31

EXPERIMENTAL DESIGN CONSIDERATIONS IN PHARMACOKINETIC STUDIES

WILLIAM W. HOPE, VIDMANTAS PETRAITIS, AND THOMAS J. WALSH
National Cancer Institute, National Institutes of Health, Bethesda, Maryland

Contents

31.1 Introduction
31.2 Defining the Overall Goal of the Experiments
31.3 Experimental Platforms
31.4 Impact of Drug Measurement on Study Design
31.5 Choosing the Dosage Range and the Number of Dosages Used to Define Pharmacokinetic Relationships
31.6 Sampling Times
31.6.1 Defining the Number and Timing of Informative Sampling Points
31.6.2 Peak Concentrations
31.6.3 When Precisely to Acquire Pharmacokinetic Data
31.6.4 Optimal Sampling and D-Optimal Design
31.7 Determination of Drug Concentrations in Tissues and Bodily Fluids
31.8 Conclusion
References

31.1 INTRODUCTION

Pharmacokinetics is the study of the spatial and temporal distribution of drugs. Pharmacokinetic studies are conducted in experimental contexts for two major reasons. The first is to define pharmacokinetic relationships for new drugs that may

Preclinical Development Handbook: ADME and Biopharmaceutical Properties,
edited by Shayne Cox Gad
Copyright © 2008 John Wiley & Sons, Inc.

be beneficial for patients. This process is important for preclinical drug development where one wants to develop candidate molecules with pharmacokinetic profiles that are likely to be favorable for clinical use or that meet predefined developmental targets. A further understanding of the pharmacokinetics in experimental systems is required to obtain prior information before proceeding to human Phase I and II studies. The second reason to conduct pharmacokinetic studies is to further understand the concentration–effect relationships (i.e., pharmacodynamic relationships). For both these purposes, an accurate description of pharmacokinetics is vital in order to obtain appropriate conclusions regarding drug behavior and utility. To achieve this goal, appropriate experimental design is essential.

This chapter outlines some of the issues in the design of experimental models, which enable pharmacokinetic relationships to be established. The objective is to estimate the pharmacokinetic relationships as precisely as possible, at minimum cost, and using the minimum possible number of animals and analytical samples. Although this subject matter is vast, we focus our discussion on antimicrobial pharmacology from which broader principles may be extrapolated.

31.2 DEFINING THE OVERALL GOAL OF THE EXPERIMENTS

The experimental goals and aims will, to a large extent, dictate the design of the pharmacokinetic experiments. One obviously wishes to design the experiment to yield data to address the problems in hand. In this regard, a clear idea as to the overall experimental goal is required. This along with at least some preliminary pharmacodynamic data will determine (1) the concentrations of drug that are likely to induce a pharmacological effect of interest and are therefore relevant for the pharmacokinetic experiments; (2) the length of the experimental period over which one requires pharmacokinetic information; (3) the likely schedules of drug which will be employed; (4) the experimental conditions that will be used (e.g., severity of the model, immune status of the model, or other physiological derangements).

Studying the appropriate dosages and schedules for the right period of time and in the right model are important because (1) the pharmacokinetics may change as a function of dose (i.e., become nonlinear, especially at higher dosages when clearance mechanisms become saturated); (2) one needs to know about the pharmacokinetics over the entire course of the experimental period rather than for a mere fraction; (3) there may be drug accumulation with different schedules of administration, which may have a bearing on the pharmacodynamic relationships; and (4) the pharmacokinetic relationships may be different in different models. The immunological status, model severity, or degree of physiological derangement may significantly alter the pharmacokinetics of any given compound. In the circumstances in which the severity of the model precludes intensive blood sampling, an approach may be to define pharmacokinetic parameters in healthy animals and cross reference data from a select number of informative points from the animals with the disease in question—this is discussed in more detail in Section 31.6.4.

Thus, the experimental conditions used in the pharmacokinetic model should replicate as closely as possible those used to define the exposure–response relationships. Given the cost of pharmacokinetic experiments, a prudent approach is to

perform only these experiments when one is reasonably sure of the experimental conditions that will be used for other aspects of the study.

31.3 EXPERIMENTAL PLATFORMS

The design of the pharmacokinetic experiment will vary according to the type of experimental platform. In small animals, such as mice, repeated sampling from a single animal is extremely difficult. In this circumstance, pharmacokinetic data can be acquired by designing a serial sacrifice study, in which a cohort of animals receiving a given dose of drug are sacrificed at predefined time points over the course of the experimental period. Blood from mice can be obtained from a terminal cardiac puncture after the administration of a general anesthetic. Alternatively, while technically more challenging, serial blood samples can be obtained in the same mouse from the saphenous vein or from a retro-orbital puncture. The principal disadvantage of such an approach is the relatively small volume of blood that can be procured from each mouse. The number of mice sacrificed at each time point depends on the confidence with which one wishes to estimate the pharmacokinetic relationships. Usually a minimum of three animals is required at each time point, but four to six may be appropriate if the drug concentrations are likely to be variable, or precise estimates of the pharmacokinetic parameters are required. Using such a design, one has point estimates of measures of central tendency (e.g., mean concentration) and the associated dispersions at each time point for each dose. The observed variance from the replicate samples can be used as a weighting function in the pharmacokinetic modeling process. A similar "serial sacrifice" design obviously applies for other experimental platforms in which the experiment is destroyed in the sampling process.

In larger animals, such as rabbits, or *in vitro* models such as hollow fiber systems, multiple samples may be taken from a single animal or via a sampling port. Clearly, however, there is a limit in terms of the volume of blood that can be drawn from an animal over time; in rabbits, for example, this volume is approximately 7 mL/kg. Studying pharmacokinetics in this manner is similar to intensive pharmacokinetic sampling strategies in humans. The data from such an experimental design can be analyzed in one of two ways: (1) the mean and standard deviation for a group of individual animals receiving a given dose of drug in which blood has been drawn at the same time point can be determined; the analysis in this circumstance is similar to murine models, described above, in which the mean drug concentration can be weighted using the inverse of the observed variance at each observation point; (2) a population methodology can be employed; in this case there is no attempt to calculate the mean and standard deviation of the pharmacokinetic data between groups of rabbits. Rather, the data from each rabbit are modeled on an individual basis. In this case, weighting is assumed to be inversely proportional to the estimated assay variance. This relationship can be established by a regression of the mean values and the corresponding standard deviations of samples of known concentration, which encompass the dynamic range of the assay and which have been run in at least quadruplicate. The principal advantage of this approach is that one is able to obtain estimates for both the central tendencies (mean and median values) and dispersions for the population as a whole, *in conjunction* with an optimal set of

pharmacokinetic parameters for each individual animal. Such an approach may be advantageous when one wishes to directly link drug exposure with effect within the same animal. The ability to sample repeatedly from one animal means that fewer large animals are required to establish pharmacokinetic relationships than is the case in smaller animals such as mice. Furthermore, the sacrifice of a larger animal may not be necessary after an appropriate washout period, depending on the compound in question.

31.4 IMPACT OF DRUG MEASUREMENT ON STUDY DESIGN

The practical limitations of drug analysis have an important bearing on the design of experimental pharmacokinetic experiments. Drug levels are most frequently measured using high performance liquid chromatography (HPLC). Other methods such as bioassay or ELISA may be used. Increasingly, however, mass spectrometry is used because of its analytical sensitivity and the possibility of high throughput analysis. One aspect of drug measurement that is critical to the design of pharmacokinetic experiments is the lower limits of detection and quantification. Knowledge of these limits may have an influence on the design of pharmacokinetic experiments in the following ways: (1) there is little point in studying dosages that produce concentrations of drug beneath the limit of detection; (2) as one approaches the limit of quantification, the variance in most analytical assays increases, thus directly influencing the confidence with which the pharmacokinetic parameters can be estimated; and (3) the inability to measure low concentrations of drug may lead to erroneous estimates of the pharmacokinetics of a given drug. This may lead to underestimations of the magnitude of drug exposure that develops following the administration of an otherwise efficacious dose; these concepts are depicted in Fig. 31.1.

If it is clear that the analytical sensitivity of the assay is suboptimal and precludes the ability to accurately describe the pharmacokinetics, then a number of approaches

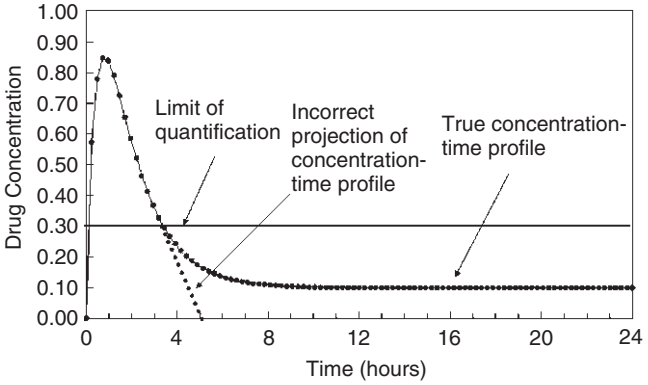


FIGURE 31.1 The impact of the limit of quantification on the ability to accurately describe the terminal phase of distribution. The figure shows the incorrect projection of the concentration–time profile at times beneath the limit of quantification, which leads to an underestimate of total drug exposure.

are possible. First, an attempt can be made to improve the limit of quantification via the optimization of the chosen analytical method (e.g., for HPLC analysis changing the mobile phase, detection settings, or the sample injection volume may improve the analytical sensitivity and the limit of quantification). Second, a more sensitive analytical method can be tried—in the majority of cases this means resorting to mass spectrometry. Third, tissue levels of drug can be measured; such an approach may be particularly useful for drugs that undergo extensive tissue distribution and that also exhibit prolonged mean residence times within tissues. By simultaneously comodeling serum and tissue drug concentrations, one may be able to estimate serum concentrations of drug that are well below the limit of detection. Such an approach is exemplified by a recent paper by Louie and colleagues [1] who were able to obtain accurate estimates of the antifungal agent caspofungin, by comodeling serum and kidney drug concentrations.

31.5 CHOOSING THE DOSAGE RANGE AND THE NUMBER OF DOSAGES USED TO DEFINE PHARMACOKINETIC RELATIONSHIPS

The range of dosages used to determine the pharmacokinetic relationships should encompass those that will be used to define the pharmacodynamic relationships. The importance of studying more than one dose is to determine whether the pharmacokinetics are linear or nonlinear. Following the administration of progressively higher dosages, clearance mechanisms may become saturated, and nonlinear pharmacokinetics will be observed. This is especially important if nonlinear kinetics are observed with dosages that are planned for the pharmacodynamic experiments. Ideally, a minimum of three dosages should be studied, but depending on the drug more may be appropriate.

An insight into whether a drug displays linear pharmacokinetics can be achieved in the following ways: (1) the presence of a linear system can be inferred using the principle of superposition; in this case, the drug concentrations following different dosages divided by the administered dose should be superimposable; (2) there should be a linear relationship between the area under the concentration–time curve (AUC) and the different dosages; (3) the pharmacokinetic parameters determined for each dosage group should not vary significantly from dose to dose; and (4) a more sophisticated method involves fitting and comparing both linear and nonlinear pharmacokinetic models to the entire dataset. A fit of one model compared with another, which is superior in a statistically significant manner, can be used to infer whether the pharmacokinetics are linear or nonlinear. An example of this process applied to human data is illustrated in a study by Lodise and colleagues [2], where an attempt was made to distinguish whether the beta-lactam piperacillin was better accounted for by a linear of nonlinear pharmacokinetic model.

31.6 SAMPLING TIMES

31.6.1 Defining the Number and Timing of Informative Sampling Points

The identification of sampling times is a critical component in the ability to obtain confident estimates of the pharmacokinetic parameters. In general, given the

considerable time and resources involved in determining pharmacokinetic relationships, one wishes to sample as little as possible, while still ensuring acceptable estimates of the pharmacokinetic parameters will be obtained. The key in this regard is the identification of informative sampling points. The information content at various time points throughout the dosing interval is not constant. The distribution of information content is a function of the shape of the concentration–time profile of a drug and the structural pharmacokinetic model that is used to obtain parameter estimates. Importantly, the trough concentration, which is frequently used in therapeutic drug monitoring, is the least informative data point for describing pharmacokinetics, since it provides little information regarding the shape of the preceding concentration–time profile. Some idea as to likely informative points can be obtained from prior knowledge of drug behavior, even if acquired in other experimental platforms. Several experiments may be required to fully characterize the concentration–time profile for a drug in early development or to define the pharmacokinetics within a new experimental system. Informative points tend to occur at points of inflection in the concentration–time curve and can be formally identified using optimal design theory (see Section 31.6.4).

31.6.2 Peak Concentrations

Peak concentrations, unlike troughs, tend to be information rich. In order to capture this information, sampling in the immediate period following drug administration is frequently performed. While this approach is standard and useful, there are some important caveats. (1) The use of compartmental pharmacokinetic modeling techniques requires an assumption of full compartmental mixing; this refers to the presence of homogeneous concentrations of drug throughout the compartment in question. The time required for full mixing to occur, even for drugs that are injected intravenously, is not instantaneous. Sampling should be delayed until full mixing is likely to have occurred; this time depends on both the route of administration and the blood circulation time. A balance must be struck between waiting for mixing to occur, but not waiting too long that important information contained within the period immediately following drug administration is lost. (2) Drug concentrations are changing very rapidly in the early period following drug administration. Consequently, relatively small errors in the time of sampling are likely to have a significant impact on the observed plasma drug concentrations. This may be an issue for experimental reproducibility and fitting pharmacokinetic models to the data obtained immediately following drug administration. (3) In attempting to capture peak concentrations, there may be considerable logistical issues in administering drug to a relatively large number of animals, and then sampling 5–15 minutes later. This is especially the case for mice, where the number of animals tends to be high, and blood samples are usually obtained by cardiac puncture. Even in the hands of experienced personnel, the time taken to obtain a blood sample from an individual mouse is approximately 1 minute. Small departures from this demanding schedule may significantly disrupt the ability to obtain samples at the desired times. Thus collecting data immediately following drug administration requires careful planning, organization, and cooperation among co-workers.

31.6.3 When Precisely to Acquire Pharmacokinetic Data

An issue arises as to whether pharmacokinetic relationships should be defined after the initiation of drug administration or at steady state (if the latter is achieved within the study period). There is no universally correct approach, but there are two guiding principles when planning a pharmacokinetic study. (1) An attempt should be made to collect informative data throughout the study period, regardless of the timing. Consequently, it may be appropriate to sample relatively intensively during the early period following drug administration, again at steady state, and at other select informative times between these periods. Such an approach may help to identify the extent of drug accumulation, and any nonlinearities that may appear with repeated drug administration. (2) If sampling occurs after repeated dosing (e.g., following the sixth dose of drug), the determination of the concentration of drug immediately prior to drug administration should be defined (i.e., a prelevel should be drawn). If this data point is not modeled or the preceding dosages are not recorded as inputs into the system, then accurate estimates for the pharmacokinetic parameters will not be obtained—information regarding prior system inputs and initial conditions cannot simply be ignored.

