004)).

Pigs have been an invaluable research model of malignant hyperthermia, assisting the discovery of the gene mutations most frequently associated with this syndrome in humans (MacLennan and Phillips 1992). The condition was first identified in pigs and described as “porcine stress syndrome” (PSS). It is characterised by mortality in a high proportion of animals sent to slaughter, death being preceded by high body temperatures, blotched skin, and hyperventilation. Those that survived until slaughter were also more likely to have poor quality unmarketable meat (described as “pale-soft-exudative” or PSE pork). Subsequently, it was

recognised that PSS/PSE might represent a form of malignant hyperthermia, as affected pigs also responded to inhalant anaesthetics with symptoms that typified malignant hyperthermia in people (Berman et al. 1970). Laboratory studies using muscle from affected pigs revealed an altered sensitivity to the effects of ryanodine (Mickelson et al. 1988). Ryanodine is a toxic plant alkaloid derived from the South American plant *Ryania speciosa*. The alkaloid specifically binds to and partially activates a ligand-gated calcium channel located in the sarcoplasmic reticulum referred to as the ryanodine receptor (Xu et al. 1998). Subsequent sequencing of the gene encoding the type-1 (skeletal muscle) ryanodine receptor (*RyR1*) located on porcine chromosome 6q11-q12 identified the causal variant for porcine malignant hyperthermia, an exon 17 coding region mutation (c.1843c>t) that results in a cysteine for arginine substitution at amino acid 615 (p.C615R) (Fujii et al. 1991).

Interestingly, the first mutation associated with malignant hyperthermia in humans was also located in exon 17 of the *RyR1* gene on the orthologous human chromosome 19q13.1 with a c.1840c>t that results in a similar p.C614R amino acid substitution (Gillard et al. 1991). Although *RyR1* c.1840c>t is present in as many as 5% of identified malignant hyperthermia susceptible human patients, currently there are over 100 different (in most cases spontaneous) mutations that account for the remaining 95% of cases (Robinson et al. 2006). Several other genes have also been implicated in human malignant hyperthermia, including *CACNA1S* which encodes the alpha subunit of the L-type voltage gated calcium channel also located in skeletal muscle and associates with the ryanodine receptor (Stewart et al. 2001).

The *RyR1* c.1843c>t variant is the only variant to date that has been associated with porcine stress syndrome and malignant hyperthermia in pigs. It seems to have arisen from a single founder animal and has been distributed amongst various pig breeds of commercial significance, including Landrace, Pietrain, Yorkshire, Duroc, and Poland China (Fujii et al. 1991). Porcine *RyR1* c.1843c>t is also referred to as HAL-1843, because a common phenotyping method to identify affected pigs is to observe the animal for clinical signs following exposure to a small amount of halothane, an inhalant anaesthetic and trigger agent (Ritter et al. 2008). The "halothane challenge test" has been used in an attempt to identify affected animals to avoid costly losses associated with deaths during shipping prior to slaughter. However, *RyR1* c.1843c>t has been proposed to be more accurate than the "halothane challenge test" for this purpose (Rempel et al. 1993). A recent (2008) genotyping survey indicates that 11% of commercial piggeries in the USA have pigs with this variant (Ritter et al. 2008).

An interesting difference between the human *RyR1* c.1840c>t and the pig *RyR1* c.1843c>t variants is that the pig mutation is recessive (two alleles are needed for malignant hyperthermia phenotype), while the human mutation is dominant (only one allele is needed for malignant hyperthermia phenotype) (Zhou et al. 2006). Recent work suggests that the human *RyR1* gene is regulated through epigenetic silencing, such that non-mutated gene copy is transcriptionally silent allowing predominant effects of the mutant protein (Zhou et al. 2006). Although two copies of the pig *RyR1* c.1843c>t appear to be needed for malignant hyperthermia susceptibility and fulminant porcine stress syndrome, heterozygous animals show advantageous properties

including higher feed efficiency and greater yield of lean meat, although there was a higher incidence of PSE pork (Leach et al. 1996). One production strategy has been to use a heterozygous boar with non-carrier sows in order to produce approximately 25% offspring with desirable growth performance and carcass attributes while avoiding losses from stress-related deaths (Ritter et al. 2008).

Malignant hyperthermia has been recognised as a syndrome in horses since at least 1975 (Klein 1975), with multiple reports of adverse responses to halothane anaesthesia and/or succinylcholine (Waldron-Mease et al. 1981; Hildebrand and Howitt 1983; Manley et al. 1983; Riedesel and Hildebrand 1985; Aleman et al. 2005). Enhanced responsiveness of biopsied muscle from affected horses to halothane, succinylcholine, and caffeine contracture tested in vitro (used clinically to test for malignant hyperthermia susceptibility in humans) has also been documented (Waldron-Mease et al. 1981; Klein et al. 1989). Reported affected breeds include Quarter horse, Thoroughbred, Appaloosa, Arabian, and also some pony breeds (Aleman et al. 2009). A missense mutation (c.7369c>g; p.R2454G) in exon 46 of the equine *RyR1* gene has been identified in two Quarter horses that died as the result of anaesthesia-induced malignant hyperthermia reaction (Aleman et al. 2004; Fig. 6). Sarcoplasmic reticulum preparations from these horses also showed higher affinity and density of ryanodine receptor binding sites. Interestingly, both horses were heterozygous for the mutation, suggesting a dominant mode of inheritance, like humans but unlike pigs. In addition, other clinical manifestations including non-anaesthetic-induced, exertional and non-exertional rhabdomyolysis, hyperthermia and other myopathies were also described in other Quarter horses that were heterozygous for this mutation (Aleman et al. 2009). A recent survey of 225 randomly selected Quarter horses in the US indicated a prevalence of 1.3% for *RyR1* c.7369c>g (Nieto and Aleman 2009). At present it is not known whether this same mutation occurs in other equine breeds, or whether other mutations in the *RyR1* gene have arisen independently.

Malignant hyperthermia was first reported in dogs in 1973 (Short and Paddleford 1973) and there have since been several case reports, although in most instances there were rarely specific confirmatory tests such as drug re-challenge or muscle biopsy contraction testing (Leary et al. 1983; O'Brien et al. 1983, 1990; Kirmayer et al. 1984; Nelson 1991). Canine malignant hyperthermia syndrome lacks some of the clinical features characteristic in other species, including lactic acidosis and early onset muscle rigidity, while the most prominent sign in dogs is hypercapnia. No particular breed sensitivity has been identified, with reported breeds including Pointer, Greyhound, Labrador retriever, Saint Bernard and Springer Spaniels (Brunson and Hogan 2004). Although at one time it was proposed that Greyhounds may be more susceptible to malignant hyperthermia (Leary et al. 1983), neither in vivo halothane–succinylcholine challenge nor in vitro muscle biopsy contraction tests revealed a difference in susceptibility of a randomly selected group of Greyhounds ($n = 7$) compared with mixed-breed dogs ($n = 6$) (Cosgrove et al. 1992). A mixed-breed colony of halothane–succinylcholine challenge susceptible dogs was established and studied for a number of years, yielding insights into the pathophysiology of this syndrome in humans (Roberts et al. 2001). Genetic studies

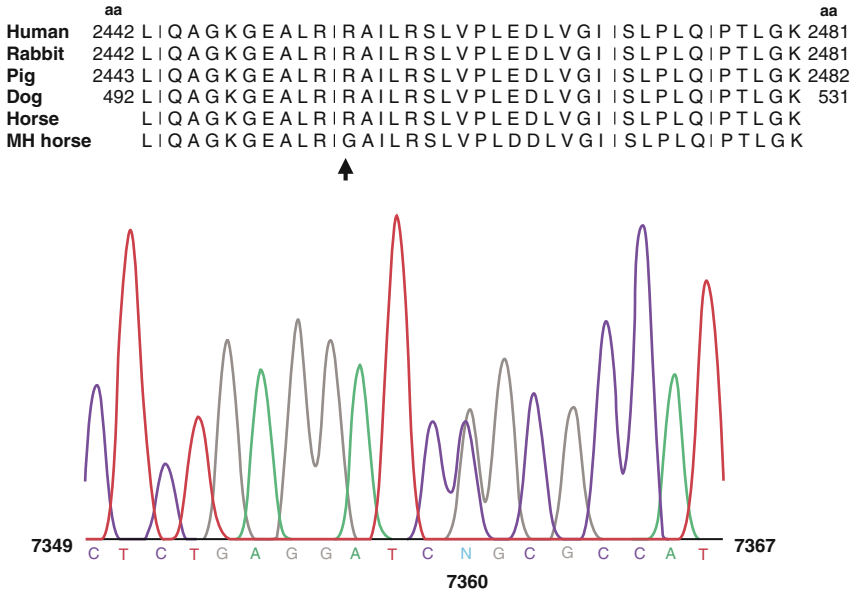

Fig. 6 Identification of a genetic polymorphism in exon 46 of the equine *RyR1* gene associated with malignant hyperthermia in Quarter horses (from (Aleman et al. 2004)). *Bottom panel* shows the sequencing chromatogram of cDNA from an affected horse that is heterozygous for C and G alleles at position 7360. As shown in the *top panel,* this mutation is predicted to result in substitution of a highly species conserved arginine (R) by a glycine (G) at amino acid position 2454, with possible effects on RyR1 function

eventually isolated the causative mutation (c.1649t>c; p.V547A) within the canine *RyR1* gene. Interestingly, like *RyR1* mutations in people and horses (but unlike pigs), the canine *RyR1* c.1649t>c also showed a dominant inheritance pattern. At present it is not known how prevalent this mutation is in the general dog population.

There have been several case reports of malignant hyperthermia in cats (de Jong et al. 1974; Bellah et al. 1989), although the genetic basis has not been determined in this species. A transgenic mouse model has been generated with a mutation that is homologous to those in humans and pigs (p.R163C) (Yang et al. 2006). This model appears to reproduce the majority of the pathophysiological features of the syndrome in people.

3.2 Warfarin Resistance in Rats

Studies of resistance to warfarin toxicity in rats have served as a useful model for understanding variability in the therapeutic anticoagulant effects of this drug in human patients. Warfarin and related anticoagulants have been used as rodenticides for over 50 years. However, currently as many as 75% of wild rats show signs of

resistance to the toxic effects of these agents (Lasseur et al. 2007). It is thought that warfarin resistance is multifactorial, largely due to mutations in the gene encoding vitamin K epoxide oxidoreductase complex subunit 1 (*VKORC1*), which is the principal target for inhibition by warfarin (Pelz et al. 2005). About 10 mutations have been found so far in *VKORC1* in rats, with different mutations or mutation combinations existing in different populations, resulting in a wide (approximately 40-fold) variability in K_i (inhibitory constant) values for inhibition by warfarin (Pelz et al. 2005; Lasseur et al. 2007). Amino acids 128 and 139 appear to be "hotspots" for mutation, with 2 and 3 different mutations at these positions, respectively, identified so far, with the most potent resistance resulting from the Y139F mutation (Pelz et al. 2005). In addition to mutations directly affecting VKORC1 inhibition potency, reduced *VKORC1* mRNA levels have been detected in some animals (Lasseur et al. 2007). In human patients being treated with warfarin, warfarin resistance, as reflected by a significantly higher dose being needed to achieve effective anticoagulation, appears primarily to be the result of a polymorphism in the *VKORC1* gene enhancer region that appears to enhance binding of a repressive transcriptional factor and decrease gene expression (Yuan et al. 2005).

4 Future Directions

The main long-term goal of pharmacogenomics for all species is "personalised medicine" – i.e. therapeutics tailored to the individual characteristics of the patient. To this end, predictive algorithms incorporating genetic profiles, as well as other critical patient information would be developed to predict drug responders, non-responders and those that may be prone to adverse drug side-effects, as has recently been developed for the treatment of human patients with the anticoagulant drug warfarin (Kangelaris et al. 2009). Therapeutic areas in veterinary medicine most likely to benefit from such an approach would involve drugs with a low therapeutic index (ratio of toxic dose to effective dose) used in the treatment of intractable disorders. Consequently, cancer therapeutics, seizure treatment and prevention, anaesthesia, pain management, and infectious disease are some of the areas likely to gain from advances in veterinary pharmacogenomics. As these are areas of importance for human pharmacogenomics, it is anticipated that advances in human, veterinary and comparative pharmacogenomics will be mutually beneficial.

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Drug Delivery Systems in Domestic Animal Species

David J. Brayden, Emilie J. M. Oudot, and Alan W. Baird

Contents

Abstract Delivery of biologically active agents to animals is often perceived to be the poor relation of human drug delivery. Yet this field has a long and successful history of species-specific device and formulation development, ranging from simple approaches and devices used in production animals to more sophisticated formulations and approaches for a wide range of species. While several technologies using biodegradable polymers have been successfully marketed in a range of veterinary and human products, the transfer of delivery technologies has not been similarly applied across species. This may be due to a combination of specific technical requirements for use of devices in different species, inter-species

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pharmacokinetic, pharmacodynamic and physiological differences, and distinct market drivers for drug classes used in companion and food-producing animals. This chapter reviews selected commercialised and research-based parenteral and non-parenteral veterinary drug delivery technologies in selected domestic species. Emphasis is also placed on the impact of endogenous drug transporters on drug distribution characteristics in different species. In vitro models used to investigate carrier-dependent transport are reviewed. Species-specific expression of transporters in several tissues can account for inter-animal or inter-species pharmacokinetic variability, lack of predictability of drug efficacy, and potential drug–drug interactions.

Keywords ABC transporters · Epithelial drug transport · Intraruminal devices · Ivermectin formulation · *P*-glycoprotein efflux · Topical delivery · Veterinary drug delivery · Veterinary drug interactions

1 Introduction to Veterinary Drug Delivery: Comparison with Drug Delivery in Human Medicine

Three of the six significant developments recently predicted to be of paramount importance in the generation of safer and more efficacious veterinary drugs in the next 20 years include drug delivery, nanotechnology and pharmacogenomics (Riviere 2007, this text, chapter, "New Technologies for Application to Veterinary Therapeutics"). Optimism with respect to future developments in drug delivery reflects recent successes in human medicine with new multi-functional polymer chemistry, assisted transdermal patch technology and novel particulate formulations all moving into the clinical development phase. It would be a mistake, however, to presume that there is a high degree of leveraging between human and veterinary drug delivery technologies. Progress in veterinary drug delivery followed developments made with species-specific delivery devices in veterinary medicine over the past 50 years. While pharmaceutical formulation expertise is the paramount skill-set required in both human and veterinary drug delivery, the latter has had a greater focus on device-led engineering science driven by physiological parameters in a particular species.

In addition to the primary objective of delivering optimal concentrations of pharmaceuticals to their site(s) of action over a required period, there are many drivers for the application of drug delivery technology to veterinary therapeutics (Table 1). In production animals, delivery technologies must be mass market and inexpensive. They are primarily restricted to antibacterial drugs, anti-parasiticides, trace elements, vitamins, and growth promoters (non-EU). For example, one-time bolus injections, drenches, or balling-gun administrations are designed to reduce costs and minimise risks of injury to animals and staff. In contrast, in companion animals, the cost issues to maintain the additional "family member" are similar to

Table 1 Target animal groups with applications ranging across experimental, therapeutic, husbandry, population management, and public health fields

Animal populations	Market requirements
Companion	Similar to human (principal differences reflect varied anatomy and (patho) physiology, together with issues of compliance)
Domestic (Food producting)	Cheap, effective, safe to administer and low residues in edible tissues
Sports	Similar to human athletes
Laboratory	Initial studies for human applications for pharmacodynamic and pharmacokinetic (drug delivery) discoveries and product development
Wild	Cheap, effective, typically challenging with respect to delivery and environmental contamination

those of human medicine and this enables premium products to be developed. Supplied on an individual basis, companion animal products cover a wide range of more expensive constituents. Examples include bronchodilating aerosols for airway disease, spot-on anti-parasiticides, and novel drugs for the management of obesity and separation anxiety. Apart from economic considerations, owner convenience and improved compliance are factors in developing formulations and devices for particular routes of delivery.

In human medicine, cost-effective, convenient, low frequency oral administration dominates the market, whereas in veterinary medicine many owners baulk at administering tablets to cats and dogs and prefer palatable or chewable formulations which can be placed in food or taken voluntarily from the hand. Likewise, while human patients are relatively accepting of nasal and pulmonary delivery for themselves, few veterinarians would advocate nasal or pulmonary delivery to cats as compliance is likely to be poor. Nonetheless, there are well-established procedures for administration of volatile (or gaseous) anaesthetics by inhalation. In addition, companion animal research has the potential to benefit from physiological similarities between monogastric animals and humans, thus enabling transfer from the more advanced human drug development sector (Riviere 2007). For example, skin patches have been highly successful in human medicine for a range of passively absorbed drugs, including nicotine, oestrogen and oxybutynin (reviewed in Ball and Smith 2008), and more recently as iontophoretic transdermal systems for fentanyl (Herndon 2007) and lignocaine (Dixit et al. 2007). On the other hand, the use in veterinary medicine of similar systems is at least partially confounded by fur, feathers or scales, the possibility of accidental oral ingestion, altered integumental physiology and high cost. Nonetheless, skin patches of fentanyl (smaller versions of the patch approved for human use, Duragesic®) are used in dogs to alleviate post-operative pain (Egger et al. 2007).

Overall, advances in veterinary drug delivery are constrained by relatively small market sizes for comparatively few classes of drugs and therefore economic considerations limit investment. Ultimately, the transfer or adaptation of human drug delivery technology is a possible option for the veterinary sector, but in practice it is uncommon due to differences in physiology and drug pharmacokinetics and a

requirement for unique device specifications for each species. Nevertheless, there are recent examples of re-formulated human SSRIs (serotonin-selective reuptake inhibitors) for dogs. Fluoxetine (Prozac®) and clomipramine (Anafranil®), marketed for separation anxiety in dogs (Rothenberg et al. 2009), and similar agents have been investigated for stress-related urination in cats (Hart et al. 2005). As a corollary, it is worth pointing out that all drug delivery systems developed for human medicine are tested in animal species prior to evaluation in human clinical trials. Transdermal patches developed for the human market are, however, evaluated in mice, guinea-pigs and rabbits in preference to cats and dogs.

The next section of this chapter reviews technologies for a range of routes of delivery in the main veterinary species. Physiological principles which underpin drug delivery in veterinary medicine are then addressed, namely how drug absorption, distribution and clearance are influenced by the expression of endogenous transporters in several species. This can explain, in part, differing pharmacokinetic (PK) profiles of the same drug across a range of species, which in turn can assist in optimising dosing schedules for delivery systems.

2 Controlled-Release Principles in Veterinary Medicine

As a consequence of dependence on passive diffusion as an important aspect of their PK profile, most agents of therapeutic value are lipophilic and many are also weak acids or bases. The pharmacological rationale for controlled- or sustained-release (CR, SR) drug delivery is based on a principle of zero order release of the active agent over time to achieve sustained plasma levels in the therapeutic range. Minimising fluctuations in plasma concentration ensures that there are reduced peaks and troughs that could lead to toxicity and sub-therapeutic delivery, respectively. Maintaining sustained plasma levels is especially important in the case of those veterinary antibacterial drugs requiring levels to be maintained above the minimal inhibitory concentration (MIC) for much or all of the inter-dose interval, noting that the area under the plasma concentration-time curve (AUC) can theoretically be the same for an immediate release (IR) versus an SR formulation, although the plasma levels attained may not be optimised for long enough in the former (Lavy et al. 2006).

Furthermore, the requirement for zero order release means that the rate-limiting step for delivery must be at the level of drug release from the device. Upon release, an assumption is made that active drug molecules can then reach the systemic circulation. For example, in the case of nicotine transdermal patches as an aid to smoking cessation in humans, patches release sustained concentrations of drug onto the skin and these are rapidly absorbed with a bioavailability of 95–98% (Gore and Chien 1998). Transdermal patches enable safe and efficacious nicotine delivery; a concentrated nicotine solution placed on the skin would normally be fatal as it is highly permeable across the *stratum corneum* due to its high lipophilicity. In veterinary medicine, SR device technology is used for drugs that cross biological

membranes passively, including anti-parasiticides, some antibacterials, steroid hormones, and growth promoters. These agents are administered to the animal once and the effects can persist for weeks, similar to a constant rate intravenous infusion. Dips, sprays, pour-on and spot-on delivery formulations are of real practical and widespread use in veterinary medicine, in contrast to transdermal patches (Walters and Roberts 1993). Hydrophilic drugs including peptides and DNA are, however, difficult to develop into SR systems for either oral or non-parenteral delivery in human or veterinary medicine, as they generally do not cross cell membranes un-aided, and are labile and expensive to produce in large quantities.

Despite these challenges, considerable research effort continues to be directed towards the development of suitable formulations for delivery of biotechnology derived molecules in human medicine. These include buccal insulin sprays containing mixed micelles (Bernstein 2008), the temporary marketing of a dry powder inhaled insulin formulation (Mitri and Pittas 2009), and the delivery by injection of bioresponsive polymer-based small interfering RNA (Schaffert and Wagner 2008). Systemic delivery of nebulised insulin was in fact also recently achieved in cats; most subjects had a 50% reduction in blood glucose after 15 min (DeClue et al. 2008). Besides achieving improved drug efficacy in animals through use of SR delivery devices and formulations, additional clinical benefits accrue as clients use more convenient dosing schedules resulting in better compliance, for example, by reducing dosing to once-a-day in companion animals or to once in an entire growing season for cattle. For production animals, improved convenience and compliance are linked to the practical and economic benefits of reduced labour costs, less risk of injury to farm workers, and reduced potential for damage to animal muscle from adverse reactions at the injection site.

3 Exogenous Regulation: Devices and Formulations

Routes of delivery used in veterinary medicine are summarised in Table 2. This section highlights devices and formulations using specific examples relating to avermectin delivery.

3.1 Oral Formulations

As an example of the differing requirements for device design in different species, intraruminal (i.r) cattle devices rely on a single administration to release anti-parasitic agents at relatively constant rate for an entire growing season, while at the same time remaining functional and intact within the confines of the rumen over that period. In contrast, gastroretentive devices for monogastric species including humans have a simpler physiological environment to cope with for a shorter period and the aim is to gradually release drug from a degradable short-term system to

Table 2 Routes of drug delivery in veterinary species with examples

Route of administration	Examples
Gastrointestinal	Liquids (solutions, suspensions, emulsions) Semi-solids (pastes) Solids (tablets, powders, granules, premixes, medicated blocks) Devices (balling guns, gastroretention) Formulation (taste masking, modified release)
Topical	Dips, spot-ons, sprays, patches, insecticidal collars, ointments, creams, ear drops
Tracheobronchial surfaces and alveoli	Inhalational anaesthetics, aerosols (including vaccines)
Parenteral	I.V, I.M, S.C, intra-articular implants, depot preparations (excipients, stabilising agents, buffers, chelating agents, surfactants, pyrogen-free)
Ocular and periodontal adhesives	Eye-drops, inserts, hydrogels, (localized delivery of non-antibiotic and antibiotic anti-microbial drugs)
Reproductive tract	Intravaginal delivery systems, applicators, sponges, controlled internal drug release (CIDR) devices, progesterone-releasing pessaries, progesterone-loaded implantable devices
Other	Intramammary infusions, ear implants (hormones)

ensure maintained drug absorption from the upper small intestine (Streubel et al. 2006). Oral i.r. devices have been successfully developed for production animals, especially cattle and sheep. Different devices are based on concepts of particulates entrapped in polymeric reservoirs, expandable devices or osmotic delivery systems (Cardinal 1997; Rothen-Weinhold et al. 2000; Rathbone and Martinez 2002; Lavy et al. 2006). Extensive details describing oral formulations that are either marketed or are in research phases for production and companion animals are available (Lavy et al. 2006). Drug classes and other compounds incorporated into oral i.r. formulations for production animals include anthelmintics, antibacterial drugs, vitamins, minerals and trace elements. In contrast, drugs formulated for oral CR for dogs or humans include β-adrenoceptor-blockers, theophylline, morphine, and a range of non-steroidal anti-inflammatory agents (NSAIDs). Device research for ruminants has followed a largely independent course from that for human use due to anatomical and physiological differences between monogastric animals and ruminants, but recent developments of gastroretentive devices for monogastric species have been described and are based on similar principles of flotation, density, osmotic gradients, expansion, and bioadhesion (Streubel et al. 2006).

Oral i.r. examples relating to ivermectin include Merck's Ivomec® SR bolus formulation for cattle (Miller et al. 2001) and Ivomec Maximizer® for sheep (Rehbein et al. 1998), releasing the drug for 135 and 100 days, respectively. Ivermectin is a hydrophobic macrocyclic lactone derivative with potent anthelminthic properties. Following administration to cattle using a balling-gun, ivermectin is released from high-density bolus devices on the basis of the principle of osmotic pressure (Fig. 1a). In the example of Ivomec® SR bolus, water enters the device via a semi-permeable membrane and the osmotic pressure establishes the rate of release of ivermectin. The onset of release is immediate and 12 mg per day is

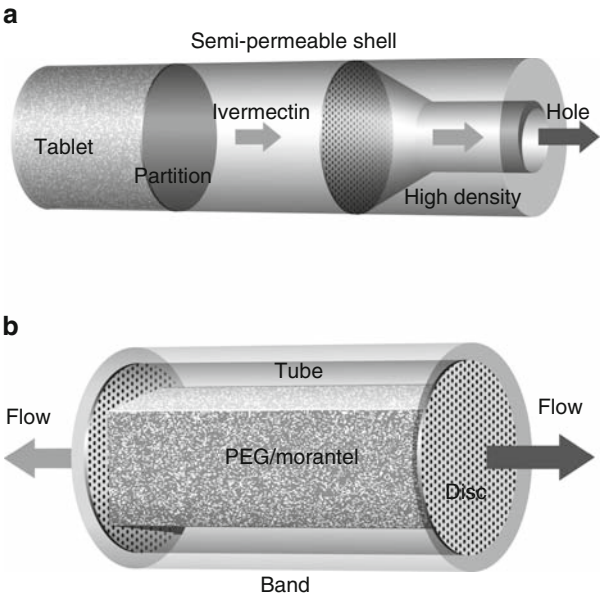

Fig. 1 Intraruminal devices. (**a**) Osmotically-driven mini-pump containing an osmotic tablet, a partition layer, the formulation and high density metal (e.g. IVOMEC-SR® bolus, Merck Corp.). (**b**) Reservoir-based PEG/drug system containing a stainless steel chamber capped at each end with polymer-impregnated polyethylene discs (e.g. Paratect® bolus morantel system, Pfizer Animal Health)

then released for approximately 135 days. The history of the development of the device components, together with the influence of dissolved gas and the radius of the exit port in achieving the required PK profile has been described in detail (Cardinal 1997). A second example is Paratect® bolus (Pfizer Animal Health). This is based on the depot principle. This oral i.r. device for cattle continuously releases morantel tartrate over a period of 90 days for the control of gastrointestinal roundworms (Jones 1983). It consists of a stainless steel tube, which has porous polyethylene discs at each end and which contain cellulose acetate. A reservoir containing morantel and polyethylene glycol (PEG) is created in the main body of the cylinder (Fig. 1b). Devices designed for pulsatile release have also been successfully developed. These systems are generally based on sequential, timed release of tablets as the outer cylinder is eroded by ruminal fluids. Several studies suggest that pulsed release of oxfendazole may be superior to SR oral formulations in prevention of parasitism in calves, perhaps in part due to decreased risk of resistance (reviewed in Cardinal 1997).

The anatomical and physiological variability between the gastrointestinal tracts of companion animals and humans, do however, mean that information on IR and CR oral formulations is limited (Sutton 2004). Intelligent formulation is the simplest example of a delivery "device". In this regard, significant advances have been made in respect of standardising testing methods for once-a-day palatable

tablets for dogs (Thombre 2004). Examples of flavoured chewable tablets designed for dogs include beef-flavoured ivermectin/pyrantel (Clark et al. 1992), beef-flavoured fluoxetine (Simpson et al. 2007), and liver-flavoured COX-2 selective NSAIDs (Pollmeier et al. 2006). Given the unpredictable flavour preferences of small animals, taste-and odour-masking technologies also have a role to play. Other options include buccal films and gels, flavoured treats and medications in fluids. As a unique equine delivery formulation, an oral paste containing a mixture of ivermectin and praziquantel achieved good efficacy and was convenient to administer (Rehbein et al. 2007). This combination is now successfully marketed as, for example, Equimax™ Paste (Pfizer Animal Health) and Zimecterin® Gold (Merial). The incorporation of carbimazole, a pro-drug of methimazole, used in the treatment of hyperthyroidism in cats, into a CR formulation provides an example of extending duration of action in companion animal medicine to allow once daily dosing while avoiding significant accumulation (Frenais et al. 2009). Bioavailability was similar for conventional and CR tablets, but was increased in fed versus fasted cats. Aragon et al. (2009) achieved sustained plasma concentrations of morphine in dogs (over 24 h) using a spheroidal formulation that provided both immediate- and CR components. However, high inter-animal variability is likely to limit the clinical use of this particular formulation.

3.2 Airway Delivery: Pulmonary and Nasal

Experimental animals have been used to investigate airway delivery of drugs, vaccines, inhalational anaesthetics, and toxins. Indeed, the image of the cigarette-smoking Beagle is one which generated a huge debate over animal experimentation as well as hazards of tobacco smoking. Use of the respiratory tract as a conduit for drug delivery to the systemic circulation in animals lags behind R&D in its human counterpart because of cost, limited markets, and technical issues of species and breed specific device design. Nonetheless, inhaled delivery of insulin to Beagles has been achieved using a modified endotracheal inhaler. This yielded insulin blood concentrations of the same order as those obtained with subcutaneous (s.c.) administration (Edgerton et al. 2009). It seems that dog models may also be used in the development pathway for pulmonary delivery in human medicine. For example, a growth-hormone-releasing factor mimetic formulated as dry powder spray-dried microparticles and designed for humans was administered intra-tracheally to dogs to yield 41% bioavailability relative to the subcutaneous (s.c.) route (Jansen et al. 2004). This type of data can be useful as a precursor to subsequent human clinical trials. Recently, inhalation of short-acting insulin aerosol by cats also reduced blood glucose concentration and it was suggested that this option could be available instead of injections, in cases where dietary changes and hypoglycaemic drugs were advocated (DeClue et al. 2008). The failure of the pulmonary human insulin product Exubera®, suggests that there are major issues for the entire field of systemic delivery by this route no matter what the species.

Inhaled drugs for small animals are, however, used to treat local conditions including canine bronchitis, kennel cough and feline asthma. The advantages are high local concentrations and reduced systemic side-effects. Rozanski et al. (2007) have outlined the major challenges for aerosol delivery to small animals. Particle size diameter is critical in order to reach different parts of the lung and this is governed in part by the type of inhaler as well as whether the particle formulation is in the format of a dry powder, liquid droplet or spray-dried. Device research for pulmonary delivery to cats and dogs involves species-specific metered dose nebulisers for usually uncooperative patients, who will not take or hold a deep breath on request. Improved devices including Aerokat® and Aerodawg® (Trudell Medical International) have become available recently and, at least in cats, owners seem only too happy to demonstrate their simplicity of use in videos available on YouTube® (www.youtube.com). Drugs that have been administered by the pulmonary route include fluoroquinolones, slow-release theophylline, β_2-adrenoreceptor agonists (albuterol), muscarinic antagonists (ipratropium) and anaesthetics (propofol) (Rozanski et al. 2007). Notable successes in equine pulmonary therapy involve use of specialised masks or hand-held inhalers to deliver aerosols such as salmeterol and pirbuterol for recurrent airway obstruction, colloquially known as "heaves" (Henrikson and Rush 2001; Derksen et al. 1996). Application of drugs, vaccines, and other biologicals in aquaculture for delivery via gills is an important topic which has been reviewed elsewhere (Shao 2001).

Nasal drug delivery in animals has had more restricted application compared to the human sector, limited to either species-specific mucosal vaccination or as veterinary medicine models for human nasal vaccination and/or drug delivery. Early research in animals suggested that nasal administration of benzodiazepenes to Beagles induced a faster onset of sleep than administration by the oral route (Lui et al. 1991), and the concept of nose-to-brain delivery of central nervous system (CNS)-active drugs is currently much in vogue in human medicine (Wu et al. 2008). Sheep have potential as a screening system for nasal drug delivery to man as relative surface areas for absorption are similar. Sheep have also been used to examine the absorption-promoting effects of the mucoadhesive polymer, chitosan, on nasal delivery of salmon calcitonin (Hinchcliffe et al. 2005). For nasal vaccine delivery, the rationale is to induce local mucosal immunity, mediated in part by secretory IgA against pathogens invading surface epithelium. To this end, live attenuated kennel cough nasal vaccines against *Bordetella bronchiseptica* have been marketed, including Nobivac Bb™ (Intervet/Schering-Plough Animal Health) for cats and Naramune-2™ (Boehringer Ingelheim) for dogs. At a research level, a range of veterinary species are being investigated for nasal vaccination against specific pathogens using a variety of novel microparticle encapsulation technologies and adjuvants (Table 3). Nanoparticulate vaccine delivery systems delivered by ballistic devices are also being extensively tested in veterinary species and in wildlife (Scheerlinck and Greenwood 2006). Airway delivery of protective vaccines remains a subject of current interest and potential targets include herds, flocks of poultry and wild animals (Ploegaert et al. 2007; Fourie et al. 2008).

Table 3 Selected nasal vaccine delivery systems in veterinary species

Species	Antigen	Delivery system/adjuvant	Reference
Feline	FIV p24Gag	*E. coli* LT(R192G)	Leavell et al. (2005)
Sheep	*T. gondii* tachyzoites	PLG microspheres	Stanley et al. (2004)
Bovine	porcine serum albumin	Alginate microparticles	Rebelatto et al. (2001)
Porcine	(OmlA) from *Actinobacillus pleuropneumoniae*	CpG ODNs in biphasic lipids	Alcon et al. (2005)
Canine	Live-attenuated *B. bronchiseptica*	None required	Davis et al. (2007)

FIV Feline Immunodeficiency Virus; *LT* Heat labile enterotoxin; *PLG* Poly(lactide)co-glycolide; *OmlA* Outer membrane lipoprotein A; *ODN* oligodeoxynucleotides

3.3 Topical Formulations and Transdermal Patches

Topical formulations including dusting powders, creams, ointments, pastes, pour-ons, drenches and spot-ons are generally designed to either kill internal parasites following absorption across the *stratum corneum*, or to kill parasites on the skin as a result of spreading out from a skin depot. In the case of topical application of anti-parasitic drugs in oily bases, such as fipronil and imidacloprid, the drug redistributes back to skin appendages, primarily pilo-sebaceous units, which in turn act as a 30–60 day reservoir for further CR in cutaneous oils onto the skin (Fig. 2) (Dryden et al. 2000). Topically-applied selamectin, formulated with isopropyl alcohol, to dogs produces 5% bioavailability (Sarasola et al. 2002). While this level of bioavailability is probably too low to cause systemic side-effects associated with the avermectin class in certain dog breeds, selamectin's mechanism of action in killing heartworm relies on reaching the circulation, as distinct from the poorly-absorbed oil-based spot-on formulations, which are designed to kill fleas on the skin. Selamectin's high potency results in acceptable efficacy despite its relatively low bioavailability. Studies following spot-on administration of radiolabelled fipronil proved that the label was concentrated in the keratinocyte layer and that it was detectable at skin sites distant from initial application, thus confirming low bioavailability and indicating spread from the initial site of application (Cochet et al. 1997). In contrast to patches, where drug release from the device is the rate-limiting step for absorption, diffusion through the *stratum corneum* is the key determinant of efficacy for topically-applied drugs (Riviere and Papich 2001). Factors influencing dermal penetration of porcine skin have been reviewed recently by Riviere and Brooks (2009). Considerable PK data are available for ivermectin pour-on products in cattle and the convenience of this route, lower cost and lack of requirement for skilled labour, is favoured by farmers over oral bolus devices. Recently, Laffont et al. (2003) demonstrated that most of the absorbed ivermectin from pour-ons resulted from skin-licking and subsequent oral absorption amongst herds. This discovery led to concerns over variable ivermectin dosing from pour-ons, unintended dosing of animals and the potential for residues in edible tissues exceeding the maximum residue limit set by the EMEA.

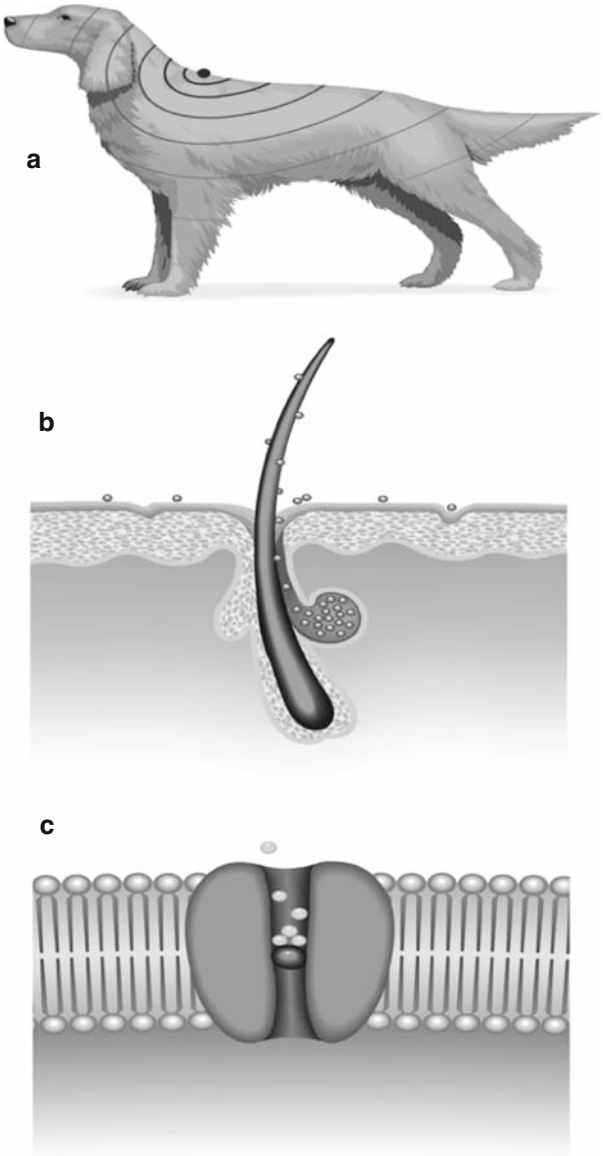

Fig. 2 Mode of action of spot-on anti-parasitic drugs (e.g. fipronil or imidacloprid). (**a**) Oil-based spot-on administered to skin behind the neck distributes over the skin gradually for 30 days. (**b**) Drugs are sequestered by sebaceous glands, which release drugs together with sebum onto skin over the therapeutic period at a level above that required to kill parasites. (**c**) Drugs act non-competitively and with high potency to inhibit flea glutamate-gated chloride channels and with lower potency to inhibit gamma amino butryric acid (GABA)-gated ones (Narahashi et al. 2007). Re-drawn by George Retseck from his original diagram with permission from the publishers of Scientific American (Fischetti 2001)

Transdermal patches delivering a range of passively-absorbed lipophilic drugs have been successfully developed for human medicine (Ball and Smith 2008). Human patches marketed over a 30-year period have incorporated scopolamine, oestrogen, clonidine, nicotine, fentanyl, buprenorphine and oxybutinin. While research in human transdermal patches has recently led to marketing of complex patches for lignocaine and fentanyl using iontophoresis to assist absorption, veterinary patch research has more limited objectives. This is due to large inter-species variation between human, ovine, porcine, bovine and murine skin structure and composition (Hammond et al. 2000), reflected by lack of cross-species prediction of barrier properties of the *stratum corneum*. Together with the presence of scales, feathers, fleece, hair or fur, uncooperative subjects, risk of oral ingestion and the lack of predictive PK-pharmacodynamic (PD) relationships (Riviere and Papich 2001), the limitations are considerable. Nevertheless, the niche opportunity to dose animals post-operatively with analgesics to achieve sustained blood levels over a 2–3 day period is attractive to veterinarians. Hofmeister and Egger (2004) carried out a meta-analysis of selected canine and feline patch studies where the mu-opioid agonist, fentanyl, was the active constituent. They concluded that, although there was large variability in the rate and extent of absorption between animals of the same species and a lack of a defined relationship between plasma concentrations and pain relief, such patches may provide an alternative option for relieving pain in small animals. The objective therefore is to provide sustained absorption for potent agents such as fentanyl, which have short elimination half-lives.

Despite this, a recent study of fentanyl patches used to reduce post-operative pain in dogs reported no analgesic benefit over and above a standard injected dose of morphine, while costs of treatment were higher for the patch system (Egger et al. 2007). Transdermal fentanyl patches have also been used in pigs (Malavasi et al. 2006) and horses (Mills and Cross 2007) with varying degrees of success. Transdermal delivery of the mu-opioid partial agonist, buprenorphine, to cats also failed to provide analgesia despite reaching significant plasma concentrations (Murrell et al. 2007). Regarding other drugs, lignocaine patches have been placed on the skin of cats (Ko et al. 2008), dogs (Ko et al. 2007) and horses (Bidwell et al. 2007) for periods of up to 72 h. In each case, although adverse events were minimal, plasma levels were low. Overall, therefore transdermal patches have not made a significant impact in veterinary medicine, but despite this, the topical route of delivery is increasingly popular. Mills and Cross (2006) have warned, however, that products need to be specifically formulated for the skin of the target species and that, in addition, extrapolation of drug PK and PD properties between species is unlikely to lead to successful outcomes. It is worth noting therefore that the increase in veterinary transdermal formulations that are compounded in unregulated US pharmacies is not based on any objective evidence of efficacy. Table 4 indicates selected formulations for veterinary topical delivery in common use, of which the majority are efficacious spot-ons. In 2008, in a disappointing assessment of veterinary pharma innovation, it was stated there were more than 12 undifferentiated iterative prescription flea-managing marketed products, the majority with similar dosing frequency, mode of administration and mechanism of action (Rothenberg et al. 2009).

Table 4 Selected technologies for topical veterinary drug delivery: Parasiticides unless stated

Formulation	Species	Example	Reference
Spot-on: oily base solution	Feline/canine	Fipronil (Frontline Top Spot®, Merial)	Hutchinson et al. (1998)
Spot-on: alcohol base	Feline/canine	Selemectin (Revolution®, Pfizer Animal Health)	Bishop et al. (2000)
Spot-on: oily base solution	Feline/canine	Imidacloprid (Advantage®, Bayer)	Arther et al. (1997)
Pour-on: dilute isopropanol solution	Bovine	Ivermectin (Ivomec® Pour-On, Merial)	Whang et al. (1994)
Flea-collar: vinyl matrix resin[a]	Feline/canine	Tetrachlorvinphos (Rabon®, Hartz)	Witchey-Lakshmanan (1999)
Transdermal reservoir patch	Canine	Fentanyl opiate (human Duragesic-50® patch, J & J, off-label use)	Kyles et al. (1996)

[a]Safer alternatives to pesticide collars and dips are recommended by the Environmental Protection Agency

3.4 Intravaginal Delivery for Fertility Regulation

Non-human primates are widely used in research on reproductive management. Recently, population control with intravaginally-administered medroxyprogesterone acetate has been achieved in baboons (Guy et al. 2008) and contraceptive vaccines have been developed for pets, farm and wild animals (Naz et al. 2005). In contrast, companion animal medicine has had a greater focus on reproductive control of hormones as a contraceptive strategy similar to humans (Winzenburg et al. 2004). Intravaginal veterinary drug administration in livestock has primarily concentrated on delivery of female steroid hormones to manage fertility and to synchronise the oestrous cycle in cattle, sheep and pigs (reviewed in Rathbone et al. 1997, 2001).

Oestrous synchrony allows all the selected females in a herd or flock to be artificially-inseminated at the same time, leading to coordinated management of parturition. This results in financial advantage for the farmer, which more than offsets the cost of the steroid device. Development of progesterone-loaded implantable systems for livestock progressed on principles of simple low-cost manufacture, easy insertion without inducing epithelial damage or stress, cattle vaginal retention rates of more than 95% and simple retrieval and immediate "switch off" of drug release (Rothen-Weinhold et al. 2000). Epithelium-permeating progesterone, synthetic progestogens or oestrogens are leached at an appropriate rate from moulded devices over periods of several weeks depending on the species, and their role is to suppress oestrous and ovulation in cyclic cattle. Device composition relied originally on polyurethane or more recently on silicone over a mould, while shape variations have progressed from sponges to coils and T-shapes.

The progesterone-releasing intravaginal device (PRID®, Ceva Animal Health) for cattle consists of micronised progesterone dispersed homogenously in silicone rubber, and cured onto a stainless steel coil to produce a cylinder (Fig. 3a). Modifications of PRID® include attachment of an oestradiol tablet to the device

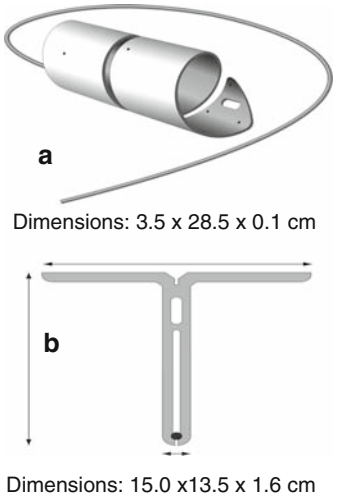

Fig. 3 Diagram of typical cattle intravaginal devices. (**a**) PRID® (progesterone-releasing intra-vaginal device); (**b**) CIDR-B® (controlled internal drug release-bovine)

in order to manage ovarian dysfunction in *postpartum* dairy cows (Kim et al. 2004). The PRID® device has evolved for successful intravaginal progesterone delivery in several species. Controlled internal drug release (CIDR®, InterAg) dispenser implants were originally developed as an alternative to sponges in sheep with the advantages of higher retention rates and a reduced mucus discharge. Full details of the manufacturing process are given in Rathbone et al. (1997); a high-temperature injection mould process is used to cure a drug-impregnated silicone matrix over a nylon spine (Fig. 3b). CIDR® designs have been marketed for sheep, goats and cattle, but different vaginal physiology makes them less suited for horses and pigs. Biodegradable CIDR® prototypes for cattle using poly caprolactone (PCL) have also been described (Rathbone et al. 2002). These have the advantage of using a cooler temperature manufacturing step, which could allow more heat labile drugs to be incorporated, as well as dealing with the environmental issue of device disposal. The PCL device is registered by Pfizer Animal Health in Australia and New Zealand. Cross et al. (2004) have also described an "intelligent breeding" intravaginal device, in which release of the drug can be controlled electronically to permit pulsed or continuous release using a gas-piston mechanism, which is triggered in response to monitored local drug concentrations. Finally, intravaginal delivery is also being investigated in companion animals for CR of anti-microbial agents from mucoadhesive tablets containing microparticles. Chitosan microspheres containing acriflavine and methacrylates were sufficiently "sticky", and released active drug for up to 8 h in vitro (Gavini et al. 2002).

3.5 Intramammary Formulations for Ruminants

CR formulations of antibiotics have the potential for intramammary administration for prevention and treatment of mastitis in cattle. The optimum period for

administration is during the dry period, when it is easier to maintain high concentrations in the teat and gland over several weeks. The goals of dry cow therapy include elimination of persistent low grade infections and prevention of colonisation in an immunologically-vulnerable period. For economic reasons, based on regulatory requirements to calculate safe withdrawal times for residues in milk, antibiotic therapy for clinical mastitis in the lactating cow requires rapid drug effects with short half-lives and the physico-chemical properties of the formulation are designed so that high concentrations are maintained in milk but for relatively short periods (Gruet et al. 2001). *Lactating* cow intramammary antibiotic aqueous-based products are formulated to achieve immediate onset of action with treatment starting for a maximum of 5–7 days. The aim is to achieve rapid elimination so as to ensure short milk withdrawal times. Drug delivery technologies for the *dry* cow are designed on the principle of inducing high local teat and tissue concentrations by parenteral injection or more usually intramammary infusions or combinations thereof. Most dry cow intramammary formulations of antibiotics rely on peanut or mineral oil-based drug suspensions in the absence of wetting agents and indeed they generally incorporate water repellant chemicals; these are more likely to ensure binding to tissue proteins and secretions and have reduced systemic bioavailability (Gruet et al. 2001). Mastitis may cause epithelial disruption and this may aid drug penetration into deeper sections of the udder, although data to confirm this remain scant (Gehring and Smith 2006). Novel intramammary delivery technologies being researched are presented in Table 5. Many are based on antibiotic or antiseptic incorporation into a range of microparticles, nanoparticles, and liposomes for uptake by phagocytes and subsequent release over several weeks. Gruet et al. (2001) have advocated a prophylactic routine of teat antiseptic wash together with local administration of SR antibiotics at the time of drying off, but have also noted that no treatments seem particularly effective against *S. aureus* and that this remains a major challenge.

Table 5 Controlled release formulations for treatment of mastitis or brucellosis*

Formulation	Comment	Reference
Alginate beads (dried gel-based particles) containing ampicillin	In vitro data on drug release	Torre et al. (1998)
Liposomes containing streptomycin	Intramammary infusions give good outcomes in combination with oxytetracycline (i.m.)*	Nicoletti et al. (1989)
PLG microspheres containing Povidone–iodine	In vitro release for 28 days with an initial 2-day burst release	Park and Han (2002)
Aqueous solution or oily suspension containing micronised benzylpenicillin	Perfused udders from lactating cows in vitro	Ehinger and Kietzmann (2000)
Polymeric microparticles of ceftiofur	In vitro data; potential as dry cow therapy	Bodmeier et al. (1997)

3.6 Ocular and Periodontal Adhesives

Eye drops are a very inefficient delivery method by which to deliver drugs systemically or locally to humans or animals as they induce a lacrimation reflex. This has led to the development of a range of solid or semi-solid degradable ophthalmic inserts and hydrogels, which enable improved contact time with the conjunctiva (Baeyens et al. 1997; Rothen-Weinhold et al. 2000). Potential active constituents comprise antibacterial, anti-fungal, anti-glaucoma and anti-inflammatory agents. Davis et al. (2004) have reviewed novel topically-applied corneal epithelium permeating formulations of carbonic anhydrase inhibitors with cyclodextrins for glaucoma treatment as well as pro-drug formulations of the antiviral agent, gangciclovir. Recently, a clinical trial for treating canine conjunctivitis used a single administration to the corneal fornix of bioadhesive ophthalmic inserts of size 5×2 mm, comprising a semi-synthetic carbomer, ethylcellulose, and hydroxypropyl cellulose soluble polymers. These inserts were impregnated with gentamicin. They were well accepted by dogs and yielded clinical outcomes of similar efficacy to gentamicin eye-drops instilled over 20 times in the same treatment period (Baeyens et al. 2002). Other soluble inserts have been based on collagen and chitosan and these have superior aqueous solubility to semi-synthetic polymers, requiring a single administration but no removal step (Baeyens et al. 1997). An example of an insoluble insert for glaucoma treatment is an alginate/ethylene vinyl acetate copolymer mixture incorporating the anti-muscarinic agent, pilocarpine (Sendelbeck et al. 1975), while pre-formed hydrogels consisting of vinyl pyrrolidone/methacrylic or acrylic acid co-polymers have been tested in rabbit eyes for the same drug (Barbu et al. 2005). In large animal medicine, diseases such as infectious bovine keratoconjunctivitis are common and require intervention with antibiotics administered in formulations and routes ranging from subconjunctival injection to systemic and topical delivery by spray or ointment (McConnel and House 2007). In all species, ophthalmic topical delivery technologies remain relatively unsophisticated and their efficacy is hindered by tear production, poor corneal permeation and excessive drainage away from the target site. Nonetheless, localised delivery by intravitreal injection of anti-VEGF (vascular endothelial growth factor) therapies for macular degeneration has led to approval of several pioneering gene-based human medicines (Wolf 2008).

Veterinary delivery systems as adjunct treatments for small animal gingivitis and periodontal disease are based on local application of non-antibiotic and antibiotic antimicrobial drugs. A biocompatible bioadhesive tablet/hygiene patch (Stomadhex®, Vetoquinol) releases the antiseptic, chlorhexidine and anti-inflammatory, niacinamide over 4 h from a tablet applied to the inside of the lower lip of dogs, resulting in reduced bacteria levels (Gruet et al. 1995). Another example for use in dogs is a CR flowable biocompatible gel containing doxycycline and *N*-methyl pyrrolidone (Doxyrobe®, Pfizer Animal Health), which is marketed as a local treatment for management of periodontal disease (Polson et al. 1996; Cleland 2001). Bioadhesive formulations of minocycline have also been tested in dogs and results suggested that

periodontal disease-associated peptidase activity was reduced over control values at 13 weeks (Hirasawa et al. 2000). These designs should permit the attainment of higher local drug concentrations than is possible from systemic delivery. Although further development of systems to improve gum contact time in small animals would be useful (reviewed in Cleland 2001), difficulties of administration for owners has the consequence that such products will be mostly limited to use by veterinarians following dental scaling and tooth extraction procedures. In a note of caution, a meta-analysis of human trials has concluded that there is rather weak evidence to date to establish that these types of local delivery systems produce significant additional clinical benefit when used as an adjunct to scaling and root canal work (Hanes and Purvis 2003).

3.7 Sustained-Release Parenteral Veterinary Drug Delivery

SR parenteral formulations for livestock and companion animals are an important sector of the pharmaceutical market in veterinary medicine, estimated at 40% of the total veterinary CR market, which in turn is approximately 15% of the total veterinary drug market (Medlicott et al. 2004). Some of the physiological differences in different species impacting on non-injected routes of delivery can be overcome by s.c. or intramuscular (i.m.) injection and a strong case can be made that long-acting injections of suspensions, solutions and implants are more convenient than oral administration. Parenterally-administered SR formulations usually yield higher bioavailability than oral ones and are the only routes currently available for peptide and protein delivery (Matschke et al. 2002). Negative aspects, however, include possible injection site irritation and inflammation, unpredictable absorption rates, lack of in vitro–in vivo release correlations, complex sterile formulation manufacturing requirements, and possible poor "syringeability". In addition, in food-producing animals, controlling the release profile must balance the need to achieve therapeutic efficacy against prolonged residual concentrations in edible tissues, so as not to lead to unduly prolonged meat withholding times. Erratic, non-linear and therefore poorly predictable depletion rates from IM injection sites of CR formulated drugs in food producing species, notably in cattle and pigs, have created major difficulties in setting meat withholding times (see chapter, "Drug Residues"). This has led to the s.c. route being favoured over i.m. injections in cattle and also to investigations into the alternative of ear injections, as this is one part of the carcass that is not used for human consumption. Specifically, extensive regulations cover the testing of SR parenteral products in animals destined for the food chain in respect of establishing reproducible, safe and effective drug release rates (Martinez et al. 2008b). A comprehensive review on the pros and cons of SR parenteral products is provided by Medlicott et al. (2004). These products range from oil- and liquid-based injections, suspensions, microparticles, and in situ forming gels to implants and they encompass selected therapeutic agents: notably antiparasiticides, antibacterials

Table 6 Long-acting injectable formulations, for cattle (unless otherwise stated)

Formulation/Product	Comment	Reference
Recombinant bovine somatotropin (Posilac®, Elanco) for increased feed conversion efficiency and milk yield	Slow release from an oil-based sterile suspension, dosed at 14 day intervals (*non-EU*)	Gulay et al. (2004)
Florfenicol antibiotic (Nuflor®, Schering-Plough) for respiratory disease and foot-rot	Solution in non-aqueous vehicle; solubility controlled; 1–2 doses in 48 h	Aslan et al. (2002)
Vitamin B12 entrapped in microspheres for lambs on cobalt-deficient pasture (SMARTShot^{-TM} B12, AgResearch)	First veterinary product using PLG microspheres; single s.c. or i.m. injection releasing B12 over many weeks	Grace et al. (2003)
Injectable steroid implants for growth promotion	Pellets containing range of growth stimulants (oestrogenic or androgenic) implanted into back of ear (s.c.) (*Non EU*)	Rossi (2006)
Long-acting ivermectin (IVOMEC®, Merial) s.c. injection as a prophylactic endectocide for sheep	Solid-dispersion of ivermectin and castor oil crushed to make aqueous suspension; 60 day persistence	Xu et al. (2007)
Long-acting oxytetracycline-poly (ethylene) glycol, PEG (Bio-Mycin® 200, Boehringer-Ingelheim) for treatment of infectious respiratory diseases	Injected (i.m., s.c) to provide therapeutic concentrations for up to 4 days. Reduced absorption rate due to properties of PEG	Dowling and Russell (2000)
Hydrogenated castor oil nanoparticle carriers for the macrolide, tilmicosin	Improved PK, maintains serum concentrations for 8 days versus 5 h normally	Han et al. (2009)

and growth promoters. The majority of long- acting parenteral products have been developed for production animal medicine and these tend to be very profitable (Medlicott et al. 2004). Table 6 presents some authorised and research examples using various types of delivery system technology for use in production animals.

Long-acting (depot) injectable formulations have made less of an impact in companion animal medicine than in livestock, as a consequence of a combination of commercial and practical issues. For example, spot-on and oral deliveries are simpler and administration by a veterinarian is not essential. Long-acting antiparasitic products for dogs include s.c. injections of moxidectin microspheres (ProHeart® 6, Fort Dodge), providing a 6 month period of CR to prevent canine heartworm (Lok et al. 2005), re-introduced in 2008 following initial safety concerns. A second example of extended duration formulations is lufenuron (Program®, Novartis Animal Health), a benzoyl urea derivative and chitin inhibitor. It was formulated as an aqueous suspension for s.c. injection to cats to give 6-month protection against fleas (Blagburn et al. 1999) and has become a viable alternative that owners might prefer to monthly oral dosing with this species. Long-acting

antibiotic injections investigated in dogs include oxytetracycline solutions with organic solvent excipients (i.m) (Kikuvi et al. 2001). Smith et al. (2008) investigated the PK of a CR liposome-encapsulated parenteral product containing the opioid analgesic drug hydromorphone. Compared to IV dosing, the apparent terminal half-life of hydromorphone in the CR formulation was increased from 0.52 h to approximately 30 h.

SR techniques and devices have been evaluated more frequently for companion animals than for production animals. One of the aims of such delivery systems is to reduce systemic side effects associated with potent drugs while maintaining efficacy. For example, biodegradable implants impregnated with cisplatin have been used with some success to prevent recurrent osteosarcoma in dogs following limb amputation (Withrow et al. 2004), and there have been attempts to implant polymethylmethacrylate beads containing gentamycin to treat canine femur osteomyelitis (Wahlig et al. 1978). As a potential contraceptive strategy, treatment with a 6-month biodegradable implant containing the gonadotrophin-releasing hormone (GnRH) agonist, deslorelin (Peptech, Australia), suppressed the activity of the pituitary-gonadal axis leading to reduced testosterone production in male dogs within 1 month (Junaidi et al. 2007). Finally, there is considerable interest in examining SR injectables in veterinary species using excipients selected from the high viscosity lipophilic sugar (sucrose acetate isobuyrate, SAIB), amphipathic molecules (PEG (poly(ethylene) glycol)-PLA (poly(lactic) acid)-PEG) co-polymers and biodegradable PLA/PLG (polylactide-co-glycolide). The SABER^{-TM} technology (Durect Corp, USA) consists of SAIB with a small amount of organic solvent with PLA polymer and its unique features permit a sol–gel conversion in situ, which forms a semi-solid depot SR implant. The simple "mix and fill" scaleable manufacturing process is a feature of this application and it is less expensive than typical microparticle technologies. In a study in mares comparing oestradiol release from microparticle and SABER^{-TM} formulations, similar plasma levels were detected (Johnson et al. 1999). Issues to address to increase acceptance of non-polymeric and polymeric in situ implant technologies in veterinary medicine include improving drug loading, replacing organic solvents and identifying sufficiently potent drugs that do not require high injection volumes (Matschke et al. 2002).

4 Endogenous Regulation: The Role of Drug Transporters in Regulating Delivery

The foregoing has examined the challenges involved in developing delivery formulations and devices for administering veterinary drugs by particular routes to optimise PK and PD profiles. The numerous endogenous transport mechanisms that contribute to drug absorption and distribution across epithelial and endothelial cell barriers comprise another important aspect of drug delivery. Thus, exogenous delivery systems and endogenous drug transporters are linked if the drug is a

substrate for transporters that influence absorption, distribution and elimination. For example, although ivermectin systemic bioavailability is sufficiently high to provide therapeutic concentrations from a range of formulations, its distribution across the blood–brain barrier (BBB) of mammalian species and its elimination via the bile duct and kidneys are regulated by the efflux transport protein, *P*-glycoprotein (*P*-gp). These transporters therefore exert significant effects on the pharmacological outcomes of drugs delivered exogenously and they may influence choice of delivery technology and route of administration. They may also be responsible for drug–drug interactions. While a range of transporters may influence drug delivery, most published literature to-date has focused on the ATP-binding cassette (ABC) superfamily of efflux transporters, probably the most significant family of membrane transport proteins (Martinez et al. 2008a). Most in vitro systems and in vivo models have been developed for studying the transport of drugs and nutrients in human rather than veterinary medicine. Consequently, more is known about transporter expression and function in laboratory animal models and human tissues than in veterinary species. As the dog is often used as a model for evaluating human formulations, there are more data on its transporters than those present in cats and livestock, but still less than in other laboratory animals (Li et al. 2008).

4.1 ABC Efflux Transporters in Veterinary Medicine

ABC efflux transporters are members of a family of ATP-dependent pumps. These membrane spanning structures are expressed widely in both prokaryotic and eukaryotic cells. Their principal function is transport of a range of substances against their concentration gradient (Davidson and Maloney 2007). There is also evidence that they may be involved in integral physiological functions, including immunological processes and cell proliferation, differentiation and death (Johnstone et al. 2000). Although their potential role in drug delivery remains to be fully determined, it is evident that regulated expression of ABC transporters confers multi-drug resistance (MDR) in tumour cells (Juliano and Ling 1976) and they are also involved in antibacterial drug resistance (Quinn et al. 2006).

The first of the ABC transporters to be discovered and characterised was *P*-gp, the *trans*-membrane pump product encoded by the *MDR1* (re-named *ABCB1*) gene. A number of these transporters play a central role in phase 0 metabolism (efflux of xenobiotics upon cell permeation) and phase III metabolism (efflux of xenobiotic metabolites), both of which are important steps in defence against xenobiotics (Dietrich et al. 2003). *P*-gp is predominantly located in apical membranes of enterocytes (gut epithelial cells), membranes of brain capillary endothelial cells at the BBB, biliary canalicular epithelial cell membranes, renal proximal tubular epithelial cells, and placental trophoblasts (Martinez et al. 2008a). Although it is not essential for normal physiology and fertility, at least in *Abcb1a* (−/−) mice, (Schinkel et al. 1994), the highly conserved nature of the *P*-gp amino acid sequence

across species together with its tissue distribution suggests an important constitutive protective function in the translocation of exogenous drugs and toxins.