31.6.4 Optimal Sampling and D-Optimal Design

D-optimal design theory has been applied extensively to humans as a means of acquiring informative data. In circumstances in which intensive sampling is not possible (e.g., neonates, patients with critical illness), D-optimal design allows for samples to be drawn at times when system information is near maximal. Such an approach allows confident estimates of the pharmacokinetic parameters using a minimal dataset. Such an approach can also be applied to experimental models, to minimize the number of samples required to be drawn and analyzed. In our laboratory, rabbits with invasive pulmonary aspergillosis (a fungal infection of the lung) are unable to tolerate intensive plasma sampling. An approach successfully employed by us is to acquire a relatively information-rich dataset in healthy animals and use these data to identify a limited number of informative sampling points in infected rabbits, thus allowing the pharmacokinetic parameters to be estimated while minimizing the burden of repeated blood sampling.

Optimal design can be performed using the sample module of the pharmacokinetic program ADAPT II of D'Argenio and Schumitsky [3]. The mean parameter values and the associated standard deviations are entered, along with the dosages of drug and the number of sampling times that are required. The identification of informative sampling times may also be important in other contexts, in which one wishes to ensure or demonstrate that the administration of a drug results in a concentration–time profile that is comparable to what was defined in a previously similar but subtly different model. Examples include the following: (1) ensuring that there is no pharmacokinetic interaction between drugs given in combination; in this circumstance, one may choose a small number of informative sampling times to demonstrate that the drug concentrations are the same as when the drugs are administered in isolation; (2) if there has been a departure from the conditions in which the original pharmacokinetic relationships were defined, but to a degree that is unlikely to have a significant effect on the pharmacokinetics, one may wish to

obtain a small number of samples in order to confirm that the concentration–time profiles are similar. For example, the original pharmacokinetic relationships may have been defined in an infection model using a particular strain or species; subsequently, one may wish to study a related species, but the experimental conditions are otherwise identical, and a full pharmacokinetic study may not be justified. In this situation it may be possible to sample at several informative points to ensure that the concentration–time profile is the same.

31.7 DETERMINATION OF DRUG CONCENTRATIONS IN TISSUES AND BODILY FLUIDS

Frequently, an assumption is made that there is a linear relationship between the concentrations of drug in the central compartment and concentrations that develop at the effect site (i.e., the site of the drug receptor). In the majority of cases this is true, meaning that serum and tissue drug concentrations do in fact track one-to-one. This enables the response at the effect site to be described in terms of the concentration–time profile within the central compartment. On occasions, however, a better understanding of drug effect can be obtained by measuring the concentration of drug at the effect site itself. Despite a linear relationship, knowledge of the concentrations at the effect site may be important for the following reasons: (1) the shape of the concentration–time profile may be different in the two compartments and for certain drugs and drug classes this may have a bearing on drug effect; (2) there may be differences in protein binding between the serum and the effect site, which could affect the free fraction of drug available to interact with its receptor; (3) there may be dissociation between the concentration–time profiles because of delayed trafficking of drug to the effect site, resulting in hysteresis and leading to an apparent dissociation between serum drug concentrations and the observed effect; (4) despite a linear relationship between the serum and the effect site, there may be an insufficient concentration of drug to elicit a biological response at the effect site; and (5) there may be unique and unappreciated transport mechanisms from the serum to the effect site—an example of this is the concentration that the macrolide group of antibiotics achieve in the epithelial lining fluid (ELF) of the lung, despite extremely low serum drug concentrations. Thus, on occasions, there may be a requirement to measure the concentration of drug in tissues or bodily fluids in order to link drug concentrations at the effect site and the drug effect itself.

The measurement of tissue drug concentrations is not straightforward. The important issues include the following. (1) Tissue from normal animals (i.e., devoid of drug) is required to generate a standard curve, which is used to determine the concentrations of drug within tissues. Ideally the assay needs to be optimized and validated for each tissue in order to define the limit of quantification. (2) In order to obtain an estimate of the time course (pharmacokinetics) of drug within the tissue compartment, serial sampling (if possible) or serial sacrifice experiments are required. The shape of the concentration–time profile may differ from the serum, meaning that different sampling times from those used in serum may be required. Animals should be serially sacrificed throughout the dosing interval. This means the numbers of animals that are required to determine the pharmacokinetics in tissues may be greater than if serum pharmacokinetics alone are determined. This may have

important implications for the cost and feasibility of such a study. (3) One should always bear in mind the complexities of distribution of drug within tissues. The effect of a drug results from an interaction with its receptor, which may only exist within subcompartments. A tissue homogenate, on the other hand, consists of a mixing of all the different compartments within tissue, including the vascular space. This principle is exemplified in a study by Groll et al. [4], where the compartmentalization of intrapulmonary lipid formulations of amphotericin B was characterized in pulmonary alveolar macrophages and epithelial lining fluid. Consequently, drug levels determined from tissue homogenates should be viewed as an approximation of the concentration of drug available to the receptor.

There are a number of additional advantages to simultaneously studying tissue and serum concentrations of drug. The comodeling of serum and tissue data may enable better estimates of pharmacokinetic parameters. A good example of this was provided by Louie et al. [1], who described the pharmacokinetics of the antifungal drug caspofungin in a murine model of invasive candidiasis. Caspofungin is cleared rapidly from the serum and is avidly taken up by tissues. The lower limit of quantification of caspofungin using HPLC is relatively high. The concentrations of drug in the kidney and serum were measured and comodeled. This approach enabled a more accurate estimate of the pharmacokinetics; the caspofungin half-life increased when the serum and tissue data were comodeled. This approach also enabled a circumvention of the problem induced by a relatively high limit of quantification mentioned earlier. Instead of moving to a more sensitive analytical assay to enable a more accurate description of the terminal elimination phase, this was achieved by measuring and comodeling drug concentrations in the kidney. In this circumstance, as drug concentrations in the serum decline beneath the lower limit of quantification, drug is still "seen" returning from peripheral compartments that are acting as a repository of drug (in this case, the kidney).

31.8 CONCLUSION

An accurate description of the pharmacokinetic properties of a drug underpins a further understanding of concentration–effect relationships. The design of pharmacokinetic experiments is critical to ensure that pharmacokinetic relationships can be accurately estimated. If the design of pharmacokinetic experiments is flawed, and the resultant pharmacokinetic models are incorrect, then erroneous conclusions regarding the concentration–effect relationship may result. This chapter summarizes our approach to the design of pharmacokinetic experiments and highlights ways in which pharmacokinetic relationships can be efficiently and accurately estimated.

REFERENCES

1. Louie A, Deziel M, Liu W, Drusano MF, Gumbo T, Drusano GL. Pharmacodynamics of caspofungin in a murine model of systemic candidiasis: importance of persistence of caspofungin in tissues to understanding drug activity. *Antimicrob Agents Chemother* 2005;49: 5058–5068.

2. Lodise TP Jr, Lomaestro B, Rodvold KA, Danziger LH, Drusano GL. Pharmacodynamic profiling of piperacillin in the presence of tazobactam in patients through the use of population pharmacokinetic models and Monte Carlo simulation. *Antimicrob Agents Chemother* 2004;48:4718–4724.
3. D'Argenio DZ, Schumitzky A. *ADAPT II. A Program for Simulation, Identification, and Optimal Experimental Design. User Manual*. Los Angeles: Biomedical Simulations Resource, University of Southern California; 1997. http://bmsr.esc.edu/.
4. Groll AH, Lyman CA, Petraitis V, Petraitiene R, Armstrong D, Mickiene D, Alfaro RM, Schaufele RL, Sein T, Bacher J, Walsh TJ. Compartmentalized intrapulmonary pharmacokinetics of amphotericin B and its lipid formulations. *Antimicrob Agents Chemother* 2006;50:3418–3423.

32

BIOAVAILABILITY AND BIOEQUIVALENCE STUDIES

ALEXANDER V. LYUBIMOV[1] AND IHOR BEKERSKY[2]

[1]*University of Illinois at Chicago, Chicago, Illinois*
[2]*Consultant, Antioch, Illinois*

Contents

32.1 Introduction
- 32.1.1 Rationale for Bioavailability/Bioequivalence (BA/BE) Studies
- 32.1.2 Need and Aim of BA/BE Studies
- 32.1.3 Role of BA/BE Studies in Drug Development and Nonclinical Studies
- 32.1.4 Regulatory Considerations and Guidelines
- 32.1.5 Importance of Safety Margins
- 32.1.6 Definitions

32.2 IND-Targeted Nonclinical Development
- 32.2.1 Objective
- 32.2.2 Species in IND-Targeted Toxicology Studies
- 32.2.3 Key Elements of IND-Target Development
- 32.2.4 Use of Animal Models for the Selection of Dosage Forms for Nonclinical Toxicology Studies
- 32.2.5 Species Differences in Bioavailability

32.3 NDA-Targeted Nonclinical Development
- 32.3.1 Toxicokinetics in Chronic Toxicity Studies
- 32.3.2 Use of Animals to Evaluate the BA/BE of Clinical Dosage Forms
- 32.3.3 Role and Impact of Nonclinical Studies on Clinical Development: Animal to Humans

32.4 Conclusions

References

Preclinical Development Handbook: ADME and Biopharmaceutical Properties,
edited by Shayne Cox Gad
Copyright © 2008 John Wiley & Sons, Inc.

32.1 INTRODUCTION

The two questions that confront the pharmaceutical scientist during the process of developing a molecule into a drug are "Is it safe?" and "Is it therapeutic?" The question of safety is an important issue that surfaces very early in development; without a clear understanding of what defines safe use, it is difficult, if not impossible, to continue development. Thus, animal toxicology/safety studies are the primary ingredient in any NDA submission. However, such studies are just as important in the conduct of an IND-enabling program, which allows the conduct of the first human clinical studies.

Today, the process of developing a new chemical entity (NCE) into a drug that is approved for marketing follows a well-defined and regulated pathway. The key issues that will determine approval are safety and efficacy in the Phase III trials. In this context, preclinical animal studies provide both the initial and subsequent "windows" on toxicity safety and thus have a direct impact on decisions as to the first clinical trials and on a dosing regimen in such trials. In fact, concerns about safety and efficacy are evaluated throughout the whole drug development process.

In spite of reservations that can be raised about the extrapolation of animal data to humans, toxicology studies provide the best currently available assessments of what can reasonably be expected in humans. The designs obtained from toxicology studies are thus of particular concern since safety predictions based on the data obtained cannot be found to be unwarranted in retrospect. The challenge to the pharmaceutical scientist therefore is to use available "tools" that provide information on toxicity and drug disposition in the animals used in toxicology/safety studies. Regardless of the stage in development, the systemic exposure (e.g., the AUC) is the metric by which the relationship of drug disposition and toxicity is evaluated.

In this context, the inclusion of biopharmaceutics and pharmacokinetics into preclinical development, specifically into the toxicological/safety studies, is a logical step since drug concentration data defines the extent of exposure to drugs.

32.1.1 Rationale for Bioavailability/Bioequivalence (BA/BE) Studies

Bioavailability and bioequivalence are pharmacokinetic (PK) terms that quickly gained importance in the "early" days of the development of pharmacokinetics as a drug development science. As such, their use was primarily targeted to clinical development. Bioavailability is defined in the *Code of Federal Regulations*, Title 21, 320.1 [1] as the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action. For drug products that are not intended to be absorbed into the bloodstream, bioavailability may be assessed by measurements intended to reflect the rate and extent to which the active ingredient or active moiety becomes available at the site of action.

In clinical use, it is not unusual that several dose strengths or different dosage forms (capsules, tablets) are used; therefore a study(ies) is (are) conducted to ascertain that the same plasma concentration time profile is obtained at the different strengths or dosage forms, thus providing the appropriate drug exposure for patient management—that is, bioequivalence. Bioequivalence is often used in clinical trials for comparison of the reference (pioneer) and generic drugs. The *Code of Federal*

Regulations, Title 21, Volume 5 [2] defines bioequivalence as the absence of a significant difference in the rate and extent to which the active ingredient or active moiety in pharmaceutical equivalents or pharmaceutical alternatives becomes available at the site of drug action when administered at the same molar dose under similar conditions in an appropriately designed study. Where there is an intentional difference in rate (e.g., in certain controlled release dosage forms), certain pharmaceutical equivalents or alternatives may be considered bioequivalent if there is no significant difference in the extent to which the active ingredient or moiety from each product becomes available at the site of drug action. This applies only if the difference in the rate at which the active ingredient or moiety becomes available at the site of drug action is intentional and is reflected in the proposed labeling, is not essential to the attainment of effective body drug concentrations on chronic use, and is considered medically insignificant for the drug.

The extent of product bioavailability is estimated by the area under the blood concentration versus time curve (AUC). AUC is most frequently estimated using the linear trapezoidal rule. Other methods for AUC estimation may be proposed and should be accompanied by appropriate literature references during protocol development.

For a single-dose bioequivalence study, AUC should be calculated from time 0 (predose) to the last sampling time associated with quantifiable drug concentration $AUC_{0\text{-}LOQ}$. The comparison of the test and reference product value for this noninfinity estimate provides the closest approximation of the measure of uncertainty (variance) and the relative bioavailability estimate associated with $AUC_{0\text{-}INF}$, the full extent of product bioavailability.

In a multiple-dose study, the AUC should be calculated over one complete dosing interval ($AUC_{0\text{-}t}$). Under steady-state conditions, $AUC_{0\text{-}t}$ equals the full extent of bioavailability of the individual dose $AUC_{0\text{-}INF}$ assuming linear kinetics. For drugs that are known to follow nonlinear kinetics, the sponsor should consult with the Center for Veterinary Medicine (CVM) to determine the appropriate parameters for the bioequivalence determination [7].

The rate of absorption is estimated by the maximum observed drug concentration (C_{max}) and the corresponding time to reach this maximum concentration (T_{max}). When conducting a steady-state investigation, data on the minimum drug concentration (trough values) observed during a single dosing interval (C_{min}) should also be collected. Generally, three successive C_{min} values should be provided to verify that steady-state conditions have been achieved. To determine a steady-state concentration, the C_{min} values should be regressed over time and the resultant slope should be tested for its difference from zero [7].

Confidence intervals should routinely be used to interpret bioequivalence data. The following bioavailability and bioequivalence standards are usually required:

- The 90% confidence interval of the relative mean AUC of the test reference formulation should be within 80–125%.
- The relative mean measured C_{max} of the test to reference formulation should be within 80–125%.
- The relative mean measured C_{min} of the test to reference formulation should be within 80–125%.

32.1.2 Need and Aim of BA/BE Studies

In preclinical toxicology studies, the selection of the dosage form is not as complicated as for clinical use and this will be addressed subsequently. The issue of doses is of importance as the selection of doses for the various types of toxicology studies is an occult process that involves varying amounts of soft data. However, in order to provide a good prospective assessment/projection regarding safety in humans based on systemic exposure, it is clear that BA/BE principles also apply to animal toxicity studies.

In terms of bioavailability, toxicology studies usually entail three or four dose levels; and BA is "relative" as systemic exposure is evaluated by the linearity (or nonlinearity) of eliminating the kinetic at each dose level and/or as a function of time during the toxicity study. Considering that the dose range in toxicology studies is usually a large multiple of the clinical dose, a relative BA is acceptable in data evaluation of drug concentration and toxic effect(s).

Bioequivalence in preclinical animal studies is a more complex issue and in practical terms applies mostly to dog and primate studies where different oral dosage forms may be evaluated (solutions, capsules of neat drug, galenic tablets) to ensure a maximal drug release profile and thus a definition of the "line" between pharmacology and toxicology.

It is important to know that the drug is bioavailable at the selected route of administration, and that the absence of toxic or pharmacologic effect is real and not just a result of the drug not being absorbed.

In short, both concepts have an application to animal toxicology studies as the exposure needs to be determined and confirmed in order to calculate the animal to human safety margins on which clinical development is dependent.

32.1.3 Role of BA/BE Studies in Drug Development and Nonclinical Studies

As stated earlier, the concepts of BA/BE were first used in clinical development as tools to define a clear and consistent therapeutic use. Considering that the objective of toxicology studies is to demonstrate the toxic potential of the drug across a wide dose range, over varying durations of administration, and across different dosage forms and at least two species, the utility of BA/BE is readily apparent as a means to provide the best prospective assessment of animal safety data in terms of a safe exposure in humans. This is especially true in early stages of development where there is limited exposure/safety data in humans available.

32.1.4 Regulatory Considerations and Guidelines

Bioavailability and/or bioequivalence studies play a key role in the drug development period for both new drug products and their generic equivalents. For both, these studies are also important in the postapproval period in the presence of certain manufacturing changes. The article by Chen et al. [3] reviews the regulatory science of bioavailability and bioequivalence and provides the FDA's recommendations for drug sponsors who intend to establish bioavailability and/or demonstrate bioequivalence for their pharmaceutical products during the developmental process

or after approval. Statistic and pharmacokinetic principles of bioavailability and bioequivalence trials are described in Rajaram et al. [4]. This document was prepared as a set of workshop material for the International Congress on Medical and Care Compunetics in June 2004 in Den Hague, The Netherlands.

Toxicokinetics (TK) is the inclusion of pharmacokinetic principles into the toxicology/safety studies conducted in animal species. The objectives of TK are to evaluate drug absorption, distribution, and elimination as a function of dose level; to aid in the selection of realistic dose levels that can be maintained over the duration of the study; to determine the duration of exposure to drug; to determine the effect of chronic drug administration on the absorption and disposition of that drug; and to aid in interpreting and extrapolating the toxicological response in different species.

With few exceptions, toxicology/safety studies are conducted in accordance with the Good Laboratory Practice (GLP) regulations [5]; thus data collection and recording are conducted in a manner that is acceptable to all dossier reviewing regulatory agencies and are subject to verification by a QA unit. In terms of providing commonly acceptable objectives and "suggestions" as to the study design, one can refer to FDA and/or ICH guidelines. A tabulation of these guidelines is presented in Table 32.1. There are no specific preclinical BA/BE in either FDA or ICH guidelines; however, most of the definitions and approaches applicable for preclinical BA/BE studies can be found in two FDA documents: *Bioavailability and Bioequivalence Studies for Oral Administered Drug Products—General Considerations* [6] and *Bioequivalence Guidance*, Center for Veterinary Medicine [7].

32.1.5 Importance of Safety Margins

As stated earlier, the extent of systemic drug exposure in animal toxicology studies is directly correlated to the determination of safety margins to calculate a safe dose in humans. The first human clinical trials are concerned with administering a safe dose without covert toxicity. The challenge is that only animal toxicity/safety data are available. This may now change with the introduction of Phase 0 studies.

A comprehensive approach to the estimation of the human dose from animal data is available [8]. In short, having NOAELs (mg/kg) from animal studies, one can extrapolate a human equivalent dose based on body surface area, from the tables provided in the guidance [8]; a safety factor is chosen and the human equivalent dose is divided by that factor.

The result is the maximum recommended starting dose. A default safety factor of 10 is normally used, and although higher factors provide more flexibility for the strength of the clinical dose, factors lower than 10 do not necessarily mean a clinical hold if other data/information are available as an aid in the assessment of the projected safety in humans. It should be kept in mind that the NOAEL is not a static number and is redetermined as the duration of the toxicology studies becomes longer in keeping with the clinical objectives and study duration. It should also be pointed out that regardless of the place in development, it is important to know the drug release profile from the dosage forms used in the toxicity/safety studies (i.e., the bioavailability) in order to estimate the animal to human safety margins with assurance.

TABLE 32.1 FDA and ICH Guidelines

Agency	Title	Date
FDA	*Bioavailability and Bioequivalence Studies for Oral Drug Products—General Considerations*	March 2003
	Bioequivalence Guidance	9 October 2002
	Exploratory IND Studies	18 January 2006
	Carcinogenicity Study Protocol Submissions	23 May 2002
	Format and Content of the Nonclinical Pharmacology/ Toxicology Section of an Application	1 February 1987
	Immunotoxicology Evaluations of Investigational New Drugs	1 November 2002
	Photosafety Testing	7 May 2003
	Single Dose Acute Toxicity Testing for Pharmaceuticals—Revised	26 August 1996
	Reference Guide for the Nonclinical Toxicity Studies of Antiviral Drugs Indicated for the Treatment of N/A Non-Life Threatening Disease: Evaluation of Drug Toxicity Prior to Phase 1 Clinical Studies	1 February 1989
	Integration of Study Results to Access Concerns About Human Reproductive and Developmental Toxicities	13 November 2002
	Nonclinical Safety Evaluation of Pediatric Drug Products	3 February 2003
	Nonclinical Studies for Development of Pharmaceutical Excipients	18 May 2005
	Statistical Aspects of the Design, Analysis, and Interpretation of Chronic Rodent Carcinogenicity Studies of Pharmaceuticals	8 May 2001
ICH	*M3—Nonclinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals*	25 November 1997
	S1A—The Need for Long-Term Rodent Carcinogenicity Studies of Pharmaceuticals	1 March 1996
	S1B—Testing for Carcinogenicity in Pharmaceuticals	23 February 1998
	S1C—Dose Selection for Carcinogenicity Studies of Pharmaceuticals	1 March 1995
	S1C(R)—Dose Selection for Carcinogenicity Studies of Pharmaceuticals: Addendum on a Limit Dose and Related Notes	4 December 1997
	S2A—Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals	24 April 1996
	S2B—Genotoxicity: Standard Battery Testing	21 November 1997
	S3A—Toxicokinetics: The Assessment of Systemic Exposure in Toxicity Studies	January 1995
	S4A—Duration of Chronic Toxicity Testing Animals (Rodent and Nonrodent Toxicity Testing)	25 June 1999
	S5B—Detection of Toxicity to Reproduction for Medicinal Products	22 September 1994
	S6—Preclinical Safety Evaluation of Biotechnology Derived Pharmaceuticals	18 November 1997
	S7A—Safety Pharmacology Studies for Human Pharmaceuticals	13 July 2001
	S7B—Safety Pharmacology Studies for Assessing the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals	14 June 2002 (Draft)
	S8—Immunotoxicity Studies for Human Pharmaceuticals	April 2006

TABLE 32.2 Acronyms and Definitions

Acronym	Definition
BA	Bioavailability means the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action. For drug products that are not intended to be absorbed into the bloodstream, bioavailability may be assessed by measurements intended to reflect the rate and extent to which the active ingredient or active moiety becomes available at the site of action (21 CFR 320.1).
BE	Bioequivalence means the absence of a significant difference in the rate and extent to which the active ingredient or active moiety in pharmaceutical equivalents or pharmaceutical alternatives becomes available at the site of drug action when administered at the same molar dose under similar conditions in an appropriately designed study.
AUC	Area Under the Curve is the integral of drug plasma concentration over a specific time (AUC_t) or to infinity (AUC_{inf}) and is a measure of drug amount absorbed (systemic absorption) into the systemic circulation.
GLP	Good Laboratory Practices Regulation.
PK	Pharmacokinetics.
NDA	New Drug Application.
QA	Quality Assurance.
NOAEL	No-Observed-Adverse-Effect-Level.
MRSD	Maximum Recommended Starting Dose.
HED	Human Equivalent Dose.
TK	Toxicokinetics.
IND	Investigative New Drug Application.
CMC	Controls, Manufacture, and Chemistry.

32.1.6 Definitions

As is the case with many texts, the use of acronyms is a common way to avoid repetitive use of a concept. A partial tabulation of acronyms and definitions of terms that are germane to the present text is presented in Table 32.2.

32.2 IND-TARGETED NONCLINICAL DEVELOPMENT

The filing of an IND is the first step in the drug development process. The IND contains nonclinical pharmacology in animal disease models, toxicology in two animal species of duration that does not exceed the duration of the proposed first clinical trial(s), preliminary ADME data, an Investigators Brochure, protocol synopsis of the proposed clinical trial, and CMC information.

32.2.1 Objective

The objective of the IND is thus to obtain FDA "approval" to proceed with the conduct of the proposed clinical trial, that is, study the molecule in the intended model—humans. Prior to the IND filing it is usual to have a pre-IND meeting with the reviewing division, where what the IND will contain is presented and FDA input

is solicited as to its "acceptance." In most cases the first clinical study, being a PK/tolerance study, is in normal volunteers. However, for some indications this study can be conducted in a small number of patients and be of a short duration. In general, the duration of the toxicology studies, the dose ranges used, the NOAELs obtained, and the safety margins that are estimated determine the final clinical protocol for the initial human study(ies).

32.2.2 Species in IND-Targeted Toxicology Studies

Although it is not our purpose to discuss the format or content of an IND dossier, it is useful to present an outline of the IND to indicate the position and role of nonclinical toxicology studies. A general outline of the IND is given in Table 32.3.

Throughout drug development, toxicology/safety studies are conducted in two species. As most drugs are intended for oral administration, the rat and dog are the primary species used for reasons of availability, costs, and long history of use. However, not all drugs are targeted for oral delivery and other routes (intravenous, dermal, etc.) and species (rabbit for reproductive toxicology, monkey) may be used (Table 32.4). In general, the current climate supports a "minimal" IND as presented in the recent FDA guidance—*Guidance for Industry, Investigators, and Reviewers: Exploratory IND Studies*. This guidance allows sponsors a great deal of flexibility in the IND content and in the toxicity studies required, provided limited human exposure and no therapeutic intent is the objective of the initial clinical trial. Thus,

TABLE 32.3 Outline of an IND

Covering Letter
Questions for the FDA
Section 1: Form 1571
Section 2: Table of Contents
Section 3: Introductory Statement
Section 4: General Investigational Plan
• Clinical Development Plan
• Planned Exposure
• Safety/Estimation of Potential Risk
Section 5: Investigators Brochure
Section 6: Protocol(s)
Section 7: Chemistry, Manufacture and Controls (CMC)
• Drug Substance
• Drug Product
Section 8: Nonclinical Pharmacology and Toxicology
• Overview of the Nonclinical Testing Strategy
• Nonclinical Pharmacology
• Nonclinical Pharmacokinetics and Metabolism
• Toxicology
• Conclusions
• References
• End of Text Tables: Summary Tabulations
• Copies of Literature Cited
• Copies of Final Study Reports

TABLE 32.4 Species and Study Types in an Exploratory IND

Species	Study Type
Mouse	Acute toxicity, subchronic toxicity (2 weeks), genotoxicity (*in vivo* micronucleus)
Rat	Acute toxicity, subchronic toxicity (2 weeks), genotoxicity
Rat	Safety pharmacology—Irwin test, and CNS effects
Rabbit	Reproductive toxicology
Dog	Acute toxicity and subchronic toxicity, safety pharmacology—QT interval prolongation
Monkey	Safety pharmacology-QT interval prolongation

a "go/no go" decision can be reached very early and is based on human, albeit limited, experience. It is important to point out that in case the data supports moving forward in development, the traditional toxicology/safety program must be conducted to continue development.

The species and studies that would be contained in such an IND are outlined below. It should also be pointed out that the route of administration in these studies would be the intended route in the clinic.

Not all of the above studies are conducted for an IND filing, and Table 32.4 presents a "menu" of what can be conducted. In general, acute and subchronic toxicity, the core genotoxicity, and safety pharmacology are required, with the most appropriate species chosen.

32.2.3 Key Elements of IND-Target Development

Throughout all drug development, safety is a factor, perhaps the key factor. The parameters that define safety are presented in the following text. It should be pointed out that as/when development moves forward with the aim of a NSA filing, safety in nonclinical studies continues to be evaluated in studies of longer duration (6 month study in rats, 9 month study in dogs/monkeys, the full panel of reproductive toxicity, carcinogenicity studies in mice and rats). However, the use of toxicokinetics and BA/BE issues remain to support the extent of exposure determinations and data interpretation. Examples of studies/data on file as well as from published reports are presented in the subsequent discussion. In this context it should be pointed out that although numerous studies germane to the present chapter are conducted, the majority are not published—thus the inclusion of data "gleaned" from personal files.

Toxicology/Safety Profile As shown in Table 32.3, Section 8 of the IND deals with toxicology, and considering that at this time in development, other sections may be rather sparse. As stated earlier, the results of the toxicity studies, in terms of NOAEL and safety margins, are key for the conduct of the first human studies. The other sections provide data that characterize the physicochemical properties of the drug, its stability, and the clinical dosage form for these studies (CMC section); the proposed clinical plan; and the Investigator's Brochure. The toxicological/safety profile is presented in detail and is comprised of nonclinical pharmacology (the rationale

for development), nonclinical pharmacokinetics and metabolism, and toxicology. For all of these components, the text contains the elements of study design, results, discussion and conclusions, and summary tabulations of the data obtained. Copies of QA final study reports are also included in the submission.

Extent of Absorption In practice, the actual (numerical) extent of absorption is not determined in the context of toxicology studies. It is important, however, to demonstrate/ascertain absorption, and this is achieved by the toxicokinetic evaluations; this data is evaluated in a relative, intradose manner and as a function of both dose and time.

Usually the level of absorption (bioavailability) is obtained from the nonclinical PK and ADME studies. Considering that these studies are conducted in the same species and with the same dosage forms as the toxicology studies, the data are thus available to provide a reasonable assessment of the bioavailability of the drug in the toxicity studies, if such an assessment is needed.

An example of a PK study in dogs for bioavailability (relative to an IV injection) of a solution and a solid dosage form (a preliminary clinical capsule) is shown in Fig. 32.1. The plasma concentration–time profiles clearly show a good oral bioavailability of the drug from the solution, whereas it was virtually quantitative from the preliminary capsule considered for the first clinical trials. The use of this capsule for the toxicity safety studies was considered as it would provide the maximum drug release profile and thus clearly define the toxicological profile and the NOAEL.

Assessment of Safety Margins Based on Systemic Exposure At the IND stage, the primary objective is to provide safety assessments, based on which the first human studies are conducted. These Phase I studies determine single-dose pharmacokinetics/tolerance in normal volunteers with the safe dose obtained in humans from the safety margins calculated from the results of the IND-enabling toxicity studies [9, 10]. A "simple" treatment of safety factor calculations can be derived from the

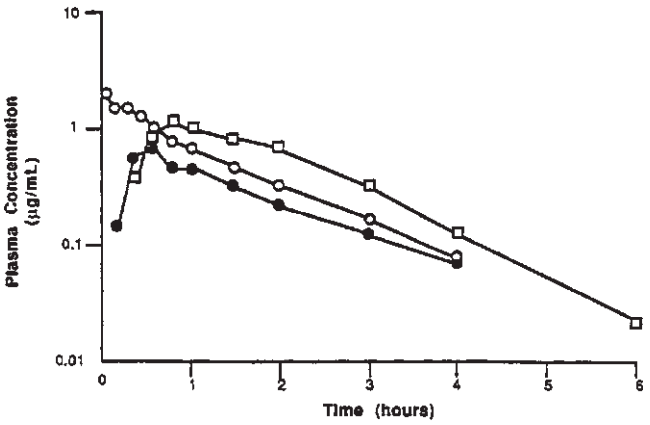


FIGURE 32.1 Mean plasma concentrations of the neuroleptic in dogs following a 5 mg/kg dose intravenously (○), as an oral solution (●), and as a clinical capsule (□). (From I. Bekersky, personal data.)

comprehensive methods presented in the guidance [8]. In brief, one can calculate the human dose expected to result in the same effect seen in the animal studies (HED); the safety margin can be estimated on a mg/kg comparison of doses, using the equation HED = Animal $NOAEL \times (W_{\text{animal}}/W_{\text{human}})^{0.33}$. This calculation takes into account both the weight and the relative body surface area.