With regard to drug delivery, the physiological distribution of ABC transporters together with the structurally diverse nature of the xenobiotics with which they interact significantly influences the pharmacokinetics, safety and efficacy of numerous clinically-relevant veterinary compounds. *P*-gp interacts with hydrophobic, hydrophilic, neutral, and charged molecules (Martinez et al 2008a). Its substrates include small molecules such as organic cations, carbohydrates, amino acids and antibiotics (Zhou 2008), although no structure–activity relationship has been clearly established. Transfected and non-transfected epithelial cell models are used to screen for ABC transporter substrates, inhibitors and potential drug–drug interactions in human drug development. Filter-grown polarised human Caco-2 intestinal epithelial cell monolayers expressing *P*-gp are considered to be the "gold standard" model for determining passive intestinal drug permeability (Hubatsch et al. 2007) and can be used to screen verapamil-sensitive polarised efflux of *P*-gp substrates (Griffin et al. 2005). However, inter-species and inter-regional differences in tissue transporter expression may occur and knowledge in this area is sparse. Extrapolation from Caco-2 and *ABCB1*-transfected monolayers, such as the MDCK (Madin-Darby Canine Kidney) cell line, to in vivo circumstances in any species is therefore questionable. Furthermore, features of the widely used Caco-2 model include over-expression of *P*-gp, or variability in *P*-gp expression together with variable activity of the principal xenobiotic-metabolising enzyme cytochrome P450 3A4 (CYP3A4) (Sun et al. 2008). As they act upon common substrates, relative contributions of *P*-gp and CYP3A4 to overall intestinal permeation of xenobiotics may be difficult to determine accurately and may result in inter-study discrepancies in reported drug PK parameters. Encouragingly however, there is evidence that *P*-gp-expressing Caco-2 monolayers have similar *P*-gp efflux activity to human (Makhey et al. 1998) and rat (Collett et al. 1999) intestinal segments. Key parameters relating to *P*-gp such as substrate affinity, saturability and capacity can be determined and are important in establishing the full influence of efflux transporter mechanisms on oral bioavailability in vivo (Tang et al. 2002).

Although the regional distribution of *P*-gp in veterinary species remains poorly documented, inter-species differences in the presence of *P*-gp in tissues is a feature (Conrad et al. 2001). There are also differences in substrate-specificity between species (Baltes et al. 2007). For example, while human MRP1 (ABCC1, humMRP1) confers resistance to the anthracyclines such as doxorubicin, an amino acid substitution at position 1089 (glutamate to glutamine) prevents interaction with the rodent and canine homologues despite 88% and 92% sequence homology with humMRP1, respectively (Stride et al. 1997; Ma et al. 2002). Further research into species- and region-specific distribution of endogenous transporters may lead to enhancement of both the efficacy and safety of species-specific drug administration and also improve the design and outcome of preclinical PK and toxicological studies in human medicine via more accurate data extrapolation between species (Ma et al. 2002; Schrickx and Fink-Gremmels 2008).

P-gp is expressed on the apical membranes of brain capillary endothelial monolayers derived from rat (Nakagawa et al. 2009), man (Poller et al. 2008), cow (Culot et al. 2008), and pig (Smith et al. 2007). *P*-gp actively reduces cerebrospinal fluid concentrations of drugs before they reach the brain. In dogs, this is a mechanism which prevents or reduces neurotoxicity of ivermectin in most breeds (Mealey 2008; Mealey et al. 2008). This same protective mechanism is one which limits distribution of useful drugs into CSF. Furthermore, as these transporters act upon a range of substrates, there is a consequent increased risk of drug–drug interactions at the level of the transporter. Pekcec et al. (2008) observed over-expression and increased function of *P*-gp at the BBB of epileptic dogs, which may contribute to resistance to and reduced efficacy of anti-epileptic drugs, such as phenobarbitone. Overcoming *P*-gp efflux function with modifying agents may be useful and even necessary when considering treatment strategies involving *P*-gp substrates for veterinary and human patients with CNS disorders. Specific examples where overcoming *P*-gp could be of benefit include delivery of chemotherapeutic agents to brain tumours (Miller et al 2008), as well as for treatment of drug-resistant epilepsy (Luna-Tortós et al. 2008).

4.2 Potential Veterinary Drug Interactions Based on Transporters

The species-specific expression of transporters has been discussed. Significant advances have been made in pharmacogenetics in human medicine as a result of the influence of patient-specific target receptor expression and metabolic enzyme activity on drug PK and PD (Martinez et al. 2008a). Although this tailored approach remains in the early stages of development in veterinary therapy, some indications of "proof of principle" have been generated through knowledge of *P*-gp expression in canine breeds. The trigger for this research was the discovery of the idiosyncratic neurological hypersensitivity of multi-drug resistant gene knock-out (*ABCB1-1Δ*) herding dog breeds to ivermectin (reviewed in Mealey 2008). The phenomenon has been observed in Collies, Australian Shepherds and Shetland sheepdogs (Martinez et al. 2008a), as well as in *Abcb1a* (−/−) mice (Schinkel et al. 1994) and in a herd of Australian Murray Grey cattle (Eagleson et al. 1987). Normally, brain concentrations of ivermectin are many times lower than those in plasma and the liver, but failure of the BBB to prevent ivermectin permeation into the CNS results in ivermectin neurotoxicity in *ABCB1-1Δ* Collies (Mealey et al. 2003). An experiment demonstrating that CNS levels of ivermectin were 100-fold greater in *Abcb1a* (−/−) mice than in their wild-type counterparts indicated that this was due to reduced efflux from the BBB (Schinkel et al. 1994). The prevalence of the *ABCB1-1Δ* homozygous genotype in Collies has been estimated to be approximately 30–40% (Mealey et al. 2002). Ivermectin-sensitive Collies develop clinical signs after administration of oral doses as low as 0.1 mg/kg (Paul et al. 1987), while non-sensitive dogs can tolerate doses up to 2.5 mg/kg (Campbell and Benz 1984). The dysfunctional phenotype is due to a spontaneous gene knock-out resulting in a 4-base pair deletion in the *ABCB1* gene (Mealey 2004), which prevents the protein from emerging from the golgi apparatus for routing to the plasma membrane of BBB

endothelia (Mealey et al. 2001; Roulet et al. 2003). Only the amino-terminus of the protein is generated (7.1% of the fully functional protein) and the ATP-binding, substrate-binding, and phosphorylation sites required for normal function are absent. For sensitive dogs, the phenotypic consequences are considerable; *P*-gp exports a wide variety of structurally-unrelated hydrophobic therapeutic drugs including loperamide, cyclosporin, digoxin and ivermectin. Atypical hypersensitivity to all such agents has been observed in *P*-gp-deficient Collies (Mealey et al. 2001, 2003). Consequently, a genetic test for the homozygous mutation is now commercially available from several international laboratories for determining the MDR1 genotype of herding-breed dogs prior to the administration of potential *P*-gp substrates.

Selamectin exhibits a wider safety margin in phenotypically-sensitive dogs than ivermectin following topical or oral administration of high doses (Bishop et al. 2000; Novotny et al. 2000). Although selamectin is equipotent to ivermectin as a *P*-gp substrate and inhibitor in vitro (Griffin et al. 2005), recent in vivo data from *Abcb1a* (−/−) mice showed that selamectin accumulates in the brain to a far lesser extent than ivermectin, even though it was confirmed as a *P*-gp substrate (Geyer et al. 2009). Even when present in high concentrations in the CNS, it did not provoke neurotoxicity in mice. Neurotoxicity is normally detected in sensitive Collies after oral consumption of either a concentrated ivermectin pour-on designed for farm livestock or from off-label high oral dose ivermectin treatment of canine demodectic mange, whereas selamectin is administered only topically to dogs in a spot-on formulation (Sarasola et al. 2002). Mealey (2008) argues, as a general principle, that administration of selamectin and the related compounds, moxidectin and milbemycin, in doses exceeding those recommended for heartworm prevention may have the potential to induce neurotoxic symptoms in *ABCB1-1Δ*dogs. In support, doramectin and milbemycin oxime caused neurotoxicosis following administration at high dose rates to ivermectin-sensitive dogs (Yas-Natan et al. 2003; Tranquilli et al. 2001), data recently confirmed for milbemycin oxime in a separate study of the treatment of demodicosis in dogs carrying the *ABCB1-1Δ* genotype (Barbet et al 2009). The argument is however unlikely to apply to selamectin, given the PK data reported by Geyer et al. (2009) in *Abcb1a* (−/−) mice. Although Paul et al. (2000) observed a wider safety margin of moxidectin in avermectin-sensitive Collies compared to ivermectin and milbemycin, cases of moxidectin poisoning have still been documented, resulting from accidental ingestion of high doses in *ABCB1-1* Δ Collies (Beal et al. 1999) and other breeds (Snowden et al. 2006).

The discovery of the *P*-gp mutation in Collies and herding breeds has implications for potential dose-dependent drug interactions between co-administered *P*-gp substrates and inhibitors in both wild-type and mutant dogs. There are numerous examples of drugs, excipients, and nutraceutical components used in veterinary medicine that are *P*-gp substrates or inhibitors or both (Mealey 2004; Martinez et al. 2008b). Selected relevant examples of substrates include doxorubucin, dexamethasone, ketoconazole and digoxin, while inhibitors include fluoxetine, verapamil, and cyclosporine. Formulation excipients that inhibit *P*-gp range from PEGs to pluronic

acids (Föger et al. 2006). Examples of nutrient-based compounds that interact with *P*-gp and other ABC transporters are Vitamin E TPGS (tocopheryl polyethylene glycol succinate) (Collnot et al. 2006) and flavonoids (Brand et al. 2006). While there is no clinical evidence to date that any food components or nutraceuticals used in veterinary species impact on drug safety at the level of *P*-gp (or indeed cytochrome P450), the human experience of toxic interactions between grapefruit juice and St John's wort and *important* drugs leaves no room for complacency (Holtzman et al 2006). Awareness of potential interactions in breeds and species with differential *P*-gp expression and increasing knowledge of inter-species differences in transporter expression and distribution will inform improved dosing regimens and enhance understanding of inherent risks in cases of polypharmacy.

Other ABC transporters, including MDR resistance associated proteins (MRPs) and breast cancer resistant protein (BCRP), are also being investigated for their potential importance in drug disposition in veterinary medicine. MRP1 (ABCC1), MRP2 (ABCC2) and BCRP (ABCG2) may be present to varying extents and impact on the BBB, bile duct and mammary glands of several species (Alvarez et al. 2006). Important discoveries were that fluoroquinolone secretion into milk was mediated by *Abcg2* in wild-type but not Abcg2 (−/−) mice (Merino et al. 2006), and the corollary that *ABCG*2 expression is up-regulated in the mammary glands of lactating sheep (Pulido et al. 2006). Knowledge of the existence and functions of such transport pathways for drugs to distribute into milk could lead to strategies to manipulate residue depletion rates. In relation to MRPs, there is evidence that ABCC1 is present on canine lymphocytes and that, in association with ABCB1, it may play a role in resistance to chemotherapeutic agents (Schleis et al. 2008). Whether ABCC1 has any practical significance for disposition of, for example, ivermectin is questionable. While ivermectin interacts with ABCC1, ABCC2 and ABCC3 in vitro, albeit with low affinity (Lespine et al. 2008), these effects do not seem to be pharmacologically relevant in the presence of a background *P*-gp-mediated efflux in MDR1- and MRP-transfected epithelial cells (Brayden and Griffin 2008), nor do they appear to act in a compensatory fashion to prevent CNS penetration of ivermectin in *Abcb1a* (−/−) mice (Schinkel et al. 1994). Studies in *Abcg2* (−/−) mice also indicate no differences in brain concentrations of either ivermectin or selamectin compared to wild-type mice (Geyer et al. 2009), further strengthening the evidence that ABCB1 is the major transporter for avermectins on the BBB. A model illustrating the influence of *P*-gp on the PK of ivermectin is shown in Fig. 4. Delivery systems that inhibit *P*-gp might restore ivermectin sensitivity in resistant target organisms (Lespine et al. 2008), although a downside could be increased CNS penetration in susceptible animals due to altered PK.

Membrane location and function of MRPs and ABCG2 on BBB endothelial cells of different species have only been explored to a limited extent; most are thought to serve as efflux pumps with overlapping and discrete sets of substrates and to be located on the apical membrane. However, research in this field has been hampered by practical difficulties in culturing isolated brain capillary endothelial cells in vitro, including rapid de-differentiation with increasing passage number, differential transporter expression to that which occurs in vivo, and unusually low

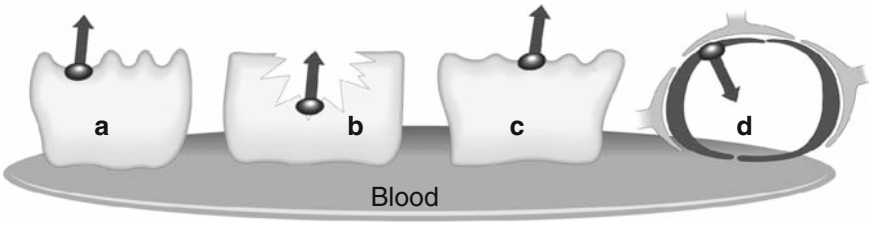

Fig. 4 The role of *P*-gp in ivermectin PK. (**a**) Oral absorption is limited by *P*-gp efflux to the intestinal lumen from intestinal enterocytes. (**b**) Increased *P*-gp-mediated secretion into bile for enterohepatic shunting occurs at the bile canaliculi bordered by hepatocytes. (**c**) *P*-gp on renal proximal tubule epithelia causes ivermectin secretion from blood into renal tubular fluid. (**d**) *P*-gp on blood–brain barrier endothelia prevents ivermectin access to the CNS from the blood. (**a–c**) act to prevent access to or to remove ivermectin from the blood. Note that blood concentrations impact on subsequent parasite exposure and that *P*-gp expression on parasite membranes may also contribute to ivermectin resistance (Lespine et al. 2008)

transendothelial electrical resistances of endothelial monolayers (even when co-cultured with astrocytes or astrocyte-conditioned media) (Grant et al. 1998; Terasaki et al. 2003). In addition, the cell isolation process is both lengthy and expensive and there is restricted tissue availability. The most complete set of mapped ABC transporters on the BBB is from rat brain microvessels (Löscher and Potschka 2005; Roberts et al. 2008), where MDR, ABCG2 and ABCC4 were expressed on the apical membrane, while ABCC1 was weakly expressed on the basolateral membrane. For companion animals, future advances will be assisted if new species-specific antibodies become available, together with further study of expression and function of ABC transporters in isolated capillaries and advanced cultured endothelial cell models (e.g. Fletcher et al. 2006).

5 Conclusions

Veterinary medical research has a long and distinguished history in the design of species-specific drug delivery devices and formulations. Development of commercially-viable and therapeutically effective implantable devices for production animals to release steroids, anthelmintics and antibacterial drugs has required creative designs using relatively cheap polymers. Spot-on formulations of anthelmintics for small animals take advantage of using the skin's sebaceous glands as a drug-secreting reservoir, notwithstanding the integumental heterogeneity of different species. Use of skin patches has been translated to a minor extent from human medicine and has found a limited niche in post-operative pain control. Long-acting SR injectable biodegradable formulations are more commonly used in veterinary medical applications than in humans. Rare, but important examples from exotic species provide both challenges and opportunities (Hunter and Isaza 2002).

Finally, endogenous control of the fate of veterinary drugs on entering the body and their subsequent distribution and elimination involve understanding the

function and regulated expression of membrane transporters which influence absorption, distribution, and elimination pathways of pharmaceuticals and biologicals. The comparative study of *P*-gp and other transporters with particular emphasis on species- and tissue-specific distribution is likely to lead to (1) a reduction in drug–drug interactions, (2) the potential to optimise drug safety and efficacy profiles, and (3) breed-specific dosing schedules based on differences in genetic make-up (see chapter, "Pharmacogenomics in Domestic Animal Species").

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Population Medicine and Control of Epidemics

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Contents

Abstract Population medicine is an important component of veterinary care in livestock (farm animals) and companion animals (pets). This chapter covers some of the chemotherapeutic approaches undertaken at population level to control infectious diseases in domestic animals. Optimisation of health, productivity and welfare in livestock commonly entails implementation of whole-herd or whole-flock strategies to effectively counter the negative impact of infectious diseases. Gastro-intestinal and liver parasites of grazing cattle and sheep are endemic in most parts of the world and can result in significant production losses. Strategically timed anthelmintic treatments are instituted with the double objective of reducing worm burdens in infected animals and ensuring reduction of pasture contamination with infective larvae. Mastitis is another major endemic problem, particularly in cattle, which causes significant economic losses to dairy farmers globally. As a painful inflammatory condition of the cow's udder, clinical mastitis also raises animal welfare concerns. Prevention of clinical mastitis requires rigorous post-milking hygiene, identification and culling of chronically infected cows, attention to the

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cow's environment and therapeutic management of udder health during the dry period. A third condition that can cause high levels of morbidity and mortality is bacterial respiratory disease. Pneumonia in young livestock is often exacerbated by stressful transportation and co-mingling of animals from different herds. The welfare consequences and production losses can be significant. Antimicrobial treatment of pneumonic animals and, when appropriate, of in-contact animals living in the same air-space is an integral part of whole-herd respiratory disease management. The role of the veterinary profession is to also ensure that principles of population medicine are understood and adhered to by pet owners. The increase in pet ownership and the importance of the human–animal bond in modern developed societies give rise to zoonotic risks, which require vigilance and intervention. Regular internal parasite control in dogs and cats, particularly in endemic areas, contributes to animal welfare and minimises public health hazards.

Keywords Bovine respiratory disease · Group therapy · Helminths · Mastitis · Public health · Swine respiratory disease

1 Introduction

Animal welfare, herd productivity and public health are the key drivers for a population-based approach to treatment and prevention of animal diseases. Veterinary practitioners, livestock producers, research scientists, pet owners and policy makers have roles to play in understanding the dynamics of disease transmission and in designing and executing group-oriented interventions to control diseases. Whilst immunisation plays a major role in the prevention of viral epidemics, minimising the impact of parasitic (usually helminth) infections and many bacterial and mycoplasmal diseases on animal production and welfare remains largely dependent on the rational use of anti-infective agents. Viral diseases are beyond the scope of this chapter; suffice it to mention that the control of their spread involves a combination of restricting animal movement, strict biosecurity measures, culling and vaccination.

This chapter focusses on the use of chemotherapeutic agents, at herd, flock and population levels, to control and prevent non-viral infectious diseases. Three areas of therapy will be considered: helminthiases in livestock and companion animals, mastitis in dairy cattle and bacterial respiratory disease in cattle and swine.

2 Helminth Control

2.1 Helminth Parasites of Ruminants

In the developed world, economic losses related to helminth (worm) infections of livestock are principally the result of treatment and prevention costs. For example,

more than 1 billion Euros are spent annually on chemical helminth control in the EU (www.parasol-project.org). The annual cost of gastro-intestinal parasites to the British sheep industry is estimated at £84 million, making parasitic gastro-enteritis (PGE) the most costly disease afflicting small ruminants (Nieuwhof and Bishop 2005). A health plan for 1,500 lambs in a 1,000 ewe lowland flock would typically include a £300 annual allocation for the treatment and prevention of gastro-intestinal nematode infections (Scott et al. 2007). In recent years, climate change and the advent of milder wetter weather in some geographical areas have resulted in a dramatic resurgence of fasciolosis (liver fluke) in sheep and cattle (Mitchell 2002; SAC Veterinary Services 2008). The emergence of anthelmintic resistance is leading, gradually, to direct production losses resulting from lack of efficacy of available anthelmintics. Resistance of gastro-intestinal nematodes and liver fluke has indeed become a global issue in sheep, and evidence is mounting that it is an emerging problem in cattle as well (Coles et al. 2001; Demeler et al. 2009; www.parasol-project.org). Another trend that adds to the economic and welfare relevance of nematode infections is the growing importance of organic farming, wherein the routine use of anthelmintics is restricted. Some cases of poor management in this production segment have resulted in clinical parasitism being reported in sheep (Waller 2006).

The life-cycle of gastro-intestinal nematodes starts with production of eggs by adult worms in the host. The eggs are expelled from the host in the faeces and contaminate the pasture. First-stage larvae hatch from the eggs and moult twice before they become infective. Third-stage larvae are capable of migrating from dung pats and soil onto moist grass. Eggs develop into infective third-stage larvae 1–2 weeks after excretion from the host. Larvae can survive up to a year on pasture. Infection occurs when third-stage larvae are consumed with the grass during the grazing season. Immature worms migrate into the gut mucosa and cause significant damage to the mucosal lining when they re-emerge as immature adults. The cycle completes when the immature forms reach the adult stage in the gastro-intestinal tract of the host. Third-stage larvae turn into adults over a period of 3–4 weeks unless they become inhibited and their development is arrested, typically for several months, in the gut mucosa (a phenomenon known as hypobiosis).

Worm control programmes rely on whole-herd anthelmintic dosing strategies, which are timed to minimise or suppress worm burdens that have resulted from exposure to infective larvae on pasture. Gastro-intestinal nematode infections of cattle involving the abomasal-dwelling species (the abomasum is the fourth and final stomach compartment in ruminants) *Ostertagia ostertagi*, *Trichostrongylus axei* and *Haemonchus placei*, and the intestinal-dwelling species *Cooperia onchophora* and *Nematodirus spp*. are ubiquitous throughout Europe. In the first grazing season calves, infection with *O*. *ostertagi* can cause severe PGE, characterised by diarrhoea, reduced feed intake and deterioration of body condition. Chemoprophylaxis with anthelmintic drugs during the first half of the grazing season (April–October) provides effective control of PGE, whilst lowering pasture contamination with nematode larvae in late summer. The use of anthelmintics in lactating dairy cows after turnout from winter housing on to pasture controls sub-clinical gastro-intestinal nematode infections and results in improved milk production (Gross et al. 1999;

Gibb et al. 2005). In sheep, within the European flock, worm infestations involving the abomasal nematode species *Haemonchus contortus* and *Teladorsagia circumcincta*, and the small intestinal parasites *Trichostrongylus colubriformis* and *Nematodirus battus* are common. They may cause slow growth and hence extended time to slaughter. Weight loss, emaciation and even death can result from acute haemonchosis and nematodirosis. Welfare concerns arise from the deterioration in body condition of emaciated sheep. Furthermore, faecal staining of the perineum, resulting from PGE-induced diarrhoea, is a major risk for cutaneous myiasis (Scott et al. 2007). This condition is the invasion of living tissues by larvae of dipteran flies in the summer. It arises as a result of female blowflies laying eggs in areas of soiled fleece and the ensuing larvae lacerating the infested skin. Timed administration of anthelmintics to ewes during the periparturient period and subsequently to grazing lambs mitigates production losses, prevents clinical disease and reduces larval pasture contamination.

Details of anthelmintic agents available for use in livestock are presented under Sect. 2.3 below and in Table 1. Although all chemoprophylactic protocols provide satisfactory control, drugs of the macrocyclic lactone (ML) group deserve special mention because they have transformed parasite control strategies in livestock over the past 28 years. These molecules are characterised by slow clearance, large volumes of distribution and long terminal half-lives (McKellar and Benchaoui 1996) (Table 2). Their prolonged persistence in the body prevents the establishment of incoming larvae for several weeks, thus allowing effective and long-lasting control of gastro-intestinal nematode infections with low frequency of drug administration. Members of this class are available in injectable form (mostly oil-based formulations for subcutaneous injection), oral drenches (aqueous solution or suspension for oral delivery), sustained release devices (e.g. osmotic bolus delivering 12.7 mg of ivermectin per day over a period of 135 days into the reticulo-rumen (forestomach) of cattle) and pour-ons (solutions for application along the midline of the back). In addition to being anthelmintic agents, MLs also have potent ectoparasiticide properties. All these attributes have made these molecules the "gold standard" of parasite control in ruminants. In food producing animals, however, drugs with long-lasting efficacy often have the drawback of persistent residues in edible tissues. Most MLs fall into this category, and edible products derived from treated animals can only enter the food chain after a relatively long withdrawal (waiting) period. The exception is eprinomectin; this molecule was specifically developed for its low partitioning into the milk (Alvinerie et al. 1999), and therefore lactating dairy cows can be dosed without the inconvenience of post-treatment milk discard.

2.2 Helminth Parasites of Dogs and Cats

2.2.1 Gastro-Intestinal Nematodes

Clinical disease caused by gastro-intestinal nematodes can range from ill-thrift and slow growth to severe haemorrhagic enteritis and anaemia, particularly in young pups and kittens (Magne 2006). From the perspective of prevalence and pathogenic

Table 1 Anthelmintic agents: spectrum of activity and mode of action

Class	Members[a]	Species	Delivery route	Spectrum of activity	Mode of action
Benzimidazoles	Thiabendazole Fenbendazole Oxfendazole Flubendazole Albendazole Mebendazole Triclabendazole[b]	Cattle, sheep, goats, pigs, poultry, cats, dogs, horses	Oral	GI nematodes, Lungworm, Liver fluke[c], Cestodes[c]	Inhibition of microtubule polymerisation, leading to disorders of intracellular homeostasis and starvation of the nematodes
Imidazothiazoles/ Tetrahydropyrimidines	Levamisole, Morantel, Pyrantel	Cattle, sheep, goats, pigs, cats, dogs, horses	Oral, SC, IM, Topical	GI nematodes, Cestodes[c], Lungworm[c], Heartworm[c]	Mimicking action of acetylcholine at the nicotinic receptors (nAchR) of the post-synaptic membrane, causing change in permeability and spastic paralysis of the worms
Macrocyclic Lactones	Ivermectin Abamectin Doramectin Selamectin Moxidectin Eprinomectin Milbemycin	Cattle, sheep, goats, pigs, cats, dogs, Horses,	Oral, SC, Topical	GI nematodes, Lungworm, Heartworm	Opening of ligand-gated chloride channels, leading to flaccid paralysis of the nematodes
Cyclic depsipeptides	Emodepside	Cats	Topical	GI nematodes	Binding to latrophilin-like receptors, causing Ca^{2+} influx and inhibition of pharyngeal pumping, leading to paralysis
Amino acetonitrile derivatives (AADs)	Monepantel	Sheep	Oral	GI nematodes	Activation of signalling via nematode-specific DEG-3 subtype nicotinic acetylcholine receptors (nAChR) (distinct from levamisole target receptors), causing hypercontraction of body wall muscles and paralysis

(*continued*)

Table 1 (continued)

Class	Members[a]	Species	Delivery route	Spectrum of activity	Mode of action
Oxindole alkaloids (Paraherquamides)	2-desoxoparaherquamide	Sheep	Oral	GI nematodes	Antagonists of the nematode nicotinic acetylcholine receptors
Salicylanilides	Closantel; rafoxanide	Sheep	Oral	Liver fluke, *H. contortus*	Uncoupling of oxidative phosphorylation in the parasite's mitochondria, leading to ATP depletion
Benzenesulphonamides	Clorsulon	Cattle	SC	Liver fluke	Interference with energy metabolism through selective antagonism of fluke phosphoglycerate kinase and mutase
Pyrazinoisoquinolines	Praziquantel, epsiprantel	Cats, dogs	Oral, topical, SC, IM	Cestodes	Putatively act through alteration of Ca^{2+} homeostasis in adult tapeworms, causing muscle contraction and tegument vacuolisation

[a]Not all members of each class are available for every animal species
[b]Efficacy limited to liver fluke. [c]Some members of the class are also active against these endoparasites

Table 2 The macrocyclic lactones: Pharmacokinetic properties in cattle

	Dose (mg/kg)	Route	AUC (ng.d/mL)	C_{max} (ng/mL)	T_{max} (days)	CL (L/h/kg)	Vdss (L/kg)	Terminal $T_{1/2}$ (days)	References
Ivermectin	0.2	IV	254	442	0	0.033	2.2	2.69	Wilkinson et al. (1985)
	0.2	SC	361	31.7	3.98	–	–	4.32	Toutain et al. (1997)
	0.5	Top.	116	12.2	3.40	–	–	5.30	Gayrard et al. (1999)
Doramectin	0.2	IV	–	–	–	0.013	1.7	3.71	Wicks et al. (1993)
	0.2	SC	511	32.6	5.31	–	–	5.39	Toutain et al. (1997)
	0.5	Top.	168	12.2	4.30	–	–	9.80	Gayrard et al. (1999)
Moxidectin	0.2	IV	195	–	–	0.046	3.0	2.97	Lifschitz et al. (2002)
	0.2	SC	162	33.5	0.369	–	–	10.3	Lifschitz et al. (2002)
	0.5	Top.	105	13.4	1.0	–	–	6.40	Sallovitz et al. (2003)
Eprinomectin	0.5	Top.	239	43.8	2.05	–	–	2.03	Alvinerie et al. (1999)

potential, the most important gastro-intestinal nematodes of dogs and cats in many urban environments are the hookworms (*Ancylostoma caninum, Ancylostoma tubaeformae, Uncinaria stenocephala*), the ascarids (*Toxocara canis, Toxocara cati*) and, in the case of dogs, the whipworms (*Trichuris vulpis*) (Bowman et al. 2003). Some of these nematode species are of public health importance because of their zoonotic potential. For example, human contact with the infective larvae of hookworms (*Ancylostoma braziliense, A. caninum, U. stenocephala*) can cause skin eruptions ranging from mild cutaneous irritation ("ground itch") to the more severe pruritic lesions of cutaneous larva migrans (creeping eruption caused by hookworm larvae migrating in the human skin) (Hendrix et al. 1996). A more insidious manifestation of zoonotic hookworm infection is eosinophilic enteritis (Kopp et al. 2008).

Ingestion of infective *Toxocara* eggs can result in visceral larva migrans, ocular larva migrans and covert toxocariasis in humans (Taylor and Holland 2001). Given the importance of the human–animal bond and the high level of pet ownership in many modern societies, it is important to minimise transmission of parasitic zoonoses. Whilst appropriate hygiene measures such as hand washing are essential, regular anthelmintic dosing of pets is also important in treating animals and controlling environmental contamination with these nematodes. As the pre-patent period (time needed for the infective larvae to become egg-producing adults) of the highly fecund *Toxocara spp.* is just over 4 weeks, monthly treatment with long acting drugs can minimise the risk of environmental contamination resulting from patent infection. This frequency of treatment is particularly relevant in high risk circumstances, wherein pets have regular access to the outdoors and especially if the household includes young children (Esccap 2006). Depending on the level of owner compliance and the risk scenarios, a de-worming frequency of at least four times a year, or alternatively less frequent treatments combined with regular faecal examinations, are generally recommended.

2.2.2 Cestodes

Echinococcus multilocularis and *Echinococcus granulosus* are zoonotic tapeworm species of major public health concern. Human alveolar echinococcosis is a hepatic

disease resembling liver cancer and is caused by the larval stage (metacestode) of *E. multilocularis*. Untreated, alveolar echinococcosis may result in severe hepatic dysfunction and metastases in other organs with high mortality. Human infection results from ingestion of *Echinococcus* eggs shed by a definitive host. The lifecycle of *E. multilocularis* is predominantly sylvatic, and includes mainly foxes but sometimes dogs and cats as definitive hosts. Small mammals, mainly rodents, are intermediate hosts (Deplazes and Eckert 2001). *E. multilocularis* is endemic in the northern hemisphere including central and Eastern Europe (Fig. 1). The prevalence rate of *E. multilocularis* in foxes in the core endemic region of central Europe is in the range of 35–65% (Torgerson et al. 2008). Inevitably, this high incidence together with the increased fox population in urban areas results in increased environmental contamination with *E. multilocularis* eggs, leading in turn to an exacerbated zoonotic risk (Dyachenko et al. 2008). Cystic echinococcosis is a condition of livestock and humans caused by the ingestion of infective eggs of *E. granulosus*. Dogs are the primary definitive hosts, and ungulates (e.g. ruminants, pigs, horses) are intermediate hosts. Humans are aberrant intermediate hosts. Infections result in the development of cysts in the liver, lungs or other organ systems, which usually require surgical removal. Prevalence of different genotypes of *E. granulosus* is high in southern and Eastern Europe (Fig. 1), North Africa, the Middle East and Asia (Budke et al. 2006).

Regular anthelmintic treatment of dogs and cats that may have access to viscera of intermediate hosts is an important component of human disease prevention. To this end, the use in household pets of effective cestodicidal compounds, such as praziquantel or epsiprantel is generally recommended (Esccap 2006). These drugs are also effective against the flea-transmitted tapeworm *Dipylidium caninum* and are generally available in combination with nematodicidal compounds, as either oral tablets or spot-on preparations and can be administered as part of a broad-spectrum de-worming programme. Anthelmintic (praziquantel) baiting campaigns

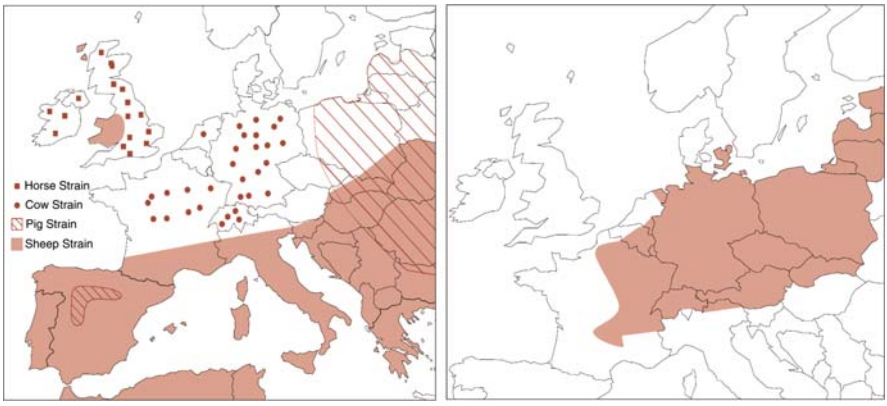

Fig. 1 Geographic distribution of *Echinococcus granulosus* (*left*) and *Echinococcus multilocularis* (*right*) in Europe (reproduced with permission from www.esccap.org)

to control *E. multilocularis* infection in wild red foxes have been undertaken in Germany (Tackmann et al. 2001), Switzerland (Hegglin et al. 2003) and Japan (Inoue et al. 2007); the long term success of these campaigns will likely depend on the continued implementation of baiting programmes and its cost effectiveness.

2.2.3 Heartworm

The mosquito-transmitted filarial nematode, *Dirofilaria immitis,* is a major cause of canine and feline disease in the USA, Africa, Australia, Japan and southern Europe. In Europe, heartworm is most prevalent in Spain, Portugal, South of France and Greece with the largest endemic area being located along the Po River Valley in northern Italy, where more than 50% of untreated dogs test positive for heartworm infection (Genchi et al. 2005). It is believed that climate change and the greater freedom of pet movement across European countries may increase the spread of arthropod-born filarial infections, including heartworm. Parasitic worms reside in the pulmonary arteries and sometimes in the right ventricle. Clinical signs can range from none to sudden death. Signs of right ventricular heart failure, occurring in heartworm-infected dogs, include coughing, reluctance to move or exercise, fatigue after moderate exercise, reduced appetite and weight loss (American Heartworm Society, www.heartwormsociety.org). Clinical manifestations of heartworm disease in cats are variable. Heartworm-infected cats may undergo spontaneous self-cure without clinical signs, but they may also suddenly show dramatic and acute symptoms. Sudden death in apparently healthy cats is not a rare event (Venco et al. 2008). *D. immitis* and the subcutaneous filarial nematode *Dirofilaria repens* have a zoonotic potential. The public health significance of *D. immitis* is not attributable to the overt signs of clinical disease in humans but rather to the severity of disease that the radiographic findings of a pulmonary coin lesion (solitary pulmonary nodule) suggest might be present (Theis 2005). Differential diagnosis includes primary or metastatic neoplasia, fungal infections, hamartomas (benign lung tumours), and tuberculosis. This lesion requires an extensive clinical work-up, which, not infrequently, culminates in a thoracotomy (Theis 2005).

In *D. immitis*-endemic areas, monthly administration of heartworm preventatives to dogs and cats throughout the high risk season is recommended (McCall 2005). The advent of the MLs – ivermectin, milbemycin oxime, moxidectin and selamectin – transformed heartworm chemoprophylaxis, introducing a new era of potent filaricidal activity, convenience afforded by long duration of action and choice of delivery route (oral, spot on and slow release injectable formulations). Combined control of flea infestations, heartworm and gastro-intestinal nematodes is another advance in companion animal parasite control, which has been achieved through the development of endectocide drugs (selamectin) or drug combinations (moxidectin + imidacloprid, milbemycin oxime + lufenuron). Experimental therapy targeting *Wolbachia,* the bacterial endosymbiont, which is essential to filarial survival, is currently under evaluation (McCall et al. 2008).

2.3 Anthelmintic Agents

Six modern classes of broad-spectrum anthelmintic agents are currently available, or are likely to become available in the near future:

- Benzimidazoles
- Imidazothiazoles/tetrahydropyrimidines
- MLs
- Paraherquamides
- Cyclic depsipeptides
- Amino-acetonitrile derivatives (AADs)

Narrow-spectrum anthelmintic classes with examples, developed for animal health prophylaxis and therapy, mainly to combat liver fluke or tapeworm infections, include the following:

- The benzimidazole compound triclabendazole
- Salicylanilides (closantel)
- Pyrazinoisoquinolines (praziquantel)
- Benzenesulphonamides (clorsulon)
- Substituted phenols (nitroxynil eglumine)

The spectrum of activity and mechanisms of action of these agents are summarised in Table 1.

2.4 Anthelmintic Resistance

Resistance of gastro-intestinal nematodes to anthelmintic agents constitutes a major threat to the worldwide small ruminant industry. Resistance to benzimidazoles, levamisole and the MLs is now widespread in all continents including Europe (Table 3). Moreover, multi-drug resistance to all three anthelmintic groups is no longer uncommon (Kaplan 2004). Whilst the most significant impact of anthelmintic resistance has been largely confined to sheep and goats, evidence of resistant *Cooperia* in cattle is emerging (Coles et al. 2001; www.parasol-project.org). In recent years, guidelines have been published to standardise the in vivo and in vitro methods available for the detection of nematode resistance (Coles et al. 2006). Resistance in liver fluke has not yet reached the scale experienced with nematodes, but it has been reported for the salicylanilides, rafoxanide and closantel, with cross resistance to the halogenated phenol, nitroxynil. A concerning trend observed since the mid-1990s is the development of resistance to triclabendazole, the principal drug used to treat liver fluke infections, because of its high activity against immature migrating stages (Brennan et al. 2007). In horses, cyathostomins resistant to the benzimidazoles and pyrantel are now widespread (Kaplan 2004; Traversa et al. 2007b); there is also evidence of *Parascaris equorum* resistance to the MLs

Table 3 Reported occurrence of anthelmintic resistance in gastro-intestinal nematodes of sheep and cattle in EU

Countries	Species	Class	References
United Kingdom	Sheep	BZ, LV, ML	Bartley et al. (2003); Sargison et al. (2001, 2005, 2007)
	Cattle	ML	Coles et al. (1998, 2001)
Denmark	Sheep	BZ, LV, ML	Bjørn et al. (1991); Maingi et al. (1997)
Sweden	Sheep	BZ	Höglund et al. (2009)
	Cattle	ML	Demeler et al. (2009)
Netherlands	Sheep	BZ, ML	Borgsteede et al. (2007)
Germany	Sheep	BZ, LV, ML	Duwel et al. (1987); Bauer et al. (1988)
	Cattle	ML	Kleinschmidt et al. (2008); Demeler et al. (2009)
France	Sheep	BZ	Palcy et al. (2010)
Italy	Sheep	LV, ML	Traversa et al. (2007a)
Spain	Sheep	BZ, LV, ML	Alvarez-Sánchez et al. (2006); Díez-Baños et al. (2008)
Slovakia	Sheep	BZ, ML	Čerňanská et al. (2006)
Belgium	Sheep	BZ	Vercruysse et al. (1989)
	Cattle	ML	Demeler et al. (2009)
Ireland	Sheep	BZ, LV	O'Brien et al. (1994); Patten et al. (2007)
Greece	Sheep	BZ	Papadopoulos et al. (2001)

BZ: Benzimidazoles; LV: Levamisole; ML: Macrocyclic Lactones

(Stoneham and Coles 2006; Traill 2008). In dogs, no reports of anthlemintic resistance were published until very recently; indeed, two isolates of *A. caninum* resistant to pyrantel have now been identified in Australia (Kopp et al. 2009). In addition, evidence of lack of efficacy has been reported for heartworm preventatives in the USA (McCall 2005); thus far, however, such treatment failures are largely imputed to owner non-compliance.

Despite the palpable need for new classes of antihelmintics to counter the mounting threat of resistance, the flow of novel molecules reaching the livestock endoparasiticide market has been disappointingly slow. The recent discovery, development and launch of a new anthelmintic class for use in sheep, the amino-acetonitrile derivatives (AADs), have been the only innovation seen since the MLs were introduced more than 25 years ago (Kaminsky et al. 2008). Control strategies that preserve the efficacy of existing classes of anthelmintics are therefore crucial to the mitigation of anthelmintic resistance. However, despite the development of a variety of measures to reduce selection for resistance, the problem continues to impact on small ruminant production worldwide. A new paradigm under discussion calls into question the whole-flock approach to anthelmintic treatment. The concept is based on leaving a proportion of animals untreated to ensure that the worm population in refugia (residing in untreated infected animals and on pasture) provides a sufficient susceptible gene pool. This unexposed nematode population is then available to dilute, and to mate with, any worms that survive treatment (van Wyk et al. 2006). A European initiative (PARAsite SOLutions, also known as PARASOL) has been established for a sustainable control of nematodes in livestock. This project proposes Targeted Selective Treatment (TST) as a strategy to slow the rate of development of anthelmintic resistance by retaining a susceptible worm population in refugia: with TST, only clinically affected animals are treated.

The approach has attracted positive comments, and it is hoped that production losses associated with sub-clinical infections will not be a deterrent to the long term implementation of this strategy by the livestock producers.

3 Mastitis Control in Dairy Cows

Mastitis, an inflammatory response of the mammary gland, usually caused by bacterial pathogens, is one of the most important endemic infectious diseases to affect dairy cattle. Mastitis has an impact on animal production, animal welfare and the quality of milk produced. Mastitis is typically recognised by clinical signs expressed as abnormalities in the milk and the udder. Infection can affect one or more of the four mammary quarters of the cow's udder. The disease is usually local but, in acute cases, may trigger systemic signs of pyrexia, anorexia and depression. Severe clinical mastitis may terminate with the cow's death or agalactia of the infected quarter(s), leading to premature culling. Based on the epidemiology of the causative bacterial pathogen, bovine intramammary infections can be categorised as contagious or environmental in origin. Contagious mastitis can be described as intramammary infection transmitted from cow to cow, wherein the primary reservoir of pathogens is infected quarters. Uninfected quarters are mainly exposed to contagious pathogens during the milking process. The bacteria responsible for such infections include streptococci (*Streptococcus agalactiae*, *Streptococcus dysgalactiae*), coagulase positive staphylococci (*Staphylococcus aureus*), coagulase negative staphylococci (CNS) and *Corynebacterium bovis*. On the other hand, the primary reservoir of pathogens responsible for environmental mastitis is the dairy cow's environment. Uninfected quarters may be exposed at any time during the cow's life, including during milking, between milkings and during the dry period (the dry period, typically 40–50 days long, is the phase of milking cessation that precedes the next calving). The primary pathogens are Gram-negative bacteria, mainly Enterobacteriaceae (*Escherichia coli*, *Klebsiella* sp., *Enterobacter* sp., *Serratia* sp., *Proteus* sp. and *Pseudomonas* sp.), environmental streptococci (mainly *Streptococcus uberis*) and enterococci (*Enterococcus faecalis*) (Oliver 1998).

Intramammary infection of the dairy cow is much less common than 40 years ago due to the implementation of udder health strategies. One such dairy health programme is the five-point plan introduced in the UK in the early 1970s. At that time, the vast majority of mastitis cases in the British herd were caused by contagious pathogens, principally *S. aureus* and *S. agalactiae*. The plan advocated treating and recording all cases of clinical mastitis, performing post-milking disinfection of all teats immediately after every milking, instituting dry cow therapy (DCT) of all cows at the end of every lactation, culling of chronic cases of mastitis and carrying out regular milking machine maintenance. As a consequence of the application of this control plan, the incidence of mastitis caused by contagious pathogens has decreased markedly (Jones and Ohnstad 2002). For example, at the Institute for Animal Health (UK) herd, 43% of mastitis cases were caused by

Table 4 Causes of clinical mastitis (% positive identifications) over time in a British dairy herd (from Hillerton and Berry 2005)

	1964	1985–1990	2000
Streptococcus agalactiae	1.9	0	0
Steptococcus dysgalactiae	22	8.3	0
Streptococcus uberis	20	43	33
Staphylococcus aureus	43	20.3	16
Coliforms	2.4	22.8	43
Arcanobacterium pyogenes	4.5	5.4	1.2

S. aureus in 1964. The percentage declined to 16% in 2000, whilst *S. agalactiae* has long been eradicated from the herd (Table 4).

The overall incidence of mastitis had also decreased by 70% by 1988 (Booth 1988), and the average somatic cell count (SCC) (number of leucocytes) of milk sold from farms declined from 550,000 cells/mL in 1972 to a national level below 200,000 cells/mL in 2004 (Hillerton and Berry 2005). Milk quality in the EU is subject to the standards set in the EC Milk Hygiene Directive (92/46), which stipulates that producers should not sell milk if the SCC exceeds 400,000 cells/mL. This standard is based on the average SCC over a 3-month period. The European farmer has a financial incentive to keep the milk SCC below this level.

The control gained over contagious pathogens causing intramammary infections over the last four decades has been accompanied by a change in the aetiology of mastitis. There has been an increase in the incidence of infections caused by environmental pathogens such as *S. uberis* and coliforms, notably *E. coli* (Table 4). Thus, despite significant overall progress, clinical and sub-clinical mastitis remain amongst the costliest diseases affecting the animal health sector, with an estimated financial burden of £300 million in the UK (Hillerton and Berry 2005).

Transition from the dry period to lactation is a vulnerable and high risk phase for the modern dairy cow (Pyörälä 2008); the biggest disease challenge at that time is mastitis, as most clinical cases occur soon after calving and in early lactation. It is not surprising, therefore, that the most common indication for antimicrobial usage in the lactating dairy cow was found to be clinical mastitis in a recent Finnish survey (Thomson et al. 2008). The use of either intramammary or systemic route (usually subcutaneous or intramuscular injection) or both to administer antimicrobial drugs for the treatment of clinical mastitis continues to be a subject of debate. It is evident that some compounds, due to their physico-chemical properties, notably low lipid solubility, have very limited distribution into the udder, i.e. there is poor penetration of the blood milk barrier (Ziv 1980), and the most efficient delivery system for such drugs is infusion into the gland via the teat canal. Such compounds include the penicillins, early-generation cephalosporins and aminoglycosides. On the other hand, macrolides, tetracyclines and fluoroquinolones distribute well into the mammary gland after systemic administration. The penetration of macrolides, which are weak organic bases, is particularly favoured by diffusion (ion) trapping in milk, which has a lower pH than plasma; it is a classic illustration of the Henderson–Hasselbalch principle of unequal distribution of drugs across biological

membranes. Fluoroquinolones have a broad-spectrum of activity extending to Gram-negative pathogens. Hence, injectable fluoroquinolones are now licenced for the treatment of acute *E. coli* mastitis in Europe. Some late-generation cephalosporins, such as cefquinome, can also be used systemically for the treatment of acute *E. coli* mastitis.

During the dry period, the presence of infection has a fundamental and lasting influence on the health and productivity of the dairy cow (Green et al. 2002). The effects are reduction in subsequent milk yield in infected quarters, reduction in milk quality (increased SCCs, decreased butterfat and protein content) at the next lactation and a greater risk of subsequent clinical mastitis. Historically, when the five-point plan was instituted, the principal value of DCT was to eliminate infections caused by *S. aureus* and various streptococcal species that persisted during lactation. Indeed, a more effective bacteriological cure is achieved by treating cows at dry off than during lactation (Smith et al. 1967). In the modern dairy herd, however, the primary objective of DCT is prevention of new intramammary infections, the prophylactic goal being a reduction in the risk of clinical mastitis during the subsequent lactation. To achieve this, the systematic use of broad-spectrum antibacterial agents at drying off is not always necessary, and a more discriminating approach can be applied (Bradley et al. 2003; Pyörälä 2008). Such an approach involves selection of the cows that are likely to be uninfected. In practice, this can be done by verifying their recent SCC records (they should be consistently lower than 200,000 cells/mL) and any mastitis history in the previous lactation (there should be no mastitis experienced in any quarter). Once identified, these cows are dried off with either (a) an antibiotic with known efficacy against the predominant environmental pathogen(s) identified within the herd or (b) an internal teat sealant infused alone, that is with no antibiotic. Using this approach, cows with SCCs greater than 200,000 cells/mL for at least two recent records, including the last one prior to drying off, are assumed to be infected, and they are investigated to identify the pathogen(s) involved; these cows receive a suitable antimicrobial drug as DCT, either alone or preferably followed by infusion of an internal teat sealant to prevent new infections.

A recently conducted UK-based field efficacy study highlighted the benefits of combination DCT in cows with high SCCs. This study in high SCC cows compared DCT with 600 mg cloxacillin alone and 600 mg cloxacillin followed by an internal teat sealant (Newton et al. 2008). Ten dairy herds were involved, and 283 cows were included in the data analysis. Clinical mastitis cases were monitored from drying off until 100 days after calving. In the quarters, given the combined treatment, there were 23 cases of clinical mastitis compared with 50 cases in the quarters that received the antibiotic alone; differences in both the mastitis episodes and quarters affected were significant ($P < 0.01$). A summary of the pathogens isolated from these cases of mastitis is given in Table 5. In the quarters treated with antibiotic alone, there were significantly more cases of clinical mastitis caused by *S. uberis* ($P = 0.02$) and coagulase-positive staphylococci ($P = 0.03$).

The likelihood of a quarter either being bacteriologically negative after calving or developing clinical mastitis in the first 100 days after calving was investigated

Table 5 Frequency of isolation of different pathogens from cases of clinical mastitis in quarters treated with cloxacillin alone or with cloxacillin plus teat sealant and the (numbers of quarters affected) (from Newton et al. 2008)

	Cloxacillin alone	Cloxacillin + Teat Sealant
Streptococcus uberis	15 (14[a])*	4 (4[b])
Escherichia coli	5 (4)*	4 (4)
Bacillus species	1(1)	
Citerobacter species	1 (1)	
Coagulase positive staphylococci	7 (7[a])	1 (1[b])
Corynebacterium species		1 (1)
Escherichia species	1 (1)	
Other *Streptococcus* species	1 (1)	
Mixed growth	3 (3)	3 (3)
No growth	16 (16)	10 (10)
Total	50 (48)[c]	23 (23)

[a, b]Rows with different superscripts are significantly different ($P < 0.05$)

*Some quarters were infected by different pathogens at different times

Table 6 Logistic regression model for the outcome of clinical mastitis: comparison between cloxacillin and cloxacillin + teat sealant (from Newton et al. 2008)

	Coefficient	Odds ratio	95% credibility interval
Intercept	–3.47		–5.07, –2.19
Reference cloxacillin alone			
Cloxacillin + teat sealant		0.47	0.26, 0.82
Reference quarter right fore			
Left fore		1.25	0.56, 2.80
Left hind		0.93	0.41, 2.17
Right hind		1.84	0.87, 4.03
Between cow variance	1.37		0.19, 3.14
Between farm variance	2.93		0.36, 11.7

by multilevel logistic regression. The quarters treated with the cloxacillin and the internal sealant were significantly more likely to be bacteriologically negative in the immediate period after calving and were significantly less likely to suffer clinical mastitis, with an odds ratio of 0.47 (95% credibility interval 0.26–0.82), than the quarters treated with cloxacillin alone (Table 6). It was also observed that there was more variation between farms, than between cows within farm, in the probability of clinical mastitis. This finding indicates that farm characteristics such as management, nutrition and prevalence of concurrent diseases are important determining factors for the occurrence of clinical mastitis.

4 Control of Respiratory Disease in Cattle and Swine

Pneumonia is a cause of major economic loss for the cattle and swine industries, due to decreased production, high levels of mortality and increased veterinary and labour costs. It is estimated that the costs of an outbreak of calf pneumonia range

from 49 to 95 Euros per affected animal, with costs rising to 118 Euros per affected animal when re-treatments are required (Potter 2007). Bovine respiratory disease (BRD) is an infectious bronchopneumonia, manifested clinically by sudden onset of depression, pyrexia, anorexia and respiratory signs, which may include dyspnoea, tachypnoea, nasal discharge and abnormal lung sounds. Young calves are particularly susceptible. Transport (sometimes over long distances), mixing, dense housing conditions and climate stress are the common risk factors. Beef calves in feedlot systems and dairy calves used for replacement or for meat production are prone to BRD when subjected to pre-disposing conditions. It seems likely that many cases of BRD are initiated by a viral pathogen (such as bovine respiratory syncytial virus (BRSV), parainfluenza 3 virus (PI3) or bovine herpes virus 1 (BHV-1)), which renders affected calves more susceptible to secondary bacterial invasion. The most commonly isolated bacterial pathogens are *Mannheimia haemolytica*, *Pasteurella multocida* and *Histophilus somni*. Field observations of clinical cases and epidemiology suggest that *Mycoplasma bovis* is also an important cause of respiratory infections in cattle; it is estimated that in France, the Netherlands, the UK, and Ireland, *M. bovis* may be isolated from approximately 20% of pneumonic lungs and involved in 20–30% of herds with BRD outbreaks (Nicholas and Ayling 2003). A recent survey investigated the prevalence of *M. bovis* in French veal calf feedlots during BRD outbreaks (Arcangioli et al. 2008). The isolation and seroconversion rates were 60–100% in affected feedlots, an indication of the importance *M. bovis* clearly has in the BRD complex.

Although good herd management practice and vaccination against respiratory viruses and *M. haemolytica* are important components of an effective BRD preventive strategy, antimicrobial therapy is commonly required to minimise the production losses and animal welfare consequences of a bacterial and/or mycoplasmal respiratory disease outbreak. Beta-lactams, tetracyclines, trimethoprim-potentiated sulfonamides, fluoroquinolones, macrolides and florfenicol are antimicrobial classes and compounds commonly used in the treatment of BRD (of these the beta-lactams and sulfonamides have no activity against mycoplasmal pathogens). The in vitro susceptibility of BRD pathogens to antimicrobials is presented in Table 7. and the main pharmacokinetic properties of commonly used agents in cattle are summarised in Table 8. In many outbreaks of calf pneumonia, there is high morbidity, and, on welfare grounds, it is advisable to treat both diseased and in-contact animals (Gibbs 2001). Metaphylactic use of tilmicosin, tulathromycin and florfenicol has been reported to reduce significantly the incidence of BRD in healthy in-contact animals during disease outbreaks (Godinho et al. 2005b; Catry et al. 2008; Van Donkersgoed et al. 2008). The use of antibacterial drugs should be integrated with other preventive measures (avoidance of animal mixing, good ventilation, appropriate stocking density, adequate nutrition, adherence to vaccination programmes), and the metaphylactic use should be limited to those situations wherein the disease outbreak is likely to become severe and affect healthy animals living in the same air space. A BRD outbreak is often defined as 10–15% of the herd becoming clinically affected within a 3-day period. When presented with an outbreak of calf pneumonia, visual assessment alone to detect respiratory signs

Table 7 In vitro susceptibility of bovine respiratory disease pathogens to antimicrobial agents

Organism	MIC_{90}* (μg/mL)							
	Ceftiofur	Amoxicillin/ clavulanic acid (2/1)	Tilmicosin	Tulathromycin	Oxytetracycline	Florfenicol	Trimethoprim/ sulfamethoxazole (1/19)	Enrofloxacin
Mannheimia haemolytica	≤0.015	0.12/0.06	4	2	≥16	2	0.25/4.8	0.03–0.06
Pasteurella multocida	≤0.004	0.32/0.16	16	1	16	0.5	0.12/2.4	≤0.016–0.125
Histophilus somni	0.03	0.06/0.03	8	4	4	0.25	0.06/1.2	0.03–0.06
Mycoplasma bovis	–	–	>128	>64	32	16	–	0.5

*Minimum drug concentration required for the inhibition of 90% of isolates

Sources: Aarestrup et al. (2004); Ayling et al. (2000); Godinho (2008); Godinho et al. (2005a); Kehrenberg et al. (2004); Priebe and Schwarz (2003); Rosenbusch et al. (2005); Salmon et al. (1996); Schwarz et al. (2004)

Table 8 Pharmacokinetics of antimicrobial agents used for the treatment of bovine respiratory disease

	Dose (mg/kg)	Route	C_{max} (μg/mL)	T_{max} (h)	AUC (μg h/mL)	Terminal $T_{1/2}$ (h)	F(%)	References
Ceftiofur*	2.2	SC	13.6	0.67–3	108	9.5	NR	Brown et al. (2000)
Tilmicosin	10	SC	0.87	0.5	17.2	29.4	NR	Modric et al. (1998)
Tulathromycin	2.5	SC	0.41	0.25	11.9	92	>90	Nowakowski et al. (2004)
Florfenicol	20	IM	3.1	3.3	70.7	18.3	78.5	Lobell et al. (1994)
Enrofloxacin**	8	SC	0.81	2.0	7.5	7.3	NR	TerHune et al. (2005)
	12.5	SC	0.96	4.8	15	6.8	NR	Davis et al. (2007)
Oxytetracycline	10	IM	3.0	4.0	61.8	9.8	99.9	Schifferli et al. (1982)

*As ceftiofur sodium

**Parameters reported for the parent compound, enrofloxacin. Enrofloxacin represents approximately 59% of the total (enrofloxacin+ciprofloxacin) drug concentrations in plasma (Davis et al. 2007) (ciprofloxacin is an active metabolite of enrofloxacin)

NR: not reported

Table 9 Susceptibility of swine respiratory disease pathogens to antimicrobial agents

Organism	MIC_{90}* (μg/mL)							
	Ceftiofur	Amoxicillin/ clavulanic acid (2/1)	Tiamulin	Tulathromycin	Oxytetracycline	Florfenicol	Trimethoprim/ sulfamethoxazole (1/19)	Enrofloxacin
Pasteurella multocida	≤0.004	0.32/0.16	32	1	1	0.5	0.12/2.4	≤0.016–0.125
Actinobacillus pleuropneumoniae	≤0.008	0.32/0.16	8	16	≥16	0.5	8/152	0.06
Mycoplasma hyopneumoniae	–	–	0.05	0.06	2	0.5	–	0.01
Bordetella bronchiseptica	16	4/2	≥32	4	≥16	4–8	4/76	0.5

*Minimum drug concentration required for the inhibition of 90% of isolates

Sources: Aarestrup et al. (2004); Godinho (2008); Godinho et al. (2005a); Hannan et al. (1997); Jones et al. (2002); Kadlec et al. (2004); Kehrenberg et al. (2004); Priebe and Schwarz (2003); Rosenbusch et al. (2005); Salmon et al. (1996); Schwarz et al. (2004); Vicca et al. (2004)

Table 10 Pharmacokinetics of antimicrobial agents used parenterally for the treatment of swine respiratory disease

	Dose (mg/kg)	Route	C_{max} (μg/mL)	T_{max} (h)	AUC (μg h/mL)	Terminal T1/2 (h)	F (%)	References
Ceftiofur*	3	IM	15.8	0.5 – 4	196	14.3	NR	Brown et al. (1999)
Tulathromycin	2.5	IM	0.62	0.25	15.2	75.6	87.7	Benchaoui et al. (2004)
Florfenicol	20	IM	3.5	1	84.3	17.2	96.9	Jiang et al. (2006)
Enrofloxacin**	2.5	IM	1.17	1.81		12.1	NR	Anadón et al. (1999)
Oxytetracycline	30	IM	15.4	1.7	399	68.5	NR	El Korchi et al. (2001)

*As ceftiofur sodium

**Parameters reported for the parent compound

NR: not reported

and depression may lead to underestimation of the extent of infection, thus resulting in animals in the early onset of the disease failing to receive therapeutic intervention. For early identification and treatment of affected calves, rectal temperature exceeding 39.6°C is often used as a threshold for initiation of antimicrobial therapy (Potter 2007). Adjunct therapy with non-steroidal anti-inflammatorry drugs (NSAIDs) can be useful in reducing pyrexia, providing analgesia and, most importantly, in limiting the acute pulmonary inflammatory response characteristic of the BRD complex (Lockwood et al. 2003; Friton et al. 2005). The lung inflammation can be so severe as to create a life threatening oedematous response.

Swine respiratory disease (SRD) is a complex condition involving a range of viral, bacterial, and mycoplasmal infections in combination with various environmental factors. Husbandary practices are also important in pathogenesis (Done and White 2003). In the UK, it has been estimated that up to 80% of lungs may show signs of enzootic pneumonia and that 20% may show signs of either acute or chronic pleurisy (Done 1991). Two of the most prevalent non-viral respiratory pathogens isolated during SRD outbreaks are *Actinobacillus pleuropneumoniae* and *Mycoplasma hyopneumoniae*, the causative agents of porcine pleuropneumonia and enzootic pneumonia, respectively. *P. multocida* is also frequently isolated from pneumonic lungs, often as a secondary agent. Atrophic rhinitis is caused by toxigenic *Pasteurella mutocida* type D, often in co-existence with *Bordetella bronchiseptica*. The latter agent causes the initial damage after which *P. multocida* attaches to the epithelium and releases its toxin (Done and White 2003). Therapeutic approaches involve broadly the same antibacterial classes as indicated above for BRD. In addition, drugs of the pleuromutilin group are also commonly used in the control of SRD. The in vitro susceptibility of SRD pathogens to antimicrobial compounds is presented in Table 9. Antimicrobial drugs are usually administered orally via water or feed for ease of delivery on a population basis and to reduce the stress of animal handling (see Chap. 1 for discussion of population pharmacokinetics). It should be noted, however, that a pig suffering from acute respiratory disease may not eat or drink initially, and those that do will normally be the animals with least signs of disease. Inter-animal variation in dosage received may therefore be considerable, and parenteral antimicrobial therapy, in these circumstances, will be a more effective drug delivery method. The main pharmacokinetic properties of parenterally administered antimicrobial agents are summarised in Table 10.

5 Conclusion

Notwithstanding the importance of vaccination, biosecurity and good husbandry practices in animal disease control, the strategic and integrated use of chemotherapeutic agents continues to play a cardinal role in mitigating the impact of many infectious diseases on welfare, production and public health.

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Interspecies Allometric Scaling

Robert P. Hunter

Contents

Abstract Lack of approved pharmaceutical agents and very limited pharmacokinetic data in the scientific literature for exotic, wildlife, and zoo species are a major issue for veterinarians treating these species. There are fewer than 15 compounds approved in the United States for zoo and wildlife species compared to nearly 300 drugs licensed for cattle. Zoo veterinarians are therefore required to extrapolate the use of approved agents (veterinary or human) to nonapproved species, often with little or no scientific basis to support drug or dose schedule selection. In general, species differences in drug absorption, metabolism, distribution, and excretion have been well documented for domestic species. However, there has been limited research to provide similar data for nondomestic species. Consequently, with the possible exception of pet bird species, there is little published information on the pharmacokinetic parameters of drugs in nondomestic species. Additionally, because of the commercial value of many zoo species, the traditional method of "trial and error" for drug and dose selection and related compliance issues is often inappropriate. There is an understandable concern, whereby the zoo veterinarian

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does not wish to be the first to administer an agent or formulation in an untested species. “One medicine” is a central concept in treating zoo species, in that vertebrate species are generally more similar than dissimilar. However, drug absorption can vary within as well as between species. Considering the anatomical differences between true monogastrics (canine and feline species), hind-gut fermentors (rodents, rabbits, horses, and elephants), fore-gut fermentors (Colobus monkeys and kangaroos), and ruminants (cattle, goats, sheep, and antelope), the potential for differences in pharmacokinetic profiles are marked. Moreover, there are potential differences between organisms in a single class. An example is the ability of several snake species to up- and down-regulate their digestive systems. This renders the time course of oral drug absorption dependent on both body temperature and time after feeding. Plasma protein binding may vary considerably between species and may also be temperature dependent. This is very significant when treating poikilothermic (reptiles, amphibians, and fish) species and when conducting pharmacokinetic studies with highly protein-bound drugs. The large body sizes of some zoo species create additional considerations for treatment with drugs and can place significant limitations on delivery of an effective drug dose.

Keywords Allometric scaling · Gastrointestinal · Interspecies · Intraspecies · Pharmacokinetics · Zoological pharmacology

1 Introduction

Although the knowledge of pharmacokinetics in veterinary and human medicine is important for dosage selection (Lees and AliAbadi 2002), there are limited data in the published literature for many drugs used in the major domestic species and serious deficiencies in pharmacokinetic information for the vast majority of minor species. Allometric scaling is an approach for dosage selection in the absence of either species-specific pharmacokinetic data or prior drug experience (Boxenbaum and DiLea 1995; Boxenbaum and Fertig 1984; Lave et al. 1997; Mahmood 2005; Obach et al. 1997). Without an understanding of the factors that influence the accuracy of these predictions, such extrapolations can lead to little or no efficacy, possibly increase the occurrence of resistance for antimicrobial or antiparasitic agents, or result in serious toxicity (Cuthbert et al. 2007; Hunter et al. 2008; Hunter and Isaza 2008). In the United States, under the animal medicinal drug use clarification act (AMDUCA), practitioners may take approved agents (veterinary or human) and extrapolate their use to nonapproved species, often with limited data to support this decision. In certain cases, because of the value of the animals individually or as part of a threatened or endangered species, the traditional method of “trial and error” for treatment selection is inappropriate.

A central tenet of veterinary medicine is “all one medicine until it is different.” Common knowledge suggests that vertebrate species, in general, are more similar than truly different. However, excluding laboratory animals, major domestic animal

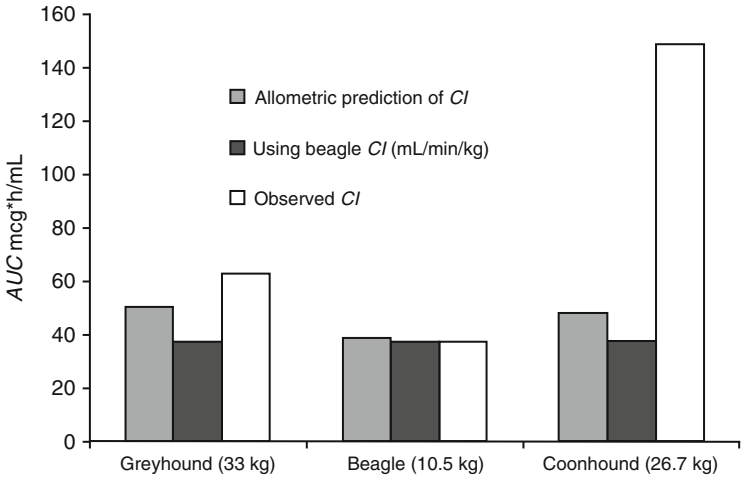

Fig. 1 Relationship between observed, allometric prediction (W^b), and linear predicted (W^1 using Beagle data) antipyrine AUC values (Martinez et al. 2009)

species, and humans, relatively little is known of the physiology of the thousands of other vertebrate species. It is known that oral absorption of a particular molecule can vary considerably within a species. Beagle and mongrel dogs have very different antipyrine clearance (Cl) (Fig. 1) but exhibit only minor differences in total body weight (Martinez et al. 2009). If one considers the anatomical differences between true monogastrics (i.e., dogs and cats), hind-gut fermentors (i.e., rodents, rabbits, and horses), fore-gut fermentors (i.e., Colobus monkeys and kangaroos), and ruminants (i.e., sheep, cattle, and goats), the potential variations in drug absorption are considerable.

When considering other vertebrate species, such as reptiles, drug metabolism may be affected by prandial state, for example, in snakes and other sporadic feeding reptiles. This mechanism allows for a minor metabolic investment of digestion to occur after a period of fasting and energy reserve depletion; thus nutrient metabolism peaks about 1 week after feeding. With variable physiological states, drug metabolism and elimination could be affected by feeding intervals. Thus, the effect of feeding in relation to time of dosing could greatly affect the way orally administered pharmacological agents are absorbed by snakes. When a snake consumes a meal, the small intestinal mucosa increases in thickness at least threefold, while the total length of the small intestine does not change. Correspondingly, villus length increases to twice prefeeding length, resulting in an increase in small intestine surface area as a result of feeding. Azithromycin absorption in ball pythons is protracted, compared to that in all mammalian species reported, on the basis of relatively large mean absorption time (MAT) and T_{max} values (Coke et al. 2003). The gastrointestinal physiology of the snake does appear to lend itself to increased metabolism of xenobiotics (Hunter et al. 2003). We hypothesized that this could be

due to the long gastrointestinal transit times of greater than one week in boid snakes (Secor et al. 1994), which allow for a xenobiotic to undergo repeated enterohepatic recirculation during the course of a single drug administration.

As azithromycin metabolism, in mammals, is mediated by several cytochrome P450 isoforms, it is possible that these systemic metabolites are formed through the action of novel cytochrome P450 isoforms, the unique gastrointestinal physiology of the ball python, or a combination of both. This may be compared with the effect that is produced by sustained-release oral formulations in mammals. This phenomenon would be expected to correspondingly prolong tissue concentrations of the parent compound and its metabolites (Hunter et al. 2003; Fig. 2). These factors are also likely to have an impact on variable bioavailability depending on time of feeding in snakes. Therefore, rate and extent of oral drug absorption may be dependent on time after feeding. In addition to differences in gastrointestinal physiology, differences in preferred optimal temperature zone could also have a role in interspecies extrapolation, but the effects of environmental temperature on pharmacokinetics are less well understood than the feeding effect on gastrointestinal physiology and drug absorption (Coke 2000).

In veterinary medicine, various methods have been used in attempts to extrapolate between species and feeding states to predict safe and effective dosage regimens. The simplest and typical method of extrapolating a dosage from a domestic

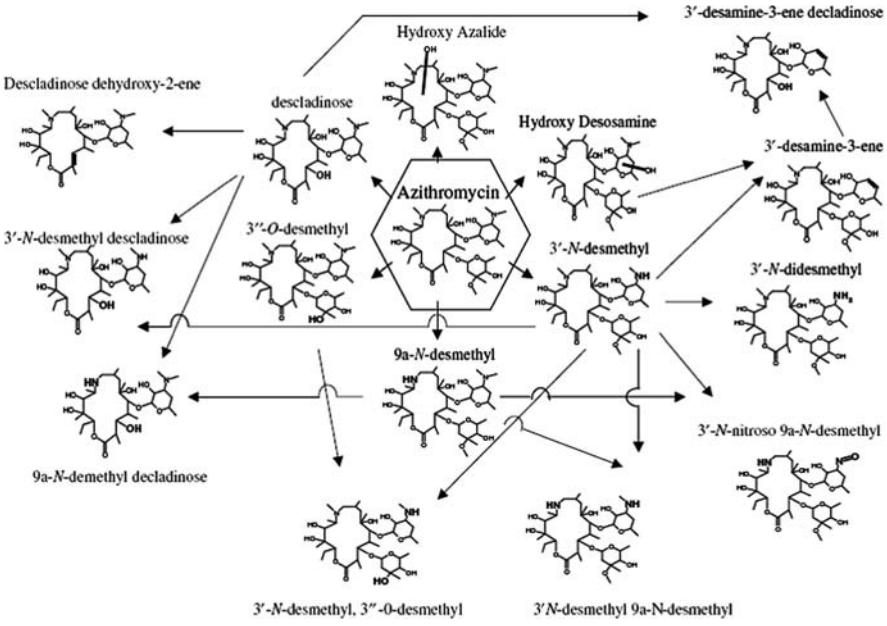

Fig. 2 Metabolite structures and possible metabolic pathways of azithromycin in the ball python (Hunter et al. 2003)

to a nondomestic species is to use a dose based on body weight (mg/kg) established in the domestic species (i.e., dog, cat, cattle, swine, chickens, or humans). This calculation results in a linear increase in the amount of drug administered as body weight increases. Although quite common, this method tends to overdose large animals and underdose small animals. A second method is similar, except that it takes the approved dose in a specific species and makes an additional assumption that links the dose to a physiological function or anatomical feature. Examples are the use of basal metabolic rate or body-surface area as the basis for dose extrapolation. Allometric scaling of pharmacokinetic parameters (V_d or Cl) is the final method of dosage extrapolation between species. This is commonly used in the human pharmaceutical industry to establish the first dosage in human drug investigations (Phase I).

The general form of the allometric equation used in scaling pharmacokinetic parameters across animals is as follows:

$$Y = aW^b,$$

where Y is pharmacokinetic parameter of interest; W is the body weight; a is the allometric coefficient; and b is the allometric exponent. The following method has been used to predict drug clearance in humans as well as in large animals. The clearance of each drug is plotted against the body weight on a log–log scale (where logs are expressed to the base 10) and the following allometric equation is applied:

$$\mathrm{Cl} = aW^b,$$

where W is the body weight and a and b are the coefficient and exponent of the allometric equation, respectively (Mahmood et al. 2006).

2 History of Scaling

The concept of scaling relative to body size has been used as the basis for comparison across species since the 1930s. The test *Vital Energetics* by Benedict (1938) appears to be the first comprehensive discussion on the topic of metabolic scaling. Benedict's work laid the foundation for the scaling of metabolic rates across a wide range of species. The log–log plots of total heat production vs. average body weight demonstrated that basal metabolic rate could be allometrically scaled and does not scale linearly with respect to body mass (Chaui-Berlinck 2006). The metabolic rate for mammals was described by Kleiber (1932, 1961) to fit the allometric equation

$$P_{\mathrm{met}} = 70\,M^{0.75},$$

where P_{met} is the metabolic power (rate of O_2 consumption if considered equivalent to the energy metabolism per unit time), 70 is the "ideal" body weight of an adult human, M is body mass, and 0.75 is the slope of the regression Kleiber used to make the mathematical calculation easier in the time period prior to the introduction of handheld calculators (Schmidt-Nielsen 1984).

Kleiber reported wide ranges of body weight and heat production within species, such as the humans, dog, and horse. The observations that body size and physiological processes are scalable by allometric equations were later summarized by Schmidt-Nielsen (1984). Various physiological processes, such as heart rate, blood volume, and glomerular filtration rate appeared to be predicted accurately for a wide variety of species, solely on the basis of the weight of the animal. Thus, body weight is generally considered to be the most predictive for allometric scaling, when raised to some exponent. From this observation, it can be reasoned that, because drug pharmacokinetic profiles are ultimately regulated by physiological functions, pharmacokinetic variables and parameters and the corresponding dosages would also follow a similar allometric scaling trend relative to body weight.

2.1 Linear Extrapolation

Linear extrapolation is the use of a single mg/kg dose established for one species and applied across all species, so that the total amount of drug increases in a linear fashion as body weight increases. For a given drug, a combination of pharmacokinetic and efficacy studies is performed to select an optimal dose for a specific species, in order to obtain an approved label claim. This method is commonly used for the target species throughout the typical weight range for the species. Given that this weight range in the dog can vary by an order of magnitude (Martinez et al. 2009), there is great potential to overdose or underdose at the extremes. The advantage of this system is the simplicity of the calculation and standardization of a single dosage for the species. However, problems may arise when this method is applied to other species without regard to species-specific pharmacological differences or to the weight range of the unapproved species (Mahmood et al. 2006; Martinez et al. 2006). The underlying assumption of this method is that any differences in species pharmacokinetics/pharmacodynamics (PK/PD) are not clinically relevant.

2.2 Metabolic Scaling

Metabolic scaling uses the ratio of a known physiological process or anatomical feature (such as basal metabolic rate or body-surface area) of two species to estimate a dosage in a species in which there is limited pharmacokinetic data.

This ratio method uses an established dosage in a specific species and links the dosage to a physiological function instead of the animal's body weight (Dorrestein 2000; Jacobson 1996; Morris 1999; Mortenson 2001; Sedgwick 1993; Sedgwick and Borkowski 1996). This calculation forces an allometric (log–log) relationship, usually with an exponent of 0.6–0.8 (Jacobson 1996; Morris 1999; Singer 2001). The assumption is that, because most physiological functions follow allometric equations relative to body weight (Kleiber 1932, 1961), pharmacological parameters (V_d and Cl_p) will also follow similar allometric relationships.

This method of dose extrapolation using basal metabolic rate ratios provides a relatively smaller dose for a large animal and a higher dose for a small animal, thus solving one of the important problems associated with using linear extrapolations. The basal metabolism method was established by and is commonly used in zoological medicine. Practical descriptions of this method and the calculations used to arrive at an extrapolated dose have been described by Sedgwick (1993) and others (Dorrestein 2000; Jacobson 1996; Morris 1999; Mortenson 2001; Sedgwick and Borkowski 1996). Briefly, all species are placed in one of five groups, termed Hainsworth's energy groups: passerine birds, nonpasserine birds, placental mammals, marsupial mammals and reptiles. The species group is used to select a predetermined K value, which is a constant for each of the five Hainsworth energy groups (Sedgwick 1993). The K value is used to calculate the metabolic rate for the selected species. A specific minimum energy cost (SMEC) value is calculated for each species, and the ratio of the target species SMEC to the SMEC of a safe, effective dose in a known species is calculated to derive an appropriate treatment regimen (Dorrestein 2000; Jacobson 1996; Morris 1999; Mortenson 2001; Sedgwick 1993; Sedgwick and Borkowski 1996). This method is simple in that the basal metabolic rate can be estimated for most species and applied to any pharmacological agent. It also facilitates extrapolation from mammalian species to avian or reptile species. Sedgwick's dosage extrapolation method has been used to establish a dose for conducting pharmacokinetic studies in new species (Downes 2002; Jacobson 1996). A commercially available computer program based on Sedgwick's methods has been distributed and used for dosage calculations (Gamble et al. 1995). An example of Sedgwick's method for the cephalosporin ceftizoxime is as follows:

Control species: mouse (Weight [W_{kg}] 0.023 kg)

Dose rate is 88 mg/kg every 1.2 h

$$\text{SMEC} = K\left(W_{kg}^{-0.25}\right) = 179$$

SMEC dose is the dose rate divided by SMEC = 88/179 = 0.5

SMEC dose = 0.5

Frequency (number of treatment intervals per 24 h) = 24/1.2 = 20

SMEC frequency is the frequency divided by *SMEC* = 10/179 = 0.1

SMEC frequency = 0.1

This provides the veterinarian with the SMEC dose and frequency adjustment. Illustrations of this approach for four species are as follows:

Subject	Calculations	Regimen
Polar bear (350 kg) K = 70	16.2 × 0.5 = 8.1	
SMEC = $70(350^{-0.25}) = 16.2$	16.2 × 0.1 = 1.6	8 mg/kg q12h
Virginia opossum (2 kg) K = 49	41.2 × 0.5 = 21	
SMEC = $49(2^{-0.25}) = 41.2$	41.2 × 0.1 = 4.1	20 mg/kg q6h
Ostrich (100 kg) K = 78	24.6 × 0.5 = 12.3	
SMEC = $78(100^{-0.25}) = 24.6$	24.6 × 0.1 = 2.4	12 mg/kg q12h
Boa (10 kg) $K = 10$	5.6 × 0.5 = 2.8	
SMEC = $10(10^{-0.25}) = 5.6$	5.6 × 0.1 = 0.5	3 mg/kg q48h

(Sedgwick 1993)

Recent data (Careau et al. 2007; Packard and Birchard 2008), however, question the assumption that basal metabolic rate can be used as a universal scaling factor. Despite its common usage in zoological medicine, this method has not been validated, and several manuscripts have illustrated failures using this method (Hunter et al. 2008; Jacobson 1996; Mahmood et al. 2006; Page et al. 1991; Table 1). Furthermore, neither companion animal veterinarians nor pharmacologists employed in human pharmaceutical development use this method to determine dosage regimens. In summary, there is no rationale published in the scientific literature that validates the use of this method for dose selection.

2.3 Allometric Scaling

Allometric scaling builds on the definition of metabolic scaling in that pharmacokinetic parameters, not simply the dose, are allometrically scalable. This method shares the assumptions that species differences in pharmacodynamics are clinically negligible and that the drug pharmacokinetics has a log–log (allometric) relationship to weight. Although allometric scaling of pharmacokinetic parameters is commonplace, little information is available for prediction of pharmacodynamic parameters across species. A general expectation is that the rates of biological turnover processes should obey basic allometric principles, while intrinsic capacity and pharmacological sensitivity may or may not agree among species owing to genetic and/or transductional differences (Lepist and Jusko 2004). Once key pharmacokinetic parameters have been estimated, then standard dose calculations can be made for the species to be treated.

Allometric scaling has become the method of choice for interspecies extrapolation in the discovery and development of drugs for human use, specifically for selecting the dose in the first human studies (Boxenbaum and DiLea 1995). This application of scaling involves three to five species. Typically these would include one rodent species (mouse or rat), a beagle dog, and a nonhuman primate species. The established pharmacokinetic parameters in this limited number of species are then used to predict human pharmacokinetic parameters, as well as intravenous first dose for human phase I studies (Brodie and Reid 1967; Mahmood and Balian 1999; Mahmood et al. 2003).

Table 1 Observed vs. predicted clearance from mammalian data to avian species[a]

Drug	Body weight (kg)	Observed CL (mL/min)	Predicted Cl (mL/min)	% error
Enrofloxacin				
Turkey	5.1	37	104	181
Ostrich	44	3,268	576	82
Chicken	0.66	18	68	278
Rhea	3	179	68	62
Red-tailed hawk	1.3	5	34	580
Salicylic acid				
Pigeon	0.45	0.52	0.09	83
Duck	3	8	1.89	76
Turkey	8	61	8.8	86
Ostrich	19	60	34.3	43
Chicken	2.2	7.7	1.14	85
Meloxicam				
Pigeon	0.45	0.29	0.35	21
Duck	3	3.05	2.85	7
Turkey	8	7.3	8.4	15
Ostrich	19	228	21.8	90
Chicken	2.2	0.48	2	317
Flunixin				
Pigeon	0.45	0.48	2.22	363
Duck	3	7	5.7	19
Ostrich	19	158	14.3	91
Chicken	2.2	0.33	4.9	1,385
Gentamicin				
Red-tailed hawk	1.3	3.1	5.5	77
Owl	1.5	1.8	4.9	172
Golden eagle	3.6	5	12.3	146
Chicken	4.7	1.6	6.7	319
Turkey	11	5	13.9	178
Chloramphenicol				
Pigeon	0.45	11.7	1.84	84
Duck	0.98	40	14.6	64
Turkey	11	132	32.4	75
Sulphadimidine				
Japanese qual	0.13	0.077	0.064	17
Pheasant	1.2	0.11	0.64	482
Chicken	1.6	0.18	0.85	372

[a]Data from Hunter et al. (2008)

Although its use in zoo medicine has been limited, this approach may have advantages over the previously described methods. Application of this method requires acquisition of species-specific pharmacokinetic data, such as clearance (Cl) and volume of distribution (V_d) from multiple species and plotting them against body weight. The form of the allometric equation used in scaling pharmacokinetic parameters across species is

$$Y = a(W)^b,$$

where Y is the pharmacokinetic parameters of interest, W is body weight, and a and b are the coefficient and exponents of the allometry. Although not yet critically evaluated in zoo medicine, this approach might provide a more accurate method for drug–dose estimation than other approaches. This has been illustrated by Mahmood (2007) for lysergic acid diethylamide (LSD) administration to an elephant. Linear extrapolation produced a total dose of 297 mg LSD for the animal. This was administered and it resulted in death within 2 h (West et al. 1962). When using allometric scaling, the total dose recommended would be 5.3 mg. Finally, when metabolic scaling was applied, the resulting total dose was between 19 and 56 mg, depending on whether human or cat data were used for the extrapolation (Mahmood 2007). It is unknown if either of these doses would have been safe and efficacious, but they are 5–15 fold less than the lethal dose derived from linear extrapolation.

Extrapolation of doses between mammalian and avian species, both qualitatively and quantitatively, is complicated by several physiological and anatomical differences (Tables 1 and 2). Anatomically, the avian renal cortex is more similar to the reptile cortex than the mammalian cortex. The physiological consequence is that glomerular filtration is not held relatively constant in birds, as it is in mammals, in the face of varying mean arterial pressure and this will impact on the pharmacokinetics of drugs in avian species (Frazier et al. 1995). Reptiles and birds have a renal portal system, so that a first-pass effect of renally cleared drugs can occur if the animal is injected in the posterior half of the body. This system is not fully understood, and it may not be as restrictive as once believed. However, until definitive data are available, reptiles and birds should be injected in the anterior portion of the body when agents dependent upon renal clearance are administered.

Table 2 Observed vs. predicted clearance from avians to avians[a]

Drug	Body weight (kg)	Observed CL (mL/min)	Predicted Cl (mL/min)	% error
Enrofloxacin				
Turkey	5.1	37	128	246
Chicken	0.66	18	53	194
Salicylic acid				
Turkey	8	61.3	25	60
Duck	3	8	7.4	8
Meloxicam				
Turkey	8	7.3	25	241
Duck	3	3	4	33
Flunixin				
Turkey	8	24	18	27
Duck	3	7	3.5	50
Gentamicin				
Red-tailed hawk	1.3	3.1	1.7	45
Golden eagle	3.6	5	4.1	18

[a]Data from Hunter et al. (2008)

While avian cytochrome P450 activities have been reported, their expression and role in avian drug metabolism are not well documented (Walker 1998). On the basis of published data (Hunter et al. 2008), it is evident that allometric scaling between mammalian and avian species produces highly biased estimates of drug clearance, irrespective of whether the drug is cleared in mammalian species primarily through renal or hepatic mechanisms.

Choice of the species to be included in the data analysis is another potential source of error. It seems likely that inclusion of at least one large animal in the scaling procedure can improve predictions (Mahmood et al. 2006; Table 3). In large animals, correction factors (used in human first-dose determination) could not be applied because there was no correlation between the exponents of the simple allometry and the correction factor used to improve dose predictions (Mahmood et al. 2006). In addition, the vast majority of large animals for which pharmacokinetic data are available are herbivores (cattle, horse, and elephant). Conversely, smaller animal species are generally either omnivores (mouse, rat, monkey, and humans) or carnivores (dogs and cats). Differences in diet can influence both drug metabolism and renal elimination (Martinez 2005). Ideally, the species selected would be similar in all the major physiological functions and differ only in size. Unfortunately, there is no method to predict, a priori, which animal species will be best suited for inclusion in the interspecies predictions for a particular species. Although data for large carnivorous species (such as lion, tiger, and polar bear) might be helpful for improving interspecies predictions, the obvious dangers hinder progress in this area of research.

3 Discussion and Conclusions

Drug–dose scaling of therapeutic agents is of particular interest to veterinarians and pharmacokineticists because this method can, when used appropriately, provide an estimate for designing therapeutic dosage regimens in an unstudied species. Sedgwick's metabolic scaling approach implies that body metabolic rate can be applied to almost any drug and any species derive a therapeutic dosage regimen (Dorrestein 2000; Sedgwick 1993; Sedgwick and Borkowski 1996). Although this would be very useful clinically, it oversimplifies the relationship between basal metabolic rate and drug pharmacokinetics. Sedgwick's method requires only that basal metabolic rate of a control species, together with a known therapeutic dose in that species, be used to calculate the dose for the unknown species for which the metabolic rate can be estimated from its weight (Sedgwick 1993). However, on the basis of the published literature (Careau et al. 2007; Jacobson 1996; Packard and Birchard 2008) Sedgwick's approach does not appear to be appropriate. This is confirmed by the work of Mahmood et al. (2006); Martinez et al. (2006); and Hunter et al. (2008).

These observations emphasize the importance of applying fundamental principles associated with allometric analysis and using appropriate caution when

Table 3 Predicted and observed clearance (mL/min) in large animals with and without inclusion of a large animal based on simple allometry[a]

Species used	Exponents	*R*	Species predicted	Observed Cl	Predicted Cl	Error
Caffeine						
Dog, human, donkey	0.727	0.998	Horse	312	340	0.92
			Camel	292	82	3.56
Dog, human, horse	0.762	0.999	Donkey	221	256	1.16
			Camel	82	323	3.94
Antipyrine						
Dog, human, camel	0.827	0.765	Horse	2,790	860	0.31
Dog, human, horse	0.951	0.795	Camel	1,613	1,038	0.64
Theophylline						
Dog, goat, horse	0.688	0.793	Camel	2,288	478	0.21
Dog, goat, camel	1.261	0.957	Horse	387	3,657	9.45
Enrofloxacin						
Dog, sheep, cow	0.784	0.897	Mare	3,331	3,552	1.07
			Camel	2,088	3,095	1.47
Dog, sheep, mare	0.676	0.969	Cow	5,775	3,063	0.53
			Camel	2,088	2,261	1.08
Dog, mare, camel	0.554	0.987	Cow	2,088	3,230	1.55
			Sheep	209	703	3.36
Cefoperazone						
Dog, human, horse	1.189	0.875	Calf	653	348	0.53
			Sheep	107	153	1.43
Dog, human, calf	0.703	0.504	Horse	4,981	758	0.15
			Sheep	107	146	1.36
Furosemide						
Dog, human, horse	1.149	0.934	Camel	1,334	1,740	1.3
Dog, human, camel	0.864	0.923	Horse	4,981	1,632	0.33
Sulphadimidine						
Dog, goat, horse	1.248	0.99	Cattle	498	737	1.48
			Camel	136	186	1.37
			Sheep	19	14	0.74
Dog, goat, cattle	1.161	0.993	Horse	253	217	0.86
			Camel	136	150	1.1
			Sheep	19	13	0.68
Dog, goat, camel	1.175	0.988	Cattle	498	555	1.11
			Horse	253	220	0.87
			Sheep	19	13	0.68
Flunixin						
Sheep, calf, donkey	1.69	0.92	Horse	492	1,868	3.8
			Mule	654	2,012	3.08
			Camel	705	2,070	2.94
Sheep, mule, donkey	1.386	0.987	Horse	492	754	1.53
			Calf	397	120	0.3
			Camel	705	820	1.16
Dog, sheep, donkey	1.173	0.973	Horse	492	707	1.44
			Calf	397	150	0.38
			Camel	29	759	1.08
			Mule	654	744	1.14
Dog, sheep, camel	1.109	0.986	Horse	492	565	1.15
			Calf	397	130	0.33
			Sheep	29	44	1.52
			Mule	654	593	0.91

[a]Data modified from Mahmood et al. (2006)

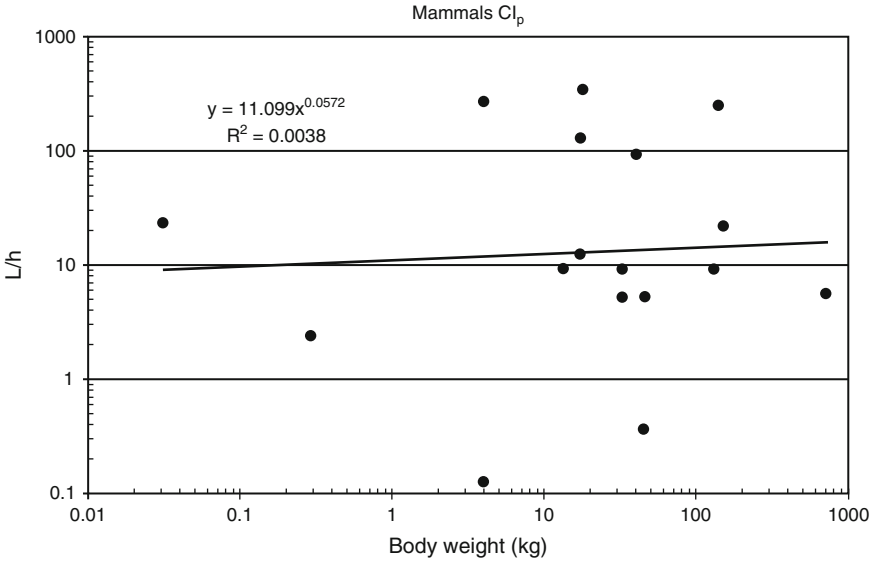

Fig. 3 Allometric plot determined for enrofloxacin plasma clearance (Cl) based on 13 mammalian species (Hunter and Isaza 2008)

extending application of the results in clinical cases. Clinicians should also carefully consider interspecies differences in the contribution of various organ systems and differences in body composition in relation to body weight. Ideally, to ensure the best therapeutic outcome, it is necessary to generate pharmacokinetic and efficacy data in each zoo species that is treated (Locke et al. 1982). Before extrapolation of any drug dose, the veterinarian should appreciate not only the mathematical assumptions but also the limitations that are associated with allometry. Careful consideration of the available literature to understand the route of elimination and the extent of metabolism of therapeutic agents will greatly assist in determining allometric relationships of pharmacokinetic parameters (Table 4). There is a continuing need to consider and apply methods for reducing the size and risk of extrapolation error, as this can affect both target animal safety and therapeutic response (Fig. 3). Data from at least one large animal (nonhuman and a body weight >70 kg) should be included to reduce potential error (Table 3).

Phylogenetic relationships are of particular importance in applying allometry in veterinary medicine (Chaui-Berlinck 2006). All extrapolation methods assume that the route of elimination is similar across all species and this is not so in many instances. This review illustrates how allometric scaling of pharmacokinetic data can be applied, and emphasizes that extrapolations between species should not be used to predict an appropriate therapeutic dosage regimen without careful consideration of the limitations. Riviere et al. (1997) estimated that of all drugs 75% are not scalable across multiple species.

Just as mammals can range from a few to thousands of kg, reptiles and birds can also vary in body weight across a wide range (Table 5). It has been suggested

Table 4 Examples of pharmaceuticals that are either good or poor candidates for allometric scaling

Poor	Good
Acetaminophen	Ampicillin
Amikacin	Apramycin
Amoxicillin	Carbenicillin
Amphetamine	Cephapirin
Antipyrine	Chlortetracycline
Cefamandole	Diazepam
Cefazolin	Erythromycin
Cephalothin	Gentamicin
Cloramphenicol	Oxytetracycline
Digoxin	Prednisolone
Fentanyl	Tetracycline
Flunixin	Thiamphenicol
Kanamycin	
Ketamine	
Ketoprofen	
Levamisole	
Lidocaine	
Meperidine	
Morphine	
Oxyphenbutazone	
Penicillin	
Phenylbutazone	
Phenytoin	
Quinidine	
Sulfadimethoxine	
Sulfamethazine	
Sulfathiazole	
Theophylline	
Ticarcillin	
Tinidazole	
Trimethoprim	
Tylosin	
Xylazine	

that it is impossible to derive a single equation correlating body mass to metabolic rate for all 6,000 species of reptiles (Funk 2000). Without knowledge of the extent and route of elimination of a drug, extrapolation of dosage regimens from one species to others is fraught with difficulty, if not impossible. Clearly, much more research is needed on the drug metabolism and excretion mechanisms and pathways in nondomestic species. Such research generated data will increase the ability of clinicians to determine more effective and safe dosage regimens for their patients (Table 5). An additional consideration when using allometry is the nonlinearity of pharmacokinetics of some drugs in some species (Manire et al. 2003; Rush et al. 2005). As stated by Fowler (1995), "no presently available chemical restraint agent is equally effective and safe for use with all 45,000+ vertebrate species." This statement can be extended from chemical restraint to therapeutic use of drugs within veterinary medicine.

Table 5 Body weight (kg) vs. organ weight (g/100 g BW). Values from Spector (1956) and adapted from Martinez et al. (2006)

Species	Genus species	Race/Breed	Number of individuals	Sex	BW (kg)	Organ weights g/100 g BW				
						Brain	Heart	Kidney	Liver	Lung
Human	*Homo sapiens*	Caucasian (US)	7	M	67	1.96	0.42	0.41	2.30	0.73
		Caucasian (EU)	4	M	49	2.53	0.64			
African Buffalo	*Syncerus caffer*		4	M,F	700	0.09	0.47	0.24	0.98	0.94
Bactrian camel	*Camelus bactrianus*		1	M	450	0.12				
Caribou	*Rangifer arcticus*		4	M,F	98	0.30	0.90	0.13	1.83	2.10
Cat	*Felis catus*		10	M,F	3.3	0.77	0.45	1.07	3.59	1.04
Cattle	*Bos tarus*	Holstein	98	F	600	0.07	0.37	0.24	1.20	0.72
Cheetah	*Acinonyx jubatus*		2	M	21	0.39	0.51	0.47	3.22	1.16
Chimpanzee	*Pan troglodytes*		2	M,F	48	0.79	0.49			
Dog	*Canis familiaris*		4	M,F	13	0.59	0.85	0.30	2.94	0.94
African elephant	*Loxodonta africana*		1	M	6,600	0.08	0.39	0.27	1.62	2.08
Gazelle	*Gazella thomsoni*		2	M	24	0.38	1.00	0.43	2.15	1.15
Guinea pig	*Cavia porcellus*		58	M	0.26	1.33	0.53	1.17	5.14	1.18
Lion	*Panthera leo*		4	M	125	0.19	0.85			2.12
Blackhowler monkey	*Alouatta paliata*		28	M, F	6.2	0.81	0.33	0.58	3.25	0.63
Rhesus monkey	*Macaca mulatta*		4	M	3.3	2.78	0.38		2.09	
Jumping mouse	*Azpus hudsonicus*		4	M,F	0.018	3.57	1.03	1.26	5.63	1.34
Meadow mouse	*Microtus drummond*		67	M,F	0.023	0.29	0.68	1.53	4.56	1.70
Rabbit		Flemmish giant	22	F	2.5	0.40	0.35	0.70	3.19	0.53
Rat	*Rattus norvegicus*	Norway	3	M,F	0.25	1.22	0.52	1.09	3.35	0.79
Swine	*Sus scrofa*		36	F	102		0.32	0.26	1.51	
Zebra	*Equus quagga*		4	M,F	280	0.20	1.42	0.35	1.67	0.80
Tawny eagle	*Aguila rapax*		5	M,F	2.4	0.59	0.63	0.50	1.82	1.04
Flamingo	*Phoeniconaias minor*		5	M,F	15	0.49	0.94	1.18	2.68	1.47
Chicken	*Gallus domesticus*		16	F	0.61	0.44	0.63	0.68	2.36	0.61
Red-tailed hawk	*Buteo borealis*		3	F	1	0.97	0.67	0.30	1.37	0.90
Pigeon	*Columba livia*		4	M,F	0.27	0.95	1.75		1.76	
European stork	*Ciconia ciconia*		3	M,F	3.3	0.47	0.92	0.65	1.92	1.11

(*continued*)

Table 5 (continued)

Species	Genus species	Race/Breed	Number of individuals	Sex	BW (kg)	Organ weights g/100 g BW				
						Brain	Heart	Kidney	Liver	Lung
Alligator	*Alligator mississipiensis*		2	F	190	0.01	0.15		0.38	0.54
Crocodile	*Crocodylus acutus*		2	M,F	110	0.01	0.12		1.02	1.00
Lizard	*Lacerta viridis*		15	M,F	0.05	0.24	0.12	0.12	5.00	
Black snake	*Coluber constrictor*		3	M,F	0.43	0.07	0.22	0.6	0.60	0.80
Green snake	*Zamenis viridis*		6	M,F	0.022	0.95		8.77	2.19	
Horned toad	*Phrynosoma cornutum*		5	M,F	0.025	0.52	0.44			
Turtle	*Testudo graeca*		30	M,F	0.32	0.09		0.48	2.66	
Cumberland turtle	*Chrysemys elegans*		21	M	0.84		0.32	0.32	5.53	1.07

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Pain and Analgesia in Domestic Animals

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Contents

Abstract The biggest challenge to the use of analgesic agents in animals is the determination of the efficacy of these agents. In humans, the verbal communication of the alleviation of pain is fundamental to the effective use of analgesics.

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In animals, the lack of verbal communication not only confounds the diagnosis and characterisation of the experience of pain, but also challenges the evaluation of the analgesic therapy. As animals possess the same neuronal pathways and neurotransmitter receptors as humans, it seems reasonable to expect that their perceptions of painful stimuli will be similar, and this is a basis for the use of laboratory animals for screening of analgesics for human use. However, as the evaluation in the laboratory animal tests is based mainly on behavioural responses, and although some physiological responses do occur, it is often difficult to separate these from stress responses.

The use of behavioural responses to evaluate analgesics in a range of species is complicated by the fact that different species show different behaviours to a similar pain stimulus, and different pain stimuli produce different pain responses in the same species. Thus behaviours may be species- and pain-specific and this can complicate analgesic evaluation. As most animals possess similar neuronal mechanisms to humans for pain perception, it is not surprising that the standard human pain control strategies can be applied to animals. For instance, local anaesthetics, opioids, non-steroidal anti-inflammatory drugs (NSAIDs), as well as other analgesics used in humans are all found to be effective for animal use. Differences in metabolism and distribution between various species, as well as financial considerations in larger animals can affect efficacy and thus limit their use. In addition, the use of any drug in a species that may be intended for human consumption will be limited by residue considerations.

The treatment of pain in animals presents many challenges, but the increasing public concerns regarding animal welfare will ensure that studies into the nature and control of animal pain will continue to have a high profile.

Keywords Pain · Pain evaluation · Pain perception · Pain recognition · Analgesic agents

1 Introduction

The concept of pain in animals has long been a source of discussion and debate. The views of Descartes (Haldane and Ross 1989) and Bentham (Bowring 1962) represent the philosophical approaches to pain, and it may be argued that this is one area in which philosophy and science most significantly overlap. As pain is a totally subjective experience, and it is not even possible to accurately establish if two human beings are experiencing a common level or depth of pain, the extrapolation to animal species is clearly a much greater challenge. What is clear is that animals consider some experiences to be noxious and they exhibit an aversive response; thus the concept of "nocioception" was developed (Sherrington 1906) to allow that animals may develop an aversive response to a noxious stimulus, without getting into the vexatious question of the nature of the experience of pain as applied to human understanding.

To most veterinarians and people working closely with animals, from both their physical and emotional responses, there is no question that animals feel something equal or very akin to the human experience of pain. From anatomical and physiological perspectives, all animals have the necessary receptors, nerves, neurotransmitters, and comparative central nervous system anatomy to be able to experience what humans describe as pain.

Histological and neurochemical evidence indicates that the neurotransmitter receptors associated with pain perception are very similar in most animals; for instance opioid receptors of all subtypes are present in the central nervous system with a very similar distribution to humans in all animal species. One major challenge to the evaluation of pain in animals derives from the enormous diversity of what is described under the broad heading of "pain" in humans. Apart from the temporal descriptions of pain, such as acute, chronic and intermittent, there is the anatomic location of the pain, or the type of pain, such as throbbing, burning or stabbing or a combination of all of these. In addition, perhaps the greatest challenge in the evaluation of pain in animals is the human concept of neuropathic pain (Mersky and Bogduk 1994), where the absent or inappropriate lesions give no indication of the real source of the pain perceived.

Other criteria have been used for characterising human pain, such as inflammatory pain and non-inflammatory pain, or more specifically physiological pain, where the pain can be beneficial to the subject (in terms of avoidance) and pathological pain where the actual pain experience is detrimental to the subject (such as arthritic pain) (Woolf and Chong 1993).

2 Animal Pain

2.1 Significance of Pain Evaluation in Animals

In the absence of verbal communication, those involved in the assessment of pain in animals have to rely on other strategies to establish the nature and intensity of the painful or nociceptive experience. One approach is to consider the strategies involved in evaluating the broad groups by which human pain is characterised, as this often interacts with the pain control strategy utilised, but the lack of verbal communication means that the evaluation will be based on observational strategies. These strategies fall into four main groups. Firstly, the observation or measurement of physiological parameters, such as pulse, temperature, respiration and feeding and drinking patterns may be useful. Secondly, the behaviour of the animal, as altered from the norm, is a widely used and powerful tool, particularly in the hands of an experienced person. Thirdly, the physical response to exacerbation of the pain stimulus, by palpation or manipulation and the increased response to this, is very informative. Finally, alteration of the presumed pain by application of analgesic strategies, utilising the three previous evaluators, is widely used.

In view of the complexity of the interacting evaluations and the species variability, a number of visual analogue scales (VAS) have been developed, but these are always species-specific and indeed often situation-specific as well (*vide infra*).

2.2 Relevance to the Human Experience

The concept of animal pain and its treatment has been based extensively on the human experience in the absence of verbal communication. However, the extension of evaluating pain in non-verbal humans has shown some interesting comparisons with the veterinary experience. Non-verbal humans fall broadly into three groups, the pre-verbal communication group, i.e. the very young, the non-communicative impaired group i.e. the severely mentally challenged and the non-communicative aged group i.e. sufferers from conditions such as strokes and dementias, a group which has increased significantly in numbers over the recent years. The evaluation and treatment of pain in these groups is often based on similar criteria to those used in veterinary species, such as behavioural responses, physiological responses and responses to analgesic therapy. Reviews of these areas have been published that provide many interesting insights into the development of treatment (Findlay and McGrath 1998; Gibson and Weiner 2005).

One interesting aspect that has arisen as a result of studies in humans is the association between memory and pain. While it is clear that people cannot recall the actual pain experienced, they can normally recall the circumstances and emotions involved. This was most lucidly documented by one Professor Wilson who had had his foot amputated at the ankle joint by James Syme, a well-known surgeon in the early nineteenth century, without the benefit of anaesthesia or analgesia. As documented by Graham (1956), he wrote "The operation was a more tedious one than some which require much greater mutilation. It necessitated much more cutting through inflamed and morbidly sensitive parts, and could not be despatched by a few strokes of the knife. I do not suppose that it was more painful than the majority of severe surgical operations are, but I am not, I believe, mistaken in thinking it was not less painful, and this is all that I wish to contend for of the agony it occasioned I will say nothing. Suffering so great as I underwent cannot be expressed in words and thus fortunately cannot be recalled. The particular pangs are now forgotten, but the black whirlwind of emotion, the horror of great darkness and sense of desertion by God and man, bordering close on despair, which swept through my mind and overwhelmed my heart, I can never forget however gladly I would do so. During the operation, in spite of the pain it occasioned, my senses were preternaturally acute, as I have been told they are in patients, in such circumstances. I still recall with unwelcome vividness the spreading out of the instruments, the twisting of the tourniquet, the first incision, the fingering of the sawed bone, the sponge pressed on the flap, the tying of the blood vessels, the stitching of the skin, and the bloody dismembered limb lying on the floor ...".

Anyone who reads this cannot doubt the emotional involvement associated with unalleviated pain. This brings us back to the question posed by Jeremy Bentham, also in the early nineteenth century, regarding animals and pain "the question is not can they reason, nor can they talk, but can they suffer?" (Bowring 1962). Thus, the concept of pain and suffering are linked and because they are regarded as

undesirable and unnecessary in humans, it was inevitable that, eventually, the concept of alleviation of pain in animals would follow.

However, this concept has been hard to implement in many cases, often associated with the variability in response to pain in various species of animals. Of all the animal species, humans are among the most demonstrative in their response to pain. They vocalise strongly, they show profound escape behaviour and they enlist the help of their peers in attempting to prevent or alleviate their pain. The group of animals which most closely parallels these responses is, not surprisingly, monkeys. However, many species show much less overt reactions to painful stimuli. This is particularly apparent in animals which live in large groups and are often subjected to predation, such as wildebeest and zebras in the wild and cattle and sheep in domesticated groups (Livingston 1994). There seems to be a survival strategy adopted by these species, in that predators in the wild become focussed on individuals of the prey species who show some abnormal behaviour. Thus, if a prey animal is showing overt signs of pain then its chances of avoiding a predator are much diminished, so the lack of overt behaviour may become a survival strategy; this is the opposite of a human who wishes to gain the support of their social group to survive. This has led to the conclusion that, if an animal does not overtly demonstrate pain then it is not feeling pain, which is clearly an error. These concepts have led to an unacceptable reluctance to treat painful situations in domestic cattle, sheep and similar species as well as non-mammalian species, all of which are non-demonstrative in behaviour and vocalisation. The level of understanding of pain in humans has progressed significantly over the past few decades, both in our knowledge of the mechanisms involved and in effective treatment strategies. Fortunately, similar advances are being made for animals as well, especially in the more difficult areas such as neuropathic pain.

2.3 Analgesic Factors

Advances in our understanding of animal pain have led to the development of strategies for assessing this pain, based on behavioural responses for several species, the difficulty being however that the behavioural indicators do not readily apply to species other than the one they were developed for. An additional factor is that the behavioural indicators that apply to acute pain do not necessarily apply to chronic or neuropathic pain. Consequently, multiple evaluations are required to adequately describe the multiple species and several pain entities that they may suffer from. Moreover, there is a need to take into account the concept of individual variation within a species in pain response, which has been well documented in relation to genetics (Critchley et al. 1986; Mogil 2004), gender (Walker and Carmody 1986) and age (Gibson and Weiner 2005). The best behavioural indicators that have been developed for animals consist of VAS, which take into account a variety of behaviours and circumstances and these, taken together, provide an estimation of the intensity and duration, and possibly the nature, of the pain from

which the animal is suffering. A major factor in the development of meaningful evaluations is the familiarity that observers have with the behavioural characteristics of the particular animal. This generally means that much more specific evaluations can be made on those species that are the closest "companions" of humans, such as dogs, cats and horses and, to a lesser extent, farm animals and other pet animals. It is only recently that pain behaviour in non-mammalian species has received significant consideration, and the information on wild species is still very limited.

2.4 Analgesic Strategies and Techniques

The strategies to deal with pain in animals have tended to follow those developed for human medicine. These fall into several groups. The first strategy has been to adopt procedures intended to lessen the development of pain; this applies particularly to surgical interventions but also to methods such as physiotherapy and other supportive measures and to techniques which lessen the likelihood of injury. One technique, which is particular to non-human animals, is that of genetic selection, the production of less aggressive breeds of dogs and cattle, the selection of chickens for better bone growth to match their weight gain and the much more recent drive to eliminate dog breed characteristics that can give rise to painful malformations (Editorial 2008).

The second strategy, which is probably the best known, is the provision of analgesic drug therapies. Most of the classes of pharmacological agents used in humans are currently used in animals, and this has led to the recognition that there are significant species differences in both the pharmacokinetics and the pharmacodynamics of particular drugs. This leads, in turn, to an imperative that dosage schedules must be developed not only for particular types and severities of pain but also on a species-by-species basis.

A third strategy is the utilisation of different routes of administration of analgesic drugs. Veterinary surgeons have traditionally preferred to use the parenteral route of administration; intramuscular, intravenous and subcutaneous routes are all commonly used. This approach is more associated with the guarantee of dose and timing compared with the convenience, but potentially greater variability, of oral administration, which is generally favoured in human medicine. In addition, the oral route is generally not preferred in animals with a functional ruminant digestive system, which can result in more complex pharmacokinetics of drugs administered in this manner. Also, the oral administration of drugs in either food or water can be associated with unreliable or unduly variable rates and extents of absorption. Moreover, the administration of drugs in tablet form to many species can represent significant challenges. Nevertheless, the oral dosing of some analgesic drug classes over short-, medium- and long-term durations has become commonplace in the companion animal species, dogs, cats and horses. This has resulted from the convenience/necessity of dosing by animal owners in these clinical subjects.

The less commonly used routes of drug administration such as intra-articular or epidural routes are nevertheless used routinely for analgesic drug administration in most domestic species. Moreover, the concept of multidrug administration to achieve "balanced analgesia", particularly in association with post surgical pain control, is now widely practised (Dobromylskyj et al. 2000).

A further strategy worthy of mention is the use of acupuncture. This is widely used in human medicine as a means of controlling pain, with increasing acceptance alongside traditional "Western" medicine approaches. The same applies to veterinary medicine. There has been general acceptance of the procedure and several studies have been published on the relevant points and meridians for some animal species, which do incidentally differ from those used in humans (Gaynor 2000). Most reported studies in animals have used needle acupuncture, although some other modalities have been reported (Gaynor 2000; Mittleman and Gaynor 2000). The evaluation of acupuncture on pain in animals has been dogged by the same problems as controlled studies in humans, namely the difficulty in ensuring acceptable controls in the form of a credible placebo, although there have been many claims based on behavioural responses, particularly in chronic pain control (Wright and McGrath 1981; Martin and Klide 1987; Klide and Martin 1989). There are also a number of other strategies that have been adopted from human practise and used with the intention of controlling animal pain. These include chiropractic and herbal therapies, as well as more esoteric therapies and more mainline ones such as massage and exercise therapies (Manning et al. 1997; Millis and Levine 1997). In some instances, the efficacy of these approaches remains to be established.

2.5 Pain Perception in Animals

The pain modality easiest to recognise and treat in domestic animals is acute pain. Like humans, this is associated in many cases with some form of trauma, either accidental or surgical, and is often clearly associated with some specific body region. In addition, the onset of acute pain can be associated with some infectious diseases, and acute inflammation in states such as pneumonia, peritonitis or cystitis may be the presenting sign. Clearly, diagnosis of the cause of acute pain is of great importance and in animals this issue is exacerbated by the lack of verbal communication. It is of interest to note that the development and extent of analgesic drug use in humans has been based to a large extent on acute responses to pain by animals. Early evaluations were performed using a murine model, based on its response to a heated hot plate of foot lifting and licking (Woolfe and McDonald 1944) or on the time taken by rats to remove their tail from hot water (D'Amour and Smith 1941). In addition, there was a commonly used test in the rat involving removal of a forepaw from a gradually increasing pressure on the footpad (Randall and Selitto 1957). These early tests comprised behavioural responses to an acute painful stimulus and they are sufficiently consistent and validated to provide the preliminary evaluation of analgesics developed for humans. It might be noted that all these

tests use an immediate response (limb withdrawal) to an acute pain stimulus. Other models utilise the pain that develops slowly at a site of acute inflammation, with the peak of hyperalgesia occurring several hours after injection of a pro-inflammatory irritant substance, such as kaolin or carrageenan.

In addition, many more animal model tests for other pain modalities have been developed using behavioural responses in laboratory animals. These include visceral pain induced by injecting irritant chemical agents into the abdomen and measuring the writhing activity of mice (Siegmund et al. 1957), and the injection of irritant agents into joints to evaluate the efficacy of agents targeted at arthritic conditions (Coderre and Wall 1987). In addition, animal models have been developed to mimic neuropathic pain states (Bennett and Xie 1988). Finally, some animal pain models are being used to study the neurological basis of pain states in humans. This includes not only models using genetically modified laboratory animals (Bölcskei et al. 2005), but also naturally occurring conditions in animals which parallel human pain states, such as interstitial cystitis (Lavelle et al. 2000) and arthritis (Lascelles et al. 2007a).

In terms of management, the control of acute pain in animals, when it is either observed or anticipated, is generally based on the procedures developed for humans, namely surgical techniques, immobilisation of traumatised regions and pharmacological intervention. In many situations the prevention or reduction of predictable pain in animals is addressed, as in humans, by the use of pre-emptive analgesia. This involves the administration of analgesics before the painful procedure is inflicted to prevent or suppress the development of central or peripheral sensitisation (Lascelles et al. 1998). Initially, there was some difficulty with this technique gaining acceptance in human medicine, as the original reports were based on retrospective studies. However, demonstration of the benefits in veterinary medicine (Slingsby and Waterman-Pearson 1998) showed clearly that the concept was a genuine physiological response to the pain stimulus rather than a placebo effect. The assumption that animals will not demonstrate a placebo effect has been fundamental in their use to evaluate analgesic strategies.

The second type of pain that animals are assumed to experience is chronic pain. Probably because of their particular affinity with chronic pain states in humans, chronic pain in animals is best studied and understood in dogs, cats and horses. Additionally, in the last few years there has been an increasing focus on chronic pain in farm animal species. The causes of chronic pain in animals are similar to those in humans. Of particular clinical importance are arthritic joint lesions. However, an appreciation of other chronically painful conditions, like cancer and spinal injury, has been receiving much more recent attention (Yazbek and Fantoni 2005), with particular focus on the dog and cat. Another major factor in relation to severe chronic unremitting pain in the major veterinary species has been the option of euthanasia, which in most societies has not been accepted as a pain alleviating option in humans. That said, there remains a significant need for therapy of chronic pain conditions in animals. This can be for companionship/sentimental reasons or for commercial reasons, such as extension of the breeding life of a valuable animal, as well as the primary consideration of animal welfare. The whole issue of the

treatment of chronic pain in animals is associated with ethical debates (Mendl et al. 2001) regarding the cost/benefit situation for both the animal and the owner (Gingerich and Strobel 2003).

Recently there has been increased interest in the occurrence of chronic post-surgical pain in humans (Kehlet et al. 2006). In particular, the incidence of ongoing post-surgical pain following a number of surgical interventions has received attention. While the existence of phantom limb pain following amputation has been long recognised, it now appears that chronic pain following a number of orthopaedic, abdominal and thoracic surgeries in man is much more frequent than was once thought, and therefore significant under-reporting was probably present. The veterinary relevance of this phenomenon should be considered as most human pain syndromes have been documented in animals. The routine ovario-hysterectomies performed on dogs and procedures such as caesarean section on farm animals, as well as surgical amputations of tails in dogs and other species for the convenience or gratification of the owner, must have the potential for some degree of post-surgical pain, although it may be that the increasing use of pre-emptive analgesia in association with surgery has had some impact on the incidence of this entity.

Another more recent human pain concept which has been described, and which may be related to post-surgical pain experience, is that of neuropathic pain. This is broadly defined as the existence of pain without obvious pathology, and at one time was regarded in some cases as a psychological phenomenon rather than the actual pain entity now recognised. As it was often misdiagnosed in humans in the past, its veterinary diagnosis in the absence of verbal communication or pathology presents particular challenges. There are indications from some clinical case reports that dogs can experience phantom limb pain on the basis of the observed stump mutilation by chewing. As dogs and cats are the only species that are commonly subject to therapeutic amputations, information in other species is limited. With regard to the other neuropathies there are less data available, with the possible exception of diabetic neuropathy which has been studied in companion animals (Mizisin et al. 2007). There are many other human conditions assessed as neuropathies with associated pain such as pancreatitis (Winston et al. 2005), but the specific diagnosis of neuropathy associated with these conditions in animals has not been reported.

3 Recognition of Animal Pain

3.1 Laboratory Animals

The most widely reported studies on the recognition of animal pain are those conducted in laboratory animals, based on tests for analgesic drugs undergoing development for human use. However, there are reports in the literature of pain control strategies for laboratory animals focused on the welfare aspects of their use (Liles and Flecknell 1992). This is also a relevant source of information for species such as guinea pigs, rabbits, rats, ferrets and mice, which are kept as pets. Recent

information on the recognition of behavioural responses to pain in these species has led to a much better understanding of appropriate treatments and dosage regimens (Roughan and Flecknell 2003). An important aspect is that these small rodents and lagomorphs show specific pain related behaviours which are not always apparent during casual observation (Roughan and Flecknell 2006), as distinct from the overt avoidance responses commonly used for analgesic drug testing. The relatively high basal heart and respiratory rates in these small mammals have presented technical difficulties in their use as physiological indices. Therefore, alternatives such as food and water intake and weight loss have been used for the evaluation of potential chronic pain, but these measures lack precision. The use of video recording, in combination with computer programme developments, has allowed for a much wider range of more specific behaviours to be assessed, and hence has facilitated the development of improved analgesic protocols (Wright-Williams et al. 2007). In laboratory animals, the behaviours associated with pain lend themselves to computerised video analysis and hence the effects of analgesics on the various behaviours associated with pain have been monitored (Roughan and Flecknell 2004). Initially, analysis of video studies was undertaken manually and while these were effective, using such systems as "Observer", they were very time-consuming and prone to error. There have been considerable advances in technology for monitoring automatic behaviour recognition programs such as "HomeCageScan". This has allowed a more rapid and bias-free approach to laboratory animal behaviour monitoring associated with pain syndromes and their alleviation with analgesics (Roughan 2008).

3.2 Dogs and Cats

Due to its close association with humans and access to veterinary treatment, the dog is a species in which pain responses have been appreciated, particularly when owners are attuned to slight variations in behaviour, appetite, weight loss, etc. Not all of these indices have been reliably interpreted. However, the various pain conditions in the dog, acute, chronic and neuropathic, have been recorded and treated. In fact, with the exception of laboratory animal studies, most of the pain treatments and preventative strategies have been developed for dogs and cats.

In the dog, as in man, pain responses are potentially modified by peripheral inputs. Thus, dogs experiencing pain post-operatively in a veterinarian's recovery room may not exhibit the same behaviours as they do within their household environment. This led to the development of the first sophisticated VAS (Holton et al. 1998). The concept of the VAS is not new, but the introduction of the multifactorial scale for the dog was a significant advance. A problem with all forms of behavioural scale has been that of quantification, and scales for pain, whether human or animal, are no exception.

Behavioural scales, irrespective of their range or complexity, are non-arithmetic. Therefore, either doubling or halving the pain experience will not be reflected in

a proportional alteration in the behaviour. This may not be the case with a physiological measurement, for which there may well be some correlation between the degree of pain and heart or respiratory rate, or the force applied to a limb. However, if the behaviour response comprises vocalisation or aggression expressed on palpation, any correlation will necessarily be imprecise. The VAS developed by Holton et al. (1998) was the first to provide an evaluation utilising multiple factors as components of the assessment. For example, a range of behaviours and interactions was assigned a score, these scores were added and the arithmetic sum indicated the level of analgesic intervention required. This VAS was modified to produce an abridged form, known as the composite measure pain score – short form (or CMPS-SF) for routine use in a clinical setting (Fig. 1). These scales have been researched to provide a response based on the precise meaning of the descriptors and interactions with individuals familiar with assessing aspects of pain such as reliability and reproducibility. However, it is important to note that, even with careful effort to validate such a VAS, each VAS can be used only for one form of pain in one species. Thus, the "Glasgow scale" applies only to acute pain in dogs, and other CMPS indices will have to be developed for other pain states and other species (Welsh et al. 1993).

As described in the section on laboratory animal pain and analgesia, the application of computer analyses of pain associated behaviours, especially video records, has been extended to dogs (Hansen Lascelles et al. 2007) and cats (Lascelles et al. 2008). Two systems have been most widely reported, "Observer" as for laboratory animals and "Ethovision". These techniques generally assess activity and movement and their application to dogs and cats has been more challenging than to laboratory animals for several reasons, not the least being the need for a more complex housing situation and a greater variability in individual animals, both in colour and size. Another area of advancement in recent years has been the use of computerised gait analysis techniques. These have become increasingly sophisticated and are used to monitor the beneficial effects of analgesics on both acute and chronic limb pain in both dogs (Waxman et al. 2008) and cats (Romans et al. 2005).

There have also been recent advances in the understanding of pain responses through the use of electroencephalography. This has evolved through the availability of appropriate computer technology (Murrell and Johnson 2006). These techniques have several advantages, in that they provide a direct measure of higher CNS responses, and hence a true reflection of pain.

Much research has been centred on dogs as the beneficiaries of companion animal pain studies and there have been fewer studies in cats. This is not logical, as humans generally regard their companion cats as highly as their companion dogs. Indeed limited studies in cats meant that until recently they were often denied analgesia, on the grounds that they could react in a dysphoric manner to opioids and perceived difficulties in metabolism of NSAIDs which led to the assumption of an increased susceptibility to toxicity. Although many recent studies have overturned these misconceptions, the recognition of pain states in cats has still not received the same level of attention, in terms of development of VAS or other numeric pain scales, as have pain states in dogs. Recently, devices to measure thermal and mechanical thresholds in cats have been developed and these have allowed

SHORT FORM OF THE GLASGOW COMPOSITE PAIN SCALE

Dog's name _____

Hospital Number _____ **Date** / / **Time**

Surgery Yes/No (delete as appropriate)

Procedure or Condition_____

_____

In the sections below please circle the appropriate score in each list and sum these to give the total score.

A. Look at dog in Kennel

Is the dog?

(i)		(ii)	
Quiet	0	Ignoring any wound or painful area	0
Crying or whimpering	1	Looking at wound or painful area	1
Groaning	2	Licking wound or painful area	2
Screaming	3	Rubbing wound or painful area	3
		Chewing wound or painful area	4

In the case of spinal, pelvic or multiple limb fractures, or where assistance is required to aid locomotion do not carry out section **B** and proceed to **C**
Please tick if this is the case ☐ then proceed to C.

B. Put lead on dog and lead out of the kennel.

When the dog rises/walks is it?

(iii)	
Normal	0
Lame	1
Slow or reluctant	2
Stiff	3
It refuses to move	4

C. If it has a wound or painful area including abdomen, apply gentle pressure 2 inches round the site.

Does it?

(iv)	
Do nothing	0
Look round	1
Flinch	2
Growl or guard area	3
Snap	4
Cry	5

D. Overall

Is the dog? *(v)*		*Is the dog?* *(vi)*	
Happy and content or happy and bouncy	0	Comfortable	0
Quiet	1	Unsettled	1
Indifferent or non-responsive to surroundings	2	Restless	2
Nervous or anxious or fearful	3	Hunched or tense	3
Depressed or non-responsive to stimulation	4	Rigid	4

Total Score (i+ii+iii+iv+v+vi) = _____

Fig. 1 The Glasgow Composite Pain Scale – Short Form. Copyright 2005 University of Glasgow www.gla.ac.uk/faculties/vet/smallanimalhospital/ourservices/painmanagementandacupuncture

quantification of the efficacy of analgesics in treating acute pain (Dixon et al. 2002, 2007). Studies of chronic pain have been focused on clinical conditions similar to human situations of chronic or neuropathic pain; they are intended more to establish the means of reducing the onset of severe pain than general alleviation of chronic pain, associated with, for example, arthritic conditions.

However, several points of interest have derived from clinical behavioural observations of cats in pain. First, cats frequently hide from their owners when experiencing pain and this action obviously hinders the recognition of pain in this species. Secondly, cats may still purr when in pain, contrary to the belief of many owners that purring is invariably a sign of contentment. The more obvious signs, as in dogs and most other species, include vocalisation, lameness, guarding of an area, inappetance, reluctance to move and failure to groom. However, if the cat or dog hides, then some or all of these responses are likely to be lost to the observer.

3.3 Horses and Other Equines

Much of the focus of pain studies in horses has been on two conditions, lameness and colic. The reasons for this are that a lame horse is of limited use to its owner both for draft purposes or for riding and horses with colic symptoms have relatively high mortality rates. However, the range of painful conditions a horse may experience is essentially as wide as for any other species.

There have been many studies on the causes and localisation of lameness in horses and there is a corresponding extensive literature which has established that equine pain can be acute, chronic or intermittent, can range from mild to severe and can have a variety of causes, including trauma, infection, allergic and genetic factors. In addition, the anatomical source can range from the spinal column to the foot and include all joints and tissues. Accurate diagnosis of the site of lameness in horses has been described extensively, as have the available treatments. In many cases a variety of remedies have been used, but far less focus has been made on the treatment of the pain than on the likelihood of obtaining a cure and hence a sound, and therefore saleable, horse.

There have, however, been many reports describing the treatment of pain in cases of colic, as "colic" signifies "abdominal pain". Several causes have been identified, ranging from the unpleasant to the fatal. Some authorities have questioned the treatment of colic pain, because of possible interference with making a correct diagnosis. Consequently, a horse might recover from what had been diagnosed as a fatal form, despite the fact that it is also possible that a horse might die from the stress of the pain associated with some forms. However, it is now widely recognised that analgesic use may allow a better clinical examination and hence facilitate the diagnosis.

Several studies on analgesic efficacy in the horse, using models such as abdominal visceral distension (Pippi and Lumb 1979), tendon injury (Chambers et al. 1993), induced arthritis (Morton et al. 2005), and local inflammation (Kamerling et al. 1985; Lees et al. 1987) have been published. The behavioural changes associated

with these models have also been described. Gait analysis in horses has dated from the earliest days of cinematography. However, the development of force plates and platforms has allowed a much more advanced, computer-based, analysis of both the forces and movements associated with lameness in horses (Barr et al. 1995). This, in turn, has facilitated studies of the efficacy of analgesic agents in many forms of lameness (Symonds et al. 2006). Horses are another species in which electroencephalograms have been used to evaluate pain and analgesic actions (Ekstrom et al. 1993; Murrell and Johnson 2006).