A similar equation uses a "conversion factor" (S) that addresses the issue of body surface area: (S factor) × ($NOAEL$ dose in mg/kg)/S factor$_{\text{human}}$ = Safe dose in humans in mg/kg (the HED). Knowing the extent of systemic exposure (AUC) from toxicokinetic assessments provides an additional level of confidence to the determination of the NOAEL, and thus to the calculation of the safety margins, and in turn, to setting a safe dose for the initial human trials.

Relationship of Absorption to Dosage Form(s) and Route of Administration The nature of the nonclinical studies that are conducted in drug development depends on the targeted disease, the route of administration in therapeutic use, and the anticipated treatment duration in humans. Thus administration by the oral, intravenous, dermal, inhalation, subcutaneous, and ocular routes in studies ranges from a single dose to a lifetime exposure. As pointed out earlier, although the rat and dog are the most frequently used species, rabbits, mice, mini- and micropigs, and monkeys are also used. The aim is to use the species that are considered to be the most relevant to humans in terms of the intended clinical use.

To discuss and/or present all possible routes of administration is not our intention here. Needless to say, the extent of absorption must be determined relative to a "standard." In this context, examples of some routes of administration are given as illustrative of this principle.

Regardless of species and route of administration, the need is to administer high, but justifiable, doses in order to establish safety margins for human exposure and to determine the target organs of toxicity. Although bioavailability for IV administration is not an issue, it is for other routes of administration. Thus the incorporation of TK into toxicity studies along with assessments of bioavailability ensures not only that the drug is present systemically, but that the extent of systemic exposure is "maximal" in order to obtain the best possible assessment of the toxicological profile.

Oral Administration The primary route of administration in many, perhaps most, nonclinical toxicity studies is oral; this is due to the fact that that is the "preferred" route of delivery in the marketplace. However, as stated earlier, other routes—intravenous, subcutaneous, inhalation, topical—are also used, both in the marketplace and thus in the preclinical toxicity studies. The absorption in all cases is determined (TK) and the extent estimated by comparison to PK studies; for example, AUC post-IV to AUC from other routes of administration.

The dosage form itself (i.e., "formulation") can have pronounced effects on the absorption and thus on the selection of the dose. Such effects are well described, especially for oral drugs. Some examples from our own studies and the literature are discussed later in several sections of this chapter.

The rat is the species most commonly used with oral administration the route of choice and is the primary rodent utilized in toxicology studies. Oral administration by gavage as a solution or suspension is the most frequently employed dosage form

and is used in studies of up to 6–9 months duration. The primary consideration is the choice of vehicle. Solutions in water are chosen if the solubility of the drug is sufficient to deliver the highest dose proposed. For drugs of low or limited solubility, a dispersing agent for use in an aqueous milieu is selected. A vehicle of 0.5% aqueous Tween 80 (i.e., polysorbate 80) has a long history of use for a wide range of drugs. Cellulose and its derivatives (e.g., methyl-, carboxy-), starch, and different natural oils (e.g., corn, sesame) are often used as a vehicle for oral gavage formulations. For studies of greater duration than 9 months (i.e., lifetime carcinogenicity studies), oral drugs are usually administered as an admixture in the diet of the rats.

The absorption in all studies is determined from the TK data and evaluated as to whether the drug release profile from the dosage form is sufficient to define the toxicological profile. An example of mean plasma concentration–time data comparing gavage administration to the administration of a similar dose as a dietary admix to rats is shown in Fig. 32.2. In this example, a composite profile was obtained using three rats per time point using alternate animals. The challenge in rodent studies is the small volume of blood that can be taken; thus an analytical method with a low LOQ is needed. Because of the small amount of blood available, rats are usually alternated through the blood collection time points and in mice blood collection is usually terminal.

Use of the dog, the most commonly utilized nonrodent species, allows a wide range of evaluations of oral dosage forms without the "liability" of limited blood sample volume. An example of comparative absorption profile in dogs is shown in Fig. 32.3, where an equivalent dose was administered as a solution and a formulated capsule. In Fig. 32.4 the absorption profile of a highly lipophilic drug formulated as two different salts of Labrafil (a fatty acid complex used to solubilize drugs) is shown. In both cases, the different absorption profile as a function of the dosage form is clearly demonstrated.

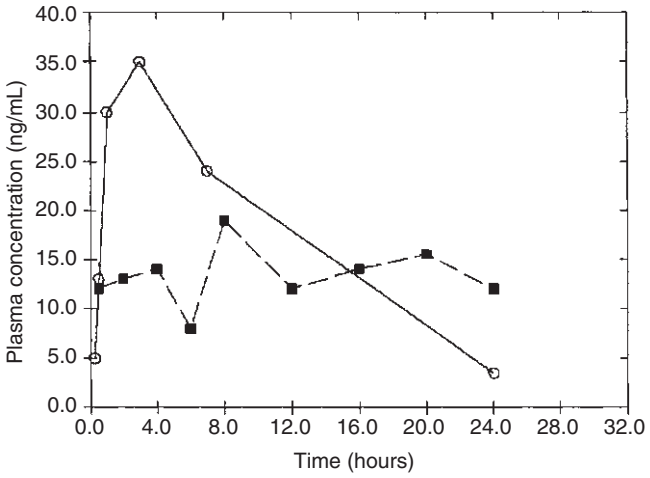


FIGURE 32.2 Plasma concentrations of drug in rats after oral administration as a solution/suspension by gavage (○) and as a dietary admix (■). (From I. Bekersky, personal data.)

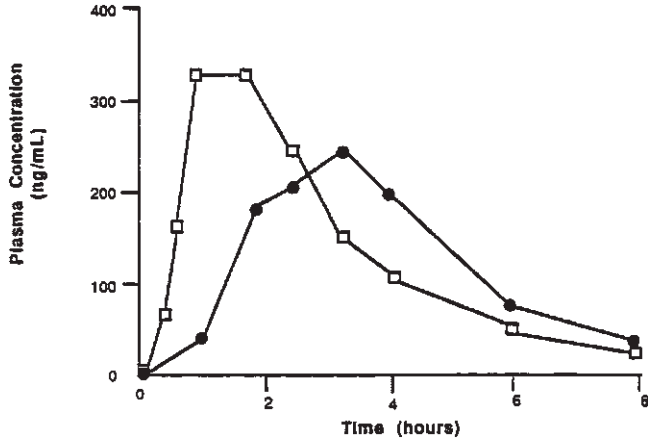


FIGURE 32.3 Mean plasma concentrations of drug in dogs after 20 mg/kg oral administrations as a solution in PEG (□) and a formulated capsule (●). (From I. Bekersky, personal data.)

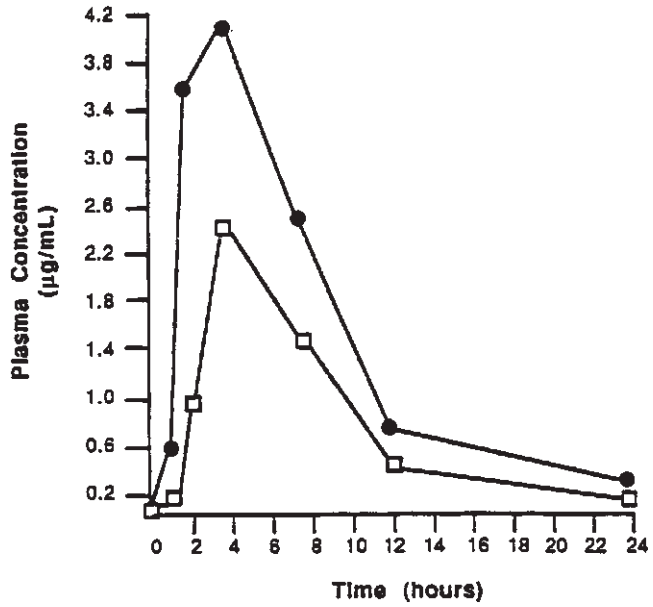


FIGURE 32.4 Mean plasma concentrations of drug in dogs after a 10 mg/kg oral administration of capsules of NMG-Larafil (□) and TEA-Labrafil (●). (From I. Bekersky, personal data.)

Low oral bioavailability of some drugs can be improved by including certain enhancers in drug formulation or special coating of the tablet/capsules. Enhancement of drug bioavailability may be studied in several animal species and can be predictable for humans. Coadministration of compound A and indinavir resulted in a 17-fold increase in oral AUC of compound A in rats due to the inhibition of the metabolism of compound A by indinavir, whereas compound A did not affect

indinavir metabolism as indicated by the unchanged indinavir AUC [11]. Similarly, the systemic exposure of compound A in dogs and monkeys was increased substantially following oral coadministration with indinavir by sevenfold and >50-fold, respectively. Enhancement in compound A systemic exposure by indinavir in humans is predicted based on the *in vivo* animal and *in vitro* human studies.

Labrasol was found to improve the intestinal absorption of gentamicin (GM) in rats. Bioavailability of GM in several different formulations containing labrasol was evaluated in beagle dogs [12].

In Wistar rats, 1.0% (w/v) *N*-trimethyl chitosan chloride (quaternized chitosan derivative) solution significantly increased the absorption of the peptide analogue, resulting in a fivefold increase of octreotide bioavailability compared with the controls (octreotide alone). Coadministration of 1.0% (w/v) chitosan hydrochloride did not enhance octreotide bioavailability [13].

In dogs dosed orally, cefoxitin bioavailability increased from 2.4% to 29% with an enteric-coated tablet containing 600 mg PCC, but uncoated tablets containing palmitoyl-DL-carnitine chloride resulted in no improvement of absorption [14]. Glycosylated bile acid analogue was also used in dogs to increase the absorption of gentamicin [15]. The increase in bioavailability depended on the site of administration. Bioavailabilities in controlled and enhanced states, respectively, were 6% and 54% when administered to the ileum, 4% and 23% for jejunal dosing, and 2% and 10% when administered orally.

Dodecylmaltoside (surfactant) increased the colonic absorption of azetirelin 8.7-fold in rats, and increased oral bioavailability in dogs from 15% to 44% when combined with citric acid in an enteric-coated capsule formulation [16]. The extent of absorption enhancement reported ranged from less than twofold to fourfold to fivefold, with maximum bioavailabilities in rats and dogs of approximately 7% and 19%, respectively [17, 18]. Intraduodenal bioavailability of calcein in rats was improved from 2% to 25–37% with microemulsion formulations containing a mixture of caprylic acid or capric acid and their sodium salts, as well as medium chain glycerides [19].

Medium chain glycerides have been delivered orally in several types of formulations. A self-emulsifying w/o microemulsion formulation containing 22% MCG was administered intraduodenally to rats (3.3 mL/kg); the bioavailability of calcein improved from 2.4% to 45%, and the bioavailability of a hydrophilic peptide improved from 0.5% to 27% [20].

To aid delivery to the lower intestine, capsules containing cefmetazole in an MCG solution were enteric coated [21]. Cefmetazole bioavailability in dogs was 65% with the enteric-coated MCG capsules, 21% with uncoated MCG capsules, and 6% with enteric-coated capsules without MCG. Enteric coating of capsules also improved bioavailability of ceftriaxone in monkeys when administered in MCG.

A vehicle containing 1.5% chitosan at pH 6.7 increased the bioavailability of intraduodenally administered buserelin in rats from 0.1% to 5.1% [22]. A 10-fold increase in the oral bioavailability of paclitaxel was observed in mice when coadministered with the P-gp inhibitor SDZ PSC 833 [23]. A similar magnitude of enhanced bioavailability was found in human subjects administered paclitaxel with 15 mg/kg cyclosporin, another P-gp inhibitor [24]. Oral absorption of digoxin in mice was increased by coadministered SDZ PSC 833, and brain concentrations and brain/plasma ratios were also increased by the P-gp inhibitor, raising the possibility that

systemic absorption of P-gp inhibitors could alter the pharmacologic and toxic effects of coadministered drugs [25].

Intravenous (IV) Administration As stated earlier, bioavailability is not an issue for IV administration as the total dose is delivered. However, there are other considerations that impact on the dose size and thus on the safety margins obtained. The IV dosage form must be a solution and thus the physicochemical properties of the drug are taken into consideration. Once the formulation is established, its compatibility with blood must be determined to establish venous and perivenous tolerance. In addition, the duration of injection (or infusion) is evaluated to establish the safety margin; however, it should be stated that this factor may not be applicable to human use because of anatomical and physiological difference.

It goes without saying that in drugs administered intravenously in the clinic, the issue of bioavailability is not a consideration. However, the species used can be an important determinant based on other factors that determine drug disposition. Examples are given in Tables 32.5 and 32.6, showing that the plasma protein binding parameter was the determinant in the selection of the appropriate species for the toxicological evaluations of ceftriaxone, a parenteral cephalosporin antibiotic. At therapeutic plasma concentrations, the extent of protein binding in human plasma is high (90–95%) and is concentration dependent (Table 32.5). The plasma protein binding in animals that were used in nonclinical development is dependent on the species [26]. In baboon plasma, protein binding (Table 32.5) is concentration dependent and the extent of binding is quite similar to that found in human plasma [26]. In dog plasma, however, protein binding is low with no meaningful relationship to drug concentration. Thus the free concentration of cefriaxone varies greatly in human, baboon, and dog over a similar plasma drug concentration range. As a consequence of the plasma protein binding, the pharmacokinetics of ceftriaxone in humans are nonlinear when based on total drug concentration, but linear if based on the free drug concentration (Table 32.6) [26, 27]. This was the case in baboons, whereas in dogs the pharmacokinetics was the same whether based on total or free drug concentration. These differences played a role in the selection of species for the toxicology studies in early development. In this context, the dog was rejected

TABLE 32.5 Protein Binding Profile of Ceftriaxone in Human and Animal Plasma

	Human		Baboon		Dog	
Drug Concentration (μg/mL)	Bound (%)	Free Concentration (μg/mL)	Bound (%)	Free Concentration (μg/mL)	Bound (%)	Free Concentration (μg/mL)
100	92.7	7.3	95.5	4.5	19.6	80.4
200	88.5	23.0	—	—	13.9	172.2
240	—	—	88.5	27.6	—	—
400	78.7	35.2	—	—	7.1	371.6
560	—	—	65.9	190.9	—	—
800	64.2	64.2	—	—	6.9	744.8
1000	—	—	51.7	483.0	—	—

Source: Adapted from Ref. 27.