In summary, there is a strong clinical impression that horses are very sensitive to pain and they show extreme distress on exposure to noxious stimuli. However, in many countries the apparently limited distress exhibited by donkeys and mules has led to both their popularity and ill-treatment and the frequent lack of treatment of painful conditions in these animals is a cause for serious concern for all persons concerned with animal welfare.

3.4 Cattle, Sheep, Goats and Camels

Ruminant species in general seem to be rather non-demonstrative to pain when they are examined superficially. However, when observed by an experienced person a variety of subtle responses are apparent. This supports the supposition referred to in Sect. 2.2, whereby these herd animals, in not showing overt abnormal behaviour, may avoid the attention of would-be predators that seek out an animal which shows some sign of "weakness". Thus, the lack of overt pain behaviour becomes a survival strategy and allows the animal to blend in with the rest of the herd.

Probably the most commonly observed pain in ruminants relates to lameness, which may be acute or chronic in nature. As in horses, the range of predisposing factors can be very wide, but they are frequently a manifestation of pain. In the case of dairy animals, one of the responses to pain is a reduction in milk production and consequently, these factors have received significant attention, focusing mainly on prevention rather than treatment of pain, because the potential problem of analgesic drug residues in milk has restricted their use (Espejo et al. 2006). However, there is a significant literature on the use of analgesics in ruminants, although there are still concerns over the accurate diagnosis of pain, particularly in cases of chronic or intermittent pain. More recently, the assessment of chronic lameness in dairy cattle has been addressed using computerised gait analysis and these studies can also be used to evaluate treatments (Flower et al. 2005).

In other ruminant species only the sheep has received significant attention in terms of pain assessment and alleviation and pain-related behaviours. This is partly due to the availability of sheep as a more easily housed and handled model for pain in cattle. Moreover, the sheep is a compliant species for pain and analgesia studies (Nolan et al. 1987a). Sheep have also been used to provide a model for chronic pain, due to the frequently occurring disease "footrot", which has enabled studies to be undertaken on the development of chronic pain in this species (Ley et al. 1989).

It also allowed the development of one of the early pain VAS scales, which included both gait impairment and the pathology of the lesions (Ley et al. 1989).

Studies in other ruminant species have been less extensive and analgesic studies are often limited to the pharmacokinetics of some of the more widely used analgesics (Ingvast-Larsson et al. 2007).

3.5 Birds

A major consideration in establishing and validating avian pain behaviours is the wide range of species, ranging from farmed ostriches to caged songbirds and from battery and egg laying hens to exotic parrots.

An important consideration influencing the study of pain behaviours in birds is that, apart from gait impairment in lame animals, they have little in common with the behaviours exhibited by mammals. This has led to the erroneous view that avian species do not feel pain in a similar manner to mammals, but apart from the inability to exhibit facial expressions in the same way as mammals, due to the presence of a beak and limited facial musculature, birds with acute pain both vocalise and exhibit strong escape behaviours (e.g. flapping of wings). Moreover, they show avoidance and preference behaviours, while in response to chronic pain, lameness and reduced food intake occur and both gait and food intake return to normal patterns when birds are treated with analgesics (McGeown et al. 1999).

Relatively few studies have been carried out on bird pain responses and many of these have focussed on the welfare of intensively farmed poultry (Duncan et al. 1989; Gentle et al. 1997). However, the nature of the welfare considerations has not always led to analgesic therapy, but rather to husbandry changes and improved genetics (Kuo et al. 1991). Flock medication with analgesic drugs is generally impracticable and, if changes can be achieved through improved husbandry, then medication may not be required.

Studies on cage birds and other species (Paul-Murphy et al. 1999, 2004) have shown that the analgesic agents used in mammals have similar effects in birds. This suggests that the enzymatic and neurotransmitter receptor mechanisms associated with pain inception and transmission have a broadly similar physiological basis, although certain physiological responses in some species do reveal differences (Machin 2005). The major difficulty in providing effective analgesic therapy in birds remains the possible failure to recognise more subtle manifestations of pain, particularly the lower grade pain states that are better recognised in mammalian companion animals and have been used in composite pain scoring and VASs.

3.6 Other Non-mammalian Vertebrates

Compared with the difficulties in recognising avian pain, described in Sect. 3.5, those associated with evaluating pain in reptiles, amphibians and fish are even

greater. The evolutionary development of tortoises, snakes and crocodiles took place before mammals evolved, and yet they are grouped together in the possible hope of achieving common pain behavioural factors for "reptiles". Not surprisingly, very limited information is available (Machin 1999; Mosley 2005), but the indications are that most mechanisms involved in pain inception and transmission are basically similar. This suggests that pain, as a mechanism of survival, developed very early in the evolution of species and has not changed in its fundamental manifestations in any significant way over the millennia. Thus, reptiles, amphibia and fish all respond to the same sort of noxious stimuli; they all exhibit species-specific behaviours in response to either acute or chronic pain conditions and they also show alleviation in the response to the stimuli in the presence of analgesic agents proven to be effective in mammals (Bennett 1998; Stevens et al. 2001). Like birds, the use of analgesics in these non-mammalian vertebrates is severely limited by the lack of information on non-overt behaviours and lack of information on the pharmacokinetics and pharmacodynamics of analgesics in these species.

4 Analgesic Agents and Their Use in Domestic Animals

4.1 Local Anaesthetics

It seems clear that the molecular genetics of neuronal sodium channels in most species have been highly conserved. Although minor variations have been identified, the general structure and function are essentially similar, which is fortunate as the ionic mechanisms of nerve conduction were initially investigated in molluscs. As the mechanism of action of local anaesthetics seems to be similar at most sodium channels, their effectiveness is correspondingly similar in most species. Hence, the efficacy demonstrated in man, where the action can depend on the anatomical structure of the nerve and local concentration of the drug, also applies in domestic species. All commonly used local anaesthetics are aminoamides. They include lignocaine, bupivacaine and mepivacaine, and have been shown to be effective in all domestic species (Dobromylskyj et al. 2000), as have the less generally used agents such as prilocaine and ropivacaine (Flecknell et al. 1990; Markham and Faulds 1996).

In addition, most of the routes of application of local anaesthetics in humans have been used in domestic animals, and their use in pre-emptive analgesia for the prevention or suppression of sensitisation has been mentioned. Topical administration in the form of gels or creams has been used for lidocaine and lidocaine/prilocaine. These agents and routes are mainly utilised in small animals for the temporary analgesia associated with the placing of needles or cannulae (Flecknell et al. 1990). Local infiltration by injection of a 1 or 2% w/v solution of lidocaine, usually with added adrenaline to prolong action by vasoconstriction, is widely used in most domestic species for superficial or regional blocks. For most veterinary

purposes, the animal will also normally be sedated with an appropriate sedative or tranquillising drug (Dobromylskyj et al. 2000). Infiltration of local anaesthesia has been used in all domestic species, from cattle to cats. Another widely used local anaesthetic technique in veterinary medicine is that of regional peripheral nerve blockade. For dental procedures in dogs, cats and horses, selective blockade of the distal branches of the cranial nerves is commonly utilised, whilst blockade of the auricular nerves is often performed in association with aural surgery (Buback et al. 1996). The cornual nerve is blocked for disbudding and dehorning procedures in cattle, and a more extensive blockade is recommended in cases of antler removal in deer (Johnson et al. 2005). In dogs and cats brachial plexus block is used to facilitate forelimb surgery (Nutt 1962) and for the hind limb the various lumbar and sacral nerves can be targeted. A paravertebral nerve block using the sites of exit of the spinal nerves from the intravertebral foramina is commonly used in the thoracic and lumbar regions of cattle to allow abdominal surgery to be performed in standing animals and this is of great benefit to both the animal and the surgeon (Cakala 1961; Moon and Suter 1993). Local blocks of limb nerves in horses have been used as a diagnostic aid to identify the source of pain in lame animals. Another veterinary use of local anaesthetics is that of intravenous regional anaesthesia. This has been reported for use in the paws of dogs and the feet of cattle (Skarda 1996), when the limb extremity is isolated by a tourniquet, and the local anaesthetic is administered intravenously distal to the tourniquet. The limb extremity is then desensitised for surgery, following which the tourniquet is removed and sensation returns over the next 20–30 min. Finally, the use of local anaesthetics at the spinal level, to provide epidural anaesthesia, has been recorded for use in animals as long as in humans (Cathelin 1901; Sicard 1901). Much of the information on epidural administration of local anaesthetics in animals is very similar to the data in humans, with the more lipid soluble drugs possessing a longer duration of action associated with slower absorption into the systemic circulation (Table 1) (Catterall and Mackie 1996). The longer duration of action of the lipid soluble anaesthetics is preceded by a slower onset of action.

Epidural administration of local anaesthetics has been documented in most domestic mammals (Torske and Dyson 2000) and there is no reason why it should not be as effective in non-mammalian species, although there are no reports of such use. Normally care is taken with local anaesthetics to avoid inadvertent intravenous administration and the consequent risk of cardio depressive effects. However, there have been reports that constant rate infusions of local anaesthetic may provide

Table 1 Epidural actions of local anaesthetics in the dog

Drug	Onset (min)	Duration (min)
Lignocaine 2%	5	45–90
Mepivacaine 2%	5	60–90
Bupivacaine 0.5%	20	120–360
Ropivacaine 0.5%	15	90–420

All drugs administered at 0.2 ml/kg. Data from Torske and Dyson (2000)

control of chronic pain following some surgeries (Cassuto et al. 1985; Doherty and Frazier 1998).

4.2 Opioids

Drugs of the opioid class have a long history in the control of pain in domestic animals. Their pharmacological actions are the same or very similar to those described in humans. The opioid receptors, described as mu, kappa and delta or OP3, OP2 and OP1, respectively, and various subtypes and orphan receptors have been identified in all domestic species. The endogenous peptides that bind to the receptors have been described (Nolan 2000).

There are, however, species differences in the distribution and location of opioid receptors and consequently differences have been described in the responses to various opioid drugs. Fortunately, the significant and major role of endogenous opioid peptides as processors of nociceptive information within the CNS is present and similar in all domestic species, including non-mammalian species. Hence, the use of opioid drugs as analgesic agents is practised across the species spectrum. Recommended dose rates in a range of species of domestic animals have been published for a wide range of opioid analgesics, including buprenorphine, butorphanol, fentanyl, methadone, morphine, nalbuphine, oxymorphone, pentazocine and pethidine and also for naloxone the opioid antagonist. The species for which clinical and dosage data are available include laboratory animals (Flecknell 1996), dogs and cats (Papich 2000) and farm animals (Thurman et al. 1996) as well as birds (Machin 2005) and reptiles (Mosley 2005). Factors determining dosage of opioids in domestic animals include hepato-metabolism, which is particularly rapid in laboratory rodents (Morris 1995), the sensitivity of dogs to the emetic actions of morphine, and an oft reported but rarely seen hypotensive effect associated with histamine release. In cats, dysphoria or other CNS effects are occasionally seen with opioid use, but this is usually associated only with very high dose rates (Papich 2000). However, in horses, excitement or stereotypical behaviour may occur, particularly with the mu agonists at lower dose rates (Mama et al. 1992). Similar effects have also been reported in ruminants (Livingston et al. 1991). Pigs seem to require relatively high doses to provide effective analgesia but other behavioural effects have not been reported. The most significant side-effect associated with opioid use in humans is respiratory depression and this is also the case in domestic animals in cases of overdose. Another major human concern with opioid use is that of its dependence/abuse. Whilst both tolerance and dependence can also be demonstrated in domestic animals, the administration of opioids is usually restricted to short-term dosing for either acute pain or perioperative use and therefore dependence is not considered to be an issue in these clinical situations. Drugs such as morphine, which have pharmacologically active glucuronide metabolites in humans, also show this characteristic in animals but, because of variations in the rates of hepatometabolism, the significance of these metabolites in animals is uncertain.

The most common routes of administration of opioids are by injection, intramuscular, subcutaneous or intravenous. In addition, oral formulations of morphine are used in dogs (Dohoo et al. 1994). The epidural route has also been reported in several species (Tung and Yaksh 1982) and the use of fentanyl transdermal patches has been reported for use in dogs, cats, horses and pigs (Kyles et al. 1996). See Tables 2–4 for opioid doses and intervals in small animals (Table 2), companion animals, birds and reptiles (Table 3) and farm animals (Table 4).

The specific opioid-reversing agents, such as naloxone, also exert the same effects in animals and they can be used to terminate the effects of opioids as in humans. In fact, a specific agent, diprenorphine, was developed to reverse etorphine (a potent opioid used for immobilisation in animals).

Table 2 Doses and intervals for opioids in small animals

Drug	Mouse	Rat	Guinea-pig	Rabbit
Buprenorphine 6–12 h	0.05–0.1	0.01–0.05	0.05	0.01–0.05
Butorphanol 4 h	1–2	1–2	–	0.1–05
Meperidine (Pethidine) 2–4 h	10–20	10–20	10–20	5–10
Morphine 4 h	2–5	2–5	2–5	2–5
Nalbuphine 4 h	2–4	1–2	1–2	1–2

All doses are in mg/kg for subcutaneous or intramuscular injection. Data from Flecknell (1996); Cowan et al. (1997); Dobromylskyj et al. (2000)

Table 3 Doses and intervals for opioids in companion animals, birds and reptiles

Drug	Dog	Cat	Bird	Reptiles
Buprenorphine 6–12 h	0.005–0.02	0.005–0.02	0.01–0.05	0.4–1
Butorphanol 2–4 h	0.2–0.6	0.2–0.8	1–4	1
Fentanyl 0.5 h (i/v)	0.001–0.005	–	0.02–0.2	–
Meperidine (Pethidine) 2–3 h	3–10	3–10	–	1–4
Morphine 4–8 h	0.1–1	0.1–0.2	200	0.05–4
Nalbuphine 3–4 h	0.3–0.5	0.3–0.5	–	–
Oxymorphone 2–4 h	0.05–0.2	0.05–0.4	–	–

All doses in mg/kg for subcutaneous or intramuscular injections. Data from Dobromylskyj et al. (2000); Machin (2005); Mosley (2005)

Table 4 Doses and intervals for opioids in farm animals

Drug	Sheep	Goat	Pig	Horse
Buprenorphine 6–12 h	0.005–0.01	0.005	0.005–0.05	0.004–0.006
Butorphanol 3–8 h	0.5	0.5	0.1–0.3	0.05–0.1
Meperidine (Pethidine) 2–4 h	2	–	2	1–2
Morphine 2–4 h	0.2–0.5	0.2–0.5	0.2–1	0.1

All doses are in mg/kg for subcutaneous or intramuscular injection. Data from Flecknell (1996); Thurman et al. (1996); Wolfensohn and Lloyd (1998); Dobromylskyj et al. (2000)

4.3 Non-steroidal Anti-inflammatory Drugs

The use of NSAIDs, initially in the form of plant extracts (e.g. willow bark), for reducing inflammation and treatment of pain in man has a long history. Similarly there have been reports on the use of salicylates in dogs and horses for many years (Abbitt et al. 1978). Their use in domestic animals was mainly associated with the treatment of musculo-skeletal pain and the alleviation of arthritic pain and this use is still significant today.

Before 1971 the mechanism of action of the NSAIDs was poorly understood (Vane 1971); however, in the decades since, many of the molecular mechanisms have been elucidated (Simmons et al. 2004). In contrast with most other analgesic agents which act on neurotransmitter receptors involved in nociceptive pathways, NSAIDs are enzyme inhibitors and have somewhat different pharmacological properties. The NSAIDs available today range from acetylsalicylic acid to the COX-2 selective coxibs. Moreover, the development of more potent agents that inhibit both cyclo-oxygenase (COX)-1 and COX-2, and the increased focus on COX-2 selective inhibitors, has led to their additional use prophylactically for the prevention of peripheral pain sensitisation, as well as the provision of long-term primary analgesia. There is also increasing evidence that NSAIDs can exert analgesic effects in the central nervous system in addition to their peripheral effects (Dolan et al. 2003). The factors associated with the generation of NSAID susceptible pain have been widely investigated and described (Lees et al. 1991) and are generally accepted as being attributable to the inhibition of prostaglandin production and hence the prevention of neuronal sensitisation by these eicosanoids. However, some NSAIDs additionally inhibit leukotriene production (Dawson et al. 1982) and, although it is now thought that this mechanism does not apply to the classical NSAIDs, drugs such as tepoxalin and licofelone inhibit COX, 5-lipoxygenase and cytokine synthesis (Ritchie et al. 1995). In addition, drugs that augment the release of nitric oxide as well as inhibiting COX have been developed as novel analgesic agents (Wallace et al. 1994).

Understanding the mechanisms of action of NSAIDs has been facilitated by studies linking their pharmacokinetic properties to their pharmacodynamic actions (PK–PD modelling) for several species (see chapter, "Species Differences in Pharmacokinetics and Pharmacodynamics" of this text). Moreover, this approach provides a rational basis for designing dosing schedules that are both safe and effective. It is likely to be extended to other analgesic drug groups in the near future. Pharmacokinetic studies of NSAIDs have led to several findings relevant to their use. Unlike most peripherally administered analgesics of other classes, for which the plasma concentrations of the drug give a good indication of the clinical effectiveness at a given instant, associated with their ability to cross the blood–brain barrier rapidly, the actions of NSAIDs on pain associated with peripheral sensitisation depends primarily on the local peripheral concentration at the site of damage (Lees and Higgins 1985). This effect has been demonstrated in several different species (McKellar et al 1994) and led to the PK–PD modelling described by Lees et al. (2004b).

Another veterinary aspect of interest is the susceptibility of cats to the risk of overdosing due to their slow rate of metabolism of some NSAIDs (Yeary and Swanson 1973). However, this does not mean they cannot be used in cats, only that the dosage interval must sometimes be extended (Glew et al. 1996). Moreover, this slow metabolism and clearance of NSAIDs does not apply to all members of this class of drug.

Orally administered salicylates are still used in farm animals based on their low cost; however oral administration of drugs to ruminants and horses can lead to high variability in bioavailability associated with variable absorption rates, associated with diet and binding to feed within the digestive tract. Injectable NSAIDs, such as carprofen, ketoprofen, meloxicam and flunixin are available for farm animal use. Parenteral dosing provides a more reliable route for administration. However, some of these drugs are non-selective for COX and others may even inhibit predominantly COX-1. They are more likely to produce the classic unwanted side-effects on the gastric mucosa and blood clotting, although reports of unwanted side-effects in farm animals are very rare (Table 5). Carprofen is selective for COX-2 in the dog and cat but not in the horse, where it is non-selective, whilst in man it is COX-1 selective (Lees et al. 2004a).

In recent years there has been a trend away from the use of NSAIDs that are non-selective COX inhibitors in cats and dogs, and a tendency either to use NSAIDs with some selectivity for COX-2, such as meloxicam or carprofen (Papich 2000) or more recently, the COX-2 selective coxibs such as firocoxib, mavacoxib and robenacoxib (Table 6). There are also now available data on the use of these drugs in smaller pets (Wright-Williams et al. 2007) and non-mammalian species (Machin 2005; Mosley 2005) (Table 7). The recent increase in the use of NSAIDs for the control of moderate to severe pain is associated with the greater tolerance to COX-2 selective drugs, an increased understanding of their role in preventing sensitisation and an increase in utilising data on their pharmacokinetics as well as pharmacodynamics (Vane and Botting 1995).

Table 5 Doses and intervals for NSAIDs in farm animals

Drug	Cow	Horse	Pig	Sheep
Acetylsalicylic acid (oral) 6–12 h	–	25	10	50–100
Carprofen 24 h	1.4	0.7	2–4	1.5–2
Flunixin 24 h	2.2	1.1	1–2	2
Ketoprofen 24 h	3	2.2	3	–
Phenylbutazone (oral) 24 h	–	2.2	–	10

All doses are in mg/kg for subcutaneous or intravenous injection except where oral administration is indicated. Data from Bishop (1998); Thurman et al. (1996) and Dobromylskyj et al. (2000)

Table 6 Doses and intervals for NSAIDs in companion animals

Drug	Dog	Cat	Rabbit
Acetylsalicylic acid (oral) 12 h	10–25	10–25 (48 h)	100
Carprofen 24 h	4	2–4	4
Flunixin 24 h	1	1	1
Ketoprofen 24 h	2	2	3
Meloxicam 24 h	0.2	0.2	0.2
Phenylbutazone (oral) 24 h	20	24 (48 h)	–
Tolfenamic acid	4	4	–
Vedaprofen	5	–	–
Deracoxib	3–4	4	–
Firocoxib	5	5	–

All doses are in mg/kg for subcutaneous or intravenous injection except where oral administration is indicated. Data from Dobromylskyj et al. (2000)

4.4 Alpha 2 Adrenergic Agonists

The use of alpha 2 adrenergic agonists in domestic animals has led to a better understanding of the mechanisms of action of these drugs. The existence of receptor subtypes has been recognised, leading to a range of actions. Three main subtypes of the alpha 2 receptor have been identified, although unlike the opioids, for which relatively pure mu agonists such as fentanyl exist, the clinically available alpha 2 agonists exert their effects on more than one receptor subtype. Unlike other analgesic classes, the human applications for alpha 2 agonists as analgesics are limited. Related to this, research data are sparse; however three agents are in common veterinary usage, for both their sedative and analgesic properties. Xylazine

Table 7 Doses and intervals for NSAIDs in small mammals, birds and reptiles

Drug	Mouse	Rat	Birds	Reptiles
Acetylsalicylic acid (oral) 24 h	120	100	–	–
Carprofen 24 h	5	5	1	2–4
Flunixin 24 h	2.5	2.5	3	0.1–0.5
Ketoprofen 24 h	–	5	2–5	2
Meloxicam 24 h	5	1	0.1	0.1–0.2

All doses are given in mg/kg for subcutaneous and intravenous injection except where oral administration is indicated. Data from Dobromylskyj et al., (2000); Machin (2005); Mosley (2005)

Table 8 Doses of alpha 2 adrenergic agonists in animals

Species	Detomidine	Xylazine	Medetomidine	Romifidine
Horse	10–80	600–3,000	–	40–120
Cattle	–	50–300	–	–
Sheep	–	50–100	–	–
Dogs	–	1,000–3,000	10–80	–
Cats	–	1,000–3,000	50–150	–
Deer	60–90	500–1,000	–	–

All doses are in μg/kg. Data from Bishop (1998)

exerts effects on both the alpha 2A and alpha 2B subtypes, as well as having some alpha 1 agonist activity. It is widely used in ruminants and the spinal effects of these agents seems to be marked (Nolan et al. 1987b), which is probably related to high receptor density in spinal nociceptive pathways (Brandt and Livingston 1990). Consequently, these drugs are more potent in ruminants than other species.

The alpha 2 agonist agent used most often in horses is detomidine (Jochle and Hamm 1986). In this species it is effective as both an analgesic and as a sedative. Medetomidine is licenced for use in dogs and cats and has a similar action (Table 8). These drugs have been administered by intrathecal and epidural routes, as well as intramuscularly and intravenously. There are reports of their synergistic interactions with opioids, presumably at the spinal sites of action (Kalso et al. 1991). The main side-effects of alpha 2 agonists are on the cardiovascular system, with complex effects on both heart rate and blood pressure (Virtainen and McDonald 1985). Profound hypoxia associated with their use has been reported in ruminants, particularly sheep (Nolan et al. 1986).

As with the opioids, specific, reversing agents are available to terminate the effects of alpha 2 agonists in animals, such as atipamezole, used to reverse the effects of medetomidine. These drugs are regarded as very useful adjuncts in clinical practise.

Most alpha 2 agonists exist as racemic mixtures, with most of the pharmacological activity associated with one enantiomer; dexmedetomidine, the enantiomer of medetomidine, is one such example.

4.5 Tramadol

Tramadol has been introduced recently into veterinary medicine, although it has been used in humans for a number of years. Its pharmacology is of interest in that its actions resemble those of opioids as well as having effects on catecholamine uptake (Kayser et al. 1992), somewhat similar to alpha 2 agonists. In combination, these actions have proved to be most effective in humans. In man, tramadol is metabolised to an active metabolite, leading to increased duration of action (Lewis and Hau 1996). Tramadol consists of a racemic mixture in which, interestingly, the two enantiomers are both pharmacologically active, but on different CNS systems. In view of its relatively high cost, tramadol's use in animals has been restricted to small animal species, in which it has a shorter duration of action (Kukanich and Papich 2004). This may be related to limited conversion to the active metabolite (McMillan et al. 2008), a property also reported in some groups of humans (Ogunleye 2001). There seems to be a range of activity in different species, which may be associated with active metabolite formation (Pypendop and Ilkew 2008) The relatively wide safety margin and limited side-effects (Scott and Perry 2000; Raffa 2001) have led to a number of clinical applications in the control of moderate to severe clinical pain, particularly for the control of post-operative pain in dogs and cats.

4.6 Treatment of Neuropathic Pain

An important development in pain detection and control in domestic animals has been the recognition that animals, like humans, experience neuropathic pain. There are several factors involved in the pathogenesis of neuropathic pain, involving glial cells (McMahon et al. 2005), neuronal calcium channels (Li et al. 2006) and neuronal sodium channels (Waxman 2007), as well as a role for glutamate-mediated pathways (Ikeda et al. 2007). This has led to the use of anticonvulsant drugs, which interact with glutamate receptors of the NMDA subtype and calcium channels, in the prevention and treatment of neuropathic pain, both as single agents and/or as adjuncts to other analgesic therapies such as NSAIDs (Caraceni et al. 1999; Blaupied et al. 2005). The two drugs most commonly used in veterinary medicine are amantadine and gabapentin (Lascelles et al. 2007b; Lamont et al. 2000). Most reports of clinical use have been in dogs. In dogs, unlike humans, in which species the drug is excreted almost totally unchanged in urine, there is significant metabolism of gabapentin (Vollmer et al. 1986). Another drug that

possesses NMDA receptor antagonist activity is ketamine, which, because of its psychogenic properties, is no longer used in humans. It is, however, still widely used in veterinary medicine as an anaesthetic induction agent. Use of this agent in animals is common as a component of multimodal analgesic strategies for prevention or reduction of post-operative neuropathic pain (Kohrs and Durieux 1998).

5 Conclusions

Elucidation of pain mechanisms and pathways and the use of analgesic drugs in animals have followed similar developments in humans. However, the use of laboratory animals and the development of models of pain in larger species have facilitated advances in studies of human pain and hence also the treatment of animal pain (Livingston 2003). The principal groups of analgesic drugs available for pain relief in animals are similar to those available for humans. Thus, opioids, NSAIDs and other analgesics such as tramadol used for treatment of neuropathic pain are generally used on the basis of human experience. However, there are some classes of analgesics, such as the alpha 2 adrenergic agonists, which have been developed mainly for veterinary use.

In human medicine, there is increasing focus on a range of factors which may influence pain perception, such as gender and race (Keogh and Herdenfeldt 2002), as well as other genetic factors. Similarly, other sensory factors which may influence mood or perception are thought to be able to influence the level of pain perceived (Rhudy and Meagher 2000). In veterinary medicine the biggest challenges are the implication of chronic pain for animal welfare in farmed animals (Mellor and Stafford 2004), the extension of surgical and other treatments for cancer to pet animals (Carsten et al. 2008) and recognition of the need for analgesic strategies in non-mammalian species (Mosley 2005). Therefore, although there are many similarities in the treatment of pain in humans and animals, there will always be some differences in approach. There are now many reports of different analgesic drugs, being used in a range of animal species; this chapter has focussed on the more common and better reported uses.

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New Technologies for Application to Veterinary Therapeutics

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Contents

Abstract The purpose of this contribution is to review new technologies and make an educated prediction as to how they will impact veterinary pharmacology over the coming decades. By examining past developments, it becomes evident that change is incremental and predictable unless either a transforming discovery or a change in societal behaviour occurs. In the last century, both discoveries and behaviours have dramatically changed medicine, pharmacology and therapeutics. In this chapter, the potential effects of six transforming technologies on veterinary therapeutics are examined: continued advances in computer technology, microfluidics, nanotechnology, high-throughput screening, control and targeted drug delivery and pharmacogenomics. These should lead to the more efficacious and safer use of existing medicants, and the development of novel drugs across most therapeutic classes

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through increases in our knowledge base, as well as more efficient drug development. Although this growth in technology portends major advances over the next few decades, economic and regulatory constraints must still be overcome for these new drugs or therapeutic approaches to become common practise.

Keywords Future · Nanotechnology · Pharmacokinetics · Pharmacology · Toxicology · Veterinary medicine

1 Introduction and Historical Background

The field of veterinary therapeutics is in a state of tremendous flux, being at the vortex of strong currents arising from rapid technological advances, altered consumer perceptions on food safety and bioengineering, and changing public attitudes towards the value of pet ownership; all occurring in an economic maelstrom which is changing corporate structure and forcing extreme cost efficiencies on the development, production and marketing of products targeted towards veterinary species. Veterinary pharmacology has evolved over past decades in response to, and to cope with, dramatic shifts in societal views and circumstances and scientific advances, including changes such as the replacement of horses with automobiles for transportation, the elevation of dogs from working animals to family pets, the birth of modern chemistry, and acceptance of the germ theory of infectious disease. This rich history has been well documented in several reviews (Jones 1977; Davis 1982; Parascandola 1992; Andersen and Higby 1995). What can be gleaned from a close examination of history is that quantum advances have occurred in association with paradigm shifts and rapid advances in basic biology and chemistry, technological breakthroughs and societal upheavals. These drivers for change will continue to shape veterinary therapeutics in future decades.

The history of pharmacology parallels the development of modern medicine with the recognition that natural products of plant or microbial origin may cure specific diseases. When one examines centuries of historic compilations of botanical, mineral and other natural substance remedies listed in the world's *Materia Medica,* it is revealed that little development occurred for thousands of years until the growth of chemistry and the resultant birth of experimental pharmacology almost two centuries ago. At the turn of the nineteenth century, the French physiologists–pharmacologists, Megendie and Pelletier, studied the effects of intravenous injections of plant extracts containing opiates, strychnine and other substances and compiled their preparation and dosages into a formulary. Shortly thereafter, in 1813, the Spanish physician Orfila published the results of his experiments in a book entitled *Toxicologie Generale*. This was followed mid-century by the pioneering studies of Claude Bernard and others who extracted active chemical principles from classic remedies, thereby firmly establishing a chemical basis for their action. The transforming element of these early studies was that they employed the experimental paradigm to demonstrate biological activity, establishing both the

principles and methods upon which the discipline of modern pharmacology continues to be based. What differs two centuries later is that technology allows us to create more selective drugs, enables more rapid screening for activity and fosters integrated mathematical analysis of all levels of biological and chemical activity. In addition, we possess a much deeper understanding at the molecular and genomic level of both, how drugs work and how their actions may result in toxicological as well as pharmacological effects.

Modern developments in the science of pharmacology are based on improved knowledge of basic mechanisms of drug action and of the molecular basis of disease, together with an explosion of technological advances in multiple fields, including analytical chemistry, computational sciences, molecular biology, genomics and material engineering. It is the projection of these transforming technologies onto our current knowledge base that provides a realistic approach to predicting what the discipline may well resemble in the future. We must rely on market forces to determine whether these predictions will come to fruition.

2 Major Dynamics Affecting Veterinary Pharmacology

Several factors will determine what will emerge as new animal drug products in the decades ahead. These factors relate to economic, societal and technological issues. Economic and societal factors are the principal drivers in commercial decisions, as in the absence of a market; new products will not be developed. Corporate consolidation in food animal production and pharmaceutical industries, the recent occurrence of high profile food safety incidents (e.g. melamine adulteration of food, Salmonella pet food contamination), the adoption of a vegetarian lifestyle by increasing numbers of individuals, coupled with marked expansion in the size and diversification of the companion animal market, are factors which have had a major impact on the profitability of companion animal compared to production animal drugs. The substitution of melamine in protein supplements, as a fraudulent source of protein in pet foods and human infant formula in China, is a new development which necessitated the screening of all components of pet food for so-called economic adulterants. This need for increased chemical and microbial surveillance is especially felt in products destined for food-producing animals and alters the profitability of such items. Such incidents also illustrate the global connectivity, and hence vulnerability, of food and pharmaceutical distribution systems. The reduced regulatory cost of developing companion animal products, due to the lack of a requirement to establish human food safety endpoints, has resulted in the development of many more products targeted for use in small companion animal species. Identification of the human–animal bond (Lagoni et al. 1994) and its relation to ageing of the human population in developed countries further supports the development of many more products for small animal therapeutics.

The physiological similarities between humans and some companion animal species (notably dogs and cats) and their disease states facilitate the identification of

many drug candidates as an offshoot of human drug development. Direct advertising of products to pet owners in some countries enlarges this market segment. The commonality of the underlying science, highlighted in the recent reemergence of the "one medicine – one health" concept linking human and veterinary medicine, further propels this synergy (Enserink 2007). This linkage is additionally supported by the development of so-called "life-style" drugs for managing health and behaviour of companion animals. Therefore, together with their financial profitability, novel drug development is facilitated by similarities in physiology. This is evidenced by the number of behaviour-modifying drugs currently being developed from psychopharmacology research for the treatment of conditions including aggressive behaviour, separation anxiety or obsessive–compulsive disorders, as well as by the recent introduction of dirlotapide, a drug designed and introduced to reduce obesity in dogs (Overall 2001; Wren et al. 2007).

A consequence of societal trends on animal health drug development is that the companion animal market can bear a wider range of directed health products than is possible for the food animal production market where physiological differences are greater in many species (notably ruminants and birds), while profit margins remain narrow amidst intense global competition for food. These financial considerations do not exclude development of food animal drugs, delivery systems or diagnostic screens, provided they are sufficiently economical on a per-unit basis and can perform well in this much larger market segment. Technological breakthroughs tend to promote such novel developments. One area, wherein the development of food animal drugs may be potentially profitable, is non-hormonal growth regulating agents, developed to increase the efficiency of feed conversion and to increase lean body mass of food animals for consumption by health-conscious populations in developed countries (Sillence 2004). Paradoxically, meat consumption in developing countries is also increasing (Nellemann et al. 2009), thereby further enlarging, yet differentiating, the food animal market. For example, agents designed to reduce environmental impact of animal agriculture in developing countries have a large potential market. However, increased regulatory and safety issues concerning the impact of animal agriculture on human food safety (such as antimicrobial resistance, toxic drug and pesticide residues and bovine spongiform encephalopathy – BSE) creates market entry barriers that are higher than those occurring in the companion animal market. For the latter the only significant human safety issues relate to the individual animal owner or veterinarian in handling or administering animal products.

Regulatory policies and efforts to achieve global harmonisation in all major animal health drug markets are major determinants of which products are profitable for development. Should major changes occur in regulatory policies that either increase the time required for product approval or increase the cost of meeting regulatory demands, product submissions are likely to decrease, as both factors increase development costs and subsequent profit margins are reduced. In contrast, should a major pandemic crisis occur, regulatory control could be eased to allow the use of high-throughput technologies, as discussed later, to facilitate rapid development of diagnostic and therapeutic responses to the emerging threat.

The occurrence of such episodic transforming events and their impact on diagnostic and therapeutic requirements cannot be predicted.

3 Technological Developments

This analysis focusses on technological innovations already occurring in the first decade of the twenty-first century that might impact on product development two decades hence. It builds on an analysis first reported in a review of this area (Riviere 2007). One way to approach this topic would be to review novel approaches to therapy and assess how drugs might target critical processes. This approach has been extensively used and reviewed during the compilation of the latest edition of a broad-based veterinary pharmacology textbook (Riviere and Papich 2009), but would only identify incremental advances in the discipline. A second approach which has been adopted is to assess how technology facilitates both rapid screening for pharmacological activity and more controlled and targeted delivery of existing drugs, as well as expediting and increasing the focus of the drug development process. The exponential growth of all areas of biological and medical sciences has led to an increased understanding of the molecular basis of both disease and drug action. For example, increased knowledge of and greater quantitation of how metabolism and drug transporters affect absorption, distribution, excretion and metabolism (ADME) processes, as well as their subsequent pharmacodynamic activity, has increased markedly in recent years (Yengi et al. 2007), as discussed in chapters, "Pharmacogenomics in Domestic Animal Species" and "Drug Delivery Systems in Domestic Animal Species" of this text. These developments, together with the growth in, and increased sophistication of, computational pharmacology, will greatly accelerate drug development, as well as changing the nature of the discipline.

The following six areas of endeavour are particularly likely to significantly affect future drug developments in veterinary medicine:

(a) Further advances in computer technology
(b) Microfluidics
(c) Nanotechnology
(d) High-throughput screening
(e) Increased control and targeting of drug delivery
(f) Increased knowledge of pharmacogenomics

Major breakthroughs in expediting drug development would be facilitated by the combination of two or more of these areas to yield both novel products and development strategies. The possibility of interactions inevitably increases the uncertainty of predicting what will emerge in the coming decades from interactions between multiple independent pathways. How interactions between diverse systems can transform a discipline is exemplified in the developments of DNA microarray technology and of novel drugs specifically designed for modern drug delivery

approaches. The DNA microarray technology was developed by adaptation of ink-jet printer technology to "print" oligonucleotides on glass plates, which then underwrote the whole field of genomics (Fodor et al 1991; Schena et al. 1995). Similarly, transdermal microneedle technology that allows topical drugs to bypass the normally restrictive stratum corneum barrier was enabled by independent developments in material science and microfabrication engineering. Again, transforming technological innovations such as these occurring in fields far removed from medicine and even biology are almost impossible to predict.

Various combination products, including drug and delivery devices, implanted physiological feed-back systems and nanoparticle drug carriers, require decisions on whether the parts of a system or the whole system should be evaluated at the regulatory level. Several fundamental questions may be raised. How is the human food safety profile of residues from an implanted drug carrier or delivery technology determined? How stable and robust are mathematical algorithms embedded in drug delivery devices? Can misuse by an owner of a new product be dangerous either to the pet being treated or to the owners? Should novel manufacturing methods be evaluated for drug safety under existing guidelines or are new guidelines required? The 2009–2011 Research Plan for the Center of Veterinary Medicine of the US Food and Drug Administration (Food and Drug Administration 2009) indicates that research to anticipate both novel drugs and development strategies is beginning to be explored. Studies linking in vitro data to in vivo drug metabolism, the development of ultra-sensitive multiclass drug residue analytical screens, genomic screens for identifying foreign animal proteins in feed, computational databases of microbial drug resistance patterns and research in the fields of immunopharmacology and pharmacokinetics, all suggest that this regulatory agency is both starting to anticipate novel products and, equally important, applying new technologies, usually developed for other uses, to veterinary therapeutics. This translation and application of technology is likely to accelerate future developments.

Technological issues arose with the advent of genetically modified foods and with biotechnology products produced using transgenic animals (Rudenko et al. 2006). Both biochemically and analytically many of these biotechnology products do not differ either from their natural counterparts or those produced using classical chemistry approaches. However, large sections of society seem to view them as being fundamentally different, as a consequence of the methods used in their production. This is evidenced by European non-acceptance of genetically modified foods and limitations to the effectiveness of the broader global impact of the Green movement. What similar impact would these attitudes have on therapeutic items targeted at companion animals? The answer to this question is not yet forthcoming, but could have a major impact on the future development of veterinary therapeutics. Nanotechnology potentially faces a similar bias.

A major limitation to the transformation of pharmacology based on the application of new technologies is the lack of movement of young scientists trained in these disciplines to industry and regulatory agencies, and the need to cross-train scientists in traditional fields of medicine, pharmacology and toxicology to interpret and

integrate these new approaches. The responsibility of academia is not only to train scientists in the very specialised techniques of genomics, analytical chemistry and bioinformatics, but also in the traditional disciplines so that these novel data can be interpreted and applied to the design of actual drugs. The paradox is that we need more scientists at the cutting edge of modern biology and technology, but at the same time we require generalists who can adapt and integrate these specialised disciplines as the basis of a novel product development. This is a major challenge that academia has not yet successfully addressed in this era of specialisation and increasingly narrowly focused research.

Similarly, the animal health industry and the corresponding regulatory agencies must appoint individuals with new perspectives and expertise, while retaining others with the experience to integrate them into the drug development and regulatory assessment systems. A more daunting challenge is to first update and then harmonise regulations developed for mid- and late-twentieth century drugs and technology to effectively regulate twenty first century products. This has not yet been achieved. Adoption of a new evaluation paradigm requires an almost absolute certainty that the new approach is equivalent to, or better than, the traditional approach, which in itself may be seriously flawed. Only time and perseverance can overcome these problems.

4 Continued Advances in Computer Technology

One of the most pervasive influences, having a transformational impact on all areas of medicine, is the continuing developments in computer technology, comprising the speed of data processing, the amount of data that can be dealt with and the integration of existing separate and independent systems to allow automation of processes and devices previously not thought possible only a decade or so ago. The portability of diverse software between different computing systems has accelerated the development of many novel systems that can be expected to have great impact on the separate processes of drug discovery, development and regulatory approval. The dramatic improvements in wireless communication coupled to the growth of the so called “cloud computing” will facilitate and accelerate this change. A good example of this is the modern cell phone, which combines phone, internet and personal digital assistant into a single integrated device. Similar integration of once diverse functions and devices is occurring in the pharmaceutical arena.

A prime illustration of the enabling power of computational sciences on biology was the development some 20 years ago of the basic local alignment search tool (BLAST). This allowed non-mathematicians to rapidly comprehend the vast increase in the number of gene sequences being generated by sequence analysis studies (Altschul et al. 1990). The simultaneous emergence of increasingly economical gene sequencers with the computational tools to interpret them enabled significant advances in understanding the genetic basis of modern medicine to be made. This development will translate into new approaches to therapy in the near

future. In the absence of integrated computational methods, large genomic datasets could be analysed only by professional programmers possessing advanced computer programming skills.

Also of great significance have been advances in the sophistication, range and "user-friendliness" of pharmacokinetic modelling software programmes. There have been further parallel developments of pharmacodynamic models. Together these permit an increased throughput in the conduct of ADME and explorative studies that previously were time, as well as cost, prohibitive. Of equal if not greater significance, this increased ability to conduct such studies, using techniques which can be grouped under the umbrella term of *pharmacometrics* (Ette and Williams 2007), increases our understanding of the biological determinants of ADME processes. This, in turn, facilitates development of structure–activity relationships (SAR) for ADME as well as pharmacodynamic endpoints.

It is not possible to track all the innumerable developments occurring in these areas across the fields of pharmacology and toxicology, where such developments fall under the description of computational toxicology (Elkins 2007). A pivotal report from the U.S. National Research Council (2007) outlined a mechanistic-based and quantitative approach for toxicology in the twenty first century that melds in vitro testing with pharmacokinetic and pharmacodynamic modelling to create a more realistic, defensible and precise approach to chemical risk assessment. However, the integration of such diverse approaches is tedious, expensive, time consuming and fraught with difficulties when attempts to adopt such approaches are made by regulatory authorities. This is especially difficult for constantly evolving techniques. Despite their use for formal regulatory approval, they are outstanding tools for probing mechanisms of disease and drug action, as well as screening for and developing safe and effective drug candidates for submission through the traditional regulatory channels. Some such techniques may not be used for formal drug approval, but will nevertheless be adapted to compounds and devices that perform better in regulatory testing as well as in the market place.

One example of enablement through the development of such powerful software is the increasing application of mechanistic physiological based pharmacokinetic models (PBPK) to more compounds, leading to integration of biological processes with quantitative endpoints (Reddy et al. 2005). PBPK models, rather than being founded on empirical curve-fitting approaches, are constructed using biological data from organs linked by systemic blood flow. They lend themselves well to extrapolations between species, as species-specific physiological and metabolic variables (e.g. blood flow to specific organs, hepatic biotransformation enzymes) can readily be incorporated. Recent examples of the PBPK approach in veterinary medicine include the prediction of drug withdrawal times for oxytetracycline in sheep and sulfamethazine in swine (Craigmill 2003; Buur et al. 2006). These approaches allow simulation of the effect of inter- and intra-species variability on the ultimate outcome. They can greatly improve the design of experimental studies and even of clinical trials. As seen with the compound melamine, they also allow cross species extrapolations to be made based on underlying physiology in the face of minimal data (Buur et al. 2008). As discussed earlier, melamine was the

chemical adulterant of pet food that resulted in widespread kidney failure in pet animals in Asia and North America. Contaminated pet food was subsequently used as feed for food animals necessitating determination of melamine withdrawal times to ensure that the entire chemical was eliminated from animals before human consumption. A PBPK model allowed data collected in laboratory animals to be combined with sparse swine data to make realistic estimates of withdrawal times.

A further adaptation of this approach comprises integrating genomic and proteomic data with PBPK models to create systems biology approaches that attempt to describe chemical and pharmacological actions by building models from the receptor up to the whole animal (Kitano 2002). These models are in the early developmental stages but, as they become more refined, they will have the potential to dramatically advance the field of comparative pharmacology and toxicology. There will be a resultant increase in drugs better targeted on receptors and disease mechanisms and with improved safety profiles. Integration of sophisticated quantitative SAR (QSAR) models as input into such approaches will greatly increase our understanding of drug actions at the molecular level. The challenge and limitation to developing such models in the present times is the lack of data in biological systems reflecting the chemical diversity seen in QSAR models. However, systems biology approaches will allow data to be evaluated at multiple levels and may provide a method to expand their chemical inference space, that is the multiple properties of a chemical (molecular weight, solubility, etc.) for which available data might be applicable. Once validated, they would allow the so-called "in silico" trials, that is simulated trials undertaken entirely on a computer, which could potentially develop lead drug compounds using much fewer preclinical laboratory animal studies. Although a smaller number of live animal studies would still have to be conducted before approval to validate these predictions, these would be reduced in number, as a consequence of the efficiency and accuracy inherent to the robustness of the in silico analysis together with to the automated battery of pre-clinical safety and efficacy tests.

Another field in which computational power has already exerted a major impact is in the application of advanced statistical tools to analyse the much increased sets of integrated data. In pharmacology, population pharmacokinetic models are now being used to define the population factors that determine drug disposition and activity (Ette and Williams 2007). It is increasingly recognised that effective products must be based on the determinants of individual and sub-group susceptibility. Population pharmacokinetic models allow the integration of kinetic models describing ADME parameters to be linked to statistical models defining co-variates identifying the source of variability in a population response. These models were introduced to veterinary pharmacology a decade ago (Martin-Jimenez and Riviere 1998) but only recently have they begun to be considered in drug development and therefore in regulatory submissions. These models offer the prospect of identifying factors such as age, weight, gender, disease and breed, which significantly modify drug disposition or activity. The role that inter- and intra-species differences in drug transporters and metabolising enzymes exert on ADME parameters is being actively researched (see chapters, "Pharmacogenomics in Domestic Animal

Species" and "Drug Delivery Systems in Domestic Animal Species" of this text). There is now a need to determine which factors increase activity and decrease inter-individual variability. The adoption of population models has become commonplace in human medicine and their adoption in veterinary medicine will be increasingly forthcoming as more user-friendly software continues to be developed (see chapter, "Species Differences in Pharmacokinetics and Pharmacodynamics" for further discussion).

5 Microfluidics

Computer processor miniaturisation and continuing advances in micro-scale engineering have led to the development of so-called microfluidic devices, which enable complete analytical chemistry platforms to be created on the size of a postage stamp, described as the "lab on a chip" (Manz et al. 1990; McClain et al. 2003). In addition to the markedly reduced material costs, these systems, which are also referred to as micro Total Analysis Systems (microTAS) devices, reduce considerably reagent volumes and sample sizes required for both detection and quantitation. Moreover, micro electromechanical systems (MEMS) have opened up possibilities of implantable feed-back controlled drug delivery devices on a scale much smaller than that which is currently available. One major advantage of miniaturisation is reduction in power requirements, permitting battery technology not to be rate-limiting. Indeed, it is likely that the future will witness the development of devices powered by absorbing ionic substances from the animal in which the device is implanted to energise internal batteries!

There are many applications for such devices, which were in the realm of science fiction a mere decade ago. These devices can miniaturise traditional wet chemistry assays, thereby greatly reducing required sample sizes, volumes of reagents and, most importantly, the generation of hazardous waste. Microfluidic cell culture systems are currently being developed and utilised (Rhee et al. 2005; Kim et al. 2006). In veterinary medicine, microfluidic analytical devices might be used for the determination of biomarkers of disease or adverse drug effects in individual animals, allowing true individualisation of drug therapy. Moreover, their small size would reduce the cost of expensive species-specific reagents. The veterinarian could have a clinical chemistry laboratory contained within a unit of the size of a match-box! This could greatly increase the accuracy of diagnosis, and in turn increase the efficacy and safety of drug therapy. Tests for chemicals and drug residues in food animals could also be readily created to provide accurate and sophisticated analytical capabilities to the range or the feedlot. These approaches could be accomplished with current technology.

More advanced devices could be created with incremental improvements. Real-time microbiological devices searching for specific genetic determinants of drug resistance could allow selection of the appropriate antimicrobial drug for each patient, thus enhancing significantly the use of drugs in a rational manner. All that is

required for the production of such devices is miniaturisation and better definition of the demands of the clinical marketplace. Should the occurrence of drug-induced microbiological resistance become so widespread as to limit the use of antimicrobial drugs in the face of widespread infectious disease, such tailor-made approaches might become necessary and thus economically feasible. If such systems were also coupled to microcomputers containing pharmacokinetic algorithms which are already readily available, minimum inhibitory concentrations (MIC) could be determined and drug dosage regimens directly calculated. For those drugs with narrow therapeutic indices, a drug concentration analysis could be performed to dictate adjustment of dosage regimens if needed. Such developments are within our ability to accomplish. However, the absolute need and market requirements are not yet in place to foster their development.

Particularly valuable and innovative would be the use of implantable drug delivery devices containing both micro TAS and MEMS components within a single device. An obvious application would be for insulin delivery in conjunction with an on-board glucose sensor, or cardiovascular arrhythmia control via a heart rate monitor and controlled drug release device. In large animal medicine, applications could be developed for reproductive synchronisation, for automatic detection of specific microbial resistance determinants and for controlled drug release, based on an endogenous triggering signal. Finally, it is possible also to envisage the development of implantable endocrine organs that would radically alter the management of chronic diseases.

At the present time the development of units of this kind for veterinary patients would not be cost-effective. However, in the foreseeable future, manufacturing costs will be reduced and even now their manufacture is within the capability of most biomedical engineering graduate students. The major unknown challenge is if and how regulatory systems will adapt to the assessment of subject-individualised devices. Independent clinical chemistry or drug analysis units could be approved under current regulations, but it is the integration of these with drug delivery on an individual animal basis for which the regulatory path is unclear. Similarly, approving individual animal drug dosages based on an automated feedback system will require the development of new regulatory guidelines.

6 Nanotechnology

Nanotechnology is another transforming technology that has the potential to alter dramatically drug therapy. It may be defined as manufactured materials that are $<$100 nm across one dimension and possess unique physical properties due to this small size (National Research Council 2006, 2009; Leduc et al. 2007; Booker and Boysen 2005). Potential applications range from use as drug carriers to truly futuristic possibilities, including nanofactories, artificial ribosomes and wholly manufactured cells. This account will focus on shorter-term, more realistic developments that could lead to novel products within the next two decades.

The properties of nanomaterials that are unique relate to their structural stability and quantum-scale reactivity, which open up many exploitable possibilities, including targeted drug delivery and the creation of microscale biological sensors. Autofluorescent quantum dots (QD) are currently being developed for use in imaging applications. In the field of biosensors, a chemical reaction on the surface of a nanofibre may alter the electrical properties of the material, rendering them ideal for use as biological sensors when linked to specific antibodies. In theory, such devices could be used to provide an early warning of the onset of many diseases or for the presence of toxic chemicals. Nanomaterials may also be developed to contain tissue-targeting biomolecules, enabling a reduction in the dose required to provide efficacy. Multifunctional nanoparticles containing tumour-seeking sensors, imaging agents and receptor-triggered release of toxins that could revolutionise the therapy of cancer are currently under consideration (Service 2005). Some workers have conceptualised what are essentially synthetic viruses that would function as intelligent cellular delivery devices but could not replicate to produce adverse effects.

Nanomaterials may also be constructed from block-polymers of drugs themselves, as drug carrying dendrimers (repeatedly branching polymers) or, alternatively, incorporated into nano-shells that would allow accurate controlled release of drug at the tissue target site. The selective, or in some cases restricted, transport of nanomaterials further increases their potential for targeted or restricted drug delivery. The extremely high surface reactivity of nanomaterials on a weight basis also creates the possibility of scavenging applications, ranging from the removal of toxic substances to scavenging free-oxygen radicals generated in various disease processes.

As well as facilitating implantation into animals, nanoscale material science will further reduce the size of microprocessors and microfluidic devices (discussed above) and thereby facilitate the development of nanoscale computer processors. These will increase further the power as well as decreasing the size and cost of analytical instruments, microarray platforms and computers.

A cautionary comment must also be made, as the toxicology of nanomaterials has yet to be clearly defined (Monteiro-Riviere and Tran 2007). The parameters for characterising the properties of nanomaterials must be determined to ensure that any therapeutic platform utilising nanoscale material does not result in adverse events.

A specific issue that has been recognised is that the pharmacokinetic properties of nanomaterials thus far studied are significantly different from small organic molecules (Riviere 2009; Lee et al. 2009). Using a PBPK model for QD in mice and rats, our group has shown that tissue uptake cannot be modelled by flow-limited approaches but rather should take into consideration actual mechanism of cellular uptake. This is most likely to be due to vesicular cellular transport mechanisms. Another issue is that for many nanoparticles, excretion from the body does not readily occur, making concepts such as bioaccumulation and tissue withdrawal periods problematic. What are the dose metrics for nanoparticles: mass, particle number, particle size, surface charge? Self-agglomeration of nanomaterials or

complex formation with other macromolecules, alter their size and properties as a function of site within the body. The impact of these variables on biodistribution and elimination is not known. Finally, most current pharmacokinetic approaches are based on biodistribution within the vascular system as the distribution compartment. However, the particulate and hydrophobic nature of some nanomaterials may target them to the lymphatic system. Although this may be beneficial when targeting immune modulating drugs, it may be disadvantageous for other nanomaterials.

7 High-Throughput Screening

Many aspects of modern chemistry and biology have been automated and this enables the biological activity of drugs, as well as their adverse effects, to be screened rapidly without a requirement for detailed hypothesis-driven research. All aspects of early drug discovery and development are influenced by this increasingly automated "brute-force" approach. Combinatorial chemistry allows the generation of large numbers of study compounds with the potential for use for specific therapeutic targets. These libraries of compounds may contain tens of thousands of chemical entities sharing a common chemical motif. High throughput screens can then be used to select compounds with desirable pharmacokinetic (ADME) properties or for the greatest efficacy or potency in in vitro tests (e.g. cloned receptor assays, micro-arrays directed against specific therapeutic targets). Additional tests directed at the assessment of toxicological potential can then be used to further exclude less desirable compounds as candidate drugs. These approaches are currently in use for many drug classes developed for use in humans.

The future holds great promise as components of these systems are validated and transferred to other applications. As this pyramid of increasingly sophisticated tests is explored and developed, whole cell in vitro assays may then be used to assess whether similar responses are obtained in more complex biological systems. In vitro tests to assess the biotransformation of candidate drugs by specific enzymes can be rapidly accomplished. In vivo trials can then be conducted frequently with several compounds being dosed simultaneously using the so-called cassette pharmacokinetic designs, to obtain an initial estimate of ADME parameters (Hsieh and Korfmacher 2006). Individual compound studies will then be conducted to validate these rapid screening tests which assume that drug–drug interactions do not occur at the low doses used. The rapid advances in analytical chemistry that allow quantification with increased sensitivity levels as well as specificities allow these multiple drug studies to be conducted in economical and well characterised laboratory animals, selected for similarity to the veterinary target species, of interest. Finally, the miniaturisation of all aspects of the several automated processes, discussed above in microfluidic and nanomaterial sections, will further increase speed, whilst decreasing the size and cost of integrated systems. As these approaches become more successful for human therapeutics and, as a result, cost and ease of use further decrease, increased application to targeted veterinary applications could occur.

An in vitro high-throughput membrane coated fibre array technique using gas chromatography/mass spectrometry (GC/MS) has been developed in our laboratory to assess absorption of chemicals and drugs through skin. The approach is well suited for high throughput screening and is predictive of dermal chemical absorption as well as chemical mixture interactions (Xia et al. 2007; Baynes et al. 2008). Further refinement of such approaches will allow rapid identification of drug candidates optimised for delivery by a selected route, as well as assessing the effect of formulation excipients on rate and extent of delivery without a requirement to conduct in vivo trials.

The dramatic impact of automation in transforming drug development in the pharmaceutical industry in only two decades is remarkable. It is difficult to project forward these developments into future forecasts, but it is certain that very large libraries of compounds with specific activities screened for adverse effects will become available for clinical development in veterinary therapeutics. An example of how technology has enabled rapid advances in genomics is the fact that a mere 20 years ago it took one year to manually sequence a gene with 10,000 base pairs. This can now be accomplished in less than 2 weeks and, moreover, at a fraction of the cost. This technology will continue to be optimised, so that time and cost per base-pair will continue to decrease. As indicated above, developments in bioinformatics in association with in silico pharmacology and toxicology analyses will allow data to be interpreted with greater speed and accuracy, thus enabling identification of more candidate drugs with appropriate safety and efficacy profiles in target species.

8 Increased Ability to Control and Target Drug Delivery

Although each of the above developments, with the exception of the continued development of high-throughput systems, could revolutionise veterinary therapeutics, the area with the greatest likelihood of impacting within the next two decades is controlled and targeted drug delivery. This topic is reviewed in chapter, “Drug Delivery Systems in Domestic Animal Species” of this volume. Innovations now close to adoption include the addition of polymer groups, such as polyethylene glycol (PEG), to protein and peptide drugs to prolong systemic residence times (Greenwald 2001). In addition, increased SAR knowledge has allowed the selection of drug molecules with longer terminal half-lives, avoiding the need for frequent administration.

When increased knowledge of the determinants of the mechanisms of cellular uptake of nanoparticles is obtained (Ryman-Rasmussen et al. 2007), nano-based therapeutics, targeted to specific cell types will become possible. Electrically-assisted transdermal delivery systems developed a decade ago and now in use for some human applications, may also have applications in veterinary medicine (Riviere and Heit 1997). One innovation was the transdermal delivery of charged or peptide drugs not otherwise able to penetrate the stratum corneum barrier. Controlled-release transdermal patches and formulations for compounds such as

fentanyl used in humans have also been used in dogs. However, there may be a need for re-formulation to ensure optimal use in individual species. This will become possible as our knowledge of species differences in drug absorption expands (Riviere and Papich 2001). Innovative approaches that have been developed experimentally include the use of multicompartment microchips containing either multiple doses of a drug or multiple drugs, which may be released by several mechanisms (Langer 2001). Radio control of such devices introduces further possibilities.

Advances in technology approaching commercial application are microneedles. These are structures of such small diameter that puncturing the skin does not introduce the chance of contamination, nor does it produce any sensation and this drug delivery mode is therefore classified as non-invasive. Such systems would permit construction of drug patches for controlled delivery of substances not normally able to penetrate intact skin (Verbaan et al. 2007). Potential applications range from delivery of therapeutic proteins to genes; the latter could be used to transform skin cells to secrete systemically available polypeptide hormones, such as growth hormone and clotting factors (Khavari et al. 2002). The development and commercial application of such systems would fundamentally change the treatment of chronic deficiency diseases in veterinary patients. Moreover, novel applications might be particularly appropriate for food producing animals, as chemical residues associated with traditional dosage forms would not exist. An example of an application, already taken to proof-of-concept stage, is coating solid microneedles with antigens for non-invasive vaccine delivery (Widera et al. 2006). A problem to be overcome with all these approaches, when applied to many animal species compared to humans or pigs that serve as human models, is increased fur density and variability in skin thickness. These variables make standardisation difficult.

Novel approaches to improved targeting and controlled rate of drug delivery are already available and have been adopted in human medicine. The additional hurdles to using such approaches in veterinary medicine are related to economics, involving the cost of development and sometimes also the cost of manufacture. In addition, the technology which comes to dominate in human medicine (e.g. electrical delivery versus microneedles) will determine which of these approaches become practicable to use in veterinary medicine, a less than desirable manifestation of the one-medicine concept.

9 Increased Knowledge of Pharmacogenomics

As discussed above and reviewed in chapter, “Pharmacogenomics in Domestic Animal Species” of this text, advances in the genomic sciences have led to significant advances in comparative medicine (Cunningham 2000; Semizarov and Blomme 2009). As the genetic code of more species, breeds and microorganisms is revealed, specific targeting of drugs to species and disease specific endpoints will become possible. As more data are collected in several species with defined

diseases, the determinants of what are now called idiosyncratic drug effects will become apparent. The application of statistical algorithms to gene expression data is a relatively recent development (Wen et al. 1998). Further development of these bioinformatic techniques will greatly increase the value and application of these data sets. Increased understanding of mechanisms underlying susceptibility in one species may be directly translated to others of special veterinary interest. Deficiencies in biotransformation or drug transport due to lack of an enzyme or drug transporter, as for example the sensitivity of Collies and related canine breeds due to the absence of the MDR-1 gene coding for *p*-glycoprotein transporter (Mealey 2004), will be better defined and screened.

Genomics, proteomics, and metabolomics are being integrated into many approaches to screening for toxicological effects (Riviere 2006). This can be expected to reduce the drop-out rate of lead drug candidates. As experience interpreting these systems is gained, integrated databanks will be generated which allow more accurate SAR studies to be conducted. As these relationships are validated, they will enable the development of more robust in silico pharmacology and toxicology screens. What was previously described as exercises in data-mining is now the field of analytics, for which graduate training programmes for specialists in analysing such integrated databases are now in place.

10 Potential Impact on Veterinary Medicine

All of the approaches and techniques described in this chapter streamline and optimise the drug discovery process and are potentially applicable to all therapeutic classes. It is unlikely that novel therapeutic classes of drugs replacing existing compounds will be developed, for example oligonucleotides or exotic material nanodrug devices, as replacements for organically synthesised drugs. Rather, there will be a refinement of drugs based on more selective activity and with reduced potential for untoward side-effects applying high-throughput analysis to combinatorial chemistry libraries, or alternatively QSAR to design drugs with highly selective properties. Components of these increasingly efficient screens will include separate assays for ADME, efficacy and toxicity, as well as resistance for antimicrobial and anthelmintic drug classes to ensure that viable drug candidates emerge.

Drugs formulated with specific targeting moieties will become more common. The controlled delivery of drugs to target tissues will be enhanced using technologies already in the process of being commercialised. Examples are the use of advanced polymer chemistry, nanodrug formulations, microneedles and/or electrically-assisted delivery techniques. Species-specific drug delivery devices will become more common. The benefit will be to render administering drugs to animals easier and less frequent. Cats are an obvious target species for many of these approaches due to the difficulties owners have in administering oral medications on a daily basis to this species.

In food animal pharmacology, microbial resistance will continue to be a dominant concern arising from antimicrobial drug use, although improved vaccines may well better control the diseases they are currently used to treat. This would further decrease market share and hence potentially the development of novel compounds. The limited profitability of antimicrobial drugs is also a major consideration in human medicine. Another likely advance is the emergence of non-hormonal growth regulating technologies. Moreover, novel drugs approved in food animal species will have short or even zero withdrawal times based on better SAR, whilst drug delivery devices will be less invasive and have minimal impact on carcass quality or safety. Tracking individual animals using implanted microchips may generate the epidemiological databases that would facilitate control of drug resistance problems. Implantation of biochemical sensors based either on nanotechnology or microfluidics together with wireless alert devices might permit sentinel monitoring of specific genetic markers or metabolites of selected bacteria (e.g. enteropathogenic *E. coli* H0157) or chemical exposures to prevent such animals from entering the food supply.

Although such advances may be readily predicted from existing developments in several newer technologies, the emergence of products with marketing authorisations will ultimately be critically dependent on regulatory acceptance of such novel compounds and systems. History informs us that regulatory agencies have been inflexible in adapting to new technologies; good examples being the difficulty of replacing visual meat inspectors with specific microbial screening tests (National Research Council 2001), or batch product testing with real-time individual-unit product manufacturing technologies (e.g. Process Analytical technology (PAT)) (Rantanen 2007). One may therefore ask, how will combination products (for example a novel drug in a computer controlled microfluidic device) be tested, judged and approved? Differences in organisation and policy of regulatory agencies across different jurisdictions regarding combination products are large. A similar division occurs when food safety is handled under a different agency than companion drug approval. Such agency structure may be deeply enshrined in tradition and legal precedence, rendering major changes difficult even within a single country and even more difficult globally. Likewise, the question can be put, will regulatory agencies allow product development for specifically targeted populations. How narrowly can such populations be defined and what will the data requirements be for approval in subpopulations? Existing experiences in the United States with minor species drug approvals suggest this area will not be easy to effect change. Will regulatory agencies have the knowledge base and guidelines to regulate novel products and their method of manufacture? For example, the definition of "nano" is context specific with many existing formulations containing particles that might be considered nano-size, yet they do not fit the strict material science definition of a "true" nanomaterial , in which one dimension is less than 100 nm and the particle has resulting unique physicochemical properties. Regulations designed to prevent widespread environmental exposure and occupational exposure may inadvertently raise the hurdle to the approval of a novel nanotherapeutic. As well as hurdles within any one regulatory environment, there is uncertainty as to whether international

harmonisation will simplify or complicate these developments? Differences in time from submission to market between regulatory jurisdictions (e.g. United States and European Union) could have major negative impacts on how these many advances are brought into practical therapeutics. The global harmonisation of animal drug regulations would, at a stroke, remove impediments to rapid drug approval and subsequent marketing.

11 Conclusion

So we may ask, where will veterinary pharmacology be in 20 or 30 years time? As can be appreciated from the discussion in this chapter, this question is particularly difficult to answer. Any paradigm shift in the knowledge of disease control or transformational change in the structure of drug regulatory agencies could dramatically change the nature of the mid-twenty-first century armamentarium. Global disease outbreaks could either decimate animal agriculture or alternatively speed approval of novel therapeutic agents or even both. Increased development of ever smaller and faster computing platforms linked to microfluidic platforms could individualise drug therapy in ways difficult to comprehend today. The goal of this review has not been to make iron-clad predictions but rather to stimulate thinking in relation to harnessing developing technologies to improve animal health and, in the case of food animals, without adversely risking human food safety.

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Part II
The Interface of Veterinary Pharmacology and Man

Genetically Modified Animals and Pharmacological Research

Dominic J. Wells

Contents

Abstract This chapter reviews the use of genetically modified animals and the increasingly detailed knowledge of the genomes of the domestic species. The different approaches to genetic modification are outlined as are the advantages and disadvantages of the techniques in different species. Genetically modified mice have been fundamental in understanding gene function and in generating affordable models of human disease although these are not without their drawbacks. Transgenic farm animals have been developed for nutritionally enhanced food, disease resistance and xenografting. Transgenic rabbits, goats, sheep and cows have been developed as living bioreactors producing potentially high value biopharmaceuticals, commonly referred to as "pharming". Domestic animals are also important as a target as well as for testing genetic-based therapies for both inherited and acquired disease. This latter field may be the most important of all, in the future development of novel therapies.

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1 Introduction

The technology that allows the addition to or inactivation of specific genes in an animal's genome opens up a huge range of opportunities. Genetic manipulation can be used to create animal models of the human disease, investigate specific gene function, improve resistance to disease and treat inherited or spontaneous disease. Before reviewing the use of transgenic animals, it is important to understand the basics of the production of genetically modified animals and the potential pitfalls associated with their analysis.

The first method of producing genetically modified animals was reported in the mouse in 1980 by Gordon and colleagues. The authors used a small pipette to hold a single cell fertilised egg and another very fine glass pipette to inject a dilute DNA solution into one or other of the pronuclei. This technology has subsequently been used to generate a very wide range of genetically modified mammals, including mouse, rat, rabbit, pig, sheep, goat and cattle (Hammer et al. 1985). The process of integration of the DNA into the host genome is random, generally occurring at only one site in each embryo but often with multiple copies being integrated at that site (Brinster et al. 1985). A high copy number is often associated with a reduced level of expression of the transgene (Garrick et al. 1998). The random nature of the integration means that not all of the transgenic animals will express the transgene as in a proportion of cases the transgene integrates into heterochromatin and so is silenced. The transgene may also integrate close to a strong promoter/enhancer or repressor and this may modify the pattern of expression. Finally, the transgene may integrate into a critical gene that may be responsible for the phenotypic change in the transgenic rather than the transgene. Consequently, it is generally regarded as necessary to produce several microinjection transgenics with the same phenotype to have confidence that the phenotype is due to the transgene. The transgene cassette design also plays a significant role in determining the level of expression. The presence of introns generally improves expression compared to a cDNA (Whitelaw et al. 1991) and some sequences upstream of the promoter, locus control regions, have been shown to confer site-independent expression (e.g. Talbot et al. 1989). Therefore, careful transgene design can improve the efficiency of this technology.

An alternative to microinjection is to use recombinant lentiviruses to transfer a transgene into an embryo. This approach was first published for mice by Lois et al. (2002) and its use in farm animals has been recently reviewed (Whitelaw et al. 2008). Although this method of transgenesis is much more efficient than microinjection, the lentiviral vectors have a limited packaging capacity accommodating a maximum 8 kb transgene and integration occurs at multiple sites in the genome. Subsequent breeding of the transgenic founder will lead to segregation of these sites

and several generations are likely to be required to yield animals possessing just one site of integration and thus generate a stable transgenic line with consistent expression. Consequently, this approach has not been widely used in mammals but has been extensively used in the production of transgenic chickens where good alternative methods of transgenics are not available. Lentiviral transgenesis has also been used to generate the first transgenic non-human primates that exhibited germline transmission (Sasaki et al. 2009).

The second technological development was reported by Robertson and colleagues in 1986. They showed that mouse totipotent embryonic stem (ES) cells could be genetically modified and made to contribute to the embryo. This opened up the possibility of specific genetic modification of ES cells in culture and generation of mice derived from these specifically modified cells. This gene targeting is currently undertaken via homologous recombination using a targeting vector isogenic with the target locus in the ES cells apart from the specific modification being introduced. Thus, this technique overcomes the problems associated with microinjection; the genetic modification is precisely located and is a single copy. This technique is particularly well suited to disrupting specific genes (knockout) for functional studies or for the generation of models of inherited monogenic disease in man. The disadvantage of the ES approach is that the generation of transgenics takes longer than by pronuclear microinjection and greater molecular and cell biology skills are required for successful gene targeting and maintenance of the totipotency of the ES cells.

However, it has not been possible to extend stem cell technology to many other species. This problem has been overcome by the development of a third technology, nuclear cloning. In this technique the maternal DNA is removed from an oocyte and is replaced by a nucleus taken from a cultured cell. Initially this was performed with nuclei from early embryos but later studies used cells from adult sources. These donor cells could be genetically modified in culture, using the homologous recombination approach noted above, before nuclear transfer in the oocyte and implantation into the foster mother. This technology was first demonstrated with non-embryonic cells in the sheep by Campbell and colleagues in 1996 with the production of the famous sheep, Dolly, using the nuclei from cultured cells. This was shortly followed by the demonstration of mice derived from the nuclei of somatic cells (Wakayama et al. 1998) and calves also derived from nuclei of somatic cells (Kato et al. 1998; Vignon et al. 1998). Over a period of 10 years, a wide range of mammals were cloned from adult somatic cells: pigs (Polejaeva et al. 2000), goats (Zou et al. 2001), cats (Shin et al. 2002), horses (Galli et al. 2003) and dogs (Lee et al. 2005).

However there have been significant problems associated with cloning adult farm animals (Edwards et al. 2003). The major limitation is the extreme inefficiency of the process in terms of live offspring from the number of embryos generated. This problem does not vary significantly between species or the somatic cell type. Cloned embryos and foetuses die at all stages of the pregnancies. In addition, a high proportion of clones are larger than normal and die soon after birth. Despite these problems some clones are physiologically normal and can reproduce

without problems. It has also been reported that clones can age prematurely and have shorter telomeres that would be expected for a normal animal of the same age. One solution to the ageing issue in cloned animals is to use adult stem cells as the source of the nuclei. The use of porcine skin-derived stem cells has recently been reported with successful generation of live piglets (Hao et al. 2009).

The very recent development of induced pluripotent stem (IPS) cells offers the prospect of an increased efficiency of production of gene targeted domestic animals. IPS cells are cells that have been treated with a cocktail of transcription factors found in ES cells and show high telomerase activity and totipotent properties. The technology was originally developed by Takahashi and Yamanaka (2006) who showed that the addition of just four transcription factors could re-programme adult fibroblasts into pluripotent stem cells. Domestic animal IPS cells are currently being produced for domestic species such as the pig (Wu et al. 2009).

2 The Potential of Detailed Animal Genomes

The genomes of many of the major veterinary species have been sequenced in the last decade. In 2004 the dog became only the fifth mammal to have its entire genome fully sequenced when the information was publicly released (Lindblad-Toh et al. 2005). This followed the earlier work in which molecular markers were used to study genetic relationships between 85 domestic dog breeds and microsatellite genotypes were used to correctly assign canine genomes to specific breeds (Parker et al. 2004). An initial sequence and comparative analysis of the Abyssinian cat genome was published in 2007 by Pontius and colleagues. An improved coverage of the bovine genome was recently published (Zimin et al. 2009) and the genomes of alpaca, sheep and pig are currently being sequenced (e.g. Humphray et al. 2007). Beginning from approximately 300 mapped markers scattered on the 31 pairs of autosomes and the X chromosome in 2001, the horse genome is now among the best-mapped in domestic animals (Chowdhary and Raudsepp 2008). This includes approximately 1.5 million single nucleotide polymorphisms (SNPs) from a variety of breeds.

Although the cost of sequencing has fallen dramatically and the rate of sequencing risen rapidly since the start of sequencing the human genome, the coverage and assembly of genomes often contain small gaps and numerous errors which will take many years to improve. Thus work will need to continue to revise and refine the genome maps of the various species.

Dogs provide important models of human disease as well as being an accessible source of spontaneous diseases that can be used to test a variety of gene therapy approaches. Such studies are enhanced by a detailed understanding of the canine genome and the associations of specific diseases with different dog breeds (Karlsson and Lindblad-Toh 2008). There are over 450 inherited diseases in the dog of which at least half have very similar clinical presentations compared to man (Tsai et al. 2007). Examples include a variety of breeds with haemophilia due to

mutations in the Factor VIII or IX genes (reviewed by Øvlisen et al. 2008) and the Briard with retinitis pigmentosa due to a RPE mutation (Aguirre et al. 1998). The canine genome has also been useful for understanding genetic changes that affect body shape and size. Chondrodysplasia, the short-legged phenotype typical of breeds such as dachshund, corgi, and basset hound, has been shown to be due to an evolutionarily recent retrotransposition of fibroblast growth factor 4 (Parker et al. 2009). A SNP for IGF-1 is likewise associated with the difference in body size between breeds (Sutter et al. 2007). In addition, dogs can be used to study the genetics of complex traits using both specific breeds and the canine population as a whole (Parker and Ostrander 2005). A good example is the investigation of cardiovascular disease genes in the dog (Parker et al. 2006).

Knowledge of an animal's genome, and in particular the variation in that genome, such as the SNPs, has a number of important consequences. Genetic markers such as specific SNPs can be linked to production traits and aid in structured breeding programmes in farm animals (e.g. Ibeagha-Awemu et al. 2008). Analysing traits that are economically important to horse owners, such as fertility and sex determination will be an important consequence of a better understanding of the equine genome (Chowdhary et al. 2008).

3 Use of Transgenic Mice to Reveal Drug Targets

The technology outlined above has been widely used to generate animal models of the disease and to investigate specific gene function. By comparing normal animals with animals in which specific genes have been inactivated it has been possible to elucidate the contribution of a wide variety of genes to various disease processes and so define specific drug targets with great accuracy. There are numerous examples of such experiments and the following paragraphs discuss a few specific examples that demonstrate the importance and utility of this use of genetically modified animals. In view of the relative ease of ES cell work in mice compared to other species, together with the cost effectiveness of working with a small mammal with short reproductive intervals between generations, most of these studies have been conducted in mice. It should be noted that with standard gene knockouts the animal develops in the absence of the specific gene function and other genes may play a substitute role thus confusing interpretation. A good example is the neuropeptide Y gene (NPY). This peptide is a potent stimulator of feeding behaviour and levels fall when animals have been fed ad lib. However, the knockout displays no abnormal feeding phenotype (Erickson et al. 1996). The use of inducible conditional knockouts in which the gene can be inactivated at a precise time and in a specific tissue by drug activation of a recombinase is in many cases more informative regarding the effect of acutely blocking specific gene activity, as would be the case with many pharmacological approaches.

A huge range of mice have been generated as models of human disease and these can be used to test various therapeutic approaches. However, these models do not

always accurately reflect the human condition and care must be taken to understand the limitation of such models. For example a large number of mouse models of cystic fibrosis have been independently generated by inactivation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene (reviewed in Grubb and Boucher 1999). Although these mice develop digestive problems they do not develop the marked lung pathology seen in man and this was thought to reflect the specific pathogen free conditions in which mice, unlike man, are commonly housed for experimental work. However, only with repeated addition of respiratory tract pathogens do the mice develop lung damage (Davidson et al. 1995). Thus treatment of CFTR mice does not accurately reflect all the issues likely to be encountered in treating the human disease, i.e. thick mucus, chronic infection and lung damage.

Genetically manipulated mice have proved useful in trying to understand the mechanisms of anti-depressive drugs by manipulations of specific genes such as those for the 5-HT (serotonin) transporter and the G protein coupled receptors, 5-$HT1_A$, 5-$HT1_B$ and $5HT_4$. Downstream targets such as the TWIK-1 related K+ channel appear to offer possible targets for new anti-depressants. For such studies, it is vitally important that the genetic background is defined, as different genetic backgrounds in mice show very different behavioural performances (reviewed by Gardier et al. 2009).

Knockout mice have also played a very important role in understanding the regulation of appetite and obesity and have provided a number of targets for pharmacological intervention. Many of these models have been reviewed by Speakman et al. (2008). Another example is the knockout of the RIP140 gene, a nuclear receptor co-repressor (Leonardsson et al. 2004). These mice are lean, show resistance to high-fat diet-induced obesity and hepatic steatosis, and have increased oxygen consumption. Thus, this gene could be a target for the development of drugs to treat the metabolic syndrome in man.

4 The Development and Use of Transgenic Farm Animals

The first transgenic farm animals were pigs that expressed human growth hormone (Hammer et al. 1985) and further pigs were produced by other groups. However the effects on growth rate were variable although feed conversion efficiency was increased and fat deposition was reduced compared to non-transgenic littermates. Unfortunately the over-expression of the growth hormone was linked with the development of lethargy, lameness, and gastric ulcers and thus this was not an effective method to improve pork production (Pursel et al. 1990).

Farm animals have also been genetically modified to improve food quality. For example transgenic cattle have been produced containing additional β- and κ-casein genes which increased the casein content of the milk (Brophy et al. 2003). This was used to make cheese with increased levels of essential amino acids. Transgenic pigs have been generated that produce increased levels of omega-3 fatty acids in the meat (Lai et al. 2006); these fatty acids have been associated with prevention of

coronary heart disease. Progress and prospects in this field have recently been reviewed by Laible (2009).

Infection of the mammary gland (mastitis) is the most important disease in dairy cows with a huge annual cost in lost milk production. As mastitis resistance has not been substantially improved by selective breeding there have been a number of attempts to generate transgenic cows with increased resistance. Transgenic cows expressing lysostaphin have been shown to be resistant to *Staphylococcus aureus* infection (Wall et al. 2005) but cows producing lactoferrin were not resistant to *Escherichia coli* mastitis (Hyvönen et al. 2006).

Finally there has been considerable interest in developing genetically modified pigs for xenografting into humans to overcome the acute shortage of human heart, liver, kidney and pancreatic transplants. If a standard pig heart is transferred to a man there is a hyperacute immune rejection as soon as blood flow is established. The reason for this is that all mammals, apart for humans, primates and old-world monkeys, and some bacteria have a galactose alpha 1,3 galactose epitope on the surface of most cells. Humans carry pre-existing antibodies to this epitope, probably as the result of exposure to bacteria, and these antibodies cause rapid complement activation that destroys the foreign organ. In order to address this problem a number of different approaches have been taken including addition of human decay accelerating factor (hDAF, CD55) to block the complement activation and removal of the alpha 1,3 galactosyltransferase gene (Gal-KO) in pigs to reduce this hyperacute reaction. Such modifications aid survival of the graft in the short term but there is still medium term rejection that cannot be controlled by immunosuppressive drugs, suggesting that further genes need to be added for a protective function and/or other endogenous genes need to be inactivated. Progress in this field has been recently reviewed by Petersen et al. (2009).

5 Farm Animal Pharming

Therapeutic proteins have a wide number of uses but have historically proved difficult to produce in sufficient quantities. Production of many mammalian proteins in bacterial culture is ineffective as the biological activity of the protein can be dependent on post-translational modifications which are not performed in bacteria. An exception is human insulin which has been produced in bacteria by Eli-Lilly and marketed under the trade name Humulin N. To circumvent the post-translational problem, proteins can be produced in mammalian cell cultures but 20 years ago there were substantial technical problems in producing and purifying such proteins. Thus in the early 1990s there was considerable interest in collecting high value therapeutic proteins from the milk of transgenic animals, essentially using the animals as bioreactors, and this led to the phrase “pharming”.

The first demonstration that it was possible to express a transgene in the mammary gland of a farm animal was reported for transgenic pigs by Wall and colleagues in 1991. Subsequently transgenic rabbits, goats, sheep and cows

have been generated which secrete biopharmaceuticals in their milk (Wright et al. 1991). The choice of species used is based on subtle differences in post-translational modification, the quantity of product that the market would require and the time taken to expand the transgenic colony to the point that it would satisfy the quantity requirement. Products currently under consideration are recombinant human antithrombin III, human inhibitorC1, human fibrinogen, human albumin, human α-antitrypsin, as well as potential protein vaccines including rotavirus VP2/VP6 and malaria antigen (Houdebine 2009).

Kind and Schnieke have recently examined both the history and the likely future of animal pharming (Kind and Schnieke 2008). In the last 20 years cell based manufacturing has produced a series of highly profitable proteins, such as Epogen (recombinant erythropoietin) and Enbrel (recombinant anti tumour necrosis factor) whereas the pharming industry has only had one product approved, recombinant human antithrombin III (ATryn) marketed by GTC Biotherapeutics. Approval was granted in Europe in August 2006 and in the USA in February 2009. The contrast between the successes of the cell based manufacturing and the pharming industries is somewhat surprising as pharming would appear to have cost advantages. It has been estimated that cell-based manufacturing is 3–30 times more expensive than pharming and it also requires a very large initial capital outlay to build a culture facility of sufficient size to produce a protein on the scale necessary for regular therapeutic use (Farid 2007).

So why has animal pharming struggled to compete? A significant problem was that the early projects used microinjection transgenic technology and the transgenes proved unstable in a number of cases. Such variation was unacceptable to the regulatory authorities. This problem can now be addressed by specific gene targeting, using nuclear transfer. A possible growth area for the future will be the generation of transgenic cattle that can produce humanised polyclonal antibodies. Such antibodies would be of great benefit in treating venoms and pathogens that mutate rapidly and would be a large market that could only be provided by pharming (Kind and Schnieke 2008).

6 Gene Therapy for Diseases of Companion and Farm Animals

As noted in the previous sections, transgenic animals, which are genetically modified at the level of the germline and so transmit the genetic modification to subsequent generations, have a large number of uses in pharmacology. Genetic modification can also be performed on somatic tissues, treating just the individual. Somatic gene transfer requires a system (vector) to transfer the genetic material into the cell. There are two broad classes of vectors, viral and non-viral. The former are derived from a range of viruses and have been engineered to retain the highly efficient mechanisms for delivering genetic material into the target cell while preventing replication by deleting some or all of the viral genes. Viral vectors

have been derived from a range of viruses including adenovirus, adeno-associated virus (AAV), herpesvirus and retrovirus including lentivirus. These vectors can be produced in cell lines in which the viral genes required for replication are provided in a form that cannot be packaged into the subsequent virus. The room provided by deletion of viral genes is used to accommodate the therapeutic gene(s). Delivery of therapeutic genes, either to substitute for dysfunctional genes, or to produce products that help fight acquired disease, is termed gene therapy. Non-viral gene delivery uses purified circular plasmid DNA that is prepared in bacteria. It can be delivered in two versions, either as a complex with lipids and/or peptides or as a naked molecule with a physical driving force. Examples of the latter include high pressure/volume injections (hydrodynamic), the application of a series of electrical pulses (electroporation) and the use of ultrasound combined with microbubbles. All these methods cause transient pores in the target cell membrane and so aid the entry of the plasmid into the cell (Wells 2004). Viral vectors tend to be more efficient but the presence of viral surface proteins often leads to an acquired immune response that limits the possibility of repeated administrations.

A wide range of animals have been used as model systems for developing gene therapy treatments for human disease. As in pharmacological research, the majority of these have been rodents but large animal models, commonly dogs and non-human primates, have also been used, often as a final test before taking the treatment to human clinical trial. In a number of these cases natural spontaneous mutants have been used such as the golden retriever with muscular dystrophy (Cooper et al. 1988; Valentine et al. 1988), the German shepherd with haemophilia A (Parry et al. 1988) and the Plott Hound with Hurler disease (Spellacy et al. 1983).

An alternative approach to testing gene therapies that might be used in man is exemplified by treatments for cancers. Studies in laboratory rodents mostly use inducible tumour models in inbred experimental animals. These are poor models for human disease and thus there has been an increasing interest, both for experimental and veterinary reasons, in testing gene therapy treatments in spontaneous animal tumours. This was first highlighted by Vail and MacEwen (2000) and a number of reports have subsequently been published examining treatments in dogs (e.g. Dow et al. 1998, 2005; Bergman et al. 2003; Kamstock et al. 2006; Cutrera et al. 2008; Finocchiaro and Glikin 2008) and cats (Jahnke et al. 2007; Hüttinger et al. 2008). It is interesting to note that most of the above have used non-viral gene transfer methods. The majority of studies have reported a modest improvement with some partial and some complete remissions.

Other gene therapies for spontaneous disease that have been tested in pets include treatments for weight loss and anaemia associated with cancer and kidney disease. Electroporation of growth hormone-releasing hormone (GHRH) plasmid has been used to treat cats and dogs with chronic renal failure (Brown et al. 2009). Treated animals showed an increase in body weight, improved appetite and activity, maintained kidney function and survived longer than the untreated controls. This group have also used the same treatment for cancer associated anaemia in dogs (Bodles-Brakhop et al. 2008). They noted improvements in red blood cell numbers,

haemoglobin content, haematocrit levels and improved survival compared to untreated controls.

DNA vaccination has also been used in dogs to control seasonal allergy to Japanese cedar pollen (Masuda 2005) and immunostimulatory liposome–plasmid complexes have been used to treat refractory atopic dermatitis in dogs (Mueller et al. 2005).

Delivery of porcine GHRH plasmid by electroporation has been shown to decrease morbidity and mortality in treated sows and their offspring over three consecutive pregnancies (Person et al. 2008). This technology was approved for use in food animals in Australia in January 2008 under the name of LifeTide SW 5 marketed by VGX Animal Health Inc.

7 Summary

Genetic modification of laboratory and farm animals has enabled a greater understanding of gene function, development of animal models of human disease, insight into new targets for pharmacological development, the production of biopharmaceuticals, disease resistant farm animals, a possible source of transplants and genetic therapies for man and companion animals. These developments can only increase with a detailed knowledge of the genome for each of the domestic species. It is important to understand the technology and thus limitations to these genetic modifications. The development of IPS cells presents an opportunity to improve the efficiency of genetic modification and thus make transgenic farm animals a more attractive option for the future provided the marketing of meat and milk is accepted by the regulatory authorities and the public. The most promising area for future development appears to be the use of gene therapies in companion animals as this has the potential to produce beneficial results for both animals and man.

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Antimicrobial Drug Resistance

Marilyn Martinez and Peter Silley

Contents

Abstract This chapter provides an overview of our current understanding of the mechanisms associated with the development of antimicrobial drug resistance, international differences in definitions of resistance, ongoing efforts to track shifts in drug susceptibility, and factors that can influence the selection of therapeutic intervention. The latter presents a matrix of complex variables that includes the

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mechanism of drug action, the pharmacokinetics (PK) of the antimicrobial agent in the targeted patient population, the pharmacodynamics (PD) of the bacterial response to the antimicrobial agent, the PK/PD relationship that will influence dose selection, and the integrity of the host immune system. Finally, the differences between bacterial tolerance and bacterial resistance are considered, and the potential for non-traditional anti-infective therapies is discussed.

Keywords Antimicrobial resistance · PK/PD · Dose selection · Antimicrobial tolerance

1 Introduction

Bacteria are incredibly resilient life-forms that have proven their ability to survive across a variety of environmental conditions. There are examples of bacterial species that can survive temperatures below freezing and above the boiling point of water, species that can survive in highly acidic environments or in alkaline conditions with pH values greater than 12, and others that thrive in aqueous media containing 30% NaCl. *Deinococcus radiodurans* is a species of bacteria that can survive in the presence of a thousand times more radiation than can be tolerated by humans (Benitez de Cruz 2008).

Facilitated by their potential for rapid growth, bacterial populations evolve over short periods of time, promoting their survival under adverse conditions. Typical of this survival capability is their adaptation to life in the presence of antimicrobial agents, where, the expression of resistance mechanisms helps to ensure their continued growth (Epstein et al. 2004). Furthermore, bacteria can communicate with each other through elaborate signalling systems that facilitate their survival in hostile environments. These communication systems promote synchronised changes in community structure and composition under adverse conditions (e.g. the formation of biofilms) or signal the presence of environmental conditions favourable for colony growth.

Given their multitude of potential survival mechanisms, can we use drugs in a manner that will prevent the development of antimicrobial resistance? The answer is NO. However, we can minimise the risk of selecting for resistant microbial strains by carefully choosing the antimicrobial agent, the product formulation, the dosage regimen, and the duration of dosing. Therapeutic strategies should be grounded in pathogen identification, an understanding of the susceptibility characteristics of that pathogen, and knowledge of the relationship between drug dose, PD, and effect. The relationship between PK and PD and the corresponding PK/PD target for dose selection is a function of the mechanism(s) of drug action, specific drug–bug interactions, the natural growth rate of the pathogen, and the mechanisms through which resistance (or tolerance) can occur.

2 Antimicrobial Mechanisms of Action

Antimicrobial agents (i.e. drugs that exert bacteriostatic (limits bacterial growth) or bactericidal (killing) effects) typically function through one of five mechanisms of action. These comprise (Chambers 2006):

- Inhibition of cell wall synthesis. These agents are generally bactericidal.
- Alteration of the 30s and 50s ribosomal subunits, resulting in a reversible inhibition of protein synthesis. These agents are generally bacteriostatic rather than bactericidal.
- Inhibition of bacterial protein synthesis or the synthesis of aberrant proteins via the binding of drug to the 30s ribosomal subunit. This ultimately results in cell death (bactericidal).
- Alteration of nucleic acid metabolism. This usually leads to cell death (bactericidal).
- Anti-metabolite activities. While this usually leads to bacteriostasis, it can result in bactericidal effects under some circumstances.

Newer agents, such as daptomycin, interfere with cell membrane electrical potential, leading to membrane depolarisation and cell death (Steenbergen et al. 2005).

It should be noted that the classical division of drugs into bacteriostatic and bactericidal is somewhat artificial. There is a growing body of evidence that under certain situations (e.g. 4–5 times the MIC) or for some pathogens, bacteriostatic drugs may exert bactericidal activity. Conversely, at low concentrations relative to the MIC (or even at one time the MIC) some bactericidal agents exert only bacteriostatic effects.

While the magnitude of the inhibitory effect for some agents is primarily dependent upon the duration of drug exposure, for others it is largely a function of the rate and/or extent of drug exposure. This phenomenon was first recognised by Shah et al. (1976) who, upon studying in vitro bacterial responses to constant drug concentrations for variable periods of time, observed that the effects could be classified as either time or concentration-dependent. In reality, most drug effects are a function both of extent and duration of exposure. For example, fluoroquinolones, which are typically classified as concentration-dependent agents, can appear to act in a time-dependent manner if drug concentrations at the site of action decrease below bactericidal drug concentrations (i.e. the drug concentrations needed to exert a killing effect) for a duration that exceeds the pathogen's duration of post-antibiotic effect (PAE). PAEs are discussed later in this section.

Drug classification of concentration-dependent or time-dependent killing is primarily a function of the shape of its concentration-effect curve. The steeper the curve, the smaller the impact of increasing drug concentrations on the antimicrobial response. Conversely, the more shallow the curve, the greater the relationship between the rate of bacterial kill versus the antimicrobial drug concentration.

As discussed by Toutain (2002), this concentration–effect relationship is frequently described by the sigmoidal E_{max} model (also known as the Hill equation):

$$E(t) = E_0 + \frac{E_{max} \times C(t)^h}{EC_{50}^h + C(t)^h}, \quad (1)$$

where,

E(t) is the effect observed for a given concentration (*C*) at time *t*
E_{max} is the maximal effect attributable to the drug
EC_{50} is the plasma concentration producing 50% of E_{max}
h is the Hill coefficient, which describes the steepness of the sigmoidal relationship between log of the concentration and effect
E_0 is the rate of spontaneous cure.

When $h = 1$, the Hill model reduces to the E_{max} model, which corresponds to a hyperbolic function.

For drugs exhibiting time-dependent killing (e.g. the β-lactams), the duration of exposure needed to achieve a targeted log-reduction in colony forming units (CFUs) is a function of the magnitude of the PAE (Nicolau 2001). Mouton et al. (2005) defined the in vitro PAE as the period of suppression of bacterial growth after the drug has been removed following a short duration of exposure to that antimicrobial compound (unit = time). However, as discussed by Owens and Ambrose (2007), although these predictions have often proven useful, the sudden on–off modality of these in vitro tests may not adequately reflect in vivo conditions where concentrations are constantly changing with time. Therefore, they defined an in vivo PAE as the difference in the time needed for the number of bacteria in a tissue of treated versus control animals to increase by tenfold once the drug concentrations in serum or at the infection site have decreased below the MIC (unit = time). Accordingly, the in vivo PAE includes any effect associated with drug concentrations that are less than the MIC (sub-MIC effects).

For many compounds, the duration of the in vivo and in vitro PAEs is substantially greater for Gram-positive than for Gram-negative pathogens. As the duration of the in vitro and the in vivo PAE of β-lactams tends to be negligible for Gram-negative species, it is often recommended that the concentrations of drug remain above the MIC of those pathogens ($T >$MIC) for $\geq$ 80% of the dosing interval. In contrast, a $T >$MIC of about 40% is normally considered to be adequate for Staphylococcal species. This difference in the duration of the in vitro and in vivo PAE also appears to be a reason why the in vivo AUC/MIC for fluoroquinolones tends to be less for Gram-positive as compared to Gram-negative organisms (Forrest et al. 1993; Ibrahim et al. 2002; Preston et al. 1998; Wright et al. 2000).

A summary of the nature of the drug effect (killing versus stasis), the mechanism through which the drug effect occurs, and the relationship of the drug effect to duration versus extent of exposure, is provided in Table 1.

Table 1 Relationship between drug, drug effects, and the PD surrogate most closely aligned to its clinical response (Martinez et al. 2006; Chambers 2006; Steenbergen et al. 2005; Safdar et al. 2004)

Mechanism of action	Drug	Activity	Bacterial effect	Duration of in vitro PAE	PD parameter
Agents affecting the function of 30s and 50s ribosomal units, resulting in a reversible inhibition of protein synthesis (and therefore generally are bacteriostatic)	Macrolide	Static	Time-dependent		
	Erythromycin, etc.	Static and cidal (e.g. cidal for *S. pneumoniae*, *S. pyogenes*)		Brief[a]	$T >$MIC
	Azalide			Prolonged	AUC_{24}/MIC
	Lincosamides (Clindamycin)			Brief	AUC_{24}/MIC
	Ketolide (Telithromycin)			Prolonged	AUC_{24}/MIC
	Chloramphenicol Florfenicol Thiamphenicol	Primarily bacteriostatic, but cidal against some pathogens. Exhibit both Gram+ and Gram– activity	Time-dependent	?	$T>$MIC
	Tetracyclines				
	Traditional (e.g. Chlortetracycline)	Static	Time-dependent	Prolonged	AUC_{24}/MIC
	Atypical (e.g. Chelocardin and Anhydrochlortetracycline)	Cidal	Time-dependent	Prolonged	AUC_{24}/MIC
Inhibition of cell wall synthesis	Beta-Lactam Penicillin Carbapenem Monobactams	Cidal	Time-dependent	Gm(–) none or brief Gm(+) may be prolonged	$T>$MIC
	Glycopeptides (e.g. Vancomycin, Teicoplanin, Bloodying)	Cidal (slower than beta lactams)	Time-dependent	Prolonged	AUC_{24}/MIC
Agents that bind to the 30s ribosomal subunit, inhibiting bacterial protein synthesis, leading to aberrant proteins and eventually cell death	Aminoglycoside (e.g. Gentamicin and Tobramycin)	Primarily cidal	Concentration-dependent	Prolonged	AUC_{24}/MIC C_{max}/MIC

(*continued*)

Table 1 (continued)

Mechanism of action	Drug	Activity	Bacterial effect	Duration of in vitro PAE	PD parameter
Alter nucleic acid metabolism: inhibit DNA gyrase, thus preventing transcription and replication	Fluoroquinolone	Cidal	Concentration-dependent	Prolonged	AUC_{24}/MIC C_{max}/MIC
Agents that act as anti-metabolites (e.g. block folate metabolism by inhibiting dihydrofolate reductase)	Trimethoprim	Static alone Cidal with sulfonamides	Time-dependent	Brief[a]	T>MIC
	Sulfonamides (PABA analogue)	Static	Time-dependent	Brief[a]	T>MIC
Inhibits initiation of protein synthesis (at 50s ribosomal subunit	Oxazolidinones (e.g. Linezolid)	Static (staphs and enterococci) Cidal (most streps)	Time-dependent	Brief[a]	T>MIC
Depolarizes bacterial cell membrane[b]	Cyclic Lipopeptide (e.g. Daptomycin)	Cidal (Gram +)	Concentration-dependent	Prolonged (>6.8 h)	AUC_{24}/MIC C_{max}/MIC

[a]Brief equals less than an hour, prolonged may be up to 6 h

[b]Unique mechanism of action leads to a near absence of microbial resistance

3 Resistance

3.1 Resistance Mechanisms

There are numerous mechanisms through which bacteria can develop resistance to antimicrobial drugs. These all involve a change in proteins made by the cell and include:

- Alteration in ribosomal binding sites)
- Gene up-regulation (e.g. synthesis of inactivating enzymes such as β-lactamases)
- Changes in target site binding (e.g. alteration in penicillin binding proteins)
- Changes in membrane permeability
  - Porin closure
  - Efflux pumps

These changes in protein synthesis are caused by changes to DNA which are

- Plasmid-mediated
- Mutational
- Conjugation
- Ingestion of DNA-materials

The mechanisms through which resistance occurs have generally been considered to be specific to the drug class and to the bacterial species against which the drug acts. However, there are an increasing number of examples where resistance mechanisms traverse drug classes, including classes such as phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A (Long et al. 2006). Table 2 provides a summary of the mechanisms of drug resistance that have traditionally been associated with specific drug classes.

3.2 Defining the Term "Resistance"

Although some bacteria have exhibited natural resistance even prior to the use of antibiotics (Davies 1994), the emergence of resistance in previously susceptible bacterial populations has been attributed to the use of antimicrobial agents. Terms such as intrinsic and acquired resistance and single, multiple and cross-resistance have been introduced to describe the nature and clinical implications of these changes in bacterial subpopulations (Prescott and Baggot 1993).

The global impact of a shrinking therapeutic arsenal has precipitated numerous efforts to track the emergence and prevalence of drug resistance. Expert panels have been convened to consider potential solutions to this problem. However, discussions of this issue and interpretation of study data have been confounded by a lack of uniformity in how the term "resistance" has been defined. To illustrate this point, Davison et al. (2000) published definitions of antibiotic resistance, confirming the

Table 2 Mechanisms of drug resistance (modified from CLSI M31-A3)

Class	Examples	Mechanisms of resistance
Aminocyclitols	Apramycin	Modifying enzymes
	Spectinomycin	Efflux
		Ribosomal mutations
Aminoglycosides	Gentamicin	Modifying enzymes
	Kanamycin	Decreased permeability
	Neomycin	Target site modification/mutation
	Streptomycin	Efflux
β-lactam/β-lactamase inhibitor combinations	Amoxicillin/clavulanic acid	Inactivating enzymes – β-lactamases
Cephalosporins	Cefadroxil	Inactivating enzymes – β-lactamases
(first-generation)	Cefazolin	Reduced permeability
	Cephalexin	Altered pencillin-binding proteins
	Cephapirin	Efflux
Cephalosporins	Cefaclor	
(second-generation)	Cefuroxime	
Cephalosporins	Cefotaxime	
(third-generation)	Cefpodoxime	
	Ceftiofur	
Cephalosporins	Cefepime	
(fourth-generation)	Cefquinome	
Carbapenems	Imipenem	
	Meropenem	
Folate Pathway Inhibitors	Sulfonamides	Decreased permeability
	Trimethoprim	Production of drug-insensitive enzymes
	Trimethoprim-sulfamethoxazole	Overexpression of sensitive enzymes
Glycopeptides	Vancomycin	Target site (cell wall) resistance
	Teicoplanin	
Lincosamides	Clindamycin	Decreased ribosomal binding
	Lincomycin	Inactivating enzymes
	Pirlimycin	
Macrolides	Erythromycin	Target site (ribosome) modification/mutation
(14-membered ring)		
Macrolides	Azithromycin	Decreased permeability
(15-membered ring)	Gamithromycin	Inactivating enzymes
	Tulathromycin	Efflux
Macrolides	Aivlosin	
(16-membered ring)	Spiramycin	
	Tilmicosin	
	Tylosin	
Class	Examples	Mechanisms of resistance
Orthosomycins	Avilamycin	Decreased ribosomal binding
Oxazolidinones	Linezolid	Ribosomal mutations
		Ribosomal modification
Penicillins	Amoxicillin	Inactivating enzymes – β-lactamases
	Ampicillin	Reduced permeability
	Penicillin	Altered pencillin-binding proteins
	Oxacillin	Efflux

(*continued*)

Table 2 (continued)

Class	Examples	Mechanisms of resistance
Phenicols	Chloramphenicol Florfencicol	Target site (ribosome) modification/ mutation Decreased permeability Inactivating enzymes Efflux
Pleuromutilins	Tiamulin Valnemulin	Ribosomal mutations Ribosomal modification Decreased Permeability
Quinolones/Fluoroquinolones	Nalidixic Acid Ciprofloxacin Danofloxacin Difloxacin Enrofloxacin Marbofloxacin Orbifloxacin	Target site (DNA gyrase, topoisomerase IV) mutation Decreased permeability Efflux Target site protection
Streptogramins	Dalfopristin Pristinamycin Virginiamycin	Target site (ribosome) modification/ mutation
Tetracyclines	Chlortetracycline Doxycycline Oxytetracycline Tetracycline	Efflux Ribosomal protection Drug detoxification Target site (ribosome) mutation
Ionophores	Monensin Narasin Lasalocid	Not known

complexity of this problem. For example, Harrison and Lederberg (1998) defined antibiotic resistance as a property of bacteria that confers the capacity to inactivate or exclude antibiotics, or as a mechanism that blocks the inhibitory or killing effects of antibiotics. Alternatively, the Committee for Veterinary Medicinal Products (CVMP) in the European Medicine Evaluation Agency (EMEA) (EMEA report: Antibiotic Resistance in the European Union Associated with Therapeutic Use of Veterinary Medicines, 1999) defined microbiological resistance as either: (a) organisms that possess any kind of resistance mechanism or resistance gene; or (b) an infection where the bacteria do not respond to therapy.

Within a clinical context, antibiotic resistance has traditionally been used to indicate the likely therapeutic outcome rather than an epidemiological attribute. In other words, resistance has historically been described in terms of expressed phenotype. However, more recently, it is being defined in terms of genotype. This change has lead to difficulties in comparing data generated using different methodological approaches. For this reason, within the context of an epidemiological study, Davison et al. (2000) have suggested that the following criteria be considered:

- Resistance must be regarded as a quantifiable (qualitative or quantitative) variable at the level of either the bacterial or host population and must be defined with respect to a reference population.

- The detection methodology must possess known and quantified sensitivity, specificity, repeatability and reproducibility.
- The target bacterial and host populations must be precisely defined.
- The sampling framework must be fully specified, indicating how the samples are selected from the bacterial or host populations or the environment, including the various levels of organisation within these populations or ecosystems and the number of units from which samples are selected.

The distinction between clinical resistance (which is related to pathogen susceptibility, PK, and the approved dosage regimen) versus epidemiological cut-off values (which is purely a function of pathogen susceptibility) is fundamental to how we consider resistance (Bywater et al. 2006; Simjee et al. 2008; Turnidge and Paterson 2007). Accordingly, the definition of antibiotic resistance will vary as a function of study objective. As such distinctions are frequently difficult to discern and/or disentangle; there is a critical need to establish a common language and common standards. This is particularly relevant when comparing resistance rates within a global context. For example, similar to the previously mentioned EMEA 1999 report, resistance is defined both from a clinical and an epidemiological perspective in the United States Food and Drug Administration (FDA) as it applies to the term "resistance" in their (FDA/CVM Guidance #152). Within that guidance document, the FDA states that "human exposure through the ingestion of antimicrobial resistant bacteria from animal-derived foods represents the most significant pathway for human exposure to bacteria that have emerged or have been selected as a consequence of antimicrobial drug use in animals". From this, it would appear that both clinical (human exposure through the ingestion of antimicrobial resistant strains) and epidemiological (have emerged or have been selected as a consequence of antimicrobial drug use in animals) interpretations have been applied within the context of this guidance.

In addition to the challenge of defining "resistance", we need to consider the source of resistance data, which in greater part, comes from surveillance studies. The World Health Organisation (WHO) published a document entitled, "Surveillance Standards for Antimicrobial Resistance" (WHO 2001), which states that for a variety of reasons, "data obtained from clinical sources are generally unrepresentative of the totality of disease within a population". The report emphasised the importance of understanding the relationship between the surveyed populations in the context of the wider population. As part of this discussion it was accepted that across a global environment, there are inconsistencies in the submission, analysis and use of microbiological specimens. Furthermore, the report points to the limited epidemiological relevance of the resulting conclusions unless the susceptibility data are linked to disease incidence.

While this WHO report focused primarily on surveillance data generated within human populations, the same principles apply to animal surveillance systems. As corroborated by Franklin et al. (2001) in a report prepared by the Office International des Epizooties (OIE) ad hoc group of experts on antimicrobial resistance, "it should be borne in mind when designing resistance monitoring and surveillance

programmes that results from diagnostic submissions may not reflect the resistance situation in the animal population, as these types of submissions tend to include specimens from severe and/or recurrent clinical cases, including therapy failures". Franklin et al. (2001) further explain that because these isolates are likely to represent biased samples, this type of susceptibility data may not indicate the true prevalence of resistance within the given animal population. Therefore, caution should be exercised when interpreting these data. The group emphasised that a mechanism for mitigating this bias would be to "consider collection of samples from primary clinical cases not previously treated with antibiotics, or the isolation of potentially pathogenic bacteria from healthy animals". For food-producing animals, the collection of samples at the time of slaughter rather than from clinical cases can be used to monitor resistance as the animal enters the human food chain. Such information will help track the global impact of antimicrobial usage in veterinary species.

Table 3 provides selected examples to highlight the differences in global surveillance systems. All have the endpoint of reporting percentage resistance. The examples have been confined to surveillance of zoonotic and veterinary pathogens. For examples of surveillance in human pathogens, all of which use clinical breakpoints, see Masterton (2008).

3.3 Monitoring Programmes: Points to Consider

It is not always appropriate to compare antibiograms of veterinary and human isolates of the same pathogen (an antibiogram is the in vitro profile of an organism's response to a panel of antibiotics, which can be used to determine the sensitivity of an isolated bacterial strain to different antibiotics). In addition to inconsistent definitions of resistance, there may be differences in the methodologies used to determine minimum inhibitory concentration (MIC) values. Furthermore, even when considering one invariant organism–agent combination, there remain multiple variables that can influence the outcome of monitoring studies. When ignoring differences in methodology to determine MIC values, definitions of the term 'resistance' or bias in sampling data (all of which substantially influence the outcome), it is clear that the most important sources of variation in the interpretation of susceptibility data appear to be country, host species and the year of isolation. Unfortunately, it is often impossible to find comparable data for which only one of these parameters differ.

An example of an initiative for generating human–veterinary comparisons comprises an attempt to combine data from Italy (Moroni et al. 2006) with that generated in Germany (Kaspar 2006). The German GERM-Vet monitoring programme was established for the purpose of determining the current status of antimicrobial susceptibility of animal pathogens in Germany. This human–veterinary information gathering initiative introduced scientific rigour to the surveillance process, addressing many of the variables that could otherwise confound interpretation of data (Wallmann et al. 2003).

Table 3 Global examples of how "resistance" has been defined

Originator	Context	Resistance definition	Reference
National Antimicrobial Resistance Monitoring System – Enteric Bacteria (NARMS)	Established in 1996 as collaboration between FDA Center for Veterinary Medicine, U.S. Department of Agriculture and the Centers for Disease Control and Prevention. Monitors changes in antimicrobial drug susceptibilities of selected enteric bacteria in humans, animals, and retail meats to a panel of antimicrobial drugs important in human and animal medicine. The ultimate goal is to prolong the lifespan of approved drugs by promoting prudent and judicious use of antimicrobial drugs and to identify areas for more detailed investigation	Clinical Breakpoints (Clinical Laboratory Standards Institute)	http://www.fda.gov /cvm/narms_pg.html
European Union	To determine trends and sources of zoonoses, zoonotic agents and antimicrobial resistance in the E.U	Epidemiological cut-off values	Bronzwaer (2008)
The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP)	DANMAP, established in 1995 by the Danish government as a coordinated national surveillance and research programme for antimicrobial consumption and antimicrobial resistance in bacteria from animals, foods and humans. Monitoring of antimicrobial resistance is based on: human and animal pathogens, zoonotic bacteria and indicator bacteria	Epidemiological cut-off values	http://www.danmap.org
Enter-Net	Enter-net is administered by the European Centre for Disease (ECDC) Prevention and Control; it is an international surveillance network for human gastrointestinal infections in Europe, covering infections with Salmonella, verocytotoxin-producing Escherichia coli O157 (VTEC) and Campylobacter. ECDC aims to maintain and develop further surveillance activities and international development in this area	Dependent on country but varies across Europe	http://ecdc.europa.eu/en/Activities/Surveillance/Enter-net/

Within a veterinary framework, Hendriksen et al. (2008a, 2008b) surveyed resistance across Europe among bacterial pathogens and indicator bacteria for pigs and cattle. While their work did not incorporate harmonisation efforts into the data collection process, it did provide a positive step forward towards the creation of national surveillance schemes.

3.4 Impact of Antibiotic Use on Resistance

Since humans share a number of pathogens with food-producing animals (where they can be pathogenic or benign), it is frequently alleged that particular resistance patterns detected in human pathogens result from veterinary antibiotic use. The use of antibiotics as growth promoters in agriculture was challenged and was subsequently banned in Europe (Phillips 2007).

Even though some of the proposed links between observed resistance in particular bacterial species and the use of particular drugs in agriculture are seemingly obvious, a detailed examination of available data reveals the presence of complex issues and disparate opinions. There are authors who believe it unlikely that agricultural usage poses a major risk to human health. These authors base their opinion either upon conclusions that there is little to no role of veterinary usage in the selection or propagation of resistant strains, or that such situations, if they exist, pose minimal risk to human health (see review of Wassenaar 2005 and Casewell et al. 2003; Phillips et al. 2004a, b; Phillips 2007). Conversely, other investigators subscribe to a different view (Aarestrup et al. 1998; Bager et al. 2002; Chiller et al. 2004; Collignon 2004; Jensen et al. 2004; McEwen and Fedorka-Cray 2002; Phillips et al. 2004a, 2004b; Phillips 2007; Tollefson 2004). This ongoing debate, as exemplified by the diversity of views in these citations, reflects the difficulty associated with assigning causality to human adverse effects as a consequence of antimicrobial resistance arising from antimicrobial drug use in animals.

Regardless of the varying opinions on this topic, recent evidence clearly supports the view that certain antimicrobial agents may contribute to the prevalence of plasmid-mediated drug and indeed chromosomal resistance in bacteria that infect both humans and animals (Turnidge 2004). Since animals and humans provide overlapping reservoirs of antimicrobial resistance determinants, the issue cannot be ignored. The concern is, therefore, that indiscriminate use of antimicrobial agents in food animal production may result in the transfer of resistant bacteria and resistance genes to human pathogens, thereby compromising the treatment of infectious diseases in people (McEwen and Fedorka-Cray 2002; O'Brien 2002; Turnidge 2004).

Despite differing opinions of the extent to which the use of antimicrobial drugs in food animals poses a threat to human health, most workers agree on the importance of minimising the potential health hazards resulting from antimicrobial resistance. One suggested solution was to eliminate the use of all antimicrobial drugs used in humans as animal growth promoters. However, some investigators

claim that the presumptive human health benefits of employing such an approach is unsubstantiated by meaningful clinical data (Pfaller 2006; Phillips 2007). Wassenaar et al. (2007) addressed the concern that fluoroquinolone-resistant Campylobacter infections could result in more severe disease than susceptible strains. In a detailed analysis of the apparent link between fluoroquinolone resistance in *C. jejuni* and its presumed increased virulence, Wassenaar et al. (2007) argued that there were no significant differences in the duration of disease between susceptible and resistant infections. However, for both resistant and susceptible infections, disease symptoms were prolonged by 1–2 days on an average in British patients that had recently travelled abroad compared to those who had not travelled. This finding illustrates that even this seemingly straightforward issue may not be as clear-cut as it originally appeared.

Threlfall et al. (2006) examined changes in the occurrence of antimicrobial resistance in other zoonotic pathogens, *Salmonella enterica* serotypes Enteritidis and Typhimurium obtained from human infections in England and Wales (2000, 2002 and 2004). These investigators showed that the incidence of strains of *S. enteritidis* with resistance to nalidixic acid, coupled with decreased susceptibility to ciprofloxacin, had more than doubled between 2000 and 2004. In contrast, the overall levels of resistance in *S. typhimurium* had fallen by approximately 25%. These data demonstrated that changes in the incidence of resistance did not correlate with veterinary usage (as measured by veterinary sales of antimicrobial drugs in the UK). Furthermore, the study showed that:

- For *S. enteritidis* the important factors associated with an increased incidence of resistance were foreign travel and the consumption of imported foods contaminated with drug-resistant strains.
- For *S. typhimurium* the most important factor was an overall decline in the occurrence of multiple drug-resistant *S. typhimurium* definitive phage type 104, reflecting a change in serotype prevalence.

In their final analysis, Threlfall et al. (2006) concluded that changes in the incidence of resistance in predominant *Salmonella* spp. in human patients in England and Wales are multifactorial. The important point is that resistance rates were linked to serotype prevalence rather than to antibiotic usage.

Wassenaar and Silley (2008) also reviewed susceptibility profiles for a range of host-specific pathogens and antimicrobial compounds, in an attempt to identify lessons learned when considering bacterial pathogens that differed in host specificity. Their survey included bacterial pathogens that were limited to a human host, those specific to particular food producing animals and those that occur in both host types. From their examination of *Staphylococcus aureus, E. coli* and *Salmonella enterica*, they noted that Salmonella resistance profiles seemed to be strongly correlated to specific serovars (where serovar, or serotype, is defined as the identification of surface antigens that allow the epidemiologic classification of organisms at the sub-species level). Fluctuations in the dominant serotype were the most important factor determining the *Salmonella* resistance level. Furthermore, they found that multidrug resistance was a more severe problem in human pathogens

than it was in veterinary pathogens. Although pathogens isolated from both human and veterinary hosts appeared to have higher incidences of resistance as compared to those restricted to a single animal host, pathogens expressing the most marked (broad) drug resistance profile were found to exclusively infect humans. Thus, differences occurred in the available genetic repertoire of a bacterial species and were reflected in the observed resistance patterns. Because of these genetic differences, bacterial species undergoing comparable selection pressures in different host species do not automatically result in similarly resistant populations. Rather, the prevalence of resistance within a bacterial species can differ between populations isolated from different hosts and, for some species; fluctuations in dominant subpopulations (for instance particular serotypes) can be the most important factor determining resistance.

4 Pharmacokinetic Considerations

4.1 General Considerations

When deriving and applying PK/PD relationships for antimicrobial drugs, it is important to recognise that it is the free (non-protein bound) drug concentrations in plasma or serum that most closely reflect active drug concentrations at the site of infection (Liu et al. 2002; Merrikin et al. 1983). Therefore, although plasma free drug concentrations do not necessarily translate directly into the concentration–time profiles at the site of infection for extracellular infections, concentrations at the infection site (the biophase) are usually represented (at least proportionally) by plasma free drug concentrations. Accordingly, it is generally recommended that PK/PD relationships should be based upon free drug concentrations (Liu et al. 2002).

While there are a number of factors that determine and contribute to the PK/PD indices (e.g. AUC/MIC, C_{max}/MIC, $T >$MIC) and to numerical PK/PD targets (e.g. AUC/MIC=100 h), there are several crucial factors that the PK/PD approach does not adequately address. For example, the in vitro MIC, which is the universally used PD component of these indices, does not provide information on the time to kill, the time to maximum kill, the log change within a fixed time, or the maximum reduction in viable bacterial counts (MacGowan and Bowker 2002). Moreover, MICs are normally determined by doubling dilutions and are therefore subject to considerable inaccuracies. There is the additional consideration, that bacterial growth and growth inhibition by antimicrobial drugs in vivo may differ considerably between artificial growth media (Illambas et al. 2009; Potter et al. 2009a, b) There are also many other drug effects in vivo that are unrelated to the MIC but which can influence clinical outcome (e.g. anti-inflammatory and immunomodulatory effects, the ability to interact with dormant phase bacteria in a biofilm, the ability to interfere with bacterial colonisation on epithelial surfaces, and the influence of the drug on toxin production and release). These additional determinants of

therapeutic outcome are difficult to assess in the absence of actual clinical data. Furthermore, plasma drug concentrations, the primary component for building the PK portion of the PK/PD index, do not necessarily reflect in a predictable manner a compound's ability to diffuse first into the site of infection and then into the bacterial cell.

It is clear that the PK/PD variable providing the most appropriate surrogate for drug effectiveness is dependent upon several factors. These include the drug's mechanism of action, whether its effects are time or concentration-dependent, and the duration of its PAE. Examples of targets proposed for a range of compounds and pathogens have been reviewed by Gunderson et al. (2001).

For drugs exhibiting concentration-dependent killing, C_{max}/MIC ratios may be particularly important when the pathogen has a high MIC value or is proliferating rapidly. Such conditions lead to a greater risk of mutational events that could lead to a resistant subpopulation (Craig and Dalhoff 1998). In infectious diseases, a high bacterial burden (inoculum effect) can also increase the risk of a mutational event due simply to the laws of probability (Drusano et al. 1993; Craig and Dalhoff 1998). Therefore, in the presence of a high bio-burden, for drugs exhibiting concentration-dependent killing, the goal is to achieve high drug concentrations and therefore rapid killing (Drusano et al. 1993; Preston et al. 1998). The therapeutic objective is to reduce bacterial numbers to a level at which the host can destroy those bacteria that are not killed directly by the antimicrobial agent (and to avoid the double step mutation).

Monte Carlo simulations (a method for generating a pseudo-random set of parameter values that conform to some a priori probability distribution, such as normal, log-normal, uniform etc.) allows for the prediction of antimicrobial effectiveness in patients whose physiological attributes are likely to be encountered under clinical conditions (Zelenitsky et al. 2005; Drusano 2007). Once a numerical value for a PK/PD target has been defined (e.g. AUC/MIC = 100 h), the goal is to estimate the dose needed to achieve that target in the patient population. The proportion of the population for which the target is achieved (e.g. the proportion of subjects in the intended patient population that are expected to achieve an AUC/MIC value of 100 h) is termed the target attainment rate (TAR). The TAR is usually set at 90% (i.e. that 90% of the patient population will achieve the targeted PK/PD target). An important component of strategic dosing paradigms is an appreciation of the population variability that exists, not only in terms of bacteria but also in terms of the host. Within veterinary medicine, the latter consideration is often ignored and PK characterisation is usually based upon data generated in normal healthy animals. The use of such data results in estimates that normally and possibly markedly under-estimate the variability likely to occur in the true patient population (Martinez and Modric 2010). As a result, dosage regimens may fail to achieve the intended TAR.

To illustrate this point, nine AUC datasets ($n = 1000$ iterations per set) were generated (using a Ln-normal distribution), each described by the same mean value (125 μg × h/mL), but with the percentage coefficient of variation (%CV) varying from 15% to 100%. This situation is comparable to the difference between studies

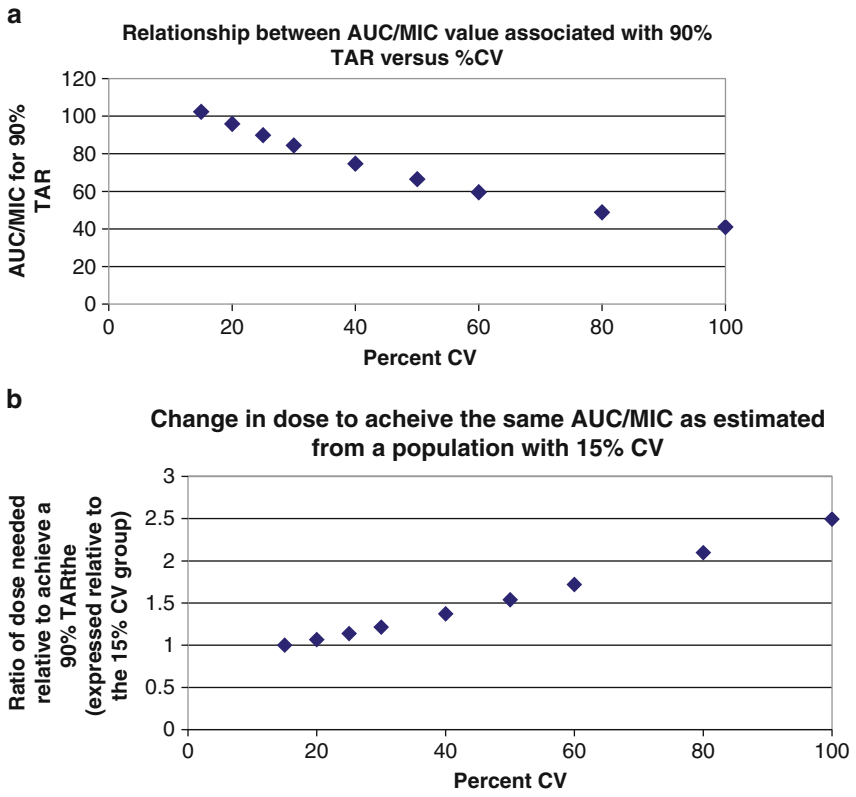

Fig. 1 (**a**) The influence of variability (expressed as %CV) on the AUC/MIC value associated with a 90% TAR. (**b**) The influence of variability on the dose needed to achieve a 90% TAR for AUC/MIC = 100 h. The *X*-axis represents the %CV of the simulated population. The *Y*-axis reflects the ratio of the dose for any %CV relative to that needed when the %CV = 15

conducted in a very homogeneous animal population under well-controlled laboratory conditions versus the wider range of PK characteristics that are likely to be observed under actual conditions of use. For simplicity, the MIC_{90} in this case was assumed to be 1 $\mu g \times mL^{-1}$, and the target AUC/MIC was assumed to be 100 h. Under these conditions, we calculated the AUC/MIC value associated with a 90% TAR. This is illustrated in Fig. 1a, which demonstrates that, as variability increases, there is a marked change in the AUC/MIC value achieved by 90% of the simulated population (i.e. the 90% TAR), even though the average AUC/MIC value in the population remained constant.

The final step shows the impact of population variability on dose selection. In other words, assuming that the goal is to have a dose that results in a 90% TAR for a target AUC/MIC of 100 h, how would the estimated dose change as population variability increases? For simplicity, all estimated dosages are expressed relative to

the dose that would be calculated if it was based upon a well-controlled PK study where the %CV was 15%. The results of this exercise are shown in Fig. 1b. This Figure shows that, within the range of variances used in the simulated dataset, the actual dose needed to achieve the targeted exposure was almost 2.5-times greater when the CV was 100% as compared to the dose needed when the %CV was 15.

4.2 Mutation Selection Window

The in vivo relevance of a mutation selection window (MSW) hypothesis was first presented by Baquero (1990), when he proposed the existence of a range of drug concentrations within which organisms which had reduced susceptibility had a survival advantage. In their review, Epstein et al. (2004) discuss how subsequent studies by Baquero and Negri (1997), Zhao and Drlica (2001), and Dong et al. (1999, 2000) served to define the boundaries for a "dangerous zone", and they described the relationship between mutational events and resistance to quinolones. Similarly, in a rabbit pneumonia model, gatifloxacin was found to pose the greatest risk of selecting for resistant strains when dosed in accordance with a regimen that produced the longest duration of exposure within the MSW (Croisier et al. 2004).

Drlica and Zhao (2007) defined the MSW from three discrete concentrations:

- The MIC of the wild-type bacteria.
- Concentrations above the MIC of the wild-type bacteria, where there is a plateau in killing due to the survival of the least susceptible microbial subpopulation.
- Concentrations at which even the least susceptible organisms are killed. The latter is termed the mutation prevention concentration (MPC). At the MPC, a cell must acquire two concurrent resistance mutations for selection of resistant strains. Such an event is highly unlikely. The MPC is estimated as the drug concentration that blocks growth when 10^{10} cells are applied to agar.

The window hypothesis is important because dosing guided by traditional PK/PD standards often places antimicrobial concentrations inside the MSW, where they can selectively enrich resistant mutant subpopulations. This can adversely affect drug response not only in the infected individual but also in subsequent hosts. Once amplified, the mutant cells can disseminate to a fresh host. Consequently, bacterial population expansion occurs and a new round of antimicrobial pressure can further enrich the mutant population (Croisier et al. 2004). Ultimately, such a scenario can lead to a predominance of the mutant subpopulation and loss of antimicrobial effectiveness over time (Epstein et al. 2004). However, Zhao and Drlica (2008) emphasised that there is a distinction between absolute clinical resistance, where the pathogen cannot be killed by the drug, versus resistant mutants, where the pathogen may be killed at higher drug concentrations. Therefore, they suggested that the indices of AUC/MPC or T>MPC may be preferable to those based upon MIC values. It might be noted that there is not a clear relationship between MIC and MPC values within and between bacterial species (Drlica et al. 2006). In fact, some

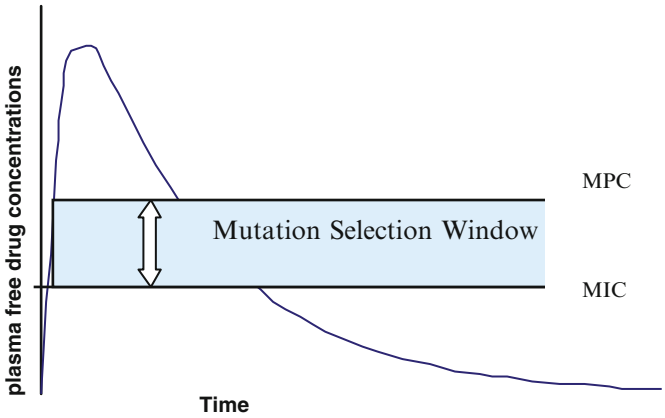

Fig. 2 Diagrammatic representation of the MSW, as defined by the upper and lower bounds, MPC and MIC, respectively

resistance mutations have a much larger effect on MPC than on MIC values (Zhao and Drlica 2008).