TABLE 32.6 Pharmacokinetic Parameters for Ceftriaxone in Humans, Dogs, and Baboon Based on Total (T) And Free (F) Drug Concentrations

Species	Dose (mg/kg)	*AUC* (μg·h/mL)	*V* (L)	CL_p (mL/min)	t_{frac12} (h)
Human	150	268 (T)10 (F)	7	10	8.6
			192	262	8.6
	1500	1978 (T)103 (F)	9	13	7.8
			162	249	7.6
Baboon	150	1573 (T)288 (F)	4.5	11	6.1
			11.9	66	2.6
	700	3913 (T)1632 (F)	5.7	21	6.5
			13.1	51	1.7
Dog	20	79 (T)68 (F)	3.8	40	1.2
			5.2	52	1.3

Source: Adapted from Refs. 26 and 27.

TABLE 32.7 Absolute Bioavailability of Drug Applied Topically to Rats and Micropigs

Species	Route	Dose	Application Area	*AUC* (ng·h/mL)	Bioavailabity, *F* (%)
Rat	IV	1 mg/kg		642	100 %
	Topical	0.5% (500 mg/m^2)	10 cm^2	68	≤5%
	Topical (abraded)	0.5% (500 mg/m^2)	10 cm^2	570	62
Micropig	IV	1 mg/kg		3900	100
	Topical	3% (3000 mg/m^2)	400 cm^2	70	≤1

Source: Adapted from Ref. 28.

based on the different pharmacokinetic profile relative to humans and was replaced by the baboon.

Dermal Administration From a toxicological end point, there are two factors that are determined in the development of topical drugs: the local toxicity at the site of application and the potential for systemic toxicity. The skin is a semipermeable barrier and absorption into the systemic circulation can thus be anticipated. In this context, the extent of systemic exposure (i.e., bioavailability) is determined following dermal application. This is determined for both intact and excoriated skin, as in clinical use the skin can be broken (such as in atopic dermatitis) with a subsequent effect on systemic exposure.

An example from a recent development of a dermal product is given in Table 32.7 [28]. Absolute bioavailability (relative to the respective IV administration) was ≤5% (rat) and ≤1% (micropig) based on AUC comparisons. Abrading the skin in a rat study increased the bioavailability to approximately 62%. The comparison of

the animal data to human IV (1 mg/kg) and topical administration (0.3%, 5000 cm^2 application area) indicated that the absolute bioavailability in atopic dermatitis patients was less than 0.5%. Interestingly, after 8 days of treatment, bioavailability decreased slightly (<0.3%), presumably reflecting an improvement in the integrity of the skin in response to treatment.

The cutaneous bioavailability of topical 2% minoxidil solution was verified in live hairless mice—minoxidil and propylene glycol deposition on the skin surface, epidermis, and dermis from the single-dose *in vivo* study [29]. Percutaneous absorption of the drug appeared to be a very small fraction of the applied dose. The pharmacokinetics of methotrexate (MTX) in rabbit skin and plasma after IV bolus and iontophoretic delivery at different current densities was studied [30]. The systemic absorption of MTX increased proportionally with increased current.

Inhalation and Ocular Administration These routes, although less frequently used, present challenges and thus warrant discussion. In the rat, the differences of the respiratory tract and the fact that rats breathe through their noses result in an inadequate model for inhalation exposure to humans. A common approach to rat inhalation studies is by exposing the nose of the animal through an opening into a cylinder filled with air containing a known concentration of the drug. Several difficulties are readily apparent: the actual dose administered and the monitoring of the systemic received. In larger animals (dogs, monkeys) inhalation is administered via a nose mask, resulting in a more discreet administration. As in all studies, a blood sampling protocol for TK analysis is included in the study for the assessment of systemic exposure.

Ocular administration is perhaps the most emotive route of administration; toxicity studies are conducted in rats, rabbits, and monkeys, with the monkey frequently used for its similarity in ocular volume to the human eye. As with topical administration, local toxicity is evaluated as well as systemic toxicity if a partitioning from the eye into the systemic circulation is demonstrated.

Preclinical studies are often performed in an attempt to improve bioavailability of nasal or ocular administered drugs. Enhancement of bioavailability of calcitonin formulated with different alkylglycosides following nasal and ocular administration was studied in rats [31]. Enhancement of bioavailability of insulin nanocomplexes in nasal formulation was also studied in rats [32]. Iontophoresis ocular drug delivery and peak concentrations determination were performed for numerous drugs in rabbit studies [33]. Hayden et al. [34] showed that focal delivery of carboplatin using subconjunctival injection or iontophoretic resulted in significantly higher peak concentrations of carboplatin in the choroid, retina, optic nerve, and vitreous body than those obtained after intravenous delivery.

Comparison of the Different Routes of Exposure Equivalence of different routes of exposure is not apparent and thus comparison of bioavailability or bioequivalence of the drug administered by the different routes of administration is often studied in various animals. This comparison is illustrated in Table 32.8 and Fig. 32.2. Other examples of such studies are presented next.

The pharmacokinetic parameters of midazolam tablets were compared after administration by the sublingual route and intravenous routes in six rabbits to

TABLE 32.8 Biopharmaceutic Parameters of a Drug in the Dog from a Dosage Form Evaluation Study

Dosage Form	C_{max} (μg/mL)	T_{max} (h)	AUC (μg·h/mL)
Intravenous (IV)			10.3
Aqueous solution	1.5	1.4	12.5
Capsule[a]	1.8	1.5	13.7
Tablet[b]	1.2	1.4	10.8
CMC suspension	0.9	1.0	5.7

[a]Soft gelatin capsule with neat drug.
[b]Tablet formulation for use in first clinical trials.
Source: I. Bekersky, personal data.

determine the bioequivalence between these routes [35]. By the sublingual route, midazolam absorption is substantial and fast. The statistical analysis, on data obtained with HPLC, shows no significant difference between pharmacokinetic parameter values calculated after intravenous and sublingual administration (0.5 mg). The absolute bioavailability was close to 100%. 1-Hydroxy-midazolam seems to have a great importance in BZD activity. To estimate the bioequivalence between intravenous and sublingual midazolam administration, it is necessary to take into account the active metabolites.

Brown et al. [36] compare the bioequivalence of the ceftiofur sodium salt in cattle after a single intramuscular (IM) or subcutaneous (SC) dose of 2.2 mg ceftiofur equivalents/kg body weight. The preestablished criterion for equivalence of the $t >$ 0.2 for IM and SC administration was satisfied. The equivalence of $AUC_{0\text{-}LOQ}$ and $t > 0.2$ for IM and SC administration of 2.2 mg ceftiofur equivalent (CE)/kg doses of ceftiofur sodium suggest similar therapeutic efficacy and systemic safety for the two routes of administration.

The pharmacokinetic properties of a long-acting formulation of chloramphenicol were determined in six yearling cattle after a single intravenous (IV) administration (40 mg/kg body weight) and after two sequential subcutaneous (SC) or intramuscular (IM) administrations [37]. The two extravascular routes were studied during a crossover trial for a bioequivalence test. Bioavailability was 19.1% after IM injection and 12.4% after SC administration. The extent of absorption from the two routes did not differ significantly. The rate of absorption was significantly lower after SC application than it was after IM injection. The time necessary for the plasma concentration to exceed 5 μg/mL was the same for the two routes. Thus, IM and SC routes were bioequivalent. In the other study [38], the pharmacokinetics of chloramphenicol were studied in sheep after three single intravenous (IV), intramuscular (IM), and subcutaneous (SC) administrations (30 mg/kg) during a crossover trial for a bioequivalence test. The two routes of absorption were not bioequivalent. Using the kinetic values, multidose regimens to maintain the therapeutic chloramphenicol blood level (5 μg/mL) were proposed: 60 mg/kg every 12 hours for 72 hours for the IM administration and 45 mg/kg for SC administration according to the same regimen. Chloramphenicol residues remained at the injection site, and 400 hours

would be necessary to obtain the level of 10 μg/kg. Determination of the creatinine phosphokinase serum values showed that the subcutaneous route induced less damage to muscle than the intramuscular route.

The disposition of spiramycin (a macrolide antibiotic) in plasma and milk after intravenous, intramuscular, and subcutaneous administration was investigated in healthy cows given a single injection of spiramycin at a dose of 30,000 IU/kg by each route [39]. The dose fraction adsorbed after intramuscular or subcutaneous administration was almost 100% and was bioequivalent for the extravascular routes, but the rates of absorption, the maximal concentrations, and the time to obtain them differed significantly between the two routes. Plasma concentrations of doramectin in 40 cattle dosed by subcutaneous (SC) or intramuscular (IM) injection (200 micrograms/kg) were compared to assess the bioequivalence of the two routes of administration [40]. The bioequivalence of the SC and IM formulation has been established.

Pietropaolo et al. [41] performed direct comparisons between injection of myelin basic protein peptide and administration by several nonparenteral routes to determine whether route impacted benefit in the treatment of murine allergic encephalomyelitis, a model for multiple sclerosis. The range of effective peptide doses spanned over 1000-fold, and route of delivery played a major role in determining optimal dose. The oral route of administration was the least effective, requiring at least 50- to 100-fold more antigen than subcutaneous injection, which in turn required at least 10-fold more antigen than delivery of peptide to the lung using an intratracheal instillation. Intratracheal delivery was also considerably more effective than inhalation of peptide and, unlike inhalation, resulted in obvious penetration of delivered material deep into the lung. The increase in therapeutic efficacy did not appear to result from slower systemic delivery of antigen.

Apomorphine (0.5 mg/kg) was administered subcutaneously and percutaneously to rabbit in order to compare the pharmacokinetic data obtained with these two different routes [42]. For the percutaneous administration, an apomorphine gel was prepared by dissolution of apomorphine in a hydroxypropylmethylcellulose gel of medium viscosity. The peak plasma concentration and the area under the curve were significantly greater with the subcutaneous route. The bioequivalence of the percutaneous route was 35% of the subcutaneous administration. One study [43] reports a paradox: the AUC of apomorphine after intravenous injection in rabbits is anomalously lower (statistically significantly) than after subcutaneous injection.

Bergeron et al. [44] examined the effect of route of administration by giving equimolar amounts of two iron chelators—NaHBED and deferoxamine (DFO)—to *Cebus apella* monkeys as either a subcutaneous (SC) bolus or a 20 min intravenous (IV) infusion. By both routes, NaHBED was consistently about twice as efficient as DFO in producing iron excretion. For both chelators at a dose of 150 μmol/kg, SC was more efficient than IV administration.

Acrylamide bioavailability after aqueous gavage was 60–98% and from the diet was 32–44%; however, first-pass metabolism or other kinetic change resulted in much higher (two- to sevenfold) internal exposures to glycidamide (its epoxide metabolite, GA) when compared to the intravenous route. A similar effect on metabolism for GA following oral administration was previously observed under an identical exposure paradigm in mice [45].

32.2.4 Use of Animal Models for the Selection of Dosage Forms for Nonclinical Toxicology Studies

Some of the issues in selecting a dosage form for animal studies have been touched upon previously. Data that is needed prior to the preparation/choosing of a dosage form are solubility, stability, compatibility with excipients, and particle size, keeping in mind that a "maximum" drug release profile is a target.

For rat studies, use of solutions or suspensions administered via gavage has been described previously. Rats are rarely used for comparison of different dosage forms. Of more illustrative value are studies with large animals (dogs, monkeys), as these species allow more flexibility in the kinds of dosage forms administered. An example of a dosage form evaluation study is given in Table 32.8 where three possible dosage forms for toxicology studies (solution, unformulated capsule, and suspension) as well as a gelenic tablet prepared for the initial clinical trials were tested. The data indicate that relative to an IV administration, the solution, capsule, and galenic tablet were acceptable for use in toxicity studies with a drug release profile equal to the IV Bioavailability of the CMC suspension was approximately half of all the other dosage forms tested.

A biopharmaceutic evaluation that involved the drug particle size and several forms of the drug is given in Table 32.9. The drug in question had excellent oral activity although the form available at the time (the free base as an oil) resulted in severe GI toxicity, which was in all probability due to the prolonged presence of unabsorbed drug substance. Capsules containing salts of the drug were then tested, indicating that the acetate salt resulted in the greatest extent of absorption relative to the other two forms. The subsequent availability of the free base in crystalline form allowed the drug to be micronized, and a second study was conducted to evaluate the absorption profile. This study indicated that reducing the particle size by micronization resulted in higher C_{max}, shorter T_{max}, and AUC values that were considerably higher than those in the first study. Interestingly, the more rapid absorption alleviated the GI toxicity found in preliminary studies, in spite of the considerably higher extent of absorption.

The selection of a formulation for nonclinical studies has received a "modest" treatment as being illustrative of the overall study design in the early stages of drug development. In order to attain a maximum drug release profile, in some cases in

TABLE 32.9 Biopharmaceutic Parameters (Mean ±SD) from Two Dosage Form Evaluation Studies

Dosage Form	C_{max} (μg/mL)	T_{max} (h)	AUC (μg·h/mL)
Nonmicronized			
Free base (oil)	1.6 ± 1.4	2.7 ± 1.2	5.9 ± 5.7
Dihydrochloride	3.6 ± 1.9	0.5 ± 0	11.9 ± 5.3
Acetate salt	4.3 ± 1.9	4.0 ± 2.0	18.9 ± 8.0
Micronized			
Free base	8.4 ± 3.5	1.8 ± 0.5	70.2 ± 6.6
Dihydrochloride	4.9 ± 2.4	2.0 ± 1.4	40.8 ± 21.9
Acetate salt	5.8 ± 2.3	2.1 ± 1.4	41.4 ± 21.6

spite of undesirable physicochemical properties, and thus clearly to define the line between pharmacology and toxicology, formulation selection has become part of the overall development of drugs. In many cases, formulation evaluation studies are not part of the toxicology/safety studies but are conducted in parallel throughout development, with the information obtained being integrated into all evaluations. Examples from the literature of studies dealing with factors that impact on bioavailability are given next.

Seven kinds of poorly water-soluble compounds as the model compounds were used to compare the plasma concentration profile of the drug following single oral administration of each compound to rats and beagle dogs as a solution, an oily solution, a suspension (or a powder), and oil/water (O/W) microemulsions [46]. Thus for six kinds of the model compounds, except disopyramide, the solubility was from 340 to 98,000 times that in water, and the AUC values in plasma concentration of the compound were equivalent to that of solution or O/W microemulsion administration, or were increased by 1.5–78 times that of suspension administration.

The bioavailability of a modified rafoxanide oral suspension was compared to the original innovator product and a generic formulation in a single-dose, randomized, parallel design study in sheep [47]. The area under the rafoxanide plasma concentration versus time curve (AUC), AUC extrapolated to infinity, and maximum plasma rafoxanide concentrations (C_{max}) were significantly smaller for both the modified and generic formulations relative to the original product. There were no significant differences between the modified and generic formulations. In terms of the calculated 90% confidence *t*-intervals of the mean % ratios, the modified and generic formulations were not bioequivalent to the original product, since they were substantially below the accepted range of 80–125%.

32.2.5 Species Differences in Bioavailability

There are many processes that determine bioequivalence of a given drug among species, including absorption from a site of administration; renal, biliary, and intestinal elimination; sequestration (in particular, binding to proteins or other macromolecules); distribution and redistribution; biotransformation; and receptor population density and uniqueness. Short [48] reviewed physiologic and pharmacologic parameters that affect drug distribution, elimination, and metabolism to make comparisons of bioequivalence of drugs between sheep and other ruminants. Interspecies differences are very important for selecting an appropriate species for preclinical studies. The following is a summary of recent literature describing interspecies differences in pharmacokinetics of a wide range of therapeutic agents.

Pharmacokinetics and pharmacodynamics of controlled release gastroretentive dosage forms (CR-GRDF), which improve the bioavailability of medications, were studied for several drugs in rats and dog models [49–52]. The bioavailability of melagatran (active form of ximelagatran) following oral administration of ximelagatran was 5–10% in rats, 10–50% in dogs, and about 20% in humans, with low between-subject variation [53]. The oral bioavailability of levovirin was 29.3% in rats, 51.3% in dogs, and 18.4% in monkeys [54]. Zebularine showed oral bioavailability of 6.7% in mice, 3.1% and 6.1% in rats, and was <1% in monkeys [55].

Extrapolation of pharmacokinetics from animals to humans may be performed based on *in vitro* metabolism studies in hepatocytes. *In vitro* degradation studies of vinpocetine with hepatocytes have shown that the activity of human hepatocytes is about one order of magnitude higher than the activity of dog hepatocytes, and two orders of magnitude higher than that of rat hepatocytes [56]. These differences can explain the differences in bioavailabilities of vinpocetine in the three species (52% in rats, 21.5% ± 19.3% in dogs, and 6.2% ± 1.9% in humans). In dogs and humans, the compound seems to be metabolized exclusively in the liver, whereas in rats extrahepatic metabolism seems also to be important. The *in vivo* clearance predicted from the activity of hepatocytes is in good agreement with the values measured *in vivo* in the case of humans and dogs.

The interspecies absorption, distribution, metabolism, and elimination (ADME) profile of *N*-geranyl-*N′*-(2-adamantyl)ethane-1,2-diamine (SQ109), a new diamine-based antitubercular drug, were characterized after intravenous and oral single administration in rodents and dogs [57]. Based on IV equivalent body surface area dose, the terminal half-life of SQ109 in dogs was longer than that in rodents, reflected by a larger volume of distribution and a higher clearance rate of SQ109 in dogs, compared to that in rodents. The oral bioavailability of SQ109 in dogs, rats, and mice were 2.4–5%, 12%, and 3.8%, respectively. The binding of [^{14}C]SQ109 (0.1–2.5 μg/mL) to plasma proteins varied from 6% to 23% depending on the species (human, mouse, rat, and dog). SQ109 was metabolized by rat, mouse, dog, and human liver microsomes, resulting in 22.8%, 48.4%, 50.8%, or 58.3%, respectively, of SQ109 remaining after a 10 min incubation at 37 °C.

Compound A (potent inhibitor of the hepatitis C virus (HCV) NS5B polymerase) exhibited marked species differences in pharmacokinetics [58]. Plasma clearance was 44 mL/min/kg in rats, 9 mL/min/kg in dogs, and 16 mL/min/kg in rhesus monkeys. Oral bioavailability was low in rats (10%) but significantly higher in dogs (52%) and monkeys (26%). Compound A was eliminated primarily by metabolism in rats, with biliary excretion accounting for 30% of its clearance. Metabolism was mainly mediated by cyclohexyl hydroxylation, with N-deethylation and acyl glucuronide formation constituting minor metabolic pathways. Qualitatively, the same metabolites were identified using *in vitro* systems from all species studied, including humans. The low oral bioavailability of compound A in rats was mostly due to poor intestinal absorption. A series of high affinity GABA(A) agonists showed good oral bioavailability in both rat and dog [59].

The pharmacokinetics of DA-6034 in rats and dogs and the first-pass effect in rats were examined [60]. The low bioavailability (approximately 0.136%) of DA-6034 at a dose of 50 mg/kg in rats could be due to considerable intestinal first-pass effect (approximately 69% of oral dose) and unabsorbed fraction from the gastrointestinal tract (approximately 30.5%). The clearance of adaphostin (potential anticancer agent) from plasma, on a square meter (m^2) basis, was equivalent for mice and rats but more rapid in dogs [61]. Oxytocin antagonist, 2′,4′-difluorophenyldiketopiperazine derivative 37, has good bioavailability (46%) in the rat and moderate bioavailability (13–31%) in the dog [62], while 5-chloroindole had good oral bioavailability in both rat and dog [63].

BMS-378806 (prototype of novel HIV attachment inhibitors) exhibited species-dependent oral bioavailability, which was 19–24% in rats and monkeys and 77% in dogs [64]. In rats and monkeys, absorption was prolonged, with an apparent terminal

half-life of 2.1 and 6.5 hours, respectively. In rats, linear pharmacokinetics was observed between IV doses of 1 and 5 mg/kg and between PO doses of 5 and 25 mg/kg. The total body clearance was intermediate in rats and low in dogs and monkeys. The steady-state volume of distribution was moderate (0.4–0.6 L/kg), contributing to a short half-life (0.3–1.2 hours) after IV dosing.

The pharmacokinetics and disposition of *N*-(2,6-dichlorobenzoyl)-4-(2,6-dimethoxyphenyl)-L-phenylalanine (TR-14035), a novel a4ss1/a4ss7 antagonist, were investigated in the rat and dog [65]. Results indicated extensive clearance of TR-14035 and low oral bioavailability, 17% and 13% in the rat and dog, respectively, at an oral dose of 10 mg/kg. A species-dependent difference in metabolism was observed. The principal metabolite, *O*-desmethyl TR-14035, observed in rat, dog, and probably human, was further conjugated with sulfate in the rat, but never in dog and human, based on *in vitro* metabolism and *in vivo* metabolite profile studies. Urinary excretion was a minor elimination route, but an interesting species difference was recognized. TR-14035 was reabsorbed from the rat renal proximal tubules and, by contrast, secreted into the tubules in the dog, probably via active transport systems.

Exposure of MGS0028, a potent group II metabotropic glutamate receptor agonist, increased proportionally (1–10 mg/kg PO) in rats, with bioavailability >60% at all doses. However, bioavailability was only approximately 20% in monkeys, and active metabolite was found in relatively high abundance in plasma. In dogs, oral bioavailability was >60%, and the metabolite was not detected. James et al. [66] found that the rat was a better *in vivo* model than the dog for the prediction of human systemic exposure in HIV-1 nonnucleoside reverse transcriptase inhibitors of the diaryltriazine and diarylpyrimidine classes of compounds [67]. The absolute bioavailability of YM466, a new factor Xa inhibitor, was 2.7–4.5% in rats, almost constant regardless of the dose levels investigated, while it was 6.9–24.6% in dogs, indicating nonlinear pharmacokinetics.

The bioavailability of SNI-2011, cevimeline, a novel muscarinic acetylcholine receptor agonist, was approximately 50% and 30% in rats and dogs, respectively. Major metabolites in plasma were both S- and N-oxidized metabolites in rats and only N-oxidized metabolite in dogs, indicating that a large species difference was observed in the metabolism of SNI-2011 [68].

Absolute oral bioavailability of a gavage dose of galantamine was high in rat (77%) and dog (78%). In mice and rats, the bioavailability of galantamine administered via the food was lower than of galantamine administered by gavage [69]. Elimination half-life of galantamine was relatively large in rat and dog and smaller in mouse and rabbit. Galantamine plasma levels after single and repeated administration of 10 mg/kg/day in all species investigated except female rat and rabbit were much higher than mean therapeutic plasma levels of galantamine obtained in humans. The pharmacokinetic profile of galantamine after repeated oral administration in rats was most similar to the profile obtained after repeated administration of 12 mg bid in humans.

Not only species but even a strain within the same species may predispose to pharmacokinetics differences especially when different strains exhibit different metabolism. Systemic exposure to oxycodone and noroxycodone was consistently higher for cytochrome P450 (CYP)2D1/2D2-deficient dark Agouti (DA) rats than for CYP2D1/2D2-replete Sprague–Dawley (SD) rats showing that strain differences predominated over diabetes status [70].

32.3 NDA-TARGETED NONCLINICAL DEVELOPMENT

When the IND is "opened" and the nonclinical and Phase I data are indicative of a "go" decision, the NDA-targeted studies are conducted. Whereas the IND contained acute and subchronic (2 and/or 4 week) toxicology studies, depending on the indication sought, an acceptable NDA will contain chronic toxicity studies in support: 6 month rat, 9 month dog (or monkey), 78 week carcinogenicity study in mice, and 24 month carcinogenicity study in rats. This would be the case for IV, PO, and inhalation drugs. For topical drugs, 52 week photocarcinogenicity and 2 year topical carcinogenicity studies are conducted.

32.3.1 Toxicokinetics in Chronic Toxicity Studies

Considering that the duration of exposure in chronic studies is severalfold that of subchronic studies, it is even more important to assess the extent of exposure. To this end, range-finding studies are conducted to ascertain that the dose range that will be used in the definitive study will provide both a NOAEL and a definition of toxicity. In general, the concepts that have been described previously hold for these studies. Toxicokinetics are an integral part of protocol designs to determine if there are changes with the duration of treatment. It is hoped that the bioavailability of the dosage form has been determined during the pre- and post-IND development, with the assurance of a consistent and demonstrable extent of systemic exposure.

It is worthwhile mentioning that the oral carcinogenicity studies are conducted with the drug administered as a dietary admix. This would require a "bridging study" comparing bioavailability of the dietary admix to a systemic administration to determine the bioavailability. Such studies entail a 5–8 sample profile over 24 hours, whereas the toxicokinetics in the definitive admix studies would consist of a single blood draw, usually at approximately 9 am.

An example of a gavage versus admix comparison using serial sampling is shown in Fig. 32.2; from such data, exposure and bioavailability based on AUC can readily be determined. Single sample monitoring over the course of a chronic study, however, is useful as accumulation or induction over the study time course can be ascertained and such data will be of help in the study evaluation.

32.3.2 Use of Animals to Evaluate the BA/BE of Clinical Dosage Forms

In the early stages of development, it is important to determine whether the dosage form targeted for clinical use has an acceptable level of bioavailability. To evaluate the dosage form in a clinical study is time consuming and costly. In this case, the clinical form was first evaluated in dogs and subsequently in humans. The results are shown in Table 32.10. Compared to IV administrations, the bioavailability of the oral capsule formulation in dog was low (27%), and based on data from ADME studies, the low bioavailability was considered to be due to extensive metabolism. Urinary excretion data indicated that only a small fraction of the dose (0.2–0.3%) was eliminated as the intact drug, with the rest excreted as a hydroxyl metabolite. It was considered that even though the bioavailability was low, the level was sufficient to continue with a clinical study. As shown in Table 32.10, the results in humans were similar: low C_{max} and low bioavailability of the intact drug (approximately

TABLE 32.10 Biopharmaceutic Comparison of a Drug in Dog and Human

Species	Dosage Form	Dose (mg/kg)	C_{max} (μg/mL)	AUC_{inf} (μg·h/mL)	F^a (%)
Dog	Capsule	7.5	0.62	66	27
Human	Tablet	10	0.08	142	37

[a]Relative to IV dose and corrected for dose.

Source: Adapted from Ref. 79.

TABLE 32.11 Comparison of Drug Release Profile of Drug from Similar Dosage Forms in Dog and Human

Species	Dosage Form	Dose (mg/kg)	T_{max} (h)	C_{max} (ng/mL)	AUC (ng·h/mL)
Dog	PEG solution	20	1	320	920
	Captex capsule	20	3	230	928
Human	PEG capsule	0.2	3	446	1524
	Neobee capsule	0.2	4	336	1004

Source: I. Bekersky, personal data.

37%). In this case, the study in dogs indicated a level of oral bioavailability that was acceptable for a clinical evaluation; a clinical study was then conducted with similar results to those found in dogs.

In Table 32.11, an evaluation of several dosage forms in dogs and subsequently in humans is shown. In dogs, there was little difference between the oral formulations tested, although the PEG solution resulted in a more rapid absorption based on T_{max}. In humans, however, the capsules containing PEG formulation appeared to be more bioavailable, an AUC of 1524 ng·h/mL versus an AUC of 1004 ng·h/mL for the Neobee formulation. Capsules containing drug as solution/suspension in PEG were subsequently developed for use in clinical trials.

In another study, preliminary experiments with an antibiotic indicated a low relative bioavailability. Physicochemical considerations suggested that ester analogues represented an approach that could potentially enhance the oral bioavailability of the parent compound. Ester analogues of the drug were synthesized and tested as solutions in dogs. The results are summarized in Table 32.12. Relative to the solution, both esters enhanced the oral bioavailability: 26% of the Bac ester, and 22% of the Piv ester; the bioavailability of the parent drug was 10%. On the basis of these results, both esters were administered to humans with results comparable to those "predicted" by the dog study.

Use of the dog to provide insights pertaining to drug absorption is not restricted to situations where information prospective to the first clinical trials is desired. Even when the clinical trials are well advanced, the dog can be utilized to ascertain a situation that may require reformulation of the clinical dosage form. An example of such a case is given in Table 32.13. The drug was unstable in the presence of oxygen and light and was formulated as a micronized suspension in oil within a soft elastic gelatin (SEG) capsule. On aging, these capsules failed to meet the dissolution speci-

TABLE 32.12 Oral Bioavailability of Drug Analogue Solutions in Dog and Human

Species	Drug Form	Dose (mg/kg)	C_{max} (μg/mL)	AUC (μg·h/mL)	Bioavailability F (%)
Dog	Parent	20	2.6	6.9	10
	Bac ester	20	9.1	18	26
	Piv ester	20	5.5	15	22
Human	Bac ester	5.3	4.4	7	29
	Piv ester	5.3	3.2	6	25

Source: I. Bekersky, personal data.

TABLE 32.13 Biopharmaceutic Evaluation of Potential Clinical Formulations in Dog and Human

Species	Administration	Approximate Dose (mg/kg)	C_{max} (μg/mL)	AUC_{inf} (μg·h/mL)	F^a (%)
Dog	Oral solution	4	3.33	12	
	"New" SEG capsule	4	1.05	4.5	39
	"Aged" SEG capsule[b]	4	1.23	5.5	47
Human	"New" SEG capsule	1	0.26	3.8	60
	"Aged" SEG capsule	1	0.29	3.7	59

[a]Bioavailability relative to respective oral solution and corrected for dose.
[b]"Aged" capsules were stored for 4 years and failed dissolution specifications.

Source: Adapted from Ref. 79.

fications for the product. To determine whether reformulation was required, these "aged" capsules were tested in dogs relative to the identical "new" formulation. In the dog study, no changes in the bioavailability were found due to aging. This was confirmed in a study in humans that gave similar results to those found in the dog study.

The dog can also be used in exploratory research. The drug in question was under development for parenteral delivery only. Oral administration to dogs resulted in very low C_{max} (3–4 μg/mL) with a bioavailability of approximately 4% relative to IV administration. However, since some oral absorption did occur, an exploratory program with dogs was initiated to study the feasibility of developing an oral dosage form for humans. The results are given in Table 32.14. The intraduodenal (ID) and rectal administration to the dog of two exploratory formulations containing an absorption enhancer indicated a significant increase in absorption as indicated by the C_{max} and AUC values. Based on these data, the concept was tested in humans. Although the bioavailability via the different routes in enteral administration was

TABLE 32.14 Biopharmaceutic Parameters of a Drug in Dog and Human Following a Single Administration by Different Routes

Species	Route	Dose	C_{max} (μg/mL)	T_{max} (h)	AUC_{inf} (μg·h/mL)	F (%)
Dog	IV	20 mg/kg	—	—	102	—
	ID[a]	50 mg/kg	47	0.5	82	32
	Rectal	50 mg/kg	28	0.2	24	10
Human	IV	500 mg	—	—	427	—
	ID	500 mg	6	0.16	24	6
	Rectal	500 mg	23	0.75	124	29

[a]ID, intraduodenal.