An illustration of a MSW is provided in Fig. 2.

With recognition of MSW concepts, there is an ongoing effort within human medicine to explore alternative dosing strategies that will minimise the emergence of resistant microbes. For example, Zhao and Drlica (2008) recommended the use of escalating dosing strategies to kill susceptible cells while preventing the amplification of resistant mutant subpopulations. Alternatively, others suggest that for difficult (severe) infections, aggressive therapy should be employed. This includes high dose – short duration (HDSD) therapies (Stein 2008; Schrag et al. 2001) or the administration of large doses of potent and highly efficacious antimicrobials for short periods of time followed by dose de-escalation (Siegel 2008).

4.3 Duration of Dosing

Relatively little experimental work has focused on the duration of therapy. In this regard, the choice of dose and duration of therapy will generally have the greatest influence in circumstances where resistance develops as a point mutation. In those situations, resistance can develop when the size of the bacterial population exceeds the inverse of the mutational frequency to resistance (Tam et al. 2007). For quinolones, target site mutations and/or efflux-pump overexpression occurs with a frequency of ~$1/10^6$–$1/10^8$.

In a study designed to examine the relationship between the duration of drug exposure and the emergence of drug resistance, Tam et al. (2007) employed in vitro infection models. In comparison with traditional rodent models, such in vitro models allow for longer therapy durations and the generation of exposure profiles

that are similar to those in humans. Using a hollow fibre system and *S. aureus*, Tam et al. (2007) demonstrated a positive connection between the duration of garenoxacin therapy and a decrease in the response to that antimicrobial agent. They showed that doses producing an AUC_{24}/MIC ratio of 114 h caused slightly greater than a 5 $\log_{10}$ (cfu $\times$ mL^{-1}) decline in the population if the administration was extended for 96 h. However, if the same dose was administered for an additional 24 h or even longer, resistant populations began to emerge. They demonstrated that the AUC_{24}/MIC ratio necessary to suppress the resistant subpopulation over a period of 48 h was 101 h whereas an $AUC_{24/}$MIC ratio of 279 h was necessary when using a full 10 days course of therapy. This reflects a nearly threefold increase in drug exposure needed to minimise the selection of resistant bacteria.

The authors suggested that one reason for their observation may be the presence of a pre-existent resistant sub-population. Amplification over time was a function of drug pressure and of the change in the MIC caused by the mutation. The authors also pointed out that, in the case of quinolones, another possible explanation is that these agents interfere with DNA replication and cause surviving staphylococci to undergo error-prone replication. Efflux-pump over-expression (by induction) may partially account for the emergence of resistance. However, there may be an interaction between these two mechanisms such that the early induction of efflux pumps provided a small survivorship advantage and therefore time for additional rounds of replication. Mutations can then arise from error-prone replication mechanisms. As the duration of therapy increases, there is the risk of a greater number of rounds of error-prone replication.

Although it may be considered appropriate to use findings such as these to establish regulatory authority guidelines regarding the duration of therapy, Tam et al. (2007) have pointed out that controlling drug dose and duration may not be consistently helpful in the prevention of resistance. When DNA is transferred by plasmids, transposons or transformation, it is unlikely that the selection of dose and duration will have an important impact. This is also true when horizontal transmission of resistant microorganisms occurs. They do, however, point out that, in circumstances in which dose and duration choice is helpful, resistance suppression means that there will be no resistant microorganisms to transmit horizontally.

In response to these studies, Drusano et al. (2009) questioned the ultimate impact of dose and duration on the relative proportions of wild-type and resistant bacteria once therapy was discontinued. To address these concerns, they generated data on the basis of four, five and six doses of the quinolone garenoxacin against the same strain of *S. aureus* used in the studies published by Tam et al. (2007). Observations continued for more than one week after the final drug exposure.

Their study clearly showed that, in all instances, the rate of initial cell kill was consistent with what had previously been observed, and the effect of drug on the resistant population was similarly in agreement with the earlier work by Tam et al. (2007). When treatment was terminated, the total population continued to decline for approximately 36 h, a finding attributed to slowly declining drug concentrations. Thus, Drusano et al. (2009) demonstrated that even non-optimal dosing regimens can be of therapeutic value if there is sufficient kill to allow host defences to

eradicate the remaining bacterial burden. Under such conditions, it is possible to use a dosing regimen without selecting for resistant strains, if the duration is short enough to avoid eradicating the susceptible population. When the susceptible population is eradicated, the resistant population begins to increase and the host defences can no longer control the infection. Clinically, this would be perceived as the emergence of resistance with clinical failure. Secondly, Drusano and colleagues also concluded that, because the growth rate of the susceptible population is always significantly greater than that of the resistant population, the resistant organisms tend to be less 'biologically fit'. The latter may have important implications with respect to the long-term prevalence of wild type versus resistant strains. Ultimately, they concluded that even sub-optimal dosing regimens will have minimal long-term impact on resistance selection pressure if the duration of dosing is short enough to avoid eradicating the susceptible subpopulation while amplifying the resistant subpopulation.

4.4 PK/PD is More Than MICs

While numerical values of PK/PD parameters have been proposed for many antibacterial drugs, most studies have evaluated the impact of antibiotic exposure on clinical efficacy rather than their impact on resistance development. It is only recently that attention has been focused on resistance prevention (Blondeau et al. 2004; Boak et al. 2007; Lees et al. 2006; Rybak 2006). In this regard, criticism has been levied against the use of MIC as the PD index, although the increasing use of MPC does help to address this deficiency (Blondeau et al. 2004).

Nevertheless, neither the MIC nor the MPC provides a complete explanation of the responses to antimicrobial agents. For example, as noted by Tam et al. (2007), PK/PD proposals relating to the prevention of resistance are difficult to substantiate, if the prevalent resistance mechanisms are the result of transmissible elements. Furthermore, MIC values provide no information on the rate of kill. In fact, even for a single drug and a single pathogen species, there can be marked variations in the killing activity across bacterial strains, even when the strains share the same MIC value. Noel et al. (2007) showed that within a bacterial species, there was strain to strain variation that impacted on the PK/PD index, and that such variability needed to be considered when targeting specific therapeutic outcomes. It is important to recall Zhao and Drlica's proposal that the PK/PD metrics for avoiding the selection of resistant strains may be better made on the basis of PK/MPC than on PK/MIC. Recent in vitro studies on strains of the pathogens *M. haemolytica* and *P. multocida* isolated from cases of calf pneumonia have also revealed the importance of recognising inter-strain variabilities in the time–kill relationships for the antimicrobial drugs amoxicillin, florfenicol, marbofloxacin, oxytetracycline and tulathromycin (Illambas et al. 2009; Potter et al. 2009a, b).

It is interesting to speculate on the best surrogate marker for such strain to strain differences. An example is the response of two bacterial species to a single

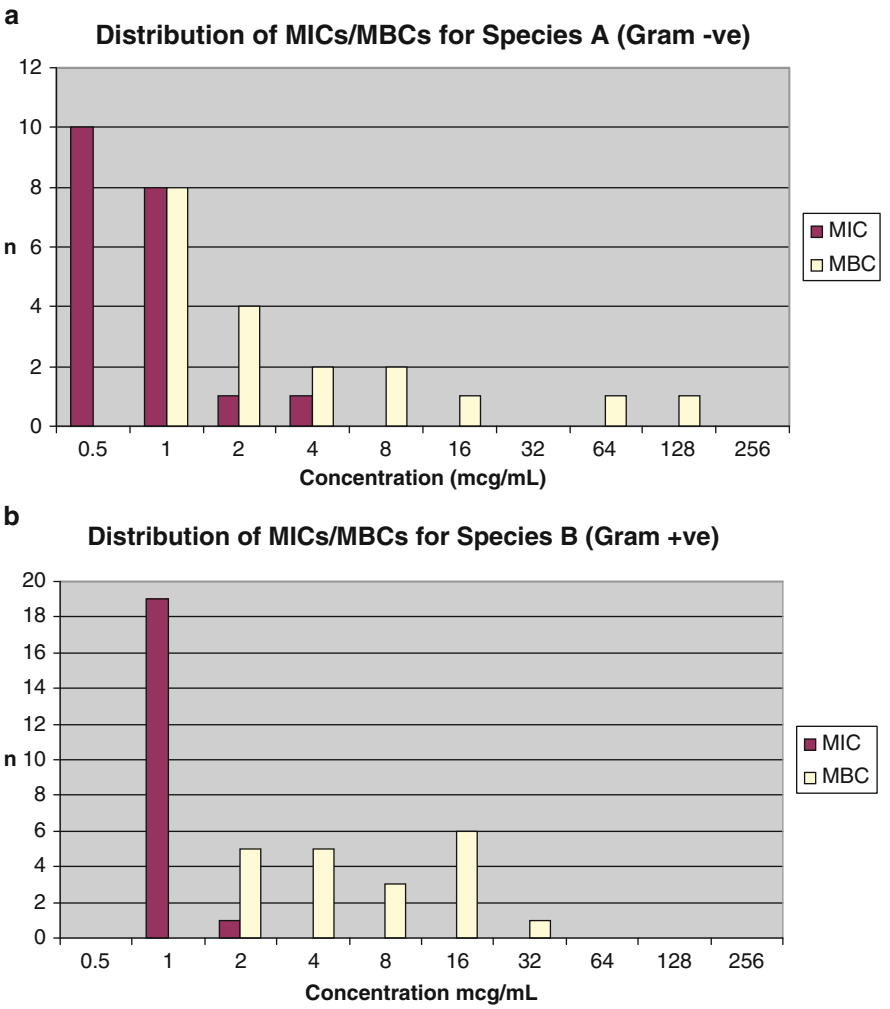

Fig. 3 (**a**, **b**) Comparison of the relationship between MIC and MBC for two microbial species: (**a**) a Gram-negative (−ve) organism; (**b**) a Gram-positive (+ve) organism

antimicrobial agent (Fig. 3a, b). It can be seen that in the first case, the minimum bactericidal concentration (MBC) distributions largely follow those of the MIC. In contrast, in the second example, the median MIC values are similar to that of the first (with the MIC values being only one dilution apart), but the MBC values have markedly different distributions. What is most disconcerting is that this kind of difference also has been observed within a bacterial species where strains exhibiting similar MIC distributions can exhibit markedly different rates of killing (P Silley, personal observation). Findings of this kind suggest the need to evaluate MBC, in addition to (or in lieu of) MIC, as a PD parameter.

In evaluating the PK/PD approach as a tool to predict the emergence of resistance, we should also be mindful of the impact of body fluids on PD. There are two issues to be considered: (1) the concentration of drug in the infected tissue; and (2) the influence of body fluid on the PD indices. For example, there can be as large as a fourfold greater concentration of many antimicrobials in gingival fluid versus serum (Conway et al. 2000; Lavda et al. 2004). This higher tissue site concentration has been attributed to the active transport of drugs, such as the fluoroquinolones and tetracyclines, by gingival fibroblasts (Yang et al. 2002).

Body fluids may impact on PD in a positive or negative manner. As an example of a positive effect, bacterial growth rate in serum tends to be slower than in broth (e.g. Bedenic et al. 2005). Therefore, studies that consider in vitro killing effects or bacteriostatic activity in artificial fluids are likely to produce conservative estimates of the killing or duration of static activity seen in vivo. An example of a negative influence is the impact of urine on fluorquinolone activity (Boy et al. 2004; Naber 2001; Well et al. 1998). Possible reasons for this include the effect of pH (ionisation reduced permeability of drug into the bacterial cell) or the effect of ions (chelating the drug and thereby reducing permeation into the bacteria (Zhanel et al. 1991). The impact of urine on antimicrobial activity has been addressed by generating data in an ex vivo model by determining the urinary bactericidal titres (Boy et al. 2004; Naber 2001; Well et al. 1998). Comparison of MIC and MBC values for amoxicillin, florfenicol, marbofloxacin, oxytetracycline and tulathromycin against several strains of the calf pathogens *M. haemolytica* and *P. multocida*, revealed differences between the matrices serum and Mueller-Hinton broth for all drugs; these were particularly marked for oxytetracycline and tulathromycin(Illambas et al. 2009; Potter et al. 2009a, b).

Another example of a negative interaction between drug and body fluid is the relationship between daptomycin concentrations in blood versus lungs and the resulting activity against pulmonary pathogens. Daptomycin has excellent in vitro activity against a large range of Gram-negative and Gram-positive bacteria. It was also found to be highly effective in reducing the number of CFUs of *S. pneumoniae* in a neutropenic mouse thigh infection model (Safdar et al. 2004). Although daptomycin is indicated for the treatment of complicated skin and skin structure infections caused by susceptible strains of Gram-positive microorganisms: *Staphylococcus aureus* (including methicillin-resistant strains), *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* subsp. *equisimilis* and *Enterococcus faecalis* (vancomycin-susceptible strains only) (FDA New Drug Application #21-572), it is not effective against community-acquired pneumonia. The reason for this is the binding and inactivation of daptomycin by human lung surfactants (Silverman et al. 2005).

4.5 Clinical Susceptibility Breakpoints

When samples are sent to the clinical laboratory for culture and susceptibility evaluation, the laboratory uses established “breakpoints” for making recommendations

of potential therapeutic agents. The classification of "susceptible" may not take full account of the ability of the antimicrobial agent to inhibit the pathogen. The PK of the drug, the formulation, the dosage regimen, and the likelihood of therapeutic success at that dosage regimen are all factors which should be considered when the susceptible (S), intermediate (I) and resistant (R) breakpoints are established for a drug. Based upon these S, I, R ratings, the clinical laboratory provides information on drugs that minimise the likelihood of therapeutic failure. Breakpoints are *not* intended as an assurance of therapeutic success.

The Veterinary Antimicrobial Susceptibility Testing Subcommittee (VAST) of the Clinical and Laboratory Standards Institute (CLSI) has recently published their M37 guidance, which describes the data and data interpretation used to establish veterinary susceptibility criteria (Clinical Laboratories Standards 2007). In veterinary medicine, these susceptibility breakpoints are disease-indication and target-animal-species specific, thereby allowing for potential differences in the susceptibility breakpoints across target animal species and within a target animal species across indications. By establishing the criteria correlating the required levels of drug exposure and the probability of success of therapy, clinically derived susceptibility breakpoints can minimise the risk of repeated exposure to suboptimal antimicrobial drug concentrations, which is a major factor contributing to the development of resistant bacterial strains.

5 Tolerance Versus Resistance

Traditionally, clonal bacterial populations have been considered to be both genotypically and phenotypically identical. However, newer techniques have caused this view to be challenged. It is now well established that phenotypic heterogeneity can exist within a clonal population due to "noise" in gene expression. This "noise" can arise as a result of stochastic variations in gene expression or in response to environmental perturbations (Jayaraman 2008). These variations cause drugs to lose, either partially or totally, their ability to fight infection (drug tolerance).

Drug resistance and drug tolerance differ, in that resistance mechanisms prevent the antibiotic from hitting a target, whereas tolerance works by shutting down the targets (Lewis 2008). Unlike resistance, which frequently involves genotypic changes, tolerance is the result of phenotypic changes. Moreover, in contrast to resistance, which is a function of individual cells, tolerance reflects a community-based synchronisation of gene expression that changes the bacteria from a growing to a slow or non-growing state (the biofilm). This conversion facilitates bacterial survival despite the presence of antimicrobial agents or adverse environmental factors without necessitating an expression of resistance mechanisms (Jefferson 2004; Keren et al. 2004).

Biofilms are densely packed communities of microbial cells that grow on surfaces and surround themselves with secreted polymers (reviewed by Nadell et al. 2009). Individual biofilms are also heterogeneous. Although the bacteria

may be genetically identical, the individual cells can express a wide variety of traits, including differences in basic metabolic activity, antibiotic tolerance spore formation, and the secretion of extracellular polymers. In fact, it is the secreted polymers that are a defining feature of biofilms.

Biofilms can include multiple bacterial species and they confer a survival advantage in several ways, including (Jefferson 2004):

- Protection against sheer forces and adverse conditions such as nutrient deprivation, changes in oxygen, changes in pH, or the presence of antibodies
- The facilitation of bacterial colonisation
- The integration of bacterial communal activities, thereby allowing for the division of labour to facilitate bacterial protection and metabolic integrity

This phenotypic transformation generally occurs deep within the biofilm and represents a slow or non-growing phase. The slow or non-growing condition leads to a reduced (or absent) sensitivity to certain compounds, particularly those that are dependent upon internal synthetic mechanisms, such as the β-lactams (Tanaka et al. 1999). For example, the reduced susceptibility to β-lactams is related to a diminished expression of penicillin-binding proteins and a decrease in the drug-induced inhibition of transpeptidases (Gilberta and Brown 1998). Such findings have led to the term "drug indifference" (Jayaraman 2008). However, biofilm-associated bacteria are not "resistant" to antimicrobials, and ultimately they revert back to a sensitive state once they convert back to the planktonic form. Furthermore, even non-growing biofilm cells remain sensitive to the fluoroquinolones or to drugs that interact with and disrupt cell membranes (Tanaka et al. 1999; Jayaraman 2008; Smith et al. 2009). It has been shown that C-8-methoxy fluoroquinolones are superior to other fluoroquinolones in their ability to kill under anaerobic conditions and they are also particularly effective even when protein synthesis is blocked (Malik et al. 2007). This may be a function of substitution at the C-8 position as pradofloxacin, with a C8-cyano grouping, has been shown to be active in the absence of protein synthesis (Körber et al. 2002). In vitro data illustrating the relationship between drug class, bacterial species, growth phase, and killing activity, is shown in Fig. 4 (based upon data from Eng et al. 1991).

Biofilm formation is now recognised to represent an important component of most human bacterial diseases including endocarditis, osteomyelitis, dental decay, middle ear infections, tuberculosis, medical device infections, and cystic fibrosis (Donlan and Costerton 2002). An example of a biofilm that has relevance in veterinary medicine comprises recurrent urinary tract (*E. coli*) infections (Opal 2007; Ball et al. 2008; Soto et al. 2006) and bovine (*S. aureus*) mastitis (Melchior et al. 2009).

In addition to biofilms, there are many circumstances in which relapses occur because surviving bacterial cells appear to have the capacity to resuscitate and divide once the concentration of the antimicrobial drug decreases below some critical value. Thus, in addition to typical slow growing or non-growing cells of the biofilm, there appear to be some bacteria that form "persister cells". These cells can survive assault from all antimicrobial agents (including fluoroquinolones and

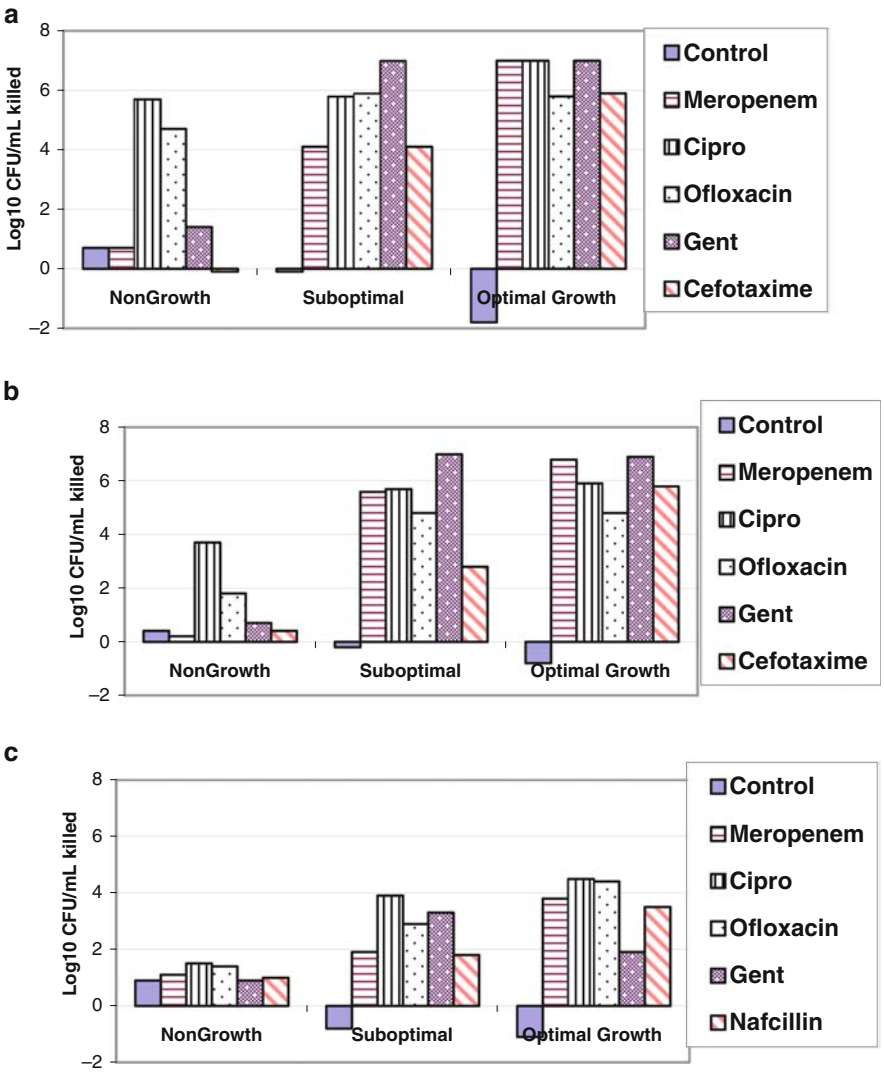

Fig. 4 (**a**–**c**) Relationship between drug, pathogen, growth phase, and antimicrobial cidal activities. Based upon data from Eng et al. (1991). Cipro = ciprofloxacin, Gent = gentamicin. (**a**) Relationship between growth phase and antimicrobial activity: *E. coli* (**b**) Relationship between growth phase and antimicrobial activity: *K. pneumoniae* (**c**) Relationship between growth phase and antimicrobial activity: *S. aureus*

those that disrupt bacterial cell membranes). They can repopulate the biofilm following discontinuation of therapy (del Pozo and Patel 2007). These cells forfeit rapid propagation to ensure survival of the bacterial population in the presence of lethal factors (Keren et al. 2004). They can exist in planktonic form or in biofilms

(Lewis 2007) and are largely responsible for the high tolerance of bacterial biofilms to antimicrobial drugs.

Persister cells are not identical to the non-growing cells within a biofilm, and drug indifference is not the same as persistence. While the lack of response to antimicrobials by dormant/slow growing cells occurs with no specific mechanistic basis, persistence is restricted to a very small subpopulation of cells whose tolerance occurs through some mechanism that as yet remains poorly understood (Lewis 2008; Jayaraman 2008).

In a typical acute infection, the initial treatment kills sensitive cells, but the persister cells are protected from the host defences and are not affected by the antimicrobial agents. It is for this reason that the recent modelling efforts support prolonged but periodic treatment protocols that address the heterogeneity in bacterial populations, rather than the continuous administration of the antibiotic typical of conventional clinical practise (De Leenheer and Cogan 2008). In other words, therapy occurs repeatedly, with each dosing period being of a short duration. This is reminiscent of the "duration" issue discussed earlier in this chapter.

Currently, it is believed that persister cells can occur both within and outside the biofilm matrix. Those that are outside the biofilm matrix (including planktonic persister cells) can potentially be killed by the host's immune system. However, persisters sometimes embed themselves in a manner that shields them from the host's immune defence system, such as those that reside in the central nervous system (*Treponema pallidum*), macrophages or granulomas (*Mycobacterium tuberculosis*) stomach (*Helicobacter pylori*), and gallbladder (*Salmonella typhi*) (Harrison et al. 2005; Jayaraman 2008). How these cells "recognise" places to hide is not known but, as discussed below, some investigators have suggested that pathogens can sense environmental conditions and communicate with other cells in a manner that maximises their likelihood of survival (Nadell et al. 2009).

Inter-communication between invading bacterial cells appears to be an important component of many infectious diseases. For example, the signalling system associated with *Pseudomonas aeruginosa* controls the production of factors implicated in virulence, and drugs that interfere with this system (e.g. azithromycin) seem to exert a significant positive effect on the clinical outcome of *P. aeruginosa* infections in cystic fibrosis patients (Winstanley and Fothergill 2009). Such cell-to-cell communication occurs via the secretion of a signalling molecule that is sensed by the overall bacterial population, leading to altered bacterial gene expression and thereby triggering the expression of virulence determinants. It has been suggested that this intercellular signalling network is responsible for coordinating the range of bacterial activities associated with the development and maintenance of biofilms (Nadell et al. 2009).

Studies to date have suggested that the communication molecules reach a critical concentration at a specific cell density (or "quorum"). Thus the term "quorum sensing" (QS) has been used to describe this signalling system (Horswill and Nauseef 2008). This sensing allows for the development of organised bacterial communities within the biofilm, which is reminiscent of a multicellular organism

(Keren et al. 2004). Clinically, relevant examples of QS in veterinary medicine have been reviewed elsewhere (Boyen et al. 2009).

QS is defined as first the capacity to detect extracellular, small molecule signals and then to alter gene expression in response to bacterial population densities. QS signalling is mediated by autoinducers that can be divided into three major classes: N-acyl homoserine lactones (AHLs) that are produced by over 70 species of Gram-negative organisms; oligopeptides that are generally produced by Gram-positive bacteria; and autoinducer-2 (AI-2) which is used by Gram-negative and positive pathogens and is thought to provide a mechanism for interspecies signalling (Kaufmann et al. 2008).

Elements of the QS apparatus of bacteria serve a variety of functions, including the coordination of gene expression within a bacterial species and either the inhibition or the activation of transcriptional programmes among competing bacterial strains and species. In response to these signals, some bacteria appear to exhibit a form of "altruistic" behaviour, undergoing a programmed cell death, releasing the sticky DNA that facilitates the formation of the biofilm matrix (Rice and Bayles 2008). This bacterial communication system can even alter transcriptional programmes in eukaryotic epithelial cells and immune effector cells (Asad and Opal 2008).

Staphylococci have QS systems in which the accessory gene regulator (agr) is genus specific and uses a post-translationally modified peptide as an autoinducing signal. *S. aureus* and *S. epidermidis* agr control the expression of a series of toxins and virulence factors and the interaction with the innate immune system. The *agr* system is a QS gene cluster that up-regulates production of secreted virulence factors and down-regulates production of cell-associated virulence factors in a cell density dependent manner. A second QS system of staphylococci, luxS, is also found in a range of Gram-positive and Gram-negative bacteria (Kong et al. 2006). Unlike many of the QS systems described in Gram-negative bacteria, agr and luxS of staphylococci reduce rather than induce biofilm formation and virulence during biofilm-associated infection. When the staphylococci are in lag phase, it is thought that staphylococci initiate infection by synthesisng surface proteins. Once colonisation is established, the bacteria multiply and enter an exponential phase, activating a density-sensing mechanism that stimulates toxic exoprotein production, thereby enabling them to spread to new sites to prevent overcrowding. The agr signalling molecule enhances biofilm detachment by up-regulation of the expression of detergent-like peptides, whereas luxS reduces cell-to-cell adhesion by down-regulating expression of biofilm exopolysaccharide (Projan and Novick 1997; Kong et al. 2006). Accordingly, it is not surprising that the *agr* gene appears to be responsible for the exponential-phase induction of toxin transcription in *Staphylococcus intermedius,* the most common cause of skin infection in dogs (Sung et al. 2006).

There are several examples in nature of mechanisms that inhibit bacterial QS (Horswill and Nauseef 2008):

- Marine algae produce compounds that compete for the AHL signalling mechanisms released by Gram-negative organisms.

- Mammalian immune cells can inactivate the QS molecules for many Gram-positive bacteria.
- Low pH can also inhibit the QS response of some Gram-positive pathogens, which may lead to an innate mechanism to minimise biofilm formation within certain body sites.

In human medicine, it has been observed that stress hormones or inflammatory cytokines can be detected by bacterial QS systems. This "timing mechanism" maximises the likelihood of successful bacterial proliferation by ensuring that bacterial growth coincides with patient vulnerability (Asad and Opal 2008). In fact, it has even been suggested that components of the host plasma proteins may exert a critical role in determining host susceptibility to bacterial infection. For example, Peterson et al. (2008) recently demonstrated that certain mammalian lipoproteins interfere with the conversion from tissue colonisation to host invasion by *S. aureus*. Interference of this switch is achieved by antagonising the *S. aureus* agr QS system that up-regulates genes required for invasive infection.

The mechanism of antagonism entails binding of the major structural protein of these lipoproteins, apolipoprotein B, to an *S. aureus* autoinducing pheromone, thereby preventing attachment of this pheromone to the bacteria and preventing subsequent signalling through its receptor, AgrC. Apoprotein B is an integral structural component of low-density lipoproteins and very low-density lipoproteins. Mice that were deficient in plasma apolipoprotein B, either genetically or pharmacologically induced, were more susceptible to invasive agr+ (an aggregation phenotype) *S. aureus* infection but not to infection with an agr deletion mutant. Therefore, apolipoprotein B at homeostatic levels in blood was concluded to be an essential innate defence effector against invasive *S. aureus* infection. This finding raises the question as to whether species differences in lipoprotein composition (Maldonado et al. 2001, 2002) may lead to interspecies differences in susceptibility to *S. aureus* infections. For example, dogs tend to have fewer problems with *S. aureus* infections compared to humans (Duquette and Nuttall 2004; Leslie 2008).

When considering these QS systems it is important not to exclude the potential for antimicrobial drugs to "interfere" with QS. Data are available that demonstrate both up- and down-regulation of protein synthesis when bacteria are exposed to sub-MIC concentrations of drugs, and that drugs such as the macrolides serve as QS inhibitors (Tateda et al. 2004).

6 New Approaches in Antimicrobial Therapy

Antimicrobial agents can exert a broad range of effects in addition to direct bacterial killing or interruption of bacterial replication. These effects, such as inhibition of bacterial toxin production via inhibition of microbial protein synthesis

(Shryock et al. 1998) and anti-inflammatory activity (Dalhoff and Shalit 2003) can play an important role in promoting a positive therapeutic outcome. For example, Slocombe et al. (1985) showed that the tissue damage associated with acute bovine pleuropneumonia, caused by *Mannheimia haemolytica,* is due to neutrophil-mediated damage to the bovine pulmonary parenchyma. In general, despite the obvious importance of the host immune response in combating acute infections, the excessive synthesis and secretion of inflammatory mediators can lead to increased morbidity and mortality. Therefore, antimicrobial drugs that minimise the secretion of these pro-inflammatory cytokines may provide a significant therapeutic benefit (Labro 1998).

Numerous classes of antimicrobials have been shown to provide therapeutic advantages that are attributable to actions in addition to their antimicrobial activities. For example, some antimicrobial drugs interfere with or induce the secretion of cytokines, thereby controlling the pro-inflammatory process (Reato et al. 2004). Mechanisms through which drugs can directly interfere with the host immune response include (van den Broek 1989):

- The inhibition of phagocytosis by binding of tetracyclines and bacitracin to divalent cations
- An interference of phagocyte H_20_2 production by sulfonamides and trimethoprim
- An increase in phagocyte uptake of *Streptococcus pyogenes* by the effect of sub-MIC concentrations of clindamycin and lincomicin on bacterial surface proteins
- A depression of chemotactic activity of granulocytes when filtrates of *Propionibacterium acnes* are exposed to gentamicin, erythromycin and minocycline
- Sub-MIC concentrations of tetracyclines decrease chemotaxis by binding to divalent cations

Because of the rapid rise in antimicrobial resistance, alternative strategies are being developed. In an attempt to minimise the proliferation of resistant strains, as encouraged through the therapeutic use of antimicrobial drugs, new therapeutic approaches are being sought. While traditional approaches target in vitro and in vivo pathogen viability (e.g. static and cidal compounds), new approaches target functions that contribute to pathogen virulence. Most importantly, such novel compounds are unlikely to present with the significant resistance concerns currently associated with traditional antimicrobial therapies. Examples of mechanisms of action currently being explored are provided in Table 4.

One of the difficulties associated with these novel approaches is that they often require that the clinician has an exact diagnosis of the cause of the infection. Moreover, traditional methods for evaluating appropriate therapies (i.e. in vitro susceptibility test procedures) will not be useful for evaluating the ability of these methods to counteract disease-causing virulence factors because such effects cannot be evaluated in the absence of the host environment. Clearly, these technologies, while holding much promise, also present many new and exciting challenges.

Table 4 Examples of virulence factors and new therapeutic approaches for targeting these factors (Periti and Mazzei 1998; Cegelski et al. 2008; Hanlon 2007; Liautard et al. 2006; Duckworth and Gulig, 2002; Clatworthy et al. 2007; Marriott et al. 2008; Lynch and Wiener-Kronish 2008; Schwegmann and Brombacher, 2008; Escaich 2008; del Pozo et al. 2009)

Factor	Mechanism of Action
Endotoxin	Neutralising activity (e.g. fluoroquinolones, aminogylcosides, glycopeptides)
Toxin	Reduce toxin production Reduce toxin delivery Reduce host response to toxin Antibody-based toxin neutralisation (anti-toxin antibodies, such as the antibodies used against botulinum neurotoxin)
Cytolysin	Counteract cytolysins (bacteria toxins that lead to lysis of host cells)=.
Adhesion factors	Inhibit bacterial formation of pili and fimbriae needed for adhesion to cell surfaces (pilicides)
Quorum sensing	See text
Cell integrity	Bacteriophage
Type III secretions	Antibodies binding to Type II injectosome (a needle like projection releasing toxins) and blocking toxin release
Bacterial species specific adhesion proteins	Reduce likelihood of biofilm formation, and therefore risk of chronic infections
Prevention of gene expression	For example, virstatin is a novel compound that inhibits expression of the toxT gene leading to the production of cholera toxin
Trojan horse strategy	Tricks bacteria to sequester a biologically inactive transition metal for $Fe3^{+}$, thereby repressing iron-dependent transcript
Inhibition of intracellular multiplication	Interfere with the synthesis of gene products needed for adaption to intracellular environment (i.e. low O_2 tension, amino acid starvation. Examples include *Legionella*, *Mycobacterium* and *Brucella*)
Electrically induced bactericidal activity	Low-intensity electrical current substantially reduced the numbers of viable planktonic bacteria or those that reside within a biofilm. This has been coined an "electricidal effect". The mechanism of action is unknown but has been suggested to result from toxic substances, the oxidation of enzymes and coenzymes, membrane damage leading to the leakage cytoplasmic constituents, and/or a decreased bacterial respiratory rate

7 Conclusions

An appreciation of the multi-faceted mechanisms through which antimicrobial drugs can exert their therapeutic effects, including the interruption of bacterial communication systems, altering host immune responses, altering host inflammatory responses, or interfering with pathogen virulence factors, will help to ensure that antimicrobial drug substance, drug product formulation and dose selection are based on the principles of prudent and rational use. Clinical and laboratory experiences point to the conclusion that the adoption of these principles will be critical in prolonging the life of our existing antimicrobial arsenal. However, in the final analysis, it is important

to remember that, it is the host immune system that is ultimately responsible for success in combating bacterial disease.

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Drug Residues

Philip T. Reeves

Contents

Abstract The use of veterinary drugs in animal production is necessary for the prevention and treatment of disease; however, such use may result in residues. Regulatory authorities administer legislative frameworks which ensure that foods derived from animals treated with approved veterinary drugs are safe for human consumption. A human food safety evaluation is conducted as follows: it estimates the risk to human health and safety – based on scientific assessment of the available information and data – formulates measures for controlling the risks identified, and communicates the findings and implications of the risk assessment to interested parties. Foods derived from animals are monitored for the presence of drug residues. The reported incidence of illegal residues from these programmes is very low. These findings reassure the public that veterinary drugs are effectively regulated and that food obtained from treated animals does not contain residues that might

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constitute a health hazard to consumers. Non-regulatory organizations, including the veterinary pharmaceutical industry, producer organisations, veterinarians and food processors, all contribute to a safe food supply. The food safety risk analysis framework is continually refined to ensure that the health of all consumers is protected.

Keywords Regulation · Veterinary · Drug · Residue · Food · Safety · Risk analysis · Trade

1 Introduction

Food-borne hazards are a worldwide public health concern (FAO 2006). Yet despite almost all food risks being microbiological in origin, the results of surveys indicate a perception that food-borne chemical hazards are a significant risk to public health (McLean 2000). Events such as the melamine-induced kidney damage and fatalities in infants and in dogs and cats resulting from the incorporation of melamine into the raw ingredients of powdered infant formula and pet food in China in 2008 reinforce these perceptions. While this is not a drug residue issue, it does emphasise the importance of effective food safety regulation. Unlike the situation with food composition, where information provided on labels allows the consumer to make an informed choice, a similar approach to chemical contamination of food is not practicable. Instead, therefore, consumers rely on regulatory authorities to ensure that foodstuffs obtained from animals treated with veterinary drug products do not contain drug-derived residues that might constitute a human health hazard. Consumers also rely on residue monitoring programmes, the published results of which provide overwhelming evidence for a safe food supply. In recent years, the issue of veterinary drug residues in animal-derived foods has become increasingly important in developing countries (Cannavan 2004). This position is not expected to change in the short term because the *per capita* consumption of meat (especially poultry) and dairy products has been estimated to increase by as much as 44% between 2002 and 2030 (Cannavan 2004).

Food-borne chemical hazards are also a major cause of trade problems internationally (FAO 2006). In this respect, effective food safety systems support the economic development of countries by providing a sound regulatory foundation for domestic and international trade in food (FAO 2006). However, regulatory frameworks for food safety are not consistent amongst jurisdictions. The differences in the national maximum residue limits (MRLs) are primarily attributable to differences in the level of risk that individual governments are prepared to accept, methodologies for establishing MRLs, and the conditions of use described in labelling of the product. The position is compounded when MRLs have not been established in countries where use of the veterinary drug is not approved. In order to address this issue and to facilitate trade, some importing countries have established “Import MRLs” for chemical–commodity combinations that are without national MRLs. The existence of differing national standards adversely affects

the international trade in animal-derived food commodities by requiring exporters to comply with a diverse range of standards imposed unilaterally by importing countries. MRLs for veterinary drugs have been developed by the Codex Alimentarius Commission (Codex) to facilitate fair practices in trade, while protecting consumer health and being compatible with established good use practices. However, Codex MRLs remain voluntary until they are accepted or used by countries. The World Trade Organisation's (WTO) "Agreement on the Application of Sanitary and Phytosanitary Measures" (SPS Agreement) specifically cites Codex standards, guidelines and recommendations as reflecting international consensus regarding the requirements to protect human health from food-borne hazards (WTO 1995). International trade agreements developed by the WTO emphasise the need for regulations governing international trade in foods to be based on science. However, other legitimate factors relevant to the health protection of consumers and the promotion of fair practices in food trade can override scientifically derived health standards (CCRVDF 2001).

The beef hormone trade dispute between the EU and the USA and Canada is one of the longest-standing disputes in the history of the WTO. A full account of the dispute can be found on the WTO website (http://www.wto.org). Of the six compounds in question, three are naturally occurring hormones (oestradiol-17β, progesterone and testosterone) and three are synthetic drugs which stimulate the actions of natural hormones (melengestrol acetate, trenbolone acetate and zeranol). While all these substances are approved for growth promotion use in cattle in the USA and Canada, the use of hormonal growth promotants has been prohibited in the EU since 1988. The importation of hormone-treated beef into the EU was subsequently prohibited on 1 January 1989. The USA, and later Canada, challenged the legality of the importation ban at the WTO in 1997. Since then, many WTO rulings relating to the dispute have been appealed and additional complaints have been filed by all parties involved. As recently as October 2008, earlier WTO decisions were overturned on appeal, allowing the EU to continue its ban on the importation of hormone-treated beef, and the USA and Canada to continue to take retaliatory measures against imports from the EU. The beef hormone trade dispute remained unresolved at the time this chapter was prepared.

2 The Food Safety Risk Analysis Framework

During the past 30 years, a risk analysis framework for food safety has been developed; it has gained wide acceptance as the preferred way to assess possible links between hazards in food and actual risks to human health (FAO 2006). Risk analysis comprises three major elements: risk assessment, risk management and risk communication. In the context of veterinary drug residues, the risk analysis framework provides for estimating the risks to consumer health, for identifying and implementing appropriate measures to manage those risks, and for communicating information on the risks and the measures applied in their management to stakeholders. Trade in

animal-derived foods is also facilitated when these principles and methods of the risk analysis framework are applied consistently amongst countries.

In this chapter, the various studies are discussed under the appropriate section of the risk analysis framework. However, there are no "hard and fast" rules in this respect. For instance, the iterative procedure for allocating MRLs in some jurisdictions involves both exposure assessment and risk characterisation.

2.1 Risk Assessment

In developed countries, veterinary drugs are regulated under legislation that requires their quality, safety and efficacy to be evaluated and deemed acceptable before marketing approval is granted. The data demonstrating the safety of residues in edible tissues and in products such as milk and eggs from treated animals relate principally to toxicology and residue chemistry. The risk assessment also draws on other disciplines, including pharmacology, microbiology, veterinary medicine, animal husbandry, epidemiology and statistics.

The assessment of dietary risk is conceptualised in the relationship:

$$\text{risk} = \text{hazard} \times \text{exposure}$$

where, "risk" is the probability of harm occurring to the consumer; "hazard" refers to the chemical residue representing a source of potential harm attributable to its intrinsic properties; and "exposure" refers to the dietary exposure to the chemical residue (Davies et al. 2003). From this relationship, it is evident that reducing hazard or exposure or both reduces risk.

The above relationship also highlights a fundamental difference between "hazard" and "risk". According to the IPCS (2004, p. 12, 13), "hazard" and "risk" are defined as follows:

Hazard is the inherent property of an agent or situation having the potential to cause adverse effects when an organism, system, or (sub) population is exposed.

Risk is the probability of an adverse effect in an organism, system, or (sub) population caused under specified circumstances by exposure to an agent.

The conduct of a food safety risk assessment requires that sufficient data of adequate quality are available. In recent years, harmonised protocols for toxicological food safety assessment have been agreed upon by the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH): these can be found on the VICH website (http://www.vichsec.org/). Additional VICH guidelines for metabolism and residue kinetic studies are currently under development.

The results of risk assessment are applicable only to the specified test substance; therefore, the chemical identity and properties of the substance are a critical component of the database. Information on chemical identity is provided by international chemical databases. Also critically important is the information relating

to the physicochemical properties of the pure active ingredient, the purity of the test substance, and any impurities present.

2.1.1 Hazard Identification

The purpose of this step is, firstly, to establish the identity of the drug molecules that are present as residues in animal-derived foods and which are capable of causing adverse effects on consumer health. Secondly, this step provides information on the effects that are considered to be adverse. Although the steps involving hazard identification and hazard characterisation often source their information from the same studies, the two steps assess different information. The preferred sources of information for the hazard identification step are human epidemiological studies, animal toxicological studies, in vitro assays, and quantitative structure–activity relationships (WHO 1995). Most commonly, a battery of tests in laboratory animals is conducted. It includes single dose toxicity studies, repeated dose (90-day) toxicity studies, reproductive toxicity studies, developmental toxicity studies, and long-term toxicity and carcinogenicity studies. A battery of genotoxicity assays is also conducted. The endpoints of these studies are the basis for identifying a No Observed Effect Level (NOEL), or a No Observed Adverse Effect Level (NOAEL), or a benchmark dose (BMD).

2.1.2 Hazard Characterisation

The hazard characterisation step in the risk assessment of veterinary drugs primarily focuses on the dose–response relationship for critical adverse effects, identification of the most sensitive animal species or strain for a given effect, and extrapolation from animals to humans. The traditional approach to toxicology data analysis assumes that a threshold dose can be identified in the dose–response relationship for a substance (situations where a threshold cannot be identified in the dose–response relationship are discussed below). At sub-threshold doses, there are no significant increases in the frequency or severity of the effect between the treated groups and the control groups. The NOEL is the highest dose that does not cause any detectable effect in the most sensitive species of test animals. Alternative approaches for characterising hazards have been reported (Crump 1984; Kroes and Kozianowski 2002); the BMD is one such approach. The BMD approach models all the available dose–response data and provides increased weighting to data near the observed dose–response range by using lower effective doses at, for example, levels causing 10% and 5% of a specified response (ED_{10} and ED_{05}). Safety factors are applied when deriving the BMD-based acceptable daily intake (ADI).

The ADI is the amount of chemical that may be consumed daily for an entire lifetime without causing an appreciable risk to human health. It is calculated by dividing the appropriate NOEL by a safety factor to account for the uncertainties encountered when extrapolating animal toxicity data to potential effects in humans

and for variation within humans. Guidance on the selection of appropriate safety factors has been published (IPCS 1987), and it is generally dependent on the nature of the critical effects and the endpoint, as well as the quality and quantity of the data used to make the safety assessment. Safety factors range in value between 10 and 2,000. Typically, a safety factor of 100 is applied when long-term animal studies are available. It allows for human beings to be, up to tenfold more sensitive than the test animals, and for tenfold variation between individuals within the human population (Rubery et al. 1990).

The value of an ADI for a chemical can differ amongst regulatory agencies due to differences in regulatory policies, or the choice of safety factors, or both. The ADI of a substance may be based on a toxicological, pharmacological or microbiological endpoint; and all relevant endpoints should be assessed when characterising the hazard. Derivation of the ADI is based on the critical endpoint observed, to result from the lowest dose. A pharmacological ADI is derived from a NOEL determined from pharmacological studies in laboratory animals or human pharmacological data, whereas a microbiological ADI is determined from studies into the effects on intestinal microflora. The studies for generating microbiological data may be conducted in humans, in vivo in gnotobiotic animals, or in vitro using species and strains of microorganisms representative of the human gut flora. The approaches used for establishing a microbiological ADI for residues in foods that are derived from animals treated with an antimicrobial agent were reviewed by Cerniglia and Kotarski (2005).

The residues of some compounds including pharmacologically active veterinary drugs are associated with short-term (acute) toxicity. To protect consumer health from such residues, some but not all jurisdictions establish an acute reference dose (ARfD) as the appropriate health standard. In addition, all jurisdictions base the ADI of substances on acute toxicity endpoints when it is appropriate to do so. Both the ARfD and the ADI protect consumers against acutely toxic residues in food. Allergenicity has not been a major issue with most of the veterinary drugs evaluated to date. An exception is benzylpenicillin, for which allergy was the determining factor in the safety evaluation conducted by the JECFA (WHO 1990). The evaluation did not establish an ADI for benzylpenicillin and instead recommended that the daily intake of penicillin from food be kept as low as practicable and always below 30 μg per person (0.5 μg/kg) of the parent drug.

The NOEL-safety factor approach is not considered to be suitable for setting acceptable intake levels for substances such as genotoxic carcinogens, whose effects involve non-threshold mechanisms. Genotoxic carcinogens may cause genetic alterations in target cells, either directly or indirectly, and they are assumed to be harmful at any level of exposure. Evidence for genotoxicity is generally obtained from mutagenicity testing. Genotoxic chemicals are banned from use in food-producing animals in many countries, while in other countries; their use is permitted at a level of risk that is sufficiently small to be deemed negligible. In contrast, non-genotoxic carcinogens per se do not cause mutations. These substances act at extra-genetic sites, causing enhanced cellular proliferation or sustained hyperfunction or dysfunction at the target sites, or both. In principle,

non-genotoxic carcinogens may be regulated using a threshold approach such as the NOEL-safety factor approach described above.

A critical consideration in the hazard characterisation step is that animal toxicological studies are conducted at concentrations several orders of magnitude higher than the concentration of chemical residues likely to be present in food. Before establishing the upper limit of the range of ADI of the residue, it is therefore necessary to determine the significance of responses detected in high-dose animal toxicological studies for low-dose human exposures. At the conclusion of this step, the upper limit of the range of ADI of the hazardous agent is known. It is then assumed that dietary intakes over a lifetime at or below this limit would not pose an appreciable risk to the health of consumers.

2.1.3 Exposure Assessment

The dietary intake of drug residues in food is estimated from the amount of the food commodity consumed, the concentration of residues present in the food consumed, and the ratio of marker residue to total residues. Only the first of these factors will be discussed here; the other two factors are discussed under risk characterisation later in this chapter.

Food consumption factors for the exposure assessment of veterinary drug residues are sourced from a widely used model. The model comprises 300 g of muscle (or muscle and skin in natural proportions in the case of fish), 100 g of liver, 50 g of kidney, 50 g of fat, 1.5 l of milk, 100 g of eggs and 20 g of honey. Fat in the model is replaced by the same amount of fat and skin in natural proportions in the case of pigs and poultry.

While all jurisdictions source food consumption factors from the model described above, the factors are used differently. Regulatory agencies in the European Union (EU) estimate the theoretical maximum daily intake (TMDI) for a 60-kg person according to (1):

$$\mathrm{TMDI} = \Sigma(\mathrm{Daily\ Intake}_i \times \ \mathrm{MRL}_i \times \mathrm{TR}_i/\mathrm{MR}_i) \tag{1}$$

where Daily Intake$_i$ (kg) is the daily consumption as defined in the model food basket; MRL$_i$ is the MRL (μg/kg) for muscle, fat, liver, kidney, eggs and honey; TR$_i$ is the total residue concentration (or pharmacological or microbiological activity where relevant) and MR$_i$ is the marker residue concentration (or pharmacological or microbiological activity where relevant) in the same tissues and commodities.

The JECFA adopted a new approach for estimating chronic dietary intake at its 66th meeting (WHO 2006). The estimated dietary intake (EDI) differs from the TMDI inasmuch as MRL used in the TMDI calculation is replaced with the median concentration of residue in the EDI calculation. In justifying the new approach, the JECFA contended that the MRL is not a realistic estimate of the residue concentration in a chronic intake scenario because it represents the estimated upper limit of a

high percentile (commonly the 95%) of the distribution of marker residue present in tissues of treated animals. By comparison, the median residue concentration represents the best point estimate of a central tendency over a prolonged period.

The EDI (μg) for a 60-kg person is determined as follows:

$$\text{EDI} = \Sigma(\text{Daily Intake}_i \times \text{Median residue concentration}_i \times \text{TR}_i/\text{MR}_i) \quad (2)$$

where Daily Intake$_i$ (kg) is the daily consumption as defined in the model food basket; median residue concentration$_i$ is the median residue concentration (μg/kg) for muscle, fat, liver, kidney, eggs and honey; TR$_i$ is the total residue concentration (or pharmacological or microbiological activity where relevant), and MR$_i$ is the marker residue concentration (or pharmacological or microbiological activity where relevant) in the same tissues and commodities.

The actual difference in value of the TMDI and the EDI varies on a case-by-case basis. For example, the FAO report of the 66th meeting of the JECFA cites TMDI and EDI values of 229 μg and 56.9 μg, respectively, for colistin and 55 μg and 29.1 μg, respectively, for erythromycin.

A recent paper published by the EMEA/CVMP noted that the EDI calculation was developed for the purpose of estimating the chronic daily intake and recommended the development of a complementary procedure for estimating acute daily intake (EMEA-CVMP 2008a). This recognises that many veterinary drugs elicit acute pharmacological effects. In Australia, the EDI calculation developed by the JECFA for estimating chronic daily intakes has been adopted and is used in conjunction with a procedure for estimating acute dietary intake. The latter has been described elsewhere (Reeves 2007). Briefly, the national estimated short-term intake (NESTI) of residues is calculated and reconciled with the ARfD of the veterinary drug. Separate NESTI calculations are performed for each of the edible tissues, and milk and/or eggs where applicable. This procedure is therefore dissimilar to an EDI calculation in which all edible tissues, and milk and/or eggs where applicable, are incorporated in a single calculation. Values of NESTI are estimated as the product of the 97.5% consumption value for Australian consumers for each edible tissue, and milk and/or eggs where applicable, and the highest residue concentrations reported in residue depletion trials. MRLs are not advanced if the point estimate of short-term dietary consumption exceeds the ARfD at the withdrawal period.

The procedure practised by the United States Food and Drug Administration (US FDA) for calculating dietary exposure is fundamentally different to those used to calculate the TMDI and the EDI. As residues are partitioned at an early step in the procedure for calculating a safe concentration of total residues, it is unnecessary to reconcile dietary intake and the ADI at a later stage. The US FDA approach uses the same food consumption factors as JECFA; however, it applies them differently. The US FDA assumes that if a person consumes 300 g of muscle tissue, then the person will not consume an allocation of liver or kidney but may consume a full allocation of milk and eggs. A safe concentration of total residues for edible tissues, and for milk and eggs where applicable, is initially calculated on the basis of the ADI and

consumption factors for the standard edible tissues (3). Only that portion of the ADI not reserved for milk or eggs is available for calculating a safe concentration of total residues in the edible tissues.

$$\text{Safe Concentration} = \text{ADI} \times 60\ \text{kg}/\text{Food Consumption Factor} \quad (3)$$

where, Safe Concentration is the safe concentration for total residues in a specified edible tissue as defined in the model food basket; ADI is the acceptable daily intake; the Food Consumption Factor is the daily consumption of the specified edible tissue.

In general, estimates of predicted dietary exposure to residues are unrealistically high compared to the actual dietary intake, which is often well below the ADI. This reflects the conservative nature of the assumptions used when estimating the predicted dietary exposure. These assumptions include the treatment of all animals at the maximum approved dose rate and duration and slaughter at the withdrawal period; the presence of residues in the edible tissues and products such as milk and eggs from all animals at MRL (for the TMDI calculation), or alternatively, at the median residue concentrations (for the EDI calculation); and the daily consumption for a lifetime of the model food basket by a 60-kg person.

2.1.4 Risk Characterisation

The risk characterisation step considers the results of the hazard identification, hazard characterisation, and exposure assessment steps of the risk assessment. In the context of veterinary drugs, the primary outcome of risk characterisation is a set of MRLs based on the ADI concept of "no appreciable health risk" and the model food basket (Arnold 2004). The established MRLs are subsequently used as tools by risk managers for protecting consumers against possible harmful effects. The MRL, known as a tolerance in the USA, is the maximum concentration of residue (expressed in mg/kg or μg/kg of food on a fresh weight basis) resulting from the authorised use of a veterinary drug that is recommended by the Codex Alimentarius Commission or national authorities to be legally permitted or recognised to be acceptable in or on food. However, not all substances leave residues that are considered to be a health risk to the consumer. While MRLs for these substances are not necessary, alternative regulatory mechanisms such as Annex II of Council Regulation (EEC) No 2377/90 in the EU and Table 5 of the MRL Standard in Australia must be complied with. Numerous excipients and some drugs such as ketoprofen for use in horses, cattle and pigs and ketamine for use in all food producing species in the EU, and ophthalmic preparations of some veterinary drugs for use in cattle and sheep in Australia, do not require MRLs. At the other extreme, the residues of certain substances constitute a health hazard to consumers irrespective of the concentration present in food. As a result, MRLs for these substances cannot be established and their use in food-producing animals is not permitted. Examples include chloramphenicol and the nitrofurans. With chloramphenicol,

there is evidence of genotoxicity in vitro; in addition, a dose–response relationship or threshold dose for the induction of aplastic anaemia in humans treated with the drug is lacking. In the case of the nitrofurans, there is evidence of genotoxicity and carcinogenicity.

Establishing MRLs requires information relating to how each drug product is to be used, including: the recommended dose and frequency of administration and application; pharmacokinetic and metabolic studies in experimental and food-producing animals; residue depletion studies with radiolabelled drugs in target animals; a description of the method of analysis for detecting and quantifying the residues, including the marker residue; and studies designed to assess the impact of residues of antimicrobial agents on food processing.

The use pattern of a drug determines its pharmacokinetic behaviour relating to absorption, distribution, metabolism and excretion (ADME), and therefore its residue kinetics. Pharmacokinetic studies, which are usually conducted in healthy animals and usually also of a similar age, weight and breed, should use the same mode of administration, product formulation, and dose and frequency of administration or application as proposed for the product's intended use(s). Concentration-time profiles of the parent drug and its metabolite(s) in tissues and body fluids are examined between the time of drug administration and the time of slaughter for human consumption. The residue data generated in these pharmacokinetic studies form the basis of comparisons with the MRLs proposed by the sponsor.

Metabolism studies in the food-producing animals are required to identify and quantify the residues before the safety of residues can be assessed. The design of these studies simulates the conditions of intended use(s) as closely as is practical. Typically, residue depletion studies use a radiolabelled drug to examine tissue profiles from zero withdrawal time to periods extending beyond the proposed withdrawal time. These studies define total residues, including free and bound residues, and major residue components. Major residues are those substances that contribute at least 10% of the total radioactivity or are present at a tissue concentration of 0.1 mg/kg or greater.

Bound residues may arise from the incorporation of residues of the drug into endogenous compounds, chemical reaction of the parent drug or its metabolites into macromolecules, or physical encapsulation or integration of radioactive residues into tissue matrices (WHO 1989). The incorporation of small fragments of the drug, usually consisting of one or two carbon units, into naturally occurring molecules is not of toxicological concern. In circumstances where bound residues are characterised and shown to be of no toxicological concern, total residues may be discounted by the equivalent amount of bound residues. This may be necessary when the bound residue comprises a significant portion of the total residue, or when a high concentration of bound residues prevents the total residue from depleting below the residue of toxicological concern and thereby precluding the assignment of a practicable withdrawal period. On this basis, the 70th meeting of the JECFA discounted bound residues of triclabendazole in tissues from cattle 28 days after treatment; total residues of triclabendazole accounted for 237% of the upper bound of the ADI compared with 31% for bioavailable residues (Reeves and Swan 2009).

A marker residue compound and a target tissue are selected on the basis of the findings of metabolism studies. A marker residue compound is selected that provides an assurance that when residues of the marker are compliant with MRL, all residues of toxicological concern are compatible with the ADI. As the marker residue may not be the only residue of toxicological or microbiological concern, its persistence in tissue must be sufficient to assure that all residues of toxicological concern have depleted prior to depletion of the marker residue. National governments and industry use the marker residue for MRL enforcement purposes.

The target tissue is the edible tissue selected for monitoring the total residue in the target animal species. The target tissue is usually, but not necessarily, the tissue with the slowest residue depletion rate. Not all jurisdictions select a single target tissue for monitoring residues: some jurisdictions regard any edible tissue for which an MRL has been established as a target tissue.

Comparative metabolism studies are conducted to ensure that the laboratory animals used in toxicity studies for deriving the ADI are exposed to the same array of substances as human consumers of edible tissues from treated animals (Ellis 2004). Auto-exposure of metabolites is taken as evidence that the safety of metabolites has been adequately assessed in the toxicological studies. In vivo comparative metabolism studies involve qualitative metabolite analysis of samples of blood, blood fractions, excreta, liver, bile, kidney and fat collected from the laboratory animal test species and the target food-animal species. Qualitative metabolite information may also be obtained from in vitro metabolism studies and structure–activity relationships.

The procedure for establishing MRLs for veterinary drugs is not consistent amongst the jurisdictions, or between the jurisdictions and the JECFA. The JECFA recommends MRLs that are no higher than necessary, that reflect the residues expected when the veterinary drug is used in accordance with good practice, and can be enforced by regulatory programmes using available analytical methodology. The JECFA procedure for establishing MRLs for veterinary drugs is shown in Fig. 1. It is an iterative process based on the data provided, and it is facilitated by the model food basket (WHO 2006). A statistical tool for establishing MRLs was agreed at the 66th meeting (WHO 2006), and it is now used routinely with all data sets that are suitable for statistical analysis. An important feature of the statistical tool is that it takes account of both the ADI and the kinetic behaviour of the residues in muscle, fat, liver and kidney. The MRLs allocated therefore maintain the tissue distributional relationships observed in pharmacokinetic studies. The statistical tool initially performs a linear regression analysis on the kinetic data describing the terminal depletion of the marker residue in edible tissues following the last administration of the drug. The results of the regression analysis are then used to estimate the upper limits of the 95% confidence interval for the upper one-sided tolerance limit on the 95% of the test animal population. Finally, the MRL is a point on the curve describing the upper one-sided 95% confidence limit over the 95%, at or beyond which the predicted median intake (EDI) is equal to, or less than, the ADI. The minimum number of food-producing animals recommended for metabolism studies varies with the animal species and the jurisdiction. In the case

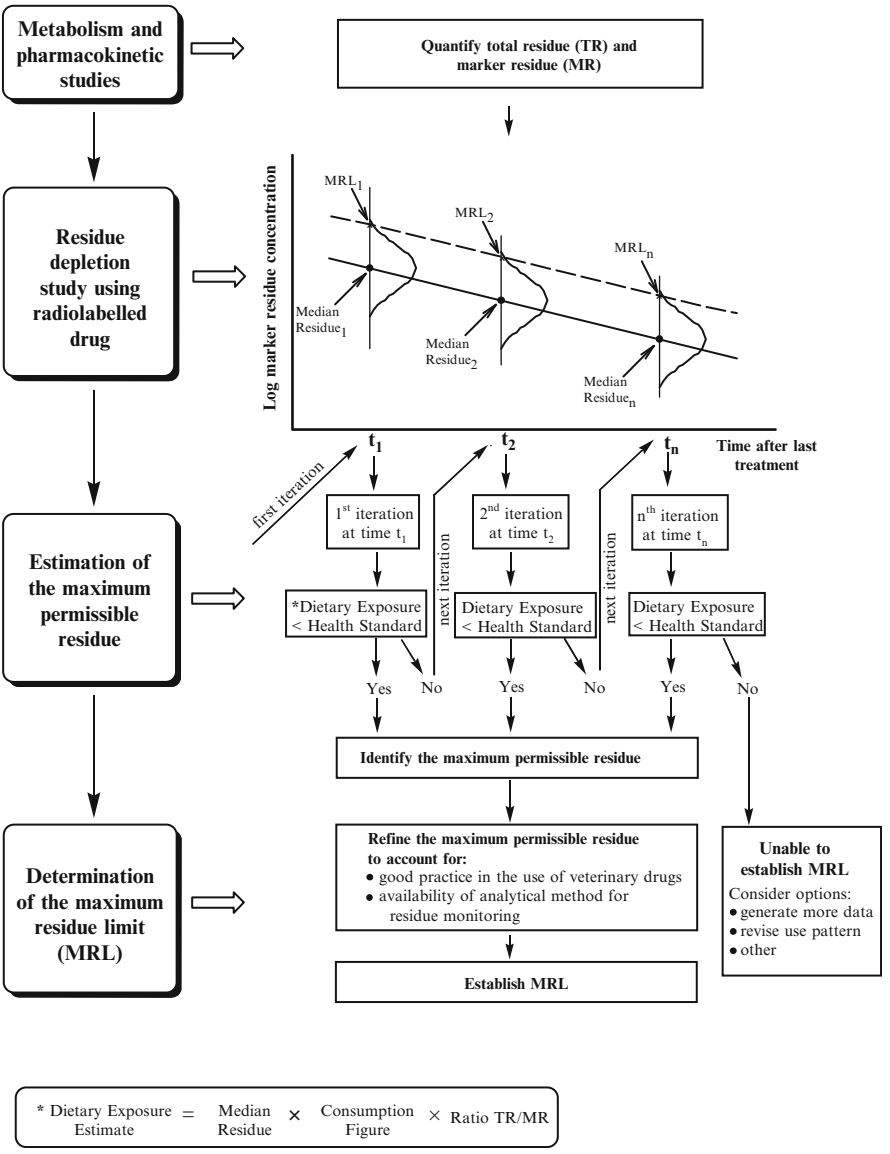

Fig. 1 Schematic diagram of the residue evaluation procedure practised by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The major elements of the procedure are depicted as *large boxes* (*left*) and the desired outcome of each process is identified (*large open arrows*). The establishment of maximum residue limits (MRLs) involves determination of the maximum permissible residue. This involves an iterative approach where residue values at time t_1 are used to estimate dietary exposure. If the estimated dietary exposure is less than the health standard, then the maximum permissible residue has been identified. Otherwise the process is repeated with values from times t_2 to t_n as required. The maximum permissible residue may be refined downwards to account for good practice in the use of veterinary drugs and the availability of suitable residue monitoring methods in establishing the MRL

of beef cattle, for example, the EMEA recommends at least twelve animals (four groups of three animals) whereas the FDA CVM recommends at least twenty animals (four groups of five animals or five groups of four animals). Presently, a VICH guideline for metabolism studies is under development, a major objective of which is to harmonise data requirements internationally.

Volume 8 of the Rules Governing Veterinary Medicinal Products in the European Community (European Commission 2003) documents the EU procedure for establishing MRLs for veterinary medicinal products. The overall approach is similar to that employed by the JECFA except for the exposure assessment (see (1) and (2)).

The US FDA food safety evaluation of veterinary drug residues calculates a tolerance (known elsewhere as a MRL; Freidlander et al. 1999). The tolerance is the concentration of marker residue at the time on the residue depletion curve corresponding to the time when total residues have decreased to the level of the safe concentration (Fig. 2; see (3) for the calculation of safe concentration). The tolerance is determined from a residue study using a radiolabelled drug, but it uses the proposed analytical method for an unlabelled drug. The analytical procedure typically uses the proposed method of analysis, and an in-line radiometric detection simultaneously quantifies the total residues and the marker residue. The ratio of the marker residue to the total residue is determined from the residue depletion curve, using the time when the concentration of total residue equals the safe concentration. The tolerance is calculated by multiplying the safe concentration by the ratio of the marker residue to the total residue.

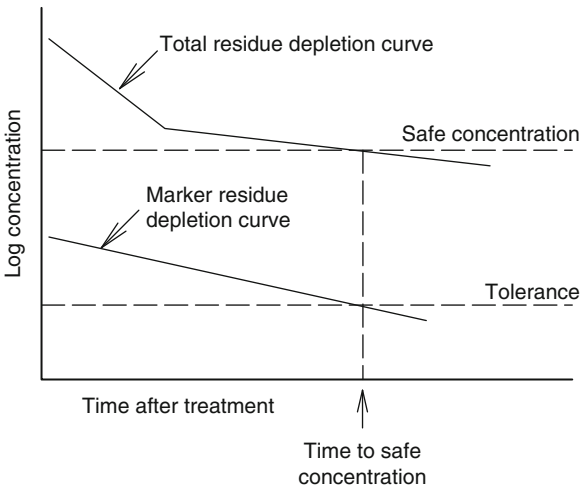

Fig. 2 Schematic diagram of the US FDA approach for determining the tolerance of a veterinary drug. The tolerance is the concentration of marker residue at the time on the marker residue curve (*lower curve*) corresponding to the time when total residues have decreased to the level of the safe concentration (*upper curve*)

Analysing for Residues of Veterinary Drugs in Depletion Studies

Methods of analysis for veterinary drug residues are required for the conduct of depletion studies in food-producing animals and the monitoring of food. Method validation provides assurance that a method is fit-for-purpose, and it produces results that can be reliably used for making decisions. The process of method validation involves a defined set of experiments to establish the performance criteria that should be achieved by an analyst using the method. The type of method and its intended use will determine the specific requirements of method validation (MacNeil 2004). In the case of analytical methods used in residue depletion studies, the performance characteristics that apply to the validation include linearity, accuracy, precision, limit of detection, limit of quantitation, specificity, stability of the analyte during analysis under stipulated conditions, and robustness of the method. Detailed information on the specific acceptance criteria relating to the performance characteristics for validation purposes can be found on the websites of the regulatory authorities. Currently, a VICH guidance on the validation of analytical methods used in residue depletion studies is under development.

In support of registration applications, sponsors typically submit to regulatory authorities a description of, and validation data for, the method used to quantify the marker residue in residue depletion studies. As data generated with this method are the basis for assigning withdrawal periods, the method must be validated across a range of marker residue concentrations, including one half and twice the MRL. The method should also be available for the enforcement of the proposed MRLs and for assisting laboratories in the development and validation of a multi-residue screening method or a confirmatory method or both.

Good Practice in the Use of Veterinary Drugs

Good practice in the use of veterinary drugs (GPVD) is defined as the official recommended or authorised usage, including withdrawal periods, approved by national authorities, of veterinary drugs under practical conditions (Codex Alimentarius Commission Procedural Manual, 18th edition, 2008). In some jurisdictions, veterinary surgeons may be subject to legislative sanction if found responsible for causing illegal residues in food (for example, *Veterinary Practice Act* 1997 (Victoria, Australia)).

Residues of Veterinary Drugs at Injection Sites

Injection site residues pose unique challenges in terms of public health and the international trade in animal-derived foods (Reeves 2007). The kinetic behaviour of drugs at injection sites is notable for high initial concentrations, which reflect the magnitude of the dose administered, followed (for some products) by slow and often erratic depletion. While the current risk assessment methodologies minimise

the dietary exposure of consumers to injection site residues, and therefore any risks associated with ingestion, they frequently result in very long withdrawal periods being assigned to the products. This discourages the development of long-acting injectable products by the veterinary pharmaceutical industry as well as their use by farmers. Possible approaches to decreasing the withdrawal period of long-acting injectable products were discussed in a recent publication (EMEA-CVMP 2008b).

At the international level, the food safety risk assessment of residues of veterinary drugs at injection sites is not consistent amongst jurisdictions (Reeves 2007). A major impediment to developing a harmonised risk assessment procedure is the scant objective information available on the probability of dietary exposure to injection site residues (EMEA-CVMP, 2005). In general terms, data are required in relation to the prevalence of injection site tissues in animals at slaughter, the fate of injection site tissues, and the incidence of residues present in injection site tissues. The prevalence of injection site tissues in animals at slaughter is confounded by differences in regional animal husbandry practices, which may prevent the findings of a survey for one category of livestock production, or for one region, being extrapolated to another. Similarly, little objective information on the fate of injection site tissues is presently available. Injection site tissue, when identified, is trimmed and discarded by meat-processing personnel to prevent it entering the human food supply; however, the proportion of the total number of injection site residues that are identified and trimmed is not known. Not all injection site tissues contain drug residues. Injection site tissue resulting from vaccination or consisting solely of scar tissue does not represent a chemical food-borne hazard for consumers.

The paper published by the Committee for Medicinal Products for Veterinary Use (CVMP) of the European Medicines Agency (EMEA) notes eleven possible approaches to the risk assessment of injection site residues. One proposal involved increasing the muscle MRL to facilitate a decrease in the withdrawal period of injectable products. The paper recognised that this would disrupt the tissue distributional relationships of residues and penalise those products administered by non-injectable routes. A different approach involved using the ARfD instead of the ADI as the permissible exposure standard. Acute toxicity considerations of the injection site residues are a current practice in Australia, Canada and the USA. The validity of this approach depends on the ingestion of injection site residues being a rare event. Acute toxicity considerations may shorten the withdrawal period, but this is only when the ARfD exceeds the ADI for the veterinary drug. From a residue surveillance perspective, this approach requires a sampling protocol that is able to distinguish between injection site muscle and non-injection site muscle. A dual sampling protocol for achieving this goal was proposed at the Codex Committee for Residues of Veterinary Drugs in Foods during the 1990s and early 2000s, but it was not adopted.

Sanquer et al (2006) demonstrated that the ADI-MRL concept for assessing chronic dietary intake of residues is not appropriate for use with injection site residues. These workers performed a qualitative exposure assessment to estimate the number of days on which an injection site, or part of an injection site, was ingested by EU consumers during 1 year. The risk assessment was modelled on all

animals being treated daily for 7 days with an antibiotic and, from official sales data, an estimate that 11.92% of these animals were administered injectable formulations. The study concluded that the maximum likelihood of a European consumer ingesting an injection site, or part of an injection site, was 4 days annually. Over the same period, 37% of European consumers would not ingest an injection site.

The sampling of injection site tissues is conducted in accordance with protocols described in regulatory guidelines (US FDA CVM 1994; EMEA-CVMP 2005). When conducting trials on multiple dosing regimens, injections are alternated between the left and right sides of animals; the last injection site is then sampled for residue analysis. In all jurisdictions, cylindrical samples weighing approximately 0.5 kg and measuring 10 cm in diameter and 6 cm in depth for intramuscular injections, and measuring 15 cm in diameter and 2.5 cm in depth for subcutaneous injections, are collected. A second concentric ring-shaped sample of approximately 0.3 kg collected from the region immediately surrounding the excised core sample is required by regulatory agencies in the EU only. All samples are homogenised thoroughly prior to sub-sampling for residue analysis.

2.2 Risk Management

2.2.1 Residues of Veterinary Drugs and Food Safety

The withdrawal period (also referred to as withdrawal time or withholding period) is the interval between the time of the last administration of a veterinary drug and the time when the animal can be safely slaughtered for food, or milk or eggs can be safely consumed. Compliance with the withdrawal period provides a high degree of assurance to both producers and consumers that the concentration of residues in foods derived from treated animals will not exceed the MRL. Withdrawal periods are typically assigned on the basis of results of a residue depletion study using non-radiolabelled drug in which the veterinary drug product proposed for marketing is administered at the highest label rate, the shortest dosing interval and for the longest duration. These conditions represent a worst-case scenario for residue depletion.

Calculating a Withdrawal Period for Edible Tissues

The EMEA published a guidance document on the establishment of withdrawal periods for edible tissues of food-producing animals (EMEA-CVMP, 1997). At the time of publication, three approaches to calculating withdrawal periods were used in Member States of the EU. The simplest method involved setting the withdrawal period at the time when residues in all tissues have depleted to below the respective MRL. An extension of this method involved addressing large variations in the

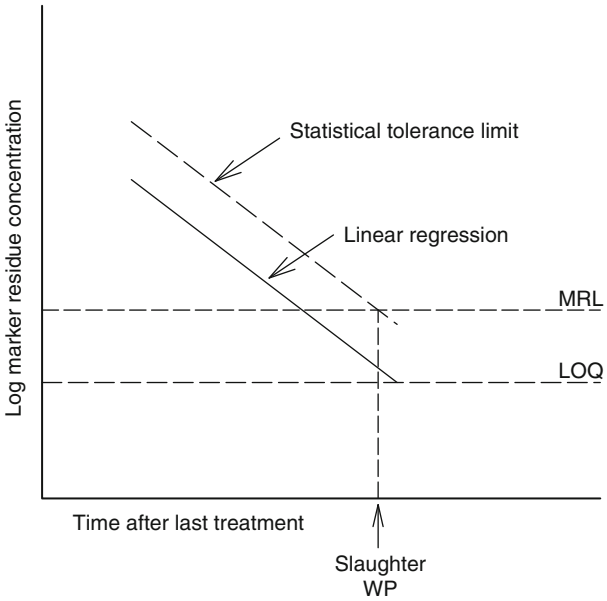

Fig. 3 Schematic diagram of the statistical approach for the calculation of a slaughter withdrawal period (WP). Residue concentration data are fitted with a least-squares regression line (*solid line*) and the upper statistical tolerance limit (*dashed line*) is calculated. The slaughter WP is the time when the upper statistical tolerance limit intersects with the maximum residue limit (MRL), rounded up to the nearest day

depletion data set, or shortcomings in the studies, by adding an extra safety period. A third method established withdrawal periods using a statistical approach. With the exception of some data sets that are not suitable for statistical analysis, the guidance recommends a linear regression technique as the method of first choice for calculating withdrawal periods in the EU.

The US FDA Center for Veterinary Medicine (1994) also utilises a statistical approach for calculating withdrawal periods for edible tissues. In general, the statistical methodology outlined by the US FDA and the EMEA are quite similar. Both use a log-linear assumption and least-squares regression to obtain a fitted line. From this fitted line, a statistical tolerance limit is calculated and its intersection with the MRL defines the withdrawal period, which is rounded up to the nearest day (Fig. 3).

Calculating a Withdrawal Period for Milk

The US FDA guidance (1994) recommends that linear regression be used to calculate withdrawal periods for milk. The method fits a regression line to the log residue concentration data of each cow before using the fitted lines to estimate the

distribution of log residue concentrations at each time. Estimates are made of between-animal variance and of measurement error variability, which are subsequently used to calculate a statistical tolerance limit at each time point. The estimated withdrawal period is the first time where the upper 95% confidence limit of the 99% of residue concentrations is at or below the MRL (Fig. 4).

The US FDA approach requires that a minimum of 20 animals are studied and milk samples are analysed in triplicate. For veterinary drug products used to treat mastitis, the method assumes that no more than one third of the milk is derived from treated animals, which is reflected in the calculation of the daily intake of milk by consumers by incorporating a factor of 1/3. The approach uses only those points in the final phase of the depletion curves when fitting the regression lines. Measurements reported as "below the LOQ" (limit of quantification), and data from time points with less than three remaining values are also excluded.

The time-to-safe-concentration (TTSC) method (EMEA-CVMP, 2000) is presented in the "Note for guidance for the determination of withdrawal periods for milk" (EMEA/CVMP/473/98-Final). It is recommended as the harmonised method for calculating milk withdrawal periods for new veterinary medicinal products in the EU. The use of alternative statistical approaches may be justified when a dataset is not suitable for analysis using the TTSC method. The latter assumes a log-normal distribution of individual times to safe concentration, and calculates the upper 95% confidence limit of the 95% of individual times to comply with MRL (i.e. the 95/95 statistical tolerance limit). The withdrawal period is calculated as the 95/95 tolerance limit (Fig. 4).

The US FDA approach and the TTSC approach are fundamentally different. In particular, the distributional assumption of the US FDA method and the TTSC

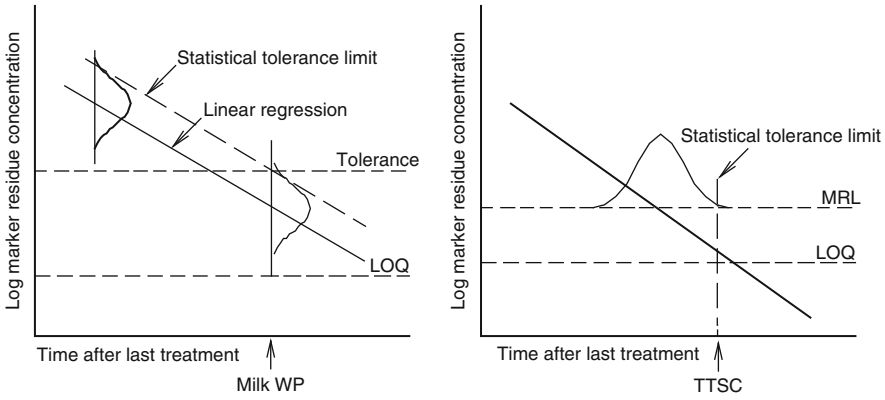

Fig. 4 Schematic diagram contrasting the US FDA approach (*left panel*) and the EMEA approach (*right panel*) for the calculation of a milk withdrawal period (WP). The US FDA approach calculates the withdrawal period as the first time where the upper 95% confidence limit of the 99% of residue concentrations is at or below the tolerance. The EMEA approach calculates the milk WP (also known as the time-to-safe-concentration [TTSC]) as the first time where the upper 95% confidence limit of the 95% of individual times complies with the MRL

method relates to the concentration of residues and the TTSC, respectively. The strength of the TTSC approach is that an assumption of linear depletion of residues from milk is unnecessary. Conceptually, the TTSC method models the withdrawal period as a function of concentration by assuming a distribution of the times to safe concentration (i.e. MRL) and calculating an upper limit of the TTSC at MRL.

The JECFA does not assign withdrawal periods for veterinary drug products. This function is performed by the relevant regulatory authority in member countries that adopt Codex MRLs, and it ensures that the depletion kinetics of individual products as well as regional practices and requirements are taken into account.

2.2.2 Residues of Veterinary Drugs in International Trade

Animal-derived foodstuffs in international trade must comply with the food standards of the importing country which, in general, are national MRLs or Codex MRLs. Situations arise with certain veterinary drugs, however, where food standards have not been established by either the importing country or the Codex Alimentarius Commission. This may reflect a prohibition on the use of the substance in food-producing animals, but more frequently, it is the result of no evaluation of data having been performed. A zero-tolerance approach to residues of such products is commonly adopted. Mechanisms for addressing this situation have been developed: they include the establishment of Import MRLs for certain substances; the development of trade agreements between trading partners; and unique to Australia, the assignment of export slaughter intervals (ESI) to veterinary products intended for use on food-producing animals destined for overseas markets (see below).

Estimating an Export Slaughter Interval

Under its legislation, the Australian Pesticides and Veterinary Medicines Authority (APVMA) must be satisfied that the registration of agricultural and veterinary chemical products will not unduly prejudice trade with countries outside of Australia. Discharge of this legislative obligation is guided by risk assessments conducted prior to the granting of marketing approvals. Compliance with the withdrawal period provides assurance that residues have depleted to below the Australian MRL. However, food standards in some markets may be more stringent than the Australian standard, or alternatively, no standard may have been established and a zero-tolerance approach to residues of the substance applies. In both of these situations, the ESI is a valuable tool for mitigating risks in trade that might otherwise arise as the result of using a veterinary chemical product that is approved for use in food-producing animals in Australia.

An ESI is the interval that elapses between administering, or applying, a veterinary chemical product to a food-producing animal and slaughter of the animal for export. An ESI may be equal to, or longer than, the withdrawal period for the

veterinary chemical product. In both situations, residues deplete to below the LOQ of the analytical method. An ESI which is shorter than the withdrawal period is never assigned as it would jeopardise food safety.

Before an ESI can be estimated for a veterinary chemical product, the risk assessment policy must determine the level of lot rejection considered acceptable. This first step in the ESI-setting procedure takes into account trade data for meat and edible offal and the level of risk acceptable to major stakeholders (producer organisations, meat processors, the veterinary pharmaceutical industry and government). The second step utilises a suite of algorithms to compute the probability of lot rejection of meat consignments when the treated animals are slaughtered at various intervals after treatment. The ESI is computed as the time when the upper tolerance limit about the regression line for the censored data intersects with the residue concentration associated with the acceptable level of risk. Where no MRL has been established in the importing country, the endpoint for the ESI determination is taken to be the LOQ of the method of analysis. Since the relationship between the probability of lot rejection for meat consignments and ESI values is displayed graphically (Fig. 5), the risk manager's task of assigning ESIs and the risk communicator's task of conveying to stakeholders the decisions taken and the basis for those decisions are greatly facilitated.

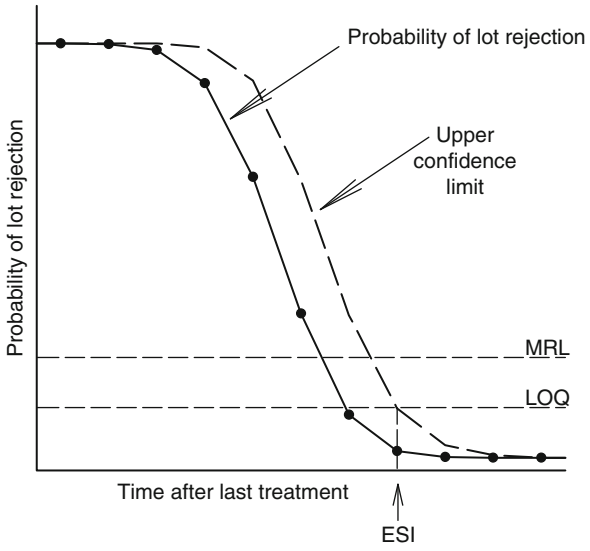

Fig. 5 Schematic diagram of the relationship between the probability of lot rejection of meat consignments and the time after the last treatment of animals with an approved veterinary product. The risk assessment policy takes into account the level of lot rejection considered acceptable. The export slaughter interval (ESI) is computed as the time when the upper confidence limit about the fitted regression line intersects with the residue concentration associated with the acceptable level of risk

2.3 Risk Communication

A critically important aspect of a food safety risk analysis is an effective two-way exchange of information throughout the process. Evaluation reports document the risks and the proposed approaches to managing the identified risks. Based on these reports and dialogue with the risk assessors, risk managers gain a clear understanding of the involved hazards and risks, the basis of decisions taken in the risk assessment, and the implications of the proposed strategy for managing the risks. The regulator must ensure that producer organisations and consumers understand the identified risks and the proposed risk mitigation strategy. Consumer engagement, including the presentation of programmes that provide an understanding of the regulatory processes, serves to assure consumers that drug residues in animal-derived foods do not pose a health risk.

Market access is the focus of risk communication relating to drug residues in international trade in animal-derived food commodities. Risk communication involves conveying to all participants along the food chain the importance of properly discharging their responsibilities in order to comply with the food standards of importing countries. Observing the directions for use including withdrawal periods in the labelling of the product is essential for ensuring that all residues comply with MRL and the health of the consumers is protected.

3 Post-approval Monitoring

3.1 Residue Control Programmes

Residue control programmes are structured in accordance with a country's needs. Programmes of developed countries generally comprise both domestic and import residue sampling programmes. Veterinary drugs for inclusion in these programmes are selected on the basis of their risk profiles. Only the domestic residue sampling programme includes steps for addressing the occurrence of violative residues in food-producing animals, on-farm. Domestic residue sampling programmes are also a trade requirement, either mandatory or as an expectation of the importing countries allowing market access to animal-derived foodstuffs. The import residue sampling programme is primarily a verification programme to determine that the domestic residue sampling programme of an exporting country is operating effectively. These programmes have two principal components: monitoring and surveillance. Residue monitoring programmes randomly sample tissues from animals at slaughter. Tissue samples are assayed for residues of veterinary drugs, pesticides and environmental contaminants, and the residues are assessed for compliance with the applicable MRL or environmental standard. The minimum sample size chosen for monitoring purposes typically provides a 95% probability of detecting at least one violation when 1% of the animal population contains residues above MRL

(FAO–WHO 1996). Surveillance programmes, in contrast, sample tissues from animals suspected of violative residues on the basis of clinical signs or herd history. If monitoring reveals a potential residue problem, the action taken will vary in accordance with the policies of the specific jurisdiction. Typically, the source of supply is traced and action is taken to avoid further occurrences. Action may include seizure and disposal of produce, additional residue testing at the cost of the producer, quarantining a farm and preventing the sale of produce until the commodity has been found to be safe for consumption and fit for sale in both domestic and export markets. Auditing of users and operators, seeking feedback from resellers, and implementing industry codes of practice may also be used to augment residue monitoring.

4 Non-regulatory Mechanisms for the Control of Drug Residues

4.1 The Role of the Veterinary Pharmaceutical Industry

Significant advances in pharmaceutical formulations and drug delivery technologies have resulted in improved efficacy and drug residue profiles of products for use in food-producing animals. Studies into specific compounds involve proprietary data and such studies are rarely published. However, one report described the process of selecting a compound (eprinomectin) from a number of potential drug candidates on the basis of optimal efficacy and residues profiles (Shoop et al. 1996). Reports such as this in the open literature highlight the considerable efforts of the veterinary pharmaceutical industry in developing products with low residues. Recent advances in pharmaceutical sciences are also providing potential solutions to residues at injection sites. For example, studies indicate that biodegradable polymers may be suitable for use as drug carriers in injectable microspheres and microcapsules (Yazar et al 2006) (see also chapter, “Drug Delivery Systems in Domestic Animal Species”). Liposomes that are potential candidates for the intramuscular delivery and sustained release of drugs in food-producing animals have also been reported (Sallovitz et al. 1998).

4.2 The Roles of Producers, Veterinarians and Food Processors

The importance of responsible use of veterinary drugs on-farm cannot be overemphasised. The implementation of quality assurance programmes by producers has resulted in a decreased incidence of violative residues (Roeber et al. 2001). This result is contributed to in part by carefully observing directions for use, including withdrawal periods in product labelling. In the case of injectable veterinary drug

products, better injection techniques have resulted in less wastage of edible meat tissue as a result of trimming blemishes. Veterinarians play an important role in minimising drug residues by instructing livestock producers on good practices in the use of approved veterinary drugs and by providing professional advice in relation to off-label (extra-label) use of veterinary drugs. The incidence of injection site residues entering the food chain is also reduced by trimming of the injection site lesions at meat processing plants.

5 Perspectives and Future Challenges

The process of managing risk in relation to drug residues in animal-derived foodstuffs is based on a risk analysis approach that is both science-based and rigorous. The desired outcome of the process is nil violations of the relevant health standards. This has been achieved, by and large, as demonstrated by the results of residue monitoring programmes that indicate the incidence of illegal drug residues in food is very low (Reeves 2005). The finding of these programmes points to effective regulation of veterinary drugs. It also provides reassurance to the public that food obtained from animals treated with veterinary drugs does not contain residues that might constitute a health hazard for consumers.

Many veterinary drugs were in therapeutic use before the current human food safety evaluation was developed and are referred to by the JECFA as "substances with a long history of use". Contemporary data packages for these compounds are not available for establishing MRLs. However, the JECFA will consider proposing MRLs for these substances if their safety can be assured to be equivalent to that for newer products. The following example demonstrates this concept. Suppose a decision is made to market an older generation parasiticide to fill a void caused by the emergence of parasitic strains resistant to newer chemotherapeutic agents. In this example, the generation of additional data to address deficiencies in the data package and the assembly of existing information and data may permit the establishment of MRLs.

The requirement for a human food safety evaluation for a generic product formulation that has been shown to be bioequivalent to a drug approved for use in food-producing animals is a common source of confusion and debate. However, drug formulations demonstrated to be bioequivalent on the basis of plasma drug concentrations and derived variables such as C_{max} and AUC may have very different tissue residue profiles. This difference can be pronounced with formulations that demonstrate "flip-flop" pharmacokinetics and has been described for a hypothetical scenario involving bioequivalent injectable formulations (Reeves 2007).

A future challenge for the risk analysis of food safety relates to the increasing complexity of the toxicological evaluation. To some extent, this is driven by certain newer drugs that are very potent and exert their actions via receptor-mediated mechanisms, or they are drugs derived from biotechnology. From a food safety perspective, modern analytical instrumentation with low limits of sensitivity can

detect and quantify the residues of these compounds. A separate issue relates to limitations of the conventional NOEL-safety factor approach to the toxicological evaluation of very potent compounds that exert their effects through receptors (for a review, see Greenlees and Hooberman 2004). Reports in the literature describe modifications to the conventional approaches to toxicological evaluations (Crump 1984) and the development of new approaches that are able to provide quantitative estimates of toxicological risk of low levels of veterinary drug residues (Kroes and Kozianowski 2002). Data analysis and interpretation with these new approaches to safety assessment will be challenging. It requires risk assessors and risk managers to interact closely. Advances in toxicology also need to be incorporated into the risk analysis framework. Toxicogenomics, which combines our ever-increasing knowledge of the genome and its processes with conventional toxicology, is a case in point.

Refinement of the exposure assessment of residues of veterinary drugs was addressed by the JECFA at its 66th meeting (WHO 2006). The new procedure yields more realistic estimates of chronic dietary intake of residues. A procedure for estimating the acute dietary exposure to residues of veterinary drugs needs to be developed to address acute exposure scenarios, which are of particular interest for pharmacologically active substances.

In developing countries, the demand for animal protein is rapidly increasing as is the desire of some of these countries to participate in international trade in animal-derived food commodities. However, many developing countries do not have the capacity required to produce food that complies with the standard required for local and international markets (Cannavan 2004). Frequently, national legislative frameworks for regulating veterinary drugs are inadequate or non-existent and residue control laboratories lack the required capability. The situation is confounded by the needs of animal production systems in developing countries, which frequently rely on the use of veterinary drugs not approved in developed countries. As a consequence, Codex MRLs have not been established for many veterinary drugs that are essential in developing countries. Examples include parvaquone and buparvaquone, which are used for treating tick-transmitted Theileria parva infection (East Coast fever) of cattle in east and central Africa, and homidium which is used for treating tsetse-transmitted trypanosomiasis (sleeping sickness) of food animals in some regions of Africa. Dossiers for all three substances were unsuccessfully sought at the twelfth session of the CCRVDF (CCRVDF 2000). The delegation of the USA has offered to assist developing countries in identifying data gaps and seeking commitments from sponsors to provide dossiers for evaluation by the JECFA (CCRVDF 2009). As a result of this collaboration, the number of MRLs for veterinary drugs required for animal production in developing countries might increase.

Finally, a series of safety guidelines have been developed and published by the VICH and other guidelines are in development (VICH website http://www.vichsec.org/). In addition to harmonising the technical requirements for veterinary medicinal product registration globally and reducing the costs associated with product development, the guidelines minimise the use of test animals.

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Veterinary Medicines and the Environment

Alistair B.A. Boxall

Contents

Abstract Veterinary medicines may be emitted either directly or indirectly into the environment, following its use. As veterinary medicines are biologically active compounds, there is a concern that their occurrence in the environment may have an adverse impact on aquatic and terrestrial organisms. This chapter reviews the major sources by which veterinary medicines enter the environment, the fate, behaviour and occurrence of veterinary medicines in the environment and the potential effects on environmental and human health. Finally, gaps in the current knowledge are identified and recommendations provided on priorities for future research.

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Keywords Veterinary medicines · Environmental fate · Ecotoxicity · Environmental risk

1 Introduction

Following administration to an animal patient, drugs are absorbed and in some instances may be metabolised. The parent compound(s) and any metabolites may then be released into the environment directly, for example, the use of medicinal products in fish farms, and indirectly, via the application of animal manure (containing excreted products) to land or via direct excretion of residues onto pasture (Jørgensen and Halling-Sørensen 2000; Boxall et al. 2003; Boxall 2004; Sarmah et al. 2006). Once in the environment, veterinary medicines and their metabolites have the potential to affect aquatic and terrestrial communities and may also enter drinking water supplies and the human food chain. This chapter reviews the present state of the understanding of the inputs of livestock medicines to the environment and synthesises the available information on the fate, transport and effects of veterinary medicines in the environment. Gaps in current knowledge are highlighted and recommendations made for future research.

2 Routes of Input to the Environment

The main routes of input to the soil and aquatic environments and subsequent transport routes are illustrated in Fig. 1. Compounds may also be released during the manufacturing process. During livestock production, veterinary drugs enter the

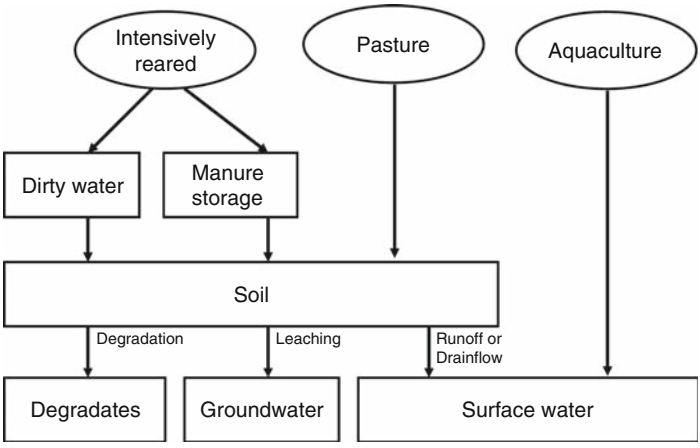

Fig. 1 Routes of entry of veterinary medicines to the environment

environment through removal and subsequent disposal of waste material (including manure/slurry and "dirty" waters), via excretion of faeces and urine by grazing animals, through spillage during external application, via wash-off from farmyard hard surfaces, or by direct exposure/discharge into the environment. For aquaculture treatments, the drug can be added directly to the aquatic environment. Limited amounts of compound may also be released to the environment from the treatment of companion animals or the improper disposal of unused products. Each of these routes of input is described in more detail below.

2.1 Manufacturing

During the manufacture of the active ingredient and formulation of the finished drug product, raw materials, intermediates or the active substance may be released into the air, into water in wastewater, and onto the land in the form of solid waste. The main route of release of both veterinary and human drugs into the environment during manufacturing is probably via process waste effluents produced during the cleaning of the active pharmaceutical ingredient and manufacturing equipment used for coating, blending, tablet compressing and packing (Velagaleti et al. 2002). Biological and chemical degradation processes, such as biotransformation, mineralisation, hydrolysis and photolysis, are thought to remove most drug residues before process waste effluents or sludge solids are discharged to surface waters/sewage treatment works or released onto the land (Velagaleti et al. 2002). In addition, a number of practises are often implemented by the industry to reduce waste generation and material losses. These include process optimisation, production scheduling, materials tracking and waste stream segregation (US EPA 1997). Manufacturing plants employ a number of treatment methodologies and technologies to control and treat emissions and minimise the amount of waste produced. These include the use of condensers, scrubbers, adsorbent filters and combustion or incineration for recovery and removal in air emissions. Neutralisation, equalisation, activated sludge, primary clarification, multimedia filtration, activated carbon, chemical oxidation and advanced biological processes may be used for treatment of waste waters (US EPA 1997).

2.2 Livestock Treatment

For veterinary products administered either orally or by injection, the major route of entry of the product into the environment is probably, via excretion following use and the subsequent disposal of contaminated manure onto land (Metcalfe et al. 2009). Many intensively reared farm animals are housed indoors for long periods at a time. Consequently, large quantities of farmyard manure, slurry or litter are produced which are then disposed of at high application rates onto the land (Montforts 1999). Although each class of livestock production has different

housing and manure production characteristics, the emission and distribution routes for veterinary medicines are essentially similar. Manure or slurry will typically be stored before it is applied to land. During this storage time it is possible that residues of veterinary medicines will be degraded. A number of studies have therefore explored the persistence of a range of substances in different manure/slurry types (e.g. Loke et al. 2000; Teeter and Meyerhoff 2003; Blackwell et al. 2005; Kolz et al. 2005). For example, macrolides and β-lactam antibiotics have been shown to be rapidly dissipated in a range of manure types whereas avermectins and tetracyclines are likely to persist for months. Available data indicate that the dissipation of veterinary medicines in manure or slurry can be very different from their dissipation behaviour in soils (e.g. Blackwell et al. 2005), possibly due to differences in the degradation mechanisms. Degradation in manure and slurry may be anaerobic whereas degradation in soils is most likely to be due to aerobic organisms. For many substances, while the parent compound appears to disappear, it may not in fact be degraded; rather it becomes associated with the manure or slurry and becomes non-extractable. There is currently considerable discussion over the nature of these non-extractable residues, as well as about the implications of these bound residues on environmental health.

Drugs administered to grazing animals or animals reared intensively outdoors may be deposited directly onto land or surface water in dung or urine, exposing soil organisms to high local concentrations (McCracken 1993; Strong and Wall 1994; Halling-Sørenson et al. 1998).

Another significant route for environmental contamination can be the release of substances used in topical formulations. Various substances are used externally on animals and poultry for the treatment of external or internal parasites and infections. Sheep in particular are host to a number of external insect parasites for which treatment and protection can be obligatory. The main methods of external treatment include plunge dipping, pour-on formulations and the use of showers or jetters. With all externally applied veterinary medicines, both diffuse and point source pollution can occur. Sheep dipping activities provide several routes for environmental contamination. In dipping practise, chemicals may enter watercourses through inappropriate disposal of used dip, leakage of used dip from dipping installations and from excess dip draining from treated animals. Current disposal practises rely heavily on spreading used dip onto land. Wash-off, of the chemicals from the fleeces of recently treated animals into the soil, water and hard surfaces may occur on the farm, during transport or at stock markets. Medicines washed off, excreted or spilt onto farmyard hardsurfaces (e.g. concrete) may be washed off to surface waters during periods of rainfall.

2.3 Aquaculture

Veterinary medicines used for aquaculture are commonly administered as medicated feed, by injection or, in the case of topical applications, as a bath formulation.

Bacterial infections in fish are usually treated using medicated food pellets which are added directly to pens or cages (Samuelsen et al. 1992a, b; Hektoen et al. 1995). When infected, cultured fish show reduced appetite and thus feed intake. Consequently, a large proportion of medicated feed is not eaten and this passes through the cages and is available for distribution to other compartments. Furthermore, the bioavailability of many antibacterial agents is relatively low and drugs may also enter the environment, via faeces and urine (Björklund and Bylund 1991; Hustvedt et al. 1991). In recent years, improved husbandry practises have reduced the amount of waste feed generated and more recently authorised medicines have greater bioavailability ($F > 95\%$). Nevertheless, deposition of drugs from uneaten feed or faeces on or in under-cage sediment can be a major route of environmental contamination for pharmaceuticals used in aquaculture (Jacobsen and Berglind 1988; Björklund et al. 1991). Once present on or in sediment, compounds may also leach back into the water column. During periods of treatment, some of the drugs entering the environment in waste feed and faeces are also taken up by exploitative wild fish, shellfish and crustaceans (Björklund et al. 1990; Samuelsen et al. 1992b; Capone et al. 1996).

Where topical applications of chemotherapeutics are made, fish are usually crowded into a small volume of water for treatment. Concentrated drugs are added directly to the water of open net-pens or ponds, net-pens enclosed by a tarpaulin or tanks. Waste effluent is then either released into the surrounding water column or subjected to local wastewater treatment and recycling (filters, settlement basins and ponds) (Montforts 1999). Sludge recovered from waste water recycling activities may be applied directly onto land or sold as fertiliser (Montforts 1999).

3 Fate and Behaviour

Once a veterinary medicine is released into the environment, its behaviour will be determined by its underlying physical properties (including water solubility, lipophilicity, volatility and sorption potential). In the following sections information on the fate and transport of veterinary medicines in the environment is reviewed.

3.1 Sorption in Soil

The degree to which veterinary medicines may adsorb to particulates varies widely. Consequently, the mobility of different veterinary medicinal products also varies widely (e.g. Fig. 2). Available data indicate that sulfonamide antibiotics and organophosphate parasiticides will be mobile in the environment whereas tetracycline, macrolide and fluoroquinolone antibiotics will exhibit low mobility. The sorption behaviour of individual veterinary medicines can also vary widely in different soil types and unlike many other classes of soil contaminant (e.g. hydrocarbons and

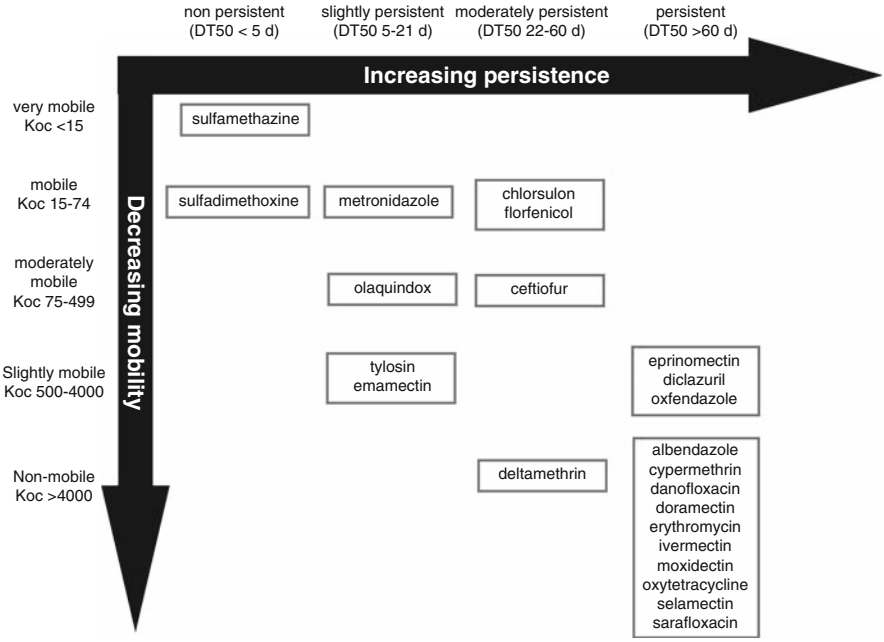

Fig. 2 Distribution of reported mobility (based on sorption coefficients) and persistence data for veterinary medicinal products (adapted from Pope et al. 2009). K_{oc} = organic carbon-normalised sorption coefficient in soil, DT50 = the time taken for degradation/dissipation of 50% of the amount of drug originally present

many pesticides), the difference in sorption of a given compound in different soils cannot be explained by variations in soil organic carbon. These large differences in sorption behaviour are explained by the fact that many veterinary medicines are ionisable with pKa values in the pH range of natural soils. Medicines can therefore occur in the environment as negative, neutral, zwitterionic and positively charged species (e.g. Ter Laak et al. 2006a, b). Depending on the chemical species, interactions with soil can occur through electostatic attraction, surface bridging, hydrogen bonding or hydrophobic interactions (Ter Laak et al. 2006b). The sorption behaviour is also influenced by the properties of the soil including pH, organic carbon content, metal oxide content, ionic strength and cationic exchange capacity (e.g. Jones et al. 2005; Sassman and Lee 2005; Strock et al. 2005; Ter Laak et al. 2006b). The complexity of the sorbate–sorbent interactions means that modelling approaches developed for predicting the sorption of other groups of chemicals (e.g. pesticides and neutral organics) are inappropriate for use in veterinary medicines. Manure and slurry may also alter the behaviour and transport of medicines. Studies have demonstrated that the addition of these matrices can affect the sorption behaviour of veterinary medicines and that they may affect persistence (e.g. Boxall et al. 2002; Thiele Bruhn and Aust 2004). These effects have been attributed to changes in pH or alterations in the nature of dissolved organic carbon in the soil/manure system.

3.2 Persistence in Soil

The main route for degradation of veterinary medicines in soils is via aerobic soil biodegradation. Degradation of veterinary medicines is affected by environmental conditions such as temperature and pH and the presence of specific degrading bacteria that have developed to degrade groups of medicines (Gilbertson et al. 1990; Ingerslev and Halling-Sørensen 2001). As well as varying significantly between chemical classes (e.g. see Fig. 2), degradation rates for veterinary medicines also vary within a chemical class. When manure is combined with soil, degradation may be enhanced for selected medicines. Depending on the nature of the chemical, other degradation and depletion mechanisms may occur, including soil photolysis and hydrolysis (e.g. Wolters and Steffens 2005). The degradation processes may well result in the formation of degradation products (e.g. Kolz et al. 2005). In some instances, these degradation products may be of greater environmental concern than the parent compound as some have similar or greater toxicity, some are more persistent and some are more mobile (Boxall et al. 2003). It is therefore important that the fate of the degradation products in soils is considered when assessing the impact of a veterinary medicine on the environment.

3.3 Transport in Soil Systems

Contaminants applied to soil can be transported to aquatic systems in surface runoff, subsurface flow and drainflow. The extent of transport via any of these processes is determined by a range of factors, including: the solubility, sorption behaviour and persistence of the contaminant; the physical structure, pH, organic carbon content and cation exchange capacity of the soil matrix, and climatic conditions such as temperature and rainfall volume and intensity. Most work to date on contaminant transport from agricultural fields has focused on pesticides, nutrients and bacteria, but recently a number of studies have explored the fate and transport of veterinary medicines. Lysimeter, field-plot and full-scale field studies have investigated the transport of veterinary medicines from the soil surface to field drains, ditches, streams, rivers and groundwater (e.g. Aga et al. 2003; Kay et al. 2004, 2005a, b, c; Burkhard et al. 2005; Hamscher et al. 2005; Kreuzig and Holtge 2005; Blackwell et al. 2007, 2009). A range of experimental designs and sampling methodologies has been used. These investigations are described in more detail below.

3.3.1 Leaching to Groundwater

The movement of sulfonamides and tetracyclines in soil profiles has been investigated at the field scale using suction probes (Hamscher et al. 2000a; Blackwell et al. 2007). In these studies, sulfonamides were found at depth but the tetracyclines were

not, which is most likely due to the high potential for tetracyclines to sorb to the soil. Carlson and Mabury (2006) reported that chlortetracycline applied to agricultural soil in manure was detected at soil depths of 25 and 35 cm, but monensin remained in the upper soil layers. There are only a few reports of veterinary medicines in groundwater (Hirsch et al. 1999; Hamscher et al. 2000a). In an extensive monitoring study conducted in Germany (Hirsch et al. 1999), while no antibiotics were detected in groundwater at most of the sites investigated, residues of sulfonamide antibiotics were detected at a few of the study sites. Contamination at two of these sites was attributed to irrigation of agricultural land with domestic sewage but the other sites were believed to have become contaminated due to the application of animal manures to the soil surface (Hirsch et al. 1999).

3.3.2 Runoff

Transport of veterinary medicines via runoff (i.e. overland flow) has been observed for tetracycline antibiotics (i.e. oxytetracycline) and sulfonamide antibiotics (sulfadiazine, sulfamethazine, sulfathiazole and sulfachloropyridazine) (Kay et al. 2005c; Kreuzig et al. 2005). Just like leaching, the transport of these substances is influenced by the sorption behaviour of the compounds, the presence of manure in the soil matrix and the nature of the land to which the manure is applied. Runoff of highly sorptive substances, such as tetracyclines, was observed to be significantly lower than the more mobile sulfonamides (Kay et al. 2005c). However, even for the relatively water soluble sulfonamides, total mass losses to surface are small (between 0.04% and 0.6% of the mass applied) under actual field conditions (Stoob et al. 2007). Manure and slurry has been shown to increase the transport of sulfonamides via runoff by 10–40 times in comparison to runoff following direct application of these medicines to soils (Burkhard et al. 2005). Possible explanations for this observation include physical "sealing" of the soil surface by the slurry and/or a change in pH as a result of manure addition that alters the speciation and fate of the medicines (Burkhard et al. 2005). It has been shown that overland transport from ploughed soils is significantly lower than runoff from grasslands (Kreuzig et al. 2005).

3.3.3 Drain Flow

The transport of a range of antibacterial substance (i.e. tetracyclines, macrolides, sulfonamides and trimethoprim) has been investigated using lysimeter and field-based studies in tile-drained clay soils (Kay et al. 2004; Boxall et al. 2006a). Following application of pig slurry spiked with oxytetracycline and sulfachloropyridazine, the test compounds were detected in drainflow water (Kay et al. 2004). Concentrations of the sulfonamide were an order of a magnitude higher than tetracycline even though the spiking concentrations for the test compounds were similar; these differences are again likely to be due to differences in sorption

behaviour. In a subsequent investigation at the same site (Kay et al. 2004), in which the soil was tilled, much lower concentrations were observed in the drainflow, which suggested that tillage may be a useful mitigation strategy in the event when a veterinary product is found to pose a risk to aquatic systems. While the pig slurry used in these studies was obtained from a pig farm where tylosin was used as a prophylactic treatment, this substance was not detected in any drainflow samples, possibly because it is not persistent in slurry (Loke et al. 2000).

3.4 Surface Waters

In the water column, substances may be degraded abiotically via photodegradation and/or hydrolysis or biotically by aerobic or anaerobic organisms. Highly sorptive substances may partition to the bed sediment. For example, mesocosm studies using ivermectin show that when added to water, the compound dissipates quickly from the water column and that this dissipation is observed as an increase in the concentration of the compound in the bed sediment (e.g. Sanderson et al. 2007).

A significant amount of information is available on the fate and behaviour of many veterinary medicines in sediment due to their use as aquaculture treatments (Jacobsen and Berglind 1988; Samuelsen 1989; Björklund et al. 1990; Samuelsen et al. 1991, 1992a, 1994; Pouliquen et al. 1992; Coyne et al. 1994; Hektoen et al. 1995; Lai et al. 1995; Lunestad et al. 1995). While many compounds degrade very quickly (e.g. chloramphenicol, florfenicol, furazolidone and ormethoprim), others persist in the sediment from months to years (e.g. flumequine, ivermectin, oxolinic acid, oxytetracycline, sarafloxicin, sulfadiazine and trimethoprim).

3.5 Uptake Into Biota

Veterinary medicines may also be taken up from soil and water into biota (Migliore et al. 2003; Kumar et al. 2005; Boxall et al. 2006b). The potential uptake of veterinary medicines into plants is receiving increasing attention. Studies with a range of veterinary medicines (Boxall et al. 2006b) showed that a number of antibiotics are taken up by plants following exposure to soil at environmentally realistic concentrations of the compounds whereas other compounds were not observed to be accumulated. The lack of uptake observed may be due to the underlying properties of the compound or other factors such as high limits of detection (LODs) or significant degradation during the study. Data also indicate that while some compounds are accumulated by some plant species, they may not be taken up by others. It is generally recognised that chemicals are taken up into plants via the soil pore water and data for pesticides and neutral organic substances shows that uptake is typically related to the octanol–water partition coefficient of the compound (Briggs 1981; Burken and Schnoor 1998). The available data indicate that these relationships may not hold true for veterinary medicines (Boxall

et al. 2006b). This is perhaps not surprising as data for other environmental processes (e.g. sorption to soil) indicate that the behaviour of veterinary medicines in the environment is poorly related to hydrophobicity but is determined by a range of factors including H-bonding potential, cation exchange, cation bridging at clay surfaces and complexation. Through controlled experimental studies it may be possible in the future to begin to understand those factors and processes affecting the uptake of veterinary medicines into plants and to develop modelling approaches for predicting uptake.

4 Occurrence

Alongside the fate experiments described above, a series of studies have monitored concentrations of veterinary compounds in different matrices. Veterinary medicines have been measured in surface waters, groundwaters, sediments, slurry/manure and biota. Monitoring studies have focused on veterinary products used in sheep dips, aquaculture and as antibiotic treatments for livestock.

4.1 Aquaculture

A number of studies have explored the occurrence of veterinary medicines arising from aquaculture treatment. For example, emamectin benzoate has been detected in sediment, water, silt, mussels, dogfish and crab species. Oxolinic acid has been detected in sediments surrounding wild fish populations and other marine animals during and after the medication of cultivated fish (Björklund et al. 1991; Samuelsen et al. 1992b). Flumequine has been detected in fish tissue (Ervik et al. 1994). Residues of ivermectin have been detected in sediments under and adjacent to salmon cages (Cannavan et al. 2000). Finally, the environmental fate of oxytetracycline following its use in aquaculture has been extensively researched (Jacobsen and Berglind 1988; Björklund et al. 1990, 1991; Samuelsen et al. 1992a; Coyne et al. 1994; Capone et al. 1996; Kerry et al. 1996) with the compound being detected in wild fauna and sediments around fish farms. In selected studies (e.g. Samuelsen et al. 1992a), the environmental impacts of the treatment have been investigated alongside the monitoring studies. Data from these studies indicate that the use of antimicrobial compounds in aquaculture is associated with the occurrence of antibiotic resistant bacteria in environmental matrices.

4.2 Livestock Treatments

Several veterinary drugs have been detected in soil that has been treated with animal manure. In three separate investigations in Germany, soil samples collected

from regions with intensive livestock production were analysed for tetracyclines (Hamscher et al. 2000a, 2000b, 2000c). Concentrations of up to 41.8 μg kg^{-1} were detected in these samples. Elsewhere, American researchers detected trace amounts (approximately 0.1–2 μg kg^{-1}) of ivermectin on the top (0–3 in.) of the soil in a cattle feedlot, housing animals treated 28 days previously (200 μg kg^{-1} body weight) (Nessel et al. 1989). The authors suggest the concentrations detected in the soil are probably as a result of the faeces being trampled into the mud and subsequently being protected from light thus retarding degradation. In a recent monitoring study in the UK, oxytetracycline, lincomycin, sulfadiazine, trimethoprim, ivermectin and enrofloxacin (and its metabolite ciprofloxacin) were monitored in soils (Boxall et al. 2006a). Concentrations of the antibacterials detected ranged from 0.5 μg kg^{-1} (trimethoprim) to 305 (oxytetracycline) μg kg^{-1}.

While monitoring sewage treatment work effluents and associated receiving surface waters for antibiotic substances in Germany, residues of chloramphenicol were detected at concentrations of 0.06 and 0.56 μg l^{-1} (Hirsch et al. 1999). As its use in human medicine is extremely limited, the authors of the paper suggested that the two positive detections were most likely from its sporadic veterinary use in fattening farms.

In a national monitoring study in the US (Kolpin et al. 2002), a wide range of medicines were monitored in watercourses. A number of substances that are used as veterinary medicines, including sulfonamides, fluoroquinolones, tetracyclines and macrolides were detected in the ng l^{-1} range. Many of these substances are also used as human medicines so the concentrations may result from a combination of inputs from both human and veterinary sources. Similar broad-scale monitoring studies have been done in other regions (including Europe and Asia) and show similar results.

The majority of surface monitoring studies involve grab sampling on a number of occasions across a variety of sites. As inputs of many veterinary medicines are likely to be intermittent, it is likely that concentrations reported in the studies are significantly lower than peak concentrations. To address this, a recent UK study used continuous monitoring of water and sediment, at farms where veterinary medicines (including oxytetracycline, lincomycin, sulfadiazine, trimethoprim, ivermectin and doramectin) were known to be in use, to determine typical exposure profiles for aquatic systems (Boxall et al. 2006a). Maximum concentrations of antibacterials in stream water ranged from 0.02 μg l^{-1} (trimethoprim) to 21.1 (lincomycin) μg l^{-1}; the parasiticides (doramectin and ivermectin) were not detected. Concentrations of antibacterials in sediment were 0.5–813 μg kg^{-1} and those for doramectin and ivermectin were 2.7 and 4.9 μg kg^{-1} respectively. Generally, these concentrations are significantly lower than those required to cause effects on organisms in the environment; this is discussed later.

There are only a few reports of veterinary medicines being detected in groundwater (Hirsch et al. 1999; Hamscher et al. 2000a). In an extensive monitoring study conducted in Germany, a large number of groundwater samples were collected from agricultural areas in order to determine the extent of contamination by antibiotics (Hirsch et al. 1999). The data show that in most areas with intensive

livestock breeding, no antibiotics were present above the limit of detection (0.02–0.05 μg l^{-1}). Sulfonamide residues were, however, detected in four samples. Whilst the source of contamination of two of these is considered to be attributable to irrigation with sewage, the authors concluded that sulfamethazine, detected at concentrations of 0.08 and 0.16 μg l^{-1}, could possibly have derived from veterinary applications, as it is not used in human medicine.

In the investigations of Hamscher et al. (2000a) soil water was collected and analysed from four separate areas of agricultural land, two belonging to livestock farms and treated with animal slurry and two where no animal manure had been applied for approximately 5 years. Chlortetracycline, oxtetracycline, tetracycline and tylosin were all found at the limit of detection (0.1–0.3 μg l^{-1}) in water samples collected at 80 and 120 cm depth, independent of soil treatment. In addition, no biologically active residues could be detected with microbiological assays that had approximately fivefold higher detection limits.

Veterinary medicines are also known to leach from landfill sites. In Denmark, high concentrations (parts per million) of numerous sulfonamides were found in leachates close to a landfill site where a pharmaceutical manufacturer had previously disposed of large amounts of these drugs over a 45-year period (Holm et al. 1995). Concentrations dropped off significantly tens of metres down gradient, most probably due to microbial attenuation. Although this is recognised as a specific problem, in the UK the disposal of smaller quantities of veterinary medicines to landfill should nevertheless be considered a potential route for environmental contamination.

5 Impacts of Veterinary Drugs in the Environment

5.1 Effects in Regulatory Studies

Guidelines are available describing how the environmental risks of veterinary products should be assessed for a range of countries (Breton and Boxall 2003; de Knecht et al. 2009). The approach used in Europe is based on the recommendations of the International Co-operation on Harmonisation of Technical Requirements for Registration of Veterinary Products (VICH) who have attempted to harmonise the environmental risk assessment requirements of veterinary products in the USA, Europe and Japan. The approach is a two-phase process (CVMP 2007). In Phase 1, the potential for the environment to be exposed to the veterinary product is determined. For compounds that do not pass the Phase 1 triggers, information is required on the effects of environmental medicines on terrestrial and aquatic organisms. Recommended aquatic studies include acute toxicity studies on daphnia and fish with mortality as the test endpoint and short-term studies into the effects of the compound on the growth of green or blue-green algae. Terrestrial tests are done on earthworms (mortality) and plants (germination and growth), the effects of the

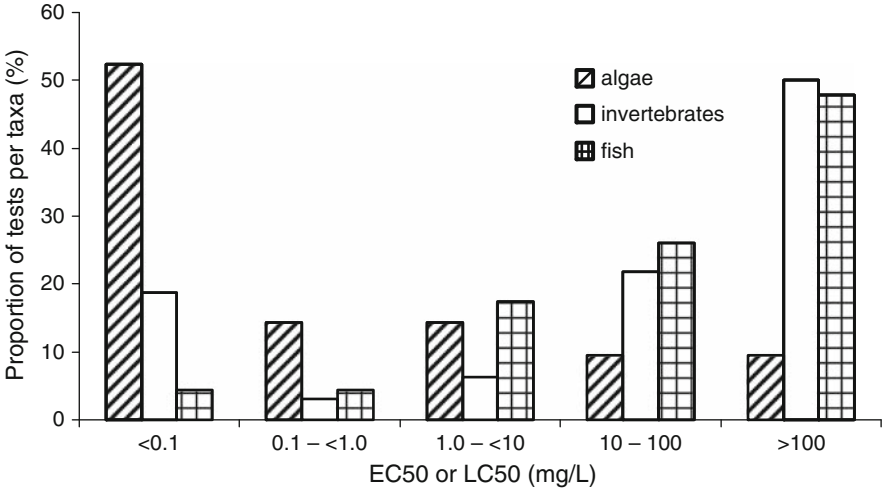

Fig. 3 Distribution of ecotoxicity test results (median effective concentrations or medial lethal concentrations) for veterinary medicines tested using algae, invertebrates or fish

test compound on the rate of nitrate mineralisation in soil is also determined to assess the impacts of the compound on soil microbes. For ecto- and endo-parasiticides, tests may also be required on dung beetles and dung flies. As a result, a large body of data is now available in the literature, regulatory assessments and in many material safety datasheets, on the effects of veterinary products in these standard studies.

An analysis of the publicly available regulatory data indicates that, in general, algae are more sensitive to veterinary medicines than fish or invertebrates (Fig. 3). When classes of medicine are considered, antibiotics are found to be particularly potent to algae (e.g. Halling-Sørenson 1999) and the parasiticides (pyrethroids, macrocyclic lactones and organophosphates) are found to be particularly toxic to invertebrates. When soil data are considered, many antibiotics are found to show high potency to plants; this is most likely due to the fact that many plants have similar biochemical pathways to those that the antibiotic affects, in microbes (Brain et al. 2008).

Obviously, just because a veterinary medicine is highly toxic to a soil or aquatic organism, it does not mean that it will pose an unacceptable risk to the environment. In order to assess environmental risks, the current regulatory guidelines for veterinary medicines recommend that the ecotoxicity data are used to estimate predicted no-effect concentrations (PNECs) and that these PNECs are then compared to exposure concentrations to establish a risk characterisation ratio. The PNEC is obtained from the ecotoxicity test endpoint using an assessment factor.

In a study to assess the risks of veterinary medicines in the UK environment, we measured concentrations of a range of frequently used medicines in areas where the medicines were known to be used (Boxall et al. 2006a). By comparing the

Table 1 Comparison of maximum measured environmental concentrations with predicted no-effect concentrations obtained using regulatory ecotoxicity studies for veterinary medicinal products (Data taken from Boxall et al. 2006a)

Veterinary medicine	Most sensitive species	Effect endpoint (mg/L)	Predicted no-effect concentration (µg/L)	Measured concentration (µg/L)	[a]Risk characterisation ratio
Oxytetracycline	Green algae	4.5	45	4.49	0.10
Sulfadiazine	Green algae	3.49	34.9	4.13	0.12
Trimethoprim	Green algae	16	1.6	0.02	0.001
Ivermectin	Daphnids	0.000025	0.00025	< 0.0002	< 0.8
Doramectin	Daphnids	0.0001	0.001	< 0.001	< 1
Lincomycin	Daphnids	379.4	379.4	21.1	0.006

[a]Risk characterisation ratio = measured environmental concentration/predicted no-effect concentration

maximum measured concentrations of these compounds in surface waters with PNECs for the study compounds, obtained from regulatory type studies, it is possible to obtain an indication of the potential risks these compounds pose to the UK environment. Maximum measured concentrations for all of the antibacterial compounds studied (oxytetracycline, sulfadiazine, trimethoprim and lincomycin) were at least an order of magnitude lower than their PNECs (Table 1) indicating that these substances do not pose a great risk to the environment. Concentrations of the parasiticides studied were below analytical LODs. As the LODs were either the same as or lower than PNECs, the data indicate that these also are unlikely to be a major concern in the water compartment (Table 1). However, many scientists are now questioning the suitability of standard regulatory studies for assessing the environmental risks of veterinary medicines and advocate the use of other more subtle and chronic endpoints.

5.2 Chronic and Subtle Effects

As veterinary medicines are biologically active substances, concerns have been raised over the relevance of the acute standard ecotoxicity studies that are employed as part of the registration process. The main concern is that if the target receptor for the veterinary medicine also occurs in an organism in the environment, it is possible that the compound will have a much more significant impact on the environment than indicated by the acute bioassays. Many scientists therefore advocate that chronic endpoints and more subtle endpoints should be investigated. Table 2 lists a number of studies that have investigated some of these endpoints and in the following section, some examples of studies exploring the chronic as well as more subtle impacts of veterinary drugs are described. Severe environmental impacts of veterinary drugs, that would not have been detected based on standard regulatory studies, are also discussed.

Table 2 Reported subtle effects of pharmaceutical compounds on aquatic and terrestrial organisms

Substance(s)	Medicine class	Reported effect	Reference
Avermectins	Parasiticide	Adult insects – loss of water balance, disruption of feeding and reduced fat accumulation, delayed ovarian development, decreased fecundity and impaired mating. Juvenile insects – delayed development, reduced growth rates, development of physical abnormalities, impairment of pupariation or emergence and a loss of developmental symmetry	Floate et al. (2005)
Tetracyclines, macrolides and streptomycin	Antibacterials	Antibacterial resistance measured in soil bacteria obtained from sites treated with pig slurry	Sengelov et al. (2003)
Cypermethrin	Ectoparasiticide	Impact on dung decomposition	Sommer and Bibby (2002)
Fenbendazole	Parasiticide	Impact on dung decomposition	Sommer and Bibby (2002)
Tylosin	Antibacterial	Impacts on the structure of soil microbial communities	Westergaard et al. (2001)
Erythromycin	Antibacterial	Inhibition of growth of aquatic plants	Pomati et al. (2004)
Tetracycline	Antibacterial	Inhibition of growth of aquatic plants	Pomati et al. (2004)
Diclofenac (used as veterinary product in Asia)	NSAID	Inihibition of basal [a]EROD activity in cultures of rainbow trout hepatocytes; effects on vulture populations	Laville et al. (2004)
Sulfamethazole	Antibacterial	Inhibition of basal EROD activity in cultures of rainbow trout hepatocytes	Laville et al. (2004)
Sulfadiazine	Antibacterial	Inhibition of degradation of human pharmaceuticals in soils press)	Monteiro and Boxall (2009)

[a]7-ethoxyresorufin-o-deethylase

5.2.1 Avermectins and Terrestrial and Aquatic Invertebrates

The avermectins are highly potent insecticides. Exposure to avermectins can elicit a number of responses, including adult and larval mortality, an effect on feeding, disruption of water balance, a reduction in growth rate, interference with moulting, inhibition of metamorphosis and/or pupation, prevention of adult emergence, disruption of mating and interference with egg production and oviposition (Strong 1993; Strong and Brown 1987). As a consequence, dung from animals treated with avermectins may not support the development of either target (e.g. *Haemotobia irritans, Musca autumnnalis, Musca domestica* and *Musca vetustissimia*) or non-target (e.g. sphaerocerids, muscids, sepsids and coleopterans) insects (Schmidt 1983; Strong and Brown 1987; Wall and Strong 1987; Ridsdill-Smith 1993; Madsen et al. 1990; Sommer et al. 1992, 1993; Strong and James 1993). The toxicity of

avermectins to dung insect populations may be associated with retardation in the rate of breakdown of pats. For example pats containing ivermectin have been shown to be intact after 340 days, whereas, untreated pats were largely degraded within 80 days (e.g. Floate 1998).