Source: I. Bekersky, personal data.

reversed in humans when compared to dogs, the potential enteral delivery in humans was ascertained.

32.3.3 Role and Impact of Nonclinical Studies on Clinical Development: Animal to Humans

During Phase I–IIa, factors such as a food effect, gender effect, and pre- and systemic metabolism are determined prior to Phase IIb in order to set/justify dose selection for the pivotal Phase III studies. Nonclinical studies have provided "windows" of information prospectively to the conduct of label–targeted human clinical pharmacology studies; examples are given next.

The bioavailability in beagle dogs and the dissolution rates of cyclandelate from five capsule preparations commercially available in Japan were measured [71]. One of the capsules that showed an extremely low bioavailability in humans also showed the lowest bioavailability in beagle dogs, although the difference in bioavailability with the highest preparation was smaller than in humans. A significant correlation was obtained between the results of the studies in humans and beagles. However, the power of the test using beagles was extremely low in comparison with that in the human study. Food enhanced the bioavailability of cyclandelate from the capsules, having the highest and lowest bioavailability in the fasted state in beagles as observed previously in the human study. The bioinequivalence of the cyclandelate capsules detected in the fasted state disappeared in the fed state in the beagle dog study, while the bioinequivalence still remained in the nonfasted state in human subjects. Thus bioequivalence testing in the fed state led to different results in both species.

Coadministration of food increases the rate and extent of cilostazol absorption [72]. After investigating the effect of concomitant food intake on the bioavailability of two nifedipine-containing modified-release dosage forms, authors concluded that a change from taking Slofedipine XL in the fed to the fasted state might result in increased systemic concentrations of nifedipine [73]. In the dog, food decreased the mean area under the plasma concentration–time curve value of compound LY354740, an analogue of glutamic acid, by approximately 34%, hence decreasing the oral bioavailability of the compound [74].

Concomitant intake of levodopa preparations with banana juice, but not with a commercial banana beverage, caused a drug–food interaction reducing levodopa bioavailability in rats [75]. Mean (±SD) peak concentrations of tepoxalin were significantly higher after feeding of low fat (1.08 ± 0.37 μg/mL) and high fat (1.19 ± 0.29 μg/mL) diets than in fasted dogs (0.53 ± 0.20 μg /mL), suggesting that feeding improves oral bioavailability [76].

Bioavailability and pharmacokinetics may be gender dependent. Jann et al. [77] showed that gender may be a significant factor in ondansetrons disposition. Gender differences in the pharmacokinetics of DA-6034 were found after intravenous but not an oral administration at a dose of 50 mg/kg to male and female Sprague–Dawley rats [78]. In rats, plasma levels of galantamine were lower in females than in males, whereas in mice, females showed higher levels than males [69]. No gender differences were observed in dogs. Sex difference was also observed in the pharmacokinetics of cevimeline in rats but not in dogs [68].

32.4 CONCLUSION

Toxicology/safety studies in animals are an integral part of any drug development program, the results of which have a direct impact on both the IND and the NDA. The calculations of safety margins for human doses are based on the measurements of systemic exposure (AUC) and the NOAEL in the animal studies. In this context, the inclusion of pharmacokinetics into the conduct/evaluation of animal toxicity studies (toxicokinetics) has become routine. Toxicokinetic evaluations ascertain that a level of systemic absorption was achieved, the intrastudy relationship of this absorption to the doses administered, and absorption changes with the duration of exposure. The inclusion of bioavailability concepts adds the element of quantity, as the level of absorption relative to a standard is obtained. As a maximum drug release profile is desired, in many cases several dosage forms are tested. Thus bioequivalence determinations of several dosage forms intended for toxicology studies provide assurance that the most appropriate dosage form is used. The bioequivalence is also determined to assess equivalence between the routes of administration, species differences, and drug derivatives preference.

Animal studies for bioavailability/bioequivalence are used both prospectively and retrospectively, and depending on the objective and study design, form an animal to human bridge in drug development.

REFERENCES

1. *Code of Federal Regulations*, Title 21, 320.1.
2. *Code of Federal Regulations*, Title 21, Volume 5.
3. Chen ML, Shah V, Patnaik R, Adams W, Hussain A, Conner D, Mehta M, Malinowski H, Lazor J, Huang SM, Hare D, Lesko L, Sporn D, Williams R. Bioavailability and bioequivalence: an FDA regulatory overview. *Pharm Res* 2001;18(12):1645–1650.
4. Rajaram L, Roy SK, Skerjanec A. Bioavailability and bioequivalence trials: statistics & pharmacokinetic principles. *Stud Health Technol Inform* 2004;159–179.

5. Good Laboratory Practice Regulations, Title 21 of the *Code of Federal Regulations*, Part 58, effective 20 June, 1979.
6. *Bioavailability and Bioequivalence Studies for Oral Administered Drug Products—General Considerations*. Washington DC: US FDA; 2003.
7. *Bioequivalence Guidance*, Center for Veterinary Medicine. Washington DC: US FDA; 2002.
8. *Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers*. Washington DC: US FDA; 2005.
9. *Guidance for Industry*, *Investigators*, *and Reviewers*: *Exploratory IND Studies*. Washington DC: US FDA; 2006 .
10. Freireich EJ, Gehan EA, Rall DP, Schmidt LH, Skipper HE. Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey, and man. *Cancer Chemother Rep* 1966:50(4):219–244.
11. Jin L, Chen IW, Chiba M, Lin JH. Interaction with indinavir to enhance systemic exposure of an investigational HIV protease inhibitor in rats, dogs and monkeys. *Xenobiotica* 2003;33(6):643–654.
12. Rama Prasad YV, Eaimtrakarn S, Ishida M, Kusawake Y, Tawa R, Yoshikawa Y, Shibata N, Takada K. Evaluation of oral formulations of gentamicin containing labrasol in beagle dogs. *Int J Pharm* 2003;268(1–2):13–21.
13. Thanou M, Verhoef JC, Marbach P, Junginger HE. Intestinal absorption of octreotide: *N*-trimethyl chitosan chloride (TMC) ameliorates the permeability and absorption properties of the somatostatin analogue *in vitro* and *in vivo*. *J Pharm Sci* 2000;89(7): 951–957.
14. Sutton SC, LeCluyse EL, Engle K, Pipkin JD, Fix JA. Enhanced bioavailability of cefoxitin using palmitoylcarnitine. II. Use of directly compressed tablet formulations in the rat and dog. *Pharm Res* 1993;10:1516–1520.
15. Axelrod HR, Kim JS, Longley CB, Lipka E, Amidon GL, Kakarla R, Hui YW, Weber SJ, Choe S, Sofia MJ. Intestinal transport of gentamicin with a novel, glycosteroid drug transport agent. *Pharm Res* 1998;15:1876–1881.
16. Sasaki I, Tozaki H, Matsumoto K, Ito Y, Fujita T, Murakami M, Muranishi S, Yamamoto A. Development of an oral formulation of azetirelin, a new thyrotropin-releasing hormone (TRH) analogue, using *N*-lauryl-*b*(-D-maltopyranoside as an absorption enhancer. *Biol Pharm Bull* 1999;22:611–615.
17. Aungst BJ, Saitoh H, Burcham DL, Huang S-M, Mousa SA, Hussain MA. Enhancement of the intestinal absorption of peptides and non-peptides. *J Control Release* 1996;41: 19–31.
18. Burcham DL, Aungst BJ, Hussain M, Gorko MA, Quon CY, Huang S-M. The effect of absorption enhancers on the oral absorption of the GPIIb/IIIa receptor antagonist, DMP 728, in rats and dogs. *Pharm Res* 1995;12:2065–2070.
19. Constantinides PP, Welzel G, Ellens H, Smith PL, Sturgis S, Yiv SH, Owen AB. Water-in-oil microemulsions containing medium-chain fatty acids/salts: formulation and intestinal absorption enhancement evaluation. *Pharm Res* 1996;13:210–215.
20. Constantinides PP, Scalart J-P, Lancaster C, Marcello J, Marks G, Ellens H, Smith PL. Formulation and intestinal absorption enhancement evaluation of water-in-oil microemulsions incorporating medium-chain glycerides. *Pharm Res* 1994;11:1385–1390.
21. Sekine M, Terashima H, Sasahara K, Nishimura K, Okada R, Awazu S. Improvement of bioavailability of poorly absorbed drugs. II. Effect of medium chain glyceride base on the intestinal absorption of cefmetazole sodium in rats and dogs. *J Pharmacobio-Dyn* 1985;8:286–295.

22. Lueßen HL, de Leeuw BJ, Langemeÿer WE, de Boer AG, Verhoef JC, Junginger HE. Mucoadhesive polymers in peroral peptide drug delivery. VI. Carbomer and chitosan improve the intestinal absorption of the peptide drug buserelin *in vivo*. *Pharm Res* 1996;13:1668–1672.
23. Van Asperen J, van Tellingen O, Sparreboom A, Schinkel AH, Borst P, Nooijen WJ, Beijnen JH. Enhanced oral bioavailability of paclitaxel in mice treated with the P-glycoprotein blocker SDZ PSC 833. *Br J Cancer* 1997;76:1181–1183.
24. Terwogt JMM, Beijnen JH, ten Bokkel Huinink WW, Rosing H, Schellens JHM. Coadministration of cyclosporin enables oral therapy with paclitaxel. *Lancet* 1998;352: 285.
25. Mayer U, Wagenaar E, Dorobek B, Beijnen JH, Borst P, Schinkel AH. Full blockade of intestinal P-glycoprotein and extensive inhibition of blood–brain barrier P-glycoprotein by oral treatment of mice with PSC833. *J Clin Invest* 1997;100:2430–2436.
26. Popick AC, Crouthamel WG, Bekersky I. Plasma protein binding of ceftriaxone. *Xenobiotica* 1987;17:1139–1145.
27. Patel IH, Kaplan SA. Pharmacokinetic profile of ceftriaxone in man. *Am J Med* 1984; 77:17–25.
28. Bekersky I, Lilja H, Lawrence I. Tacrolimus pharmacology and non clinical studies. FK506 to protopic. *Semin Cutan Med Surg* 2001;20:226–232.
29. Tsai JC, Weiner N, Flynn GL, Ferry JJ. Drug and vehicle deposition from topical applications: localization of minoxidil within skin strata of the hairless mouse. *Skin Pharmacol* 1994;7(5):262–269.
30. Stagni G, Shukla C. Pharmacokinetics of methotrexate in rabbit skin and plasma after iv-bolus and iontophoretic administrations. *J Control Release* 2003;93(3):283–292.
31. Ahsan F, Arnold J, Meezan E, Pillion DJ. Enhanced bioavailability of calcitonin formulated with alkylglycosides following nasal and ocular administration in rats. *Pharm Res* 2001;18(12):1742–1746.
32. Simon M, Wittmar M, Kissel T, Linn T. Insulin containing nanocomplexes formed by self-assembly from biodegradable amine-modified poly(vinyl alcohol)-graft-poly(L-lactide): bioavailability and nasal tolerability in rats. *Pharm Res* 2005;22(11):1879–1886.
33. Eljarrat-Binstock E, Domb AJ. Iontophoresis: a non-invasive ocular drug delivery. *J Control Release* 2006;110(3):479–489.
34. Hayden BC, Jockovich ME, Murray TG, Voigt M, Milne P, Kralinger M, Feuer WJ, Hernandez E, Parel JM. Pharmacokinetics of systemic versus focal carboplatin chemotherapy in the rabbit eye: possible implication in the treatment of retinoblastoma. *Invest Ophthalmol Vis Sci* 2004;45:3644–3649.
35. Odou P, Barthelemy C, Chatelier D, Luyckx M, Brunet C, Dine T, Gressier B, Cazin M, Cazin JC, Robert H. Pharmacokinetics of midazolam: comparison of sublingual and intravenous routes in rabbit. *Eur J Drug Metab Pharmacokinet* 1999;24(1):1–7.
36. Brown SA, Chester ST, Speedy AK, Hubbard VL, Callahan JK, Hamlow PJ, Hibbard B, Robb EJ. Comparison of plasma pharmacokinetics and bioequivalence of ceftiofur sodium in cattle after a single intramuscular or subcutaneous injection. *J Vet Pharmacol Ther* 2000;23(5):273–280.
37. Sanders P, Guillot P, Mourot D. Pharmacokinetics of a long-acting chloramphenicol formulation administered by intramuscular and subcutaneous routes in cattle. *J Vet Pharmacol Ther* 1988;11(2):183–190.
38. Dagorn M, Guillot P, Sanders P. Pharmacokinetics of chloramphenicol in sheep after intravenous, intramuscular and subcutaneous administration. *Vet Q* 1990;12(3):166–174.

39. Sanders P, Moulin G, Guillot P, Dagorn M, Perjant P, Delepine B, Gaudiche C, Mourot D. Pharmacokinetics of spiramycin after intravenous, intramuscular and subcutaneous administration in lactating cows. *J Vet Pharmacol Ther* 1992;15(1):53–61.

40. Nowakowski MA, Lynch MJ, Smith DG, Logan NB, Mouzin DE, Lukaszewicz J, Ryan NI, Hunter RP, Jones RM. Pharmacokinetics and bioequivalence of parenterally administered doramectin in cattle. *J Vet Pharmacol Ther* 1995;18(4):290–298.

41. Pietropaolo M, Olson CD, Reiseter BS, Kasaian MT, Happ MP. Intratracheal administration to the lung enhances therapeutic benefit of an MBP peptide in the treatment of murine experimental autoimmune encephalomyelitis. *Clin Immunol* 2000;95(2):104–116.

42. Durif F, Beyssac E, Coudore F, Paire M, Eschalier A, Aiache M, Lavarenne J. Comparison between percutaneous and subcutaneous routes of administration of apomorphine in rabbit. *Clin Neuropharmacol* 1994;17(5):445–453.

43. Ugwoke MI, Agu RU, Kinget R, Verbeke N. Intravenous versus subcutaneous injections of apomorphine in rabbits: a pharmacokinetic paradox. *Boll Chim Farm* 2003;142(8): 315–318.

44. Bergeron RJ, Wiegand J, Brittenham GM. HBED ligand: preclinical studies of a potential alternative to deferoxamine for treatment of chronic iron overload and acute iron poisoning. *Blood* 2002;99(8):3019–3026.

45. Doerge DR, Young JF, McDaniel LP, Twaddle NC, Churchwell MI. Toxicokinetics of acrylamide and glycidamide in Fischer 344 rats. *Toxicol Appl Pharmacol* 2005;208(3): 199–209.

46. Araya H, Nagao S, Tomita M, Hayashi M. The novel formulation design of self-emulsifying drug delivery systems (SEDDS) type O/W microemulsion I: enhancing effects on oral bioavailability of poorly water soluble compounds in rats and beagle dogs. *Drug Metab Pharmacokinet* 2005;20(4):244–256.

47. Swan GE, Botha CJ, Taylor JH, Mulders MS, Minnaar PP, Kloeck A. Differences in the oral bioavailability of three rafoxanide formulations in sheep. *J S Afr Vet Assoc* 1995;66(4): 197–201.

48. Short CR. Consideration of sheep as a minor species: comparison of drug metabolism and disposition with other domestic ruminants. *Vet Hum Toxicol* 1993;35(Suppl 2): 40–56.