The effects on other invertebrates have not been extensively investigated although investigations with annelids demonstrated no effect on population density (Wall and Strong 1987). The possible indirect effects of avermectin contaminated dung on vertebrate populations (e.g. bats and birds) have also been highlighted (e.g. McCracken 1993). Their use may result in a depletion in the quantity and quality of vertebrate food resources; this may be particularly critical during the breeding season or when young animals are foraging and fending for themselves.

5.2.2 Antibiotics and Soil Microbes

Several studies have investigated the effects of antimicrobial substances on microbes in soils and sediment (e.g. Westergaard et al. 2001). Selected substances have been shown to inhibit soil bacteria, as well as reducing the hyphe length of active moulds. Effects on the microbial composition of soils have also been demonstrated (e.g. Sommer and Bibby 2002). With the exception of a few studies (e.g. Sommer and Bibby 2002), effects on soil and sediment functioning have not been considered. Those studies that have been performed demonstrate that veterinary antibacterials may affect sulphate reduction in soil and that they inhibit the decomposition of dung organic matter in soil (e.g. Sommer and Bibby 2002. A few studies have also investigated the potential for antibiotics that are excreted by animals to select for antibiotic resistance. For example, Heuer and Smalla (2007) investigated the effects of pig manure and sulfadiazine on bacterial communities in soil microcosms using two soil types. In both soils, manure and sulfadiazine positively affected the quotients of total and sulfadiazine-resistant culturable bacteria. The results suggest that manure from treated pigs enhances spread of antibiotic resistances in soil bacterial communities. A few studies have also explored effects of veterinary antibiotics on aquatic microbes. Schallenberg and Armstrong (2004) explored the impacts of filtered water from an agricultural drain on bacteria in the lake. They showed that the drainage water reduced the abundance of aquatic bacteria in a shallow coastal lake and the data indicated that these effects may be due to antiobiotics. Finally, in a recent study, they (Monteiro and Boxall 2009), explored the indirect effects of veterinary antibiotics. In this study, the effects of sulfamethoxazole on the degradation of a range of human medicines in soil were examined. The addition of sulfamethoxazole significantly reduced the rate of degradation of the human non-steroidal anti-inflammatory drug, naproxen. This observation may have serious implications for the risks of other compounds that are applied to the soil environment such as pesticides.

5.2.3 Diclofenac and Vultures

The veterinary use of the non-steroidal anti-inflammatory drug, diclofenac, was found to be responsible for the decline in populations of three vulture species in Asia (Oaks et al. 2004). The decline in the populations was caused by renal failure and visceral gout which was attributed to the use of diclofenac in cattle. The decline in vulture populations, arising from the use of diclofenac, is thought to have serious implications for human health. As vultures are a keystone species, their population decline has a range of ecological, socio-economic, cultural and human health impacts.

For example, Markandya et al. (2008) reviewed the economic implications of the human health impacts, with regard to the decline in vulture populations. Livestock carcasses are the main food source for vultures but are also eaten by feral dogs. As the vulture populations have declined, the dog populations have increased; dogs are the main source of rabies in humans in India and so it is probable that the incidence of rabies in humans, and hence mortality, has also increased. Markandya et al. estimated that the decline in the numbers of vultures had likely caused many thousand extra deaths in the human population although this has yet to be corroborated from health monitoring data.

5.3 Human Health Risks

As well as posing a risk to aquatic and terrestrial organisms, residues of veterinary medicines in the environment may pose a risk to human health. Consumers might be exposed to these veterinary medicines in several ways, via consumption of: crops that have accumulated veterinary medicines from soils or as a result of exposure to contaminated manure or slurry; animals that have accumulated veterinary medicines through the food chain; fish exposed to treatments used in aquaculture; abstracted groundwater and surface waters contaminated with veterinary medicines. While veterinary medicines are routinely monitored in target food materials, to ensure that concentrations are below the maximum residue levels (MRLs) and hence protect human health, the magnitude of the exposure via all of the routes listed above, has not been extensively investigated.

Hughes et al. (2006) used data on the human health hazard results, from food intake surveys and modelled exposure concentrations to assess the potential risks to consumers following indirect exposure for a selection of veterinary medicines.

The results of the risk assessment were generally reassuring, with the estimated combined intake from plant-derived foodstuffs, drinking water and, where appropriate, farmed fish, amounting to less than 20% of the acceptable daily intake (ADI) for each of the sections of the population considered for approximately two thirds of the medicines selected for evaluation. A small number of substances (including albendazole, deltamethrin, florfenicol, medroxyprogesterone, tylosin, dihydrostreptomycin, salinomycin sodium, toltrazuril and nitroxinil) were identified as being of

potential concern so this is an area where further research is warranted. It is important to recognise that for many of these substances data were lacking on the major environmental fate processes so the estimates of exposure are likely to be extremely conservative.

6 Recommendations for Future Research

Over the past 10 years there has been increasing interest in the impacts of veterinary medicines in the environment and there is now a much better understanding about how veterinary medicines move around the environment, how they persist in the environment and their impacts on aquatic and terrestrial organisms. However, there are still a number of uncertainties that require addressing before there can be a full understanding of the environmental risks of veterinary medicines. Areas requiring further research include:

- Usage data are unavailable for many groups of veterinary medicines and for several geographical regions, which makes it difficult to establish whether these substances pose a risk to the environment. It is therefore recommended that usage information be obtained for these groups. Better usage data will assist in designing more robust hazard and risk management strategies that are tailored to geographically explicit usage patterns.
- Information on the potential environmental effects of veterinary medicine transformation products is lacking. It is likely that most metabolites will be less toxic than the parent compounds, but this may not necessarily always be the case.
- The relative significance of novel routes of entry to the environment from livestock treatments, such as wash-off following topical treatment and farm yard runoff, and aerial emissions, have not generally been considered. For example, the significance of exposure to the environment from the disposal of used containers or from discharge from manufacturing sites should be investigated further.
- Monitoring data are available for a small number of veterinary medicines in the soil, sediment, surface waters and groundwaters. Further targeted monitoring should be performed to determine concentrations in the environment. These data could then be used along with the existing data to evaluate current risk assessment exposure models.
- For many veterinary drugs, information is lacking in the literature on their ecotoxicity. Moreover, in instances where data are available, the studies have generally focused on short-term acute endpoints, Studies should be conducted to assess the potential impacts of those veterinary medicines for which ecotoxicity data is lacking but are seen to regularly occur in the environment. The potential sub-lethal impacts of these substances should also be explored.
- Although information is available on the direct effects of a range of veterinary medicines on aquatic and terrestrial organisms, limited information is available

on the indirect effects. The possible indirect effects of veterinary medicines should be identified. For example, concern has been raised over the possible indirect effects of anthelmintics on higher trophic levels (such as bat or bird species) that may result from the loss of dung invertebrates as a food source.

- There is increasing interest in the way in which mixtures of chemicals interact in the landscape. Although this interest is not confined to veterinary medicines, the detection of mixtures of agricultural pesticides, human and veterinary medicines and industrial chemicals in aquatic systems has challenged the traditional concept of substance-by-substance risk assessment. The high biological activity of medicines, by design, has drawn particular attention to their role in mixture toxicity within watersheds and the wider environment. Further research is therefore required on the mixture toxicity of veterinary medicines (in combination with other medicines and non-medicinal substances) and the likely occurrence and effects of these on ecosystems in natural landscapes.
- Finally, there is a need to establish the potential for veterinary medicines to bioaccumulate. It may be possible to perform these assessments using data on target animals obtained in pharmacodynamic/pharmacokinetic studies performed by manufacturers as part of the current regulatory process. In addition to this, recent research on the extrapolation of mammalian toxicity data, contained in regulatory submissions for human medicines, to predict effects on ecological receptors such as fish has shown considerable promise. This may be a cost-effective way of using existing data to predict at least some aspects of environmental hazard or risk.

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Veterinary Medicines and Competition Animals: The Question of Medication Versus Doping Control

Pierre-Louis Toutain

Contents

Abstract In racing and other equine sports, it is possible to increase artificially both the physical capability and the presence of a competitive instinct, using drugs, such as anabolic steroids and agents stimulating the central nervous system. The word doping describes this illegitimate use of drugs and the primary motivation of an equine anti-doping policy is to prevent the use of these substances. However, an anti-doping policy must not impede the use of legitimate veterinary medications

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and most regulatory bodies in the world now distinguish the control of illicit substances (doping control) from the control of therapeutic substances (medication control). For doping drugs, the objective is to detect any trace of drug exposure (parent drug or metabolites) using the most powerful analytical methods (generally chromatographic/mass spectrometric techniques). This so-called "zero tolerance rule" is not suitable for medication control, because the high level of sensitivity of current screening methods allows the detection of totally irrelevant plasma or urine concentrations of legitimate drugs for long periods after their administration. Therefore, a new approach for these legitimate compounds, based upon pharmacokinetic/pharmacodynamic (PK/PD) principles, has been developed. It involves estimating the order of magnitude of the irrelevant plasma concentration (IPC) and of the irrelevant urine concentration (IUC) in order to limit the impact of the high sensitivity of analytical techniques used for medication control. The European Horserace Scientific Liaison Committee (EHSLC), which is the European scientific committee in charge of harmonising sample testing and policies for racehorses in Europe, is responsible for estimating the IPCs and IUCs in the framework of a Risk Analysis. A Risk Analysis approach for doping/medication control involves three sequential steps, namely risk assessment, risk management, and risk communication. For medication control, the main task of EHLSC in the risk management procedure is the establishment of harmonised screening limits (HSL).The HSL is a confidential instruction to laboratories from racing authorities to screen in plasma or urine for the presence of drugs commonly used in equine medication. The HSL is derived from the IPC (for plasma) or from the IUC (for urine), established during the risk assessment step. The EHSLC decided to keep HSL confidential and to inform stakeholders of the duration of the detection time (DT) of the main medications when screening is performed with the HSL. A DT is the time at which the urinary (or plasma) concentration of a drug, in all horses involved in a trial conducted according to the EHSLC guidance rules, is shown to be lower than the HSL when controls are performed using routine screening methods. These DTs, as issued by the EHSLC (and adopted by the Fédération Equestre Internationale or FEI) provide guidance to veterinarians enabling them to determine a withdrawal time (WT) for a given horse under treatment. A WT should always be longer than a DT because the WT takes into account the impact of all sources of animal variability as well as the variability associated with the medicinal product actually administered in order to avoid a positive test. The major current scientific challenges faced in horse doping control are those instances of the administration of recombinant biological substances (EPO, GH, growth factors etc.) having putative long-lasting effects while being difficult or impossible to detect for more than a few days. Innovative bioanalytical approaches are now addressing these challenges. Using molecular tools, it is expected in the near future that transcriptional profiling analysis will be able to identify some molecular "signatures" of exposure to doping substances. The application of proteomic (i.e. the large scale investigation of protein biomarkers) and metabolomic (i.e. the study of metabolite profiling in biological samples) techniques also deserve attention for establishing possible unique fingerprints of drug abuse.

Keywords Detection time · Doping · Horse · Irrelevant urine/plasma concentration · Medication control · Risk analysis · Threshold · Withdrawal time

1 Introduction

Even though there is a debate about what exactly constitutes an animal sport, it is accepted that the three most common sporting animals are horses (racing, jumping, eventing, polo), dogs (greyhound racing, sled dog racing, coursing, hunting) and camels (racing). However, many other mammalian species, including cattle (bull-fighting, American rodeo), and birds (pigeon racing) may compete or participate in events.

In all animals participating in sports, there are requirements for high physical capability and the presence of a competitive instinct. These traits are normally acquired through training programmes and selective breeding. It is also possible to strive to reach these objectives using certain ergogenic drugs, such as anabolic steroids, and to promote stamina by administering drugs acting on the central nervous system. Thus, two major issues relating to drugs and animals in sport arise and these are sometimes difficult to delineate: the "good", that is treatment given for the best health and welfare interests of the animal (legitimate medication) and the "ugly", that is the use of drugs primarily to alter or restore athletic performance. The word "doping" is reserved for this latter illegitimate use of drugs.

The aim of this chapter is to provide an overview on doping/medication control and to summarise recent advances in terms of scientific assessments and managerial options implemented by the International Federation of Horseracing Authorities (IFHA), a body which represents the main racing authorities in the world, and by the International Equestrian Federation (FEI), which is the world governing body of equestrian sports. For a recent overview on doping control see Higgins (2006) and the earlier seminal book of Tobin (1981).

2 Rationale for Anti-doping Versus Medication Control

An anti-doping programme is characterised by a set of values, some being common to man and animals, such as ethics, fair play and honesty, chosen to ensure competition based on true merit. Other values are specific to animals and used to protect the species or breed. "A level playing field" is considered to be pivotal for both the credibility and image of the racing industry, because this sport relies on betting and the confidence of the punter is therefore essential; this explains why, for racing horses, most racing authorities in the world which operate under the medication rules of the IFHA, excluding USA, have signed the so-called Article six of the International Agreement on Breeding and Racing. This article prohibits the

presence of any substances in a horse during a race which could give a horse an advantage or a disadvantage in that race.

Whilst the primary motivation of equine anti-doping control rules has been to prevent any attempt to alter a horse's performance i.e. to actually protect a business model, it is now accepted that a goal of this policy must be, to not indirectly impede the *bona fide* use of veterinary medications. Anti-doping rules should also protect the animal and guarantee its welfare. The European Convention for the Protection of Pet Animals expresses similar values when stating that "*no substances shall be given to, treatments applied to, or devices used on a pet animal for the purpose of increasing or decreasing its natural level of performance: during competition or at any other time when this would put at risk the health and welfare of the animal*". Even in bullfighting, which is not generally regarded as a sport, but rather as a cruel activity in many countries, drug tests are performed to detect the presence of substances such as tranquillisers that are considered as "unfair" for the bull. This latter example shows how an anti-doping policy may rely on a very different set of values and is contextual.

3 Medication Versus Doping Control: Progress Towards a General Policy Giving Priority to the Welfare and Safety of the Horse

The FEI and the European Horserace Scientific Liaison Committee (EHSLC), which is the European scientific committee in charge of harmonising sample testing and policies for racehorses in Europe (Barragry 2006; Houghton et al. 2004), have established a general policy that distinguishes the control of any drug *exposure* for all illicit substances (doping control) and the control of a drug *effect* for therapeutic substances (medication control). For sport horses, the FEI qualifies in its code that a doping agent is a substance with no generally accepted medical use in competition horses but which is able either to alter a horse's performance or to mask an underlying health problem. A list of these prohibited substances is given in the FEI medication code. This list includes many drugs acting on the central nervous system (stimulants, tranquillisers), anabolic steroids and growth promoters, genetically recombinant substances (erythropoietin, growth hormone), hormonal products (natural or synthesised) etc.

In the USA, the situation differs and, until recently, the use of anabolic steroids in horse racing was largely unregulated. In 2002, to address public concerns and the lack of uniformity between American states regulations, a Racing Medication and Testing Consortium (RMTC) was formed to represent most US industry stakeholder groups. The RMTC proposed a ban on exogenous anabolic steroids and testing for endogenous anabolic steroids (testosterone, nandrolone, boldenone); these proposals will be progressively enforced in the different American states by 2009. This new US approach is based on a model rule that now recommends no race

day medication. Currently, the main differences in opinions and practises between the RMTC and countries that have signed article 6 of the IFHA are the permitted use in the USA of the loop diuretic furosemide as an "anti-bleeder" medication (*vide infra*) and the permitted plasma levels of three non-steroidal anti-inflammatory drugs (NSAIDs), namely phenylbutazone (5 μg/mL), ketoprofen (10 ng/mL) and flunixin (20 ng/mL). For these three drugs, an IV administration is permitted at least 24 h before the "post" time for the race4.

3.1 Doping Agents and Doping Control Issues

The use of furosemide, a "high ceiling" diuretic, is currently the main obstacle towards international harmonisation. It is an exemplar to show how the same drug may be classified either as a doping agent or a beneficial drug for horse welfare by different jurisdictions. Furosemide is extensively and legally used in the USA prior to racing for its putative role in the prophylaxis of exercise-induced pulmonary haemorrhage (EIPH). It is proposed that it is in the horse's best interests to race using furosemide; if so, the horse is placed on the official furosemide list and can then be treated with furosemide no less than 4 h prior to "post-time" for the race in which the horse is entered. Furosemide should be administered by the IV route, the dose should be between 150 and 500 mg per animal and plasma concentrations may not exceed 100 ng/mL (For further information see section, "RMTC: Equine Veterinary Practises, Health and Medication" in chapter, "Veterinary Medicines and the Environment").

Such use is totally forbidden by Article 6 of IFHA and FEI. In the USA, furosemide is viewed as the "modern version" of blood-letting, because a dose of 1 mg/kg produces a rapid reduction in blood volume of approximately 8–9% of total volume. Furosemide modifies the haemodynamic response to exercise (see review of Hinchcliff and Muir (1991)). It was hypothesised that furosemide could reduce the lung-fluid volume by reducing arterial wedge pressure during exercise and could thereby mitigate the risk of EIPH. While the pharmacological cardiovascular effects of furosemide are well established, their actual protective role in EIPH is more controversial. A poor repeatability of an endoscopic score after furosemide treatment was shown (Pascoe et al. 1985) and a significant difference between untreated and furosemide-treated EIPH-positive horses (Sweeney and Soma 1984) could not be detected. However, a recent investigation showed that furosemide was able to decrease the incidence and severity of EIPH in thoroughbreds (Hinchcliff et al. 2009). It should be stressed that epidemiological surveys have provided evidence that furosemide may improve racing performance (Soma and Uboh 1998). In horses, furosemide decreased the oxygen debt and the rate of blood lactate accumulation. This effect can be reversed by adding to the horse a weight compensating for the loss of body weight due to the diuresis produced by furosemide (approximately 16 kg), suggesting that changes in performance observed in bleeder horses after a furosemide treatment is due to a small reduction in body

weight and not to a selective pharmacological action on bleeding mechanisms (Soma and Uboh 1998; Zawadzkas et al. 2006). In addition, furosemide is disapproved of because it causes diluted urine, i.e. its consumption is seen as an attempt to mask other illicit substances. For all these reasons, furosemide is considered as a doping agent by the FEI and most racing authorities in the world.

Anabolic steroids with androgenic properties (testosterone, stanozolol, nandrolone, boldenone) have been used routinely in the US as performance-enhancing substances in the horse. They possess behavioural effects and are credited with increasing the competitive instinct. Testosterone, boldenone (1,2-dehydrotestosterone) and nandrolone (19-nortestosterone) are endogenous to horses and their control requires the establishment of a threshold (Table 1). In horses, 19-nortestosterone is naturally produced by the testes as well as by the ovaries. This steroid can easily be detected in mares and geldings, because its major metabolite (estranediol which is the 5-alpha-estrane-3beta,17-alpha diol) is found only in the urine of treated horses. In contrast, in colts, estranediol is found in normal urine and it was shown that the ratio of estranediol (the metabolite) over the 5-estrene-3beta,17alpha diol, (a natural related steroid which is not a metabolite of nandrolone) may be considered as evidence of the possible abuse of nandrolone (Houghton and Crone 2000), because the probability of having a ratio higher than 1 in normal post-race urine was 1 in 10,000. In the USA, a threshold of 1 ng/mL is proposed for nandrolone. The logic, advantages and drawbacks of selecting a ratio rather than a simple cut-off value to establish a threshold are discussed in Sect. 7.

Genetically recombinant substances, such as recombinant growth hormone (reGH) and recombinant erythropoietin (reEPO) as doping agents are particularly difficult to control using available analytical approaches, because their effects last much longer than their presence in detectable concentrations in body fluids. An equine recombinant growth hormone (reGH) has been marketed for horses in Australia. It has been used illegally in racing horses. It is a methionyl equine somatotrophin produced by DNA technology. There is no controlled study to demonstrate any beneficial effect of reGh administration in supra-physiological amounts on trained horses. Chronic reGH administration does not alter aerobic capacity and indices of exercise performance in unfit aged mares, so that reGH was not an ergogenic substance in a subpopulation of unfit horses (McKeever et al. 1998). GH exerts its anabolic effect in part via secretion of Insulin-like Growth Factors (IGFs) by the liver. In horses the plasma concentration of IGF is increased by GH treatment but the duration of the response is too short to be an effective approach to control GH abuse (Popot et al. 2000). Current strategies for screening GH abuse in horses rely on the long-term detection (up to 200 days) of specific anti-reGH antibodies, produced as a consequence of repeated reGH administrations (Bailly-Chouriberry et al. 2008a). A confirmatory method for reGH detection in plasma/urine is required for regulatory purposes. An analytical strategy based on LC-MS/MS through the identification of the reGH N-terminal characteristic peptide was developed but the detection time (DT) is very short (48 h) reflecting the possible delayed effects of this class of compound (Bailly-Chouriberry et al. 2008b).

Table 1 Substances for which a threshold has been adopted or proposed by different jurisdictions or organisations

Substance/jurisdiction	Threshold
Arsenic/IFHA	• 0.3 μg total arsenic per mL in urine
Boldenone/IFHA, RMTC, FEI	• 0.015 μg free and conjugated boldenone per mL in urine from entire male horses (not geldings) • No boldenone shall be permitted in geldings or female horses
Carbon dioxide/IFHA	• 36 mM available carbon dioxide per litre in plasma
Dimethyl sulphoxide/IFHA, FEI	• 15 μg/mL in urine, or • 1 μg/mL in plasma
Estranediol in male horses (other than geldings) as a biomarker of nandrolone abuse/IFHA, FEI	• 0.045 μg free and glucuroconjugated 5α-estrane-3β,17α -diol per mL in urine
Estranediol in male horses (other than geldings) as a biomarker of nandrolone abuse/Hong Kong Jockey Club,Emirates Racing Authorities, Fédération Nationale des courses françaises and some other juridictions	• The mass of free and conjugated 5*a*-estrane-3β, 17*a*-diol to the mass of (other than geldings) free and conjugated 5(10)-estrene -3β, 17*a*-idol in urine from entire male horses (not geldings) at a ratio of 1
Nandrolone/RMTC	In geldings, mare and fillies: 1 ng/mL in urine
Hydrocortisone/IFHA	• 1 μg/mL in urine
Methoxytyramine/IFHA	• 4 μg free and conjugated 3-methoxytyramine per mL in urine
Salicylic acid/IFHA	• 750 μg/mL in urine, or • 6.5 μg/mL in plasma
Salicylic acid/FEI	• 625 μg/mL in urine, or • 5.4 μg/mL in plasma
Testosterone/IFHA, RMTC	• 0.02 μg free and conjugated testosterone per mL in urine from geldings, or • 0.055 μg free and conjugated testosterone per mL in urine from fillies and mares (unless in foal)
16β-hydroxystanozolol (metabolite of stanozolol)/RMTC	• 1 ng/mL in urine for all horses regardless of sex; • Forbidden by IFHA and FEI
Theobromine/IFHA	• 2 μg/mL in urine
Caffeine/RMTC	• 100 ng/mL of serum or plasma

IFHA: International Federation of Horseracing Authorities
RMTC: Racing Medication and Testing Consortium
FEI: Federation Equestre Internationale

To overcome the limitations of traditional methods, new and sensitive methods based on fingerprint strategies are currently being considered (see Sect. 5).

Erythropoietin is a natural glycoprotein hormone, produced mainly by the kidneys. It regulates mammalian erythrocyte and haemoglobin production. There is evidence that recombinant human EPO (rhEPO), Darbepoietin, (a synthetic long-acting rhEPO) and many biosimilar (generic) rhEPOs are used in horses. The expected effect of EPO in horses is an increase in the red blood cell mass providing

improvement in oxygen-carrying blood capacity and enhancing the horse's aerobic exercise performance. The administration of rhEPO (Eprex, Janssen-Cilag at a dosage of 50 μg/kg BW, IV three times weekly for 3 weeks) increased haemoglobin concentration, haematocrit and red blood cell count by 25% in horses. Peak values were reached 1 week after the last treatment and the increased values persisted for 3–4 weeks (Lilliehook et al. 2004). In unfit horses it was shown that rhEPO enhanced aerobic capacity without either altering anaerobic power or improving exercise performance (McKeever 1996). The effects of EPO on the performance of a fit horse are unclear. Horses, in contrast to man, have an erythrocyte storage type of spleen, exerting the role of a reservoir, which can, in resting conditions, store up to 30% of the total red blood cells, and a spleenic contraction can mobilise up to 12 L of extra blood. During exercise, this reserve may be liberated immediately into the circulation by splenic contraction, thereby increasing the blood oxygen-carrying capacity. Horses may be described as "natural blood dopers". In this context, the actual effect of EPO on performance in horses remains unclear. Whatever the actual EPO effect, the prolonged half-life of RBCs (140 days in the horse) allows a putative benefit of the EPO to develop over several weeks without the risk of being detected as positive.

Using an ELISA test, the excretion profile after EPO administration to horses indicates that rhEPO may be easily detectable during the first 10 h after an IV administration but, after a delay of 48 h, EPO concentrations were indistinguishable from background levels (Tay et al. 1996). rhEPO may also be directly detectable for a few days only in horses by detecting the peptides of EPO using sensitive LC/MS/MS technology (Guan et al. 2007). Long-term use of rhEPO can be detected by screening horse plasma for EPO antibodies but no change in the level of rhEPO antibodies was observed after 3 weeks of rhEPO administration (Lilliehook et al. 2004) This immunological response to rhEPO has been responsible for an adverse response in the form of an immune-mediated anaemia and the deaths of treated horses (Piercy et al. 1998). From a mechanistic point of view, a recent study showed that rhEPO binds to the surface of the EPO receptor (EPOr) and that the rhEPO–EPOr complex is subsequently internalised into EPOr containing cells, where the rhEPO is degraded by lysosomal enzymes. RBCs possess EPOr but no lysosomal degradation system and it was shown in horses that rhEPO may accumulate in RBCs and remain elevated for up to 13 days (Singh et al. 2007). It was suggested that analysis of rhEPO in RBCs may be a better indicator of rhEPO abuse in horses. Another option to control rhEPO and all other analogues and biosimilar substances is to perform unforeseen regular controls on horses out of competition and to develop, as for eGH, new approaches to assess the imprinting of EPO using genomic resources (see Sect. 5).

3.2 Medication Issues and Medication Control

In contrast to anti-doping control, equine medication control rules seek to prevent medication violations, while protecting the welfare of the horse. In the FEI

medication code, these substances are classified in the equine Prohibited List either as Class A Medications (drugs attracting moderate sanctions and penalties) or Class B Medications (drugs attracting minor sanctions and penalties). Examples of class A medications are substances which could influence performance by relieving pain (NSAIDs, local anaesthetics, etc). Examples of class B medications include substances that have either limited performance enhancing potential (e.g. mucolytics and cough suppressants) or to which horses may have been accidentally exposed, including certain dietary contaminants (e.g. bufotenine, hordenine etc.).

The FEI acknowledges that the use of medication in a horse close to an event may be required but is inherently risky in term of medication control if insufficient time has elapsed for elimination of the drug from the horse. To support good veterinary practises, the FEI selected some twenty essential drugs that are collectively known as the FEI "Medicine Box". These are all legitimate treatments that might be used in routine clinical practise during the time closely preceding an event and for which the FEI decided to provide the information (detection times) needed for appropriate use.

Certain medications are permitted under FEI Rules. These currently include rehydration fluids, antibiotics (with the exception of procaine benzylpenicillin) and anti-parasitic drugs, with the exception of levamisole. In addition, some drugs to treat or prevent gastric ulcers may be given (i.e. ranitidine, cimetidine and omeprazole). The use of altrenogest is currently permitted for mares with estrus-related behavioural problems because altrenogest suppresses behavioural estrus in the mare within the 2–3 days following the beginning of the dosing schedule and, at the recommended dose, has no effect on dominance; hierarchy; body mass and condition score (Hodgson et al. 2005).

4 Analytical Method and Doping Testing

A sample (plasma, urine or any other matrix) that has been collected under a secure chain of custody (Dunnett 1994) must be tested by means of validated, state-of-the art drug-testing assays. Due to legal implications, all aspects of the testing procedures should be traceable and all *ad hoc* documents should be available for possible court testimony. Laboratories involved in doping control programmes should comply to a set of minimal standard as described by the AORC *Guidelines for the Minimum Criteria for Identification by Chromatography and Mass Spectrometry* to ensure that the quality and integrity of the data are defensible and fit for purpose. In addition, to conduct a referee analysis i.e. to perform a confirmatory analysis on the split (or so-called B) sample, referee laboratories should be accredited to ISO/IEC 17025 (Hall 2004), and must be member laboratories of either the association of official racing chemists (AORC) or the World anti-doping agency (WADA).

Drugs are commonly analysed and identified using chromatographic/mass spectrometric techniques, which allow for the determination of approximately 95% of

all target analytes (Thevis et al. 2008). Gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS) are techniques that can provide unequivocal evidence of the presence of a prohibited substance (Thevis and Schanzer 2007; Van Eeno and Delbeke 2003). They are considered as the sole techniques that are suitable on their own for confirmatory methods.

One of the analytical challenges for horse doping control is to distinguish hormones of endogenous vs. exogenous origin (e.g. cortisol, testosterone). Gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) is an isotopic method able to measure accurately small differences in the $^{13}C/^{12}C$ ratio of endogenous vs. synthetic steroids. In horses this technique has been explored for cortisol (Aguilera et al. 1997) and nandrolone (Yamada et al. 2007). However, this approach has a low sensitivity and requires concentrations of about 10–20 ng/mL to reliably measure the $^{13}C/^{12}C$ ratio of a molecule. In addition, it is a labour intensive and costly method to perform and is used only to provide supportive evidence of the exogenous administration of hormones.

The major scientific challenge faced today for horse doping control is the case of recombinant biological substances (EPO, GH, growth factors) having putative long-lasting effects while being difficult or impossible to detect over a few days (see Sect. 4). Innovative bioanalytical approaches are now progressing for solving these relevant emerging problems in horse anti-doping control. A promising approach is based on the analysis of gene expression in peripheral blood cells (leucocytes). There is evidence that white blood cells respond to many of these anabolic factors and this is observable for a long time after the disappearance of the substance itself. Using molecular tools, it is expected in the next future that transcriptional profiling analysis would be able to identify some molecular "signatures" of exposure to these doping substances. Resources of proteomic (i.e. the large scale investigation of protein biomarkers) and metabolomic (i.e. the study of metabolite profiling in biological samples) also deserve attention in establishing possible unique fingerprints of drug abuse.

5 Blood Versus Urine Testing and the Rationale for Selecting a Matrix for Doping and Medication Control

Currently, most controls are performed using urine but blood (plasma) should be seriously considered as a better matrix for medication control. From a pharmacokinetic/pharmacodynamic (PK/PD) point of view, the drug (free) plasma concentration is considered as the best surrogate of the drug biophase concentration. Thus, the plasma concentration is the best predictor of the drug's effect. Exceptions are diuretics for which the urine concentration is a better predictor of the drug's effect because all diuretics gain access to their target receptor directly from renal tubular fluid and not from the blood. Plasma concentrations control the amount of drug (or metabolites) excreted in urine. As such, urine drug concentrations may be viewed as

a surrogate of plasma concentrations. However, urine concentrations may also be influenced by many other factors such as urine volume and pH (for ionisable drugs) rendering the relationship between plasma and urine concentrations imprecise. The urine-to-plasma concentration ratio (Rss) varied very considerably between drugs and is also a time dependent variable. It is equal to zero just after an IV drug administration (i.e. when drug effect may be near maximal as for an anaesthetic drug) and it becomes only "invariant" i.e. a useful "parameter" after some delay i.e. when an equilibrium between plasma and urine concentrations is achieved. For a multiple dose administration regimen (and whatever the route of drug administration), the relationship between the plasma and urine concentration may be confounded by a hysteresis (lag-time between plasma and urine concentrations) and it is possible to have plasma and urine concentrations out of phase. In this situation, a peak effect may correspond to the trough urine concentrations. For some drugs, there is no (or very low) renal clearance and for that class of drugs urine is not an appropriate matrix for testing. For proteins, the renal clearance of the intact molecule is generally negligible due to the high protease activity in the proximal tubule of the nephron (some exceptions exist such as for GH and EPO) rendering urine unsuitable for monitoring many peptides or proteins of potential abuse. In addition, in man, proteases may be added fraudulently to the urethra rendering it difficult to detect protein in the urine (Thevis et al. 2008; Thevis and Schanzer 2007). Conversely, metabolic reactions of bacterial origin may occur in urine samples (for example for some corticosteroids) spuriously increasing the concentration of the analyte of interest after the sampling. For all these reasons, urine is a less robust matrix than plasma and the parent plasma drug concentration is generally the best analyte to select and to assess the systemic drug effect. The main consideration for changing from urine to plasma to enforce a medication control policy is an analytical issue, because for most drugs urine drug concentrations are higher or even much higher than plasma concentrations.

Other matrices are usable for doping control such as hair and faeces. Thanks to the major advances in analytical methodology, hair analysis may provide additional analytical evidence to that obtained from blood or urine analyses (Dunnett and Lees 2003; Popot et al. 2002). Hair is a very stable medium, in which drugs and their metabolites can be detected over prolonged periods. Hair analysis can thus provide a historical record of drug exposure for some critical drugs such as anabolic steroids. Hair seems more suitable for population surveys and investigation surveillance than for routine individual doping control. The limitation of hair as a matrix is a possible contamination of the sample from external sources such as urine, sweat from another horse etc.

It is known that endogenous steroids and different xenobiotics are eliminated by faeces and faeces may be an attractive alternative matrix to collect in yearlings for safety reasons. The presence of boldenone in horse faeces was confirmed after an oral administration of 1,4-androstadiène-3,17-dione and meclofenamic acid was detected for 6 days post-administration (Popot et al. 2004). For pigeon racing, taking blood for routine drug testing is too invasive to be acceptable for pre-race testing and faeces (actually a mixture of faeces and urine) is the appropriate matrix (de Kock et al. 2004).

6 Substances Requiring a Threshold

Horses may be regularly exposed to prohibited substances that are natural components of their feed. Salicylic acid (SA) is a stress plant substance found in many plants including alfalfa (lucerne) which explains the natural occurrence of SA in horse urine and the possible detection of SA in all post-race urine samples. As SA is the active metabolite of aspirin, a NSAID, SA is a prohibited substance and without a threshold, it would be necessary to report all these innocent positive cases. Dimethyl sulfoxide is another example of an ubiquitous natural product. Horses may also inadvertently be exposed to substances that are contaminants of manufactured feeds (e.g. theobromine due to presence of cocoa husks in feed) or by contaminants coming from the environment (e.g. arsenic). The concept of threshold was introduced to solve these unavoidable exposures of alimentary origin (Houghton 1994) i.e. when it was considered there was no other management option to solve the problem of innocent positive samples. For SA a threshold was fixed at 750 μg/mL (see Table 1) because natural exposure cannot result in a urine SA concentration above this cut-off value with a risk of about 1 in 10,000. The threshold was recently re-investigated and it was shown that a threshold of, 614 μg/mL, in urine was more suitable (Lakhani et al. 2004). For some other substances contaminating equine feed, no threshold has been fixed, because it was considered as undesirable in terms of communications for the industry to release such a threshold. This is the case for morphine (contamination by poppy seed) and for benzoylecgonine which is a metabolite of cocaine.

In addition to these exogenous substances, some endogenous hormonal substances can be administered, either to rest or a "natural" hormonal profile as is the case for testosterone in a gelding or to obtain an overexposure to achieve some pharmacological effects as is the case for cortisol which is a psychostimulant. Two approaches are used to fix a threshold: either to fix a single cut-off value as for cortisol in urine (1.0 μg/mL) or rather to use a concentration ratio between a marker of the administered compound (the substance itself or one of its metabolites) and another endogenous substance that plays the role of an "internal standard", i.e. an analyte structurally related but that is not metabolically related to the administered substance of concern. The logic of selecting a ratio rather a single cut-off value is the assumption that a ratio will be less variable regarding inter-subject differences and to possibly benefit from some negative feedback which may amplify the shift of the ratio in the case of exogenous administration. This is the case for the ratio testosterone/epitestosterone in man, used for the control of testosterone administration or for the ratio estranediol over the 5-estrene-3beta,17alpha diol for the control of nandrolone in colts. In the case of exogenous testosterone administration, the numerator (testosterone) is increased as expected, whereas the denominator (epitestosterone a substance that is produced only locally by the transformation of endogenous testosterone in the testis) is reduced by the negative feedback on the natural testosterone production in the testis. This possible advantage of a ratio should be balanced against the ability to manipulate a ratio by also administering

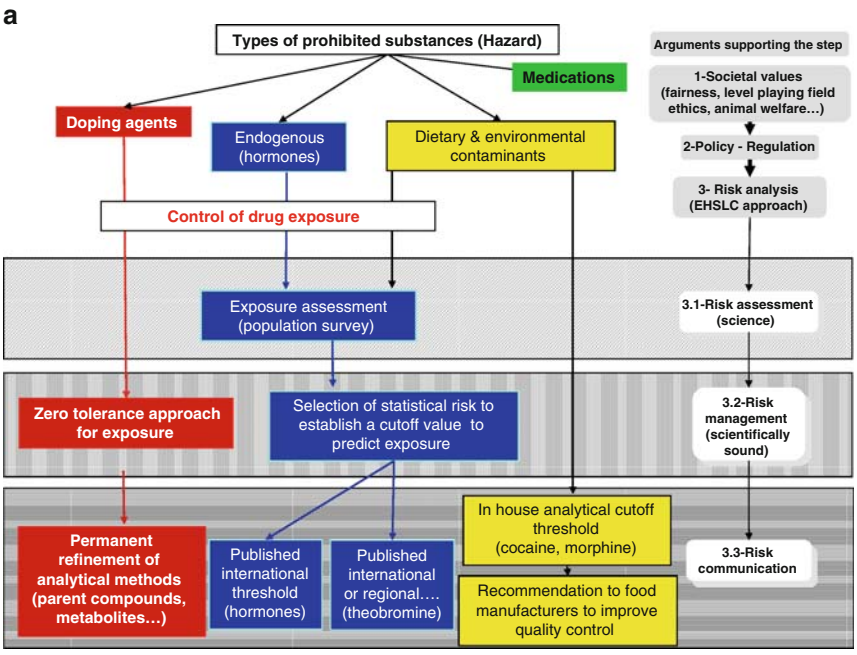
the "internal standard" to maintain the ratio value in its physiological range. In addition, the ratio approach is more challenging and time consuming from an analytical perspective, especially if one of the analytes is suppressed by negative feedback. For that reason, the principle of a single testosterone cut-off was selected in horses.

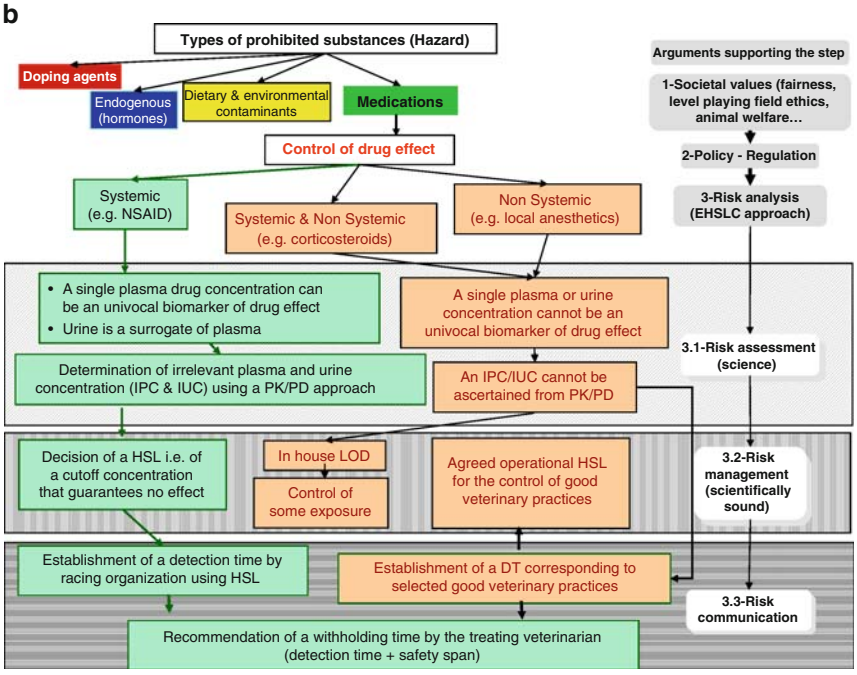
The establishment of a threshold requires the analysis of a large number of representative (international) samples (e.g. post-competition samples) collected from the future targeted population(s) and some administration/food trials. The data set is then statistically analysed with the aim of determining a critical value corresponding to a given population quantile. As generally the number of samples is too low to select directly a quantile (e.g. 1/10,000), the critical value is calculated from the observed or assumed distribution. Very often, the data are not normally distributed but positively skewed as for example for the log-normal distribution. The selection of an appropriate transformation is critical because the threshold that is subsequently calculated, for a given nominal risk, may be very different depending on the selected distribution. For example, both a log-normal and a cube root transformation were able to normalise the observed urine cortisol distribution but the cut-off value for a 1/10,000 quantile was 1,025 ng/mL (rounded to 1,000 ng/mL) with the log-normal distribution against 410 ng/mL for the cube root transformation; finally the most conservative cut-off (from the horse's perspective) was selected (Popot et al. 1997). There is no single accepted critical quantile but the case of SA likely created a precedent and quantiles lying between 1/1,000 and 1/32,000 are generally selected (Houghton and Crone 2000).

Due to regional differences in food ingested (e.g. Lucerne hay in the USA versus grass hay in Europe) and feed contamination, it may be difficult and/or unsatisfactory to fix a single international threshold covering with the same statistic routinely at risk to all horses in the world. It may be more meaningful to develop regional thresholds reflecting local practises and constraints. The logic used in establishing the theobromine threshold was different; it consisted of feeding horses with feed contaminated with different theobromine concentrations knowing that the maximal expected food contamination cannot be higher than 1.2 mg/kg. When horses were fed with this diet, the maximal urine concentrations were less than 0.60 μg/mL and the threshold was fixed to 2 μg/mL (Houghton and Crone 2000).

7 Testing Exposure and the End of a Zero Tolerance Approach for Medication Control

For doping drugs, i.e. illicit substances, with no accepted medical use in horses, the goal is to control any drug exposure (parent drug or metabolites) using the most powerful analytical methods. Although ideal for doping control, the "zero tolerance rule" is not suitable for medication control (Smith 2000; Spencer et al. 2008).


a
Types of prohibited substances (Hazard)
Medications
Doping agents
Endogenous (hormones)
Dietary & environmental contaminants
Control of drug exposure
Exposure assessment (population survey)
Zero tolerance approach for exposure
Selection of statistical risk to establish a cutoff value to predict exposure
Permanent refinement of analytical methods (parent compounds, metabolites...)
Published international threshold (hormones)
Published international or regional.... (theobromine)
In house analytical cutoff threshold (cocaine, morphine)
Recommendation to food manufacturers to improve quality control
Arguments supporting the step
1-Societal values (fairness, level playing field ethics, animal welfare...)
2-Policy - Regulation
3- Risk analysis (EHSLC approach)
3.1-Risk assessment (science)
3.2-Risk management (scientifically sound)
3.3-Risk communication


b
Types of prohibited substances (Hazard)
Doping agents
Endogenous (hormones)
Dietary & environmental contaminants
Medications
Control of drug effect
Systemic (e.g. NSAID)
Systemic & Non Systemic (e.g. corticosteroids)
Non Systemic (e.g. local anesthetics)
• A single plasma drug concentration can be an univocal biomarker of drug effect
• Urine is a surrogate of plasma
A single plasma or urine concentration cannot be an univocal biomarker of drug effect
Determination of irrelevant plasma and urine concentration (IPC & IUC) using a PK/PD approach
An IPC/IUC cannot be ascertained from PK/PD
Decision of a HSL i.e. of a cutoff concentration that guarantees no effect
In house LOD
Control of some exposure
Agreed operational HSL for the control of good veterinary practices
Establishment of a detection time by racing organization using HSL
Establishment of a DT corresponding to selected good veterinary practices
Recommendation of a withholding time by the treating veterinarian (detection time + safety span)
Arguments supporting the step
1-Societal values (fairness, level playing field ethics, animal welfare...
2-Policy - Regulation
3-Risk analysis (EHSLC approach)
3.1-Risk assessment (science)
3.2-Risk management (scientifically sound)
3.3-Risk communication

Currently, the same powerful analytical processes are used to screen for all substances, regardless of their potencies or their regulatory status. The consequence is that trace concentrations of therapeutic substances, totally irrelevant in terms of clinical or physiological effects, may now be detected for a long time (days or weeks) after their therapeutic administration. As such the zero tolerance policy is inappropriate for medication control and this opens the way to a new approach for legitimate medication based upon PK/PD principles to estimate the order of magnitude of the so-called irrelevant drug concentrations in plasma and urine (Toutain and Lassourd 2002a) and to limit the sensitivity of analytical techniques used for medication control (*vide infra*). Smith (2000) addressed the background for a

Fig. 1 Risk analysis applied to doping and medical control. Depending on the values claimed by the various organisations, prohibited substances are classified under 4 categories: doping (illicit), endogenous (hormones, CO_2, etc), dietary or environmental contaminants and medications (legitimate drugs). The final policy applied to each substance results from a risk analysis consisting of three different steps: RA, RM and RC. The RA is a wholly scientific exercise performed by risk assessors (scientists) and aimed at providing the risk manager with initial scientific data to perform the RM exercise. For doping substances, there is no scientific assessment as these drugs are a priori considered as illicit. For endogenous or dietary contaminants, scientists have to qualify the exposure in the target population (population distribution) and for medication control scientists have to provide the order of magnitude for which an exposure has an effect or not. This is done by computing irrelevant plasma (IPC) or IUC. The next step is the RM. This step is performed by risk managers (typically racing authorities) who are not scientists but nevertheless the RM should be a scientifically sound exercise. The risk manager has to decide the statistical level of risk for an endogenous product, or to decide an analytical cut-off value for screening of medications. For this, the risk manager may mitigate the IPC and IUC as determined by risk assessors considering the possibility or not of harmonising, the cost of analytical techniques, and the feasibility etc. The final decision will be a threshold value (endogenous substance or contaminant) or a harmonised screening level (HSL). For some drugs acting both locally and systemically, there is no single concentration value which covers every situation and the risk manager has to select some options (liberal or conservative). For example, to control intra-articular corticoid administration, it would be necessary to fix the HSL at a very low level (few pg/mL in urine) but in doing so, it would be impossible to use the same corticoid for a systemic administration because a urine concentration of a few pg per mL for a systemic treatment has no meaning. Conversely, determining the IPC and IUC corresponding to a systemic treatment using the PK/PD approach would not be conservative enough for an intra-articular administration. The strategy adopted by the manager may be to determine an HSL in order to support good veterinary practise and not to guarantee a lack of any effect for any route of administration for that drug (local or systemic). For example, for a local anaesthetic, good veterinary practise would consist in not using it within the 2 days preceding a race. To be consistent with this rule, the risk manager can adopt an HSL high enough to have no positive control for those observing this delay. Actually, to control good veterinary practise consists firstly, to select a withholding time (regulatory or professional considerations) and then, to establish the corresponding plasma/urine concentration. The last step is a communication step. For doping control it is explained to all stakeholders that the institution controls any exposure and that there will be a continuing improvement of the analytical techniques. For medication control, the pivotal item of communication is the detection time. Detection time is established by racing authorities (or FEI) to give to the practitioner an order of magnitude of the future withholding time. This withholding time is a veterinary decision and the practitioner should perform his own risk analysis to fix the withholding time by adding a safety span to the detection time established by racing organisations

consideration of this approach (see also Houghton (1994); Tobin et al. 1998, 1999); (Spencer et al. (2008) for reviews).

8 The Decision Making Process on No Significant Effect Levels: A Risk Analysis Integrated Approach

To solve the dilemma of whether or not to report trace levels of drugs used legitimately for therapeutic medication, the EHLSC developed a general approach following the principle of risk analysis. Figure 1 gives an overview of risk analysis for doping/medication control.

A risk analysis is a structured (formalised) approach using risk concepts. I It includes three steps: risk assessment (RA), risk management (RM) and risk communication (RC). The reasons for adopting a risk analysis are when harmonisation is a requirement, regulatory decisions need to take into account competing interests using an unbiased and transparent approach.

Before developing any risk analysis, an institution (FEI, EHLSC, IFHA) should express formally what its values and standards are, i.e. its ideal rules of conduct. For the EHSLC, this includes giving priority to protect the welfare of the horse, to defend the integrity of the sport, to protect the breed and to reassure the public.

8.1 Risk Assessment

The RA is a scientifically based process comprising the following steps: (1) hazard identification, (2) hazard characterisation, (3) exposure assessment and (4) risk characterisation.

The hazard identification consists of identifying the hazardous agent that may result in a negative (harmful) impact and the "receptors", that is the specific things or entities affected by the hazard. For medication and doping control, hazardous agents are legitimate and illegitimate drugs, contaminants and endogenous products. Receptors are horses (animal welfare), punters and public (betting, public concern), Institutions (trust and confidence in regulation) and owners and trainers (business).

Hazard characterisation is the qualitative and/or quantitative evaluation of the nature of the adverse effects associated with the hazard. For medication control, a non-experimental PK/PD approach to determine irrelevant drug plasma concentrations (IPC) and IUC has been proposed (Toutain and Lassourd 2002a, b).This non-experimental method consists of retrieving published PK parameters and variables to calculate IPC and IUC as follows: consider that an effective dose is a PK/PD hybrid variable determined by two PK parameters (plasma clearance and

bioavailability) and one PD parameter i.e. the effective plasma concentration (EPC) as given by (1)

$$\text{Effective dose rate} = \frac{\text{plasma clearance} \times \text{effective plasma concentration}}{\text{bioavailability}}. \quad (1)$$

It was suggested that (1) can be rearranged to estimate the EPC for a standard approved dosage regimen as (2):

$$\text{EPC} = \frac{\text{standard dose (per dosing interval)}}{\text{plasma clearance (per dosing interval)}}, \quad (2)$$

where plasma clearance is the genuine PK parameter that expresses the ability to eliminate a drug.

As only the intravenous route of administration was considered for this RA, bioavailability in (1) was fixed at 1. EPC may be taken as the average plasma concentrations over the dosing interval following the administration of an effective dose and, as such, EPC is a relevant surrogate of PD or clinical endpoints.

The next step involves transforming an EPC into an IPC and then into an IUC.

The IPC and IUC are defined as drug plasma (serum) or urine concentrations that guarantee the absence of any relevant drug effect.

The IPC can be deduced from the EPC by applying a safety (uncertainty) factor (SF) to EPC (3):

$$\text{IPC} = \text{EPC}/\text{SF}. \quad (3)$$

The selection of a SF is both a scientific and a regulatory decision. A default value of 500 (i.e. 50 × 10) has been suggested for the following reasons: firstly a factor of 50 was selected to transform a mean EPC into a mean IPC. This figure assumes that horse medications are marketed at a dose corresponding to (or at least similar to) their ED_{50} i.e. to a dose level able to achieve half the maximal possible effect of that drug; and that the dose effect relationship is described by a classical E_{max} (hyperbolic) model. If these two hypotheses hold, then dividing the ED_{50} by 50 leads to the estimation of an ED_2 i.e. the dose corresponding to only 2% of the maximal possible effect of that drug. Secondly, in order to take into account the inter-individual variability of PK and PD parameters in the horse population, a factor of 10 was selected (i.e. 3.3 for PK variability and 3.3 for PD variability). This factor of 3 is the common standard in toxicology and is known as the rule of 3s (CV≈ 25%) regardless of the source of uncertainty. Thus, the IPC may be viewed as a lower boundary of a population plasma concentration corresponding to a residual drug effect of 2%. It may be noted that fixing the SF to infinity would be equivalent to following the zero tolerance rule i.e. from an operational point of view, to control drug exposure with the highest performance analytical techniques, as for illicit substances.

Next, the IUC is derived from the IPC using (4):

$$\text{IUC} = \text{IPC} \times \text{Rss}. \tag{4}$$

In (4), Rss is the steady-state urine to plasma concentration ratio.

The main difficulty with IUC is the uncertainty of the retrieved Rss. Rss is seldom reported and difficult to evaluate. It is influenced by several biological factors (see Sect. 6) and a given snapshot urine concentration may correspond to very different plasma concentrations (and effects), because there is no guarantee that the horse is in a pseudo-equilibrium condition (single dose) or under steady-state conditions (multiple doses) at the time of sampling.

This inexpensive and straightforward approach requires that the marketed effective dose rate is actually appropriate. Drugs eligible for the PK/PD model must act systemically, i.e. the pharmacological effect should be directly governed by the plasma concentration. Thus, local anaesthetics, substances administered intra-articularly or by inhalation are not considered suitable candidates for analysis using this PK/PD model.

Finally, the proposed hazard characterisation method aims at determining an order of magnitude of the required sensitivity of the analytical technique, and IPC and IUC are starting values that will be used during the RM process to decide a screening limit.

8.1.1 Exposure Assessment

There is no risk without exposure, and for medication or doping control, the question of the origin of the exposure, i.e. how will exposure occur (sources assessment), is generally simple to identify. Inquiries following positive cases show that most often it is some kind of error (e.g. inappropriate large dose for intra-articular corticosteroid administration), bad veterinary practise (use of a drug without marketing authorisation and for which no information exists for rational use), lack of observance of a minimal withholding time by the trainer... and also cheating. Sometimes the source of exposure is more difficult to identify. It was observed that drugs can be detected in horse urine for a longer time than is expected from their intrinsic PK properties (Lees et al. 1986). Norgren et al. (2000) and Wennerlund et al. (2000) reported that flunixin or naproxen were detected in the urine of untreated horses that resided for several days in boxes previously allocated to horses treated with flunixin or naproxen. This suggests some cross contamination via the bedding. Possible contamination by ingesting straw contaminated by urine was also observed for dipyrone, chlorpheniramine and procaine and well documented for meclofenamic acid (Popot et al. 2007). Hence, it was concluded that spurious urinary drug rebound may lead to some positive controls after the recommended withholding time.

The question of exposure assessment is more demanding for compounds requiring the establishment of a regulatory threshold because the statistical distribution of the analyte of interest should be qualified.

8.1.2 Risk Characterisation

Risk characterisation is the last step of RA. It is an estimation of the severity of the "adverse effect" and it involves integration of the hazard, hazard characterisation, and exposure and should be expressed numerically to the risk manager. For illicit substances, it will be the "minimal" limit of detection (LOD) required for an analytical technique or the minimal performance to achieve for any other marker of exposure such as an antibody for growth hormone or EPO. For medication control, the IPC and IUC as calculated during the RA step will be provided to allow risk managers to propose a screening LOD. For endogenous analytes, a statistical distribution of the concentration of interest will be given, allowing risk managers to fix a local, regional or international threshold. For feed contaminants, it should be proposed to risk managers that they either fix a threshold (using the same approach as for endogenous product) or, alternatively, that some measure of correction be suggested to the manufacturer.

8.2 Risk Management

RM is the process of weighing policy alternatives in the light of the RA in order to minimise or reduce the assessed risk. It consists of selecting and implementing appropriate options such as prevention, control and regulatory measures. RM is not a scientific exercise but it should be scientifically sound. Sound science does not exist as a "ready for use" entity in the policy development process, so that scientific data should be subjected to a reasoned interpretation for regulatory purposes. RM is carried out by risk managers i.e. the racing authorities. They have to make decisions based not only scientific facts, but also on all relevant information from other sources including the specific values and criteria of their own organisation. For example, the FEI considers that omeprazole, a proton pump inhibitor extensively used for ulcer control, is not a prohibited substance for international horse sporting competitions and, as such, does not have to be included in a screening programme for medication control. On the other hand, it is a prohibited substance for European racing organisations. Similarly, furosemide is accepted by many US jurisdictions for the prevention of EIPH but is prohibited in Europe for reasons explained in Sect. 4. At first glance, this seems illogical and inappropriate but it should be acknowledged that science is not always able to resolve societal choices concerning what decisions to take in the case of competing interests. Science can describe the world, but science cannot determine what the world should be. Therefore, different regulatory jurisdictions may reach markedly different regulatory conclusions, based upon the same set of scientific data. For this reason, international standardisation should focus on the process of RA, which is primarily a scientific task, rather than on the harmonisation of risk criteria and RM.

For medication control, the main task at the RM step is the establishment of agreed HSL for all laboratories engaged in the EHLSC programme. The HSL is a

confidential instruction to laboratories from Racing Authorities to screen at a plasma or urine level for the presence of drugs commonly used in equine medication. The HSL is deduced from the IPC (for plasma) or from the IUC (for urine), established during the RA exercise but the HSL may be (slightly) higher or lower than the IPC/IUC to take into account other relevant factors than residual drug efficacy as the common goal to achieve harmonisation. Thus, it is verified that all countries are in a position to enforce the selected HSL. It should be stressed that HSLs are not equivalent to specific quantitative thresholds; they are decisions resulting from a RM exercise. Monitoring the HSLs through screening procedures will greatly simplify the analytical process compared to the use of absolute quantification. HSLs are not considered as threshold values.

For food contaminants, the most efficient means of avoiding inadvertent positive cases is to test the feeds. When this is impossible in practise, an analytical threshold is selected and the value selected by the risk manager is the statistical risk (usually approximately 1 in 10,000).

8.3 Risk Communication: Detection Times Versus Withdrawal Times

RC is an integral part of the risk analysis process: it is the interactive exchange of information and opinions between risk assessors, risk managers and stakeholders. Efficient RC requires the provision of meaningful, relevant and accurate information in clear and understandable terms. It is targeted at specific audiences (trainers, veterinarians, punters, etc) in order to improve the overall effectiveness of the control process. For medication control, it is evident that a screening limit does not fulfill these requirements, because a cut-off plasma/urine concentration does not comprise "ready for use" information for veterinarians who must advise owners or trainers on appropriate withholding times. Therefore, the EHLSC decided to keep this information confidential and rather to communicate the duration of the DT of the main medications when screening is performed, with the harmonised but unavailable screening limit. This led the EHLSC to embark on a programme to define DTs for the major veterinary medicinal products by conducting a series of standardised excretion studies.

A DT, according to the EHSLC definition, is the time at which the urinary (or plasma) concentrations of a drug, in all horses involved in a particular trial conducted according to the EHSLC guidance rules, are shown to be lower than the HSL when controls are performed using routine screening methods. It should be stressed that the DTs, as issued by the EHSLC (and followed by the FEI, see the FEI web site), are not synonymous with withdrawal times (WT). A DT is a raw experimental observation, whereas a WT is a recommendation and, as such, requires the professional judgement of the treating veterinarian. A WT should be longer than a DT because the WT should take into account the impact of all sources of animal variability (age, sex, breed, training, racing) as well as the variabilities associated with the medicinal product

actually administered (formulation, route of administration, dosage regimen, duration of treatment) in order to avoid a positive test.

One of the possible limitations of the information provided by published DTs in horses is the fact that they are determined from classic PK studies conducted in animals at rest and performed under laboratory conditions on a limited number of horses (generally 6 or 8). Under field conditions, training and exercise programmes may influence the rate of drug elimination. In horses, there is virtually no experimental data on the direct effect of exercise on drug disposition and hence on DT. To explore the possible influence of exercise on the DT as obtained under the EHSLC conditions, a trial was conducted to compare the PK disposition of two tests drugs [(phenylbutazone (PBZ) and dexamethasone (DXM)] under resting conditions and in conditions involving a 3-h endurance-type exercise. It was shown that a sustained mild test exercise moderately decreased the plasma clearance of both drugs (approximately 25% for DXM and 37% for PBZ). However, as the volume of distribution was correlatively decreased, the plasma terminal half-life, which is a hybrid parameter of plasma clearance and of volume distribution, remained unchanged overall (Authié et al. 2009). This is of relevance for establishing DTs and WTs, as plasma and urine half-lives, not clearance, are the main determinants of the length of the DT. More generally, it can be hypothesised that a race lasting a few minutes only will not markedly alter residual drug concentrations in plasma or urine at the control sampling times i.e. at a time when most of the drug has already been eliminated. Indeed, under European racing rules, the shortest WT is 48 h because a race horse cannot be treated with any drugs within the 2 days preceding a race.

9 From a Detection Time to a Withdrawal Time

It should be re-emphasised that a DT, as issued by the EHLSC, is not equivalent to a WT. An appropriate safety span must be considered when extrapolating a WT from a DT published by the EHLSC. The length of the safety margin required to transform a DT to a WT remains unclear. To help the veterinarian select a WT from a published DT the question of a safety span was explored using Monte Carlo Simulations (MCSs) (Toutain 2009). A Monte Carlo simulation is a numerical method with a built-in random process that involves assessing the impact of variability due to different sources. In this instance there are two main sources of variability. Firstly, there is intrinsic biological variability between horses for PK parameters controlling plasma and urine drug disposition (i.e. plasma clearance, volume of distribution, urine-to-plasma ratio). These sources of variability are explained by factors such as breed, age, sex, weight. Secondly, there are the various sources of uncertainty associated with the veterinary decision and/or trainer practise concerning the actual administered dose, uncertainty due to approximate estimation of the actual body weight, the administration of a dosage form different from that tested by the EHLSC, modalities of administration, trained/untrained conditions

etc. Using MCSs, all these sources of variability can be combined simultaneously to generate a large hypothetical population of DTs, so that the proportion (percentiles) of horses attaining a given DT value can be determined. In other words, MCSs may replace a large population survey aimed at establishing a WT experimentally.

Using MCSs, it was shown that for a low variability of PK parameters (CV = 20%), an uncertainty span of about 40% may be selected to transform a mean EHLSC DT to a WT (i.e. WT = 1.4DT), which encompasses 90% of the horse population. In contrast, for a highly variable drug (CV = 40%), the uncertainty factor is of the order of 2.1–2.2 (i.e. the WT should be approximately twice the DT). In addition, MCSs suggested that the variability in DTs will be influenced mainly by inter-animal variability and that either more or less reliable veterinary practises will have only a minimal impact on DT, because the main sources of variability for a DT are of a biological nature. A consequence of this is that DTs, as released by the EHLSC, are likely to be of generic value for other countries having different veterinary practises (slightly different dosage regimens, different formulations or routes of administration) but having similar horses to those used in the EHLSC trials. This could be a relevant argument to promote and support harmonisation between countries.

It should be stressed that the ultimate goal of the ESHLC is to propose a DT, for which a lack of drug effect can be assumed at the time of racing. For the EHLSC, the regulated parameter must be a screening LOD that guarantees a lack of drug effect at the time of racing. An HSL is a property of the drug (*substance*) that may easily be reported by a single universal (international) value while DTs are a *formulation* property (except for administration by the IV route). Consequently, there can be no universal DT value for a given drug but rather as many DTs as there are commercial formulations and indeed for a given formulation as many DTs as possible routes of administration, dosage regimens etc. This renders an international harmonisation of DT with a necessary statistical protection an unachievable illusion.

10 Conclusion

Athletes decide for themselves if they wish to take drugs, horses do not (Higgins 2006) and practically all equine organisations and jurisdictions (racing, sport) claim that horse welfare is the priority. Despite this goal substantial differences in approach still exist between America and most other countries in the world.

The pivotal aspect of these shared values is a clear distinction between doping control and medication control, with the requirement to limit the sensitivity of the analytical techniques to prevent positive cases that could be due to residual presence of legitimate drugs at concentrations without any biological relevance.

As this goal has now almost been achieved, new horizons are opening through new doping practises (including gene doping) and by the use of substances difficult to screen and/or to detect by traditional approaches.

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