49. Klausner EA, Lavy E, Stepensky D, Friedman M, Hoffman A. Novel gastroretentive dosage forms: evaluation of gastroretentivity and its effect on riboflavin absorption in dogs. *Pharm Res* 2002;19:1516–1523.

50. Klausner EA, Eyal S, Lavy E, Friedman M, Hoffman A. Novel levodopa gastroretentive dosage form: *in vivo* evaluation in dogs. *J Control Release* 2003;88:117–126.

51. Stepensky D, Friedman M, Srour W, Raz I, Hoffman A. Preclinical evaluation of pharmacokinetic–pharmacodynamic rationale for oral CR metformin formulation. *J Control Release* 2001;7:107–115.

52. Hoffman A, Stepensky D, Lavy E, Eyal S, Klausner E, Friedman M. Pharmacokinetic and pharmacodynamic aspects of gastroretentive dosage forms. *Int J Pharm* 2004;277(1–2): 141–153.

53. Eriksson UG, Bredberg U, Hoffmann KJ, Thuresson A, Gabrielsson M, Ericsson H, Ahnoff M, Gislen K, Fager G, Gustafsson D. Absorption, distribution, metabolism, and excretion of ximelagatran, an oral direct thrombin inhibitor, in rats, dogs, and humans. *Drug Metab Dispos* 2003;31(3):294–305.

54. Lin CC, Luu T, Lourenco D, Yeh LT, Lau JY. Absorption, pharmacokinetics and excretion of levovirin in rats, dogs and cynomolgus monkeys. *J Antimicrob Chemother* 2003;51(1): 93–99.

55. Holleran JL, Parise RA, Joseph E, Eiseman JL, Covey JM, Glaze ER, Lyubimov AV, Chen YF, D'Argenio DZ, Egorin MJ. Plasma pharmacokinetics, oral bioavailability, and interspecies scaling of the DNA methyltransferase inhibitor, zebularine. *Clin Cancer Res* 2005;11(10):3862–3868.

56. Szakacs T, Veres Z, Vereczkey L. *In vitro–in vivo* correlation of the pharmacokinetics of vinpocetine. *Pol J Pharmacol* 2001;53(6):623–628.

57. Jia L, Noker PE, Coward L, Gorman GS, Protopopova M, Tomaszewski JE. Interspecies pharmacokinetics and *in vitro* metabolism of SQ109. *Br J Pharmacol* 2006;147(5): 476–485.

58. Giuliano C, Fiore F, Di Marco A, Padron Velazquez J, Bishop A, Bonelli F, Gonzalez-Paz O, Marcucci I, Harper S, Narjes F, Pacini B, Monteagudo E, Migliaccio G, Rowley M, Laufer R. Preclinical pharmacokinetics and metabolism of a potent non-nucleoside inhibitor of the hepatitis C virus NS5B polymerase. *Xenobiotica* 2005;35(10–11):1035–1054.

59. Goodacre SC, Street LJ, Hallett DJ, Crawforth JM, Kelly S, Owens AP, Blackaby WP, Lewis RT, Stanley J, Smith AJ, Ferris P, Sohal B, Cook SM, Pike A, Brown N, Wafford KA, Marshall G, Castro JL, Atack JR. Imidazo[1,2-*a*]pyrimidines as functionally selective and orally bioavailable GABA(A)alpha2/alpha3 binding site agonists for the treatment of anxiety disorders. *J Med Chem* 2006;49(1):35–38.

60. Chung HJ, Choi YH, Choi HD, Jang JM, Shim HJ, Yoo M, Kwon JW, Lee MG. Pharmacokinetics of DA-6034, an agent for inflammatory bowel disease, in rats and dogs: contribution of intestinal first-pass effect to low bioavailability in rats. *Eur J Pharm Sci* 2006;27(4):363–374.

61. Li M, Wang H, Hill DL, Stinson S, Veley K, Grossi I, Peggins J, Covey JM, Zhang R. Preclinical pharmacology of the novel antitumor agent adaphostin, a tyrphostin analog that inhibits bcr/abl. *Cancer Chemother Pharmacol* 2006;57(5):607–614.

62. Borthwick AD, Davies DE, Exall AM, Livermore DG, Sollis SL, Nerozzi F, Allen MJ, Perren M, Shabbir SS, Woollard PM, Wyatt PG. 2,5-Diketopiperazines as potent, selective, and orally bioavailable oxytocin antagonists. 2. Synthesis, chirality, and pharmacokinetics. *J Med Chem* 2005;48(22):6956–6969.

63. Ahmed M, Briggs MA, Bromidge SM, Buck T, Campbell L, Deeks NJ, Garner A, Gordon L, Hamprecht DW, Holland V, Johnson CN, Medhurst AD, Mitchell DJ, Moss SF, Powles J, Seal JT, Stean TO, Stemp G, Thompson M, Trail B, Upton N, Winborn K, Witty DR. Bicyclic heteroarylpiperazines as selective brain penetrant 5-HT6 receptor antagonists. *Bioorg Med Chem Lett* 2005;15(21):4867–4871.

64. Yang Z, Zadjura L, D'Arienzo C, Marino A, Santone K, Klunk L, Greene D, Lin PF, Colonno R, Wang T, Meanwell N, Hansel S. Preclinical pharmacokinetics of a novel HIV-1 attachment inhibitor BMS-378806 and prediction of its human pharmacokinetics. *Biopharm Drug Dispos* 2005;26(9):387–402.

65. Tsuda-Tsukimoto M, Ogasawara Y, Kume T. Pharmacokinetics and metabolism of TR-14035, a novel antagonist of a4ss1/a4ss7 integrin mediated cell adhesion, in rat and dog. *Xenobiotica* 2005;35(4):373–389.

66. James JK, Nakamura M, Nakazato A, Zhang KE, Cramer M, Brunner J, Cook J, Chen WG. Metabolism and disposition of a potent group II metabotropic glutamate receptor agonist, in rats, dogs, and monkeys. *Drug Metab Dispos* 2005;33(9):1373–1381.

67. Lewi P, Arnold E, Andries K, Bohets H, Borghys H, Clark A, Daeyaert F, Das K, de Bethune MP, de Jonge M, Heeres J, Koymans L, Leempoels J, Peeters J, Timmerman P, Van den Broeck W, Vanhoutte F, Van't Klooster G, Vinkers M, Volovik Y, Janssen PA. Correlations between factors determining the pharmacokinetics and antiviral activity of HIV-1 non-nucleoside reverse transcriptase inhibitors of the diaryltriazine and diarylpyrimidine classes of compounds. *Drugs R D* 2004;5(5):245–257.
68. Washio T, Kohsaka K, Arisawa H, Masunaga H. Pharmacokinetics and metabolism of the novel muscarinic receptor agonist SNI-2011 in rats and dogs. *Arzneimittelforschung* 2003;53(1):26–33.
69. Monbaliu J, Verhaeghe T, Willems B, Bode W, Lavrijsen K, Meuldermans W. Pharmacokinetics of galantamine, a cholinesterase inhibitor, in several animal species. *Arzneimittelforschung* 2003;53(7):486–495.
70. Huang L, Edwards SR, Smith MT. Comparison of the pharmacokinetics of oxycodone and noroxycodone in male dark Agouti and Sprague–Dawley rats: influence of streptozotocin-induced diabetes. *Pharm Res* 2005;22(9):1489–1498.
71. Kaniwa N, Ogata H, Aoyagi N, Ejima A, Takahashi T, Uezono Y, Imasato Y. Bioavailability of cyclandelate from capsules in beagle dogs and dissolution rate: correlations with bioavailability in humans. *J Pharmacobiodyn* 1991;14(3):152–160.
72. Bramer SL, Forbes WP. Relative bioavailability and effects of a high fat meal on single dose cilostazol pharmacokinetics. *Clin Pharmacokinet* 1999;37(Suppl 2):13–23.
73. Schug BS, Brendel E, Chantraine E, Wolf D, Martin W, Schall R, Blume HH. The effect of food on the pharmacokinetics of nifedipine in two slow release formulations: pronounced lag-time after a high fat breakfast. *Br J Clin Pharmacol* 2002;53(6):582–588.
74. Johnson JT, Mattiuz EL, Chay SH, Herman JL, Wheeler WJ, Kassahun K, Swanson SP, Phillips DL. The disposition, metabolism, and pharmacokinetics of a selective metabotropic glutamate receptor agonist in rats and dogs. *Drug Metab Dispos* 2002;30(1): 27–33.
75. Ogo Y, Sunagane N, Ohta T, Uruno T. Banana juice reduces bioavailability of levodopa preparation. *Yakugaku Zasshi* 2005;125(12):1009–1011.
76. Homer LM, Clarke CR, Weingarten AJ. Effect of dietary fat on oral bioavailability of tepoxalin in dogs. *J Vet Pharmacol Ther* 2005;28(3):287–291.
77. Jann MW, ZumBrunnen TL, Tenjarla SN, Ward ES Jr, Weidler DJ. Relative bioavailability of ondansetron 8-mg oral tablets versus two extemporaneous 16-mg suppositories: formulation and gender differences. *Pharmacotherapy* 1998;18(2):288–294.
78. Yang SH, Bae SK, Kwon JW, Yoo M, Lee MG. Gender differences in the pharmacokinetics of DA-6034, a derivative of flavonoids, in rats. *Biopharm Drug Dispos* 2006;27(1): 47–51.
79. Crouthamel WG, Bekersky I. Preclinical evaluation of new drug candidates and drug delivery systems in the dog. In Crouthamel WG, Sarapu AC, Eds. *Animal Models for Oral Drug Delivery in Man*: *In Vitro and In Vivo Approaches*. Washington DC: American Pharmaceutical Association; 1983, pp 107–123.

33

MASS BALANCE STUDIES

JAN H. BEUMER, JULIE L. EISEMAN, AND MERRILL J. EGORIN
University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania

Contents

33.1 Introduction
33.2 Definitions
33.3 Study Design
  33.3.1 Timing of a Mass Balance Study
  33.3.2 Guidelines
  33.3.3 The Radioisotope and Its Position in the Molecule
  33.3.4 Animals
  33.3.5 Dosing
33.4 Methodology
  33.4.1 Dose Administration
  33.4.2 Sample Collection and Analysis
  33.4.3 Metabolic Profiling
33.5 Results
  33.5.1 Mass Balance Calculation
  33.5.2 Plasma Pharmacokinetics
  33.5.3 Assessment of Absorption
  33.5.4 Organ Distribution
  33.5.5 Metabolic Profiling
References

33.1 INTRODUCTION

A well-designed mass balance study significantly expands information about the pharmacological behavior of an active compound. Among many things, a preclinical

Preclinical Development Handbook: ADME and Biopharmaceutical Properties,
edited by Shayne Cox Gad
Copyright © 2008 John Wiley & Sons, Inc.

mass balance study can elucidate excretory pathways, organ distribution of parent drug and metabolites, and metabolic profiles. Moreover, a preclinical mass balance study can set the stage for its clinical counterpart, the human mass balance study.

Possible Objectives of a Preclinical Mass Balance Study

- Identify the excretory pathways of a drug (in general, urine and feces).
- Assess extent and kinetics of metabolism. Is the compound rapidly metabolized and how fast are the produced metabolites eliminated?
- Assess extent and kinetics of excretion. Is all radioactivity eventually excreted, and how long must excreta be collected?
- Elucidate the metabolic fate of a drug. How many metabolites can be observed and/or identified, and how much of the radioactivity do they account for?
- Assess organ distribution. Does (ir)reversible tissue binding occur, explaining incomplete recovery of radioactivity in excreta? Are there specific organs displaying accumulation?
- Provide information to optimally perform the human mass balance study. Is the choice of the isotope and its position in the molecule correct? Generate metabolite reference compounds and predict the radiation exposure of participants in the human mass balance study.

In general, a "mass balance study" is exactly what the term implies. This study involves the "balancing" of quantities of "mass." The "mass" that leaves the system (parent drug and metabolites excreted by various routes like urine and feces) is "balanced" against the known "mass" that was entered into the system (an amount of parent drug administered to the organism). Since most drugs undergo metabolic conversion before being excreted, a radioisotopically labeled variant of the compound is commonly administered to facilitate the quantitation of the sum of parent drug and metabolites in a variety of biological matrices. We propose the following definition for a mass balance study: "A pharmacokinetic study, aimed at characterizing the metabolic and excretory fate of a compound by collecting and analyzing excreta after administration of the compound, employing an analytical technique that allows tracing and quantitation of the compound and its products in spite of metabolic conversion."

Mass balance studies in their simplest form (as often performed in humans) are usually limited to just this particular balance, the amount that is recovered in excreta after an extended collection period, relative to the amount originally administered. However, contrary to a human mass balance study, a preclinical mass balance study allows the calculation of this mass balance at any given time by quantitating radioactivity present in various organs as well as in excreta. Thus, a preclinical mass balance study can provide information about the whereabouts of drug and metabolites inside and outside the organism at any time. This is the unique trait of the preclinical mass balance.

The relevance of the preclinical mass balance study is not limited to only preparing for the human mass balance study. It provides crucial information for the toxicokinetic evaluation of a compound. Toxicokinetic studies are aimed at integrating pharmacokinetics/metabolism and toxicity in nonclinical safety studies. Good agreement of the pharmacokinetics and metabolism of a drug in a specific animal species and humans increases the validity of the safety studies in this species for clinical safety issues [1–4]. Therefore, when there is close agreement between a specific animal species and humans with regard to excretory pathways and metabolic profile (characteristics that are best investigated by the mass balance study), this species is likely to be a better model for safety studies of the respective drug. The importance of metabolites and interspecies comparison is reflected by the recent distribution of a draft guidance for industry by the Center for Drug Evaluation and Research entitled *Safety Testing of Drug Metabolites* [5], which states the following. "Based on data obtained from *in vivo* and *in vitro* metabolism studies, when the metabolic profile of a parent drug is similar qualitatively and quantitatively across species, we can generally assume that potential clinical risks of the parent drug and its metabolites have been adequately characterized during standard non-clinical safety evaluations" [5]. Identification and pharmacologic characterization of individual metabolites is key in comparing results of preclinical studies with those of human studies [4].

The mass balance study is a unique and complicated study. It generally involves the use of radioisotopes and analysis of radioactivity, parent compound, and metabolites in a variety of matrices. As will be shown, a wealth of information can be gained from a mass balance. Fully exploiting a preclinical mass balance study requires careful design and the use of many techniques. In this chapter, the design of preclinical mass balance studies is addressed. The methodologies and expertise required for the conduct of these studies are provided, and we propose ways to express the results. Examples of the usefulness of mass balance studies in drug development are given and used to illustrate the importance of understanding the data obtained from mass balance studies.

33.2 DEFINITIONS

Mass Balance Study. A pharmacokinetic study, aimed at characterizing the metabolic and excretory fate of a compound by collecting and analyzing excreta after administration of the compound, employing an analytical technique that allows tracing and quantitation of the compound and its products in spite of metabolic conversion.

Total Radioactivity. The radioactivity content of a matrix, which consists of the sum of the parent drug and its metabolites that still contain the radioactive label.

Metabolite. Any compound being produced from the handling of a parent compound in the body.

Radioisotope. An isotope of an element capable of spontaneously emitting energetic particles by the disintegration of its atomic nuclei.

Tracer. The added (radioisotopical) portion of a compound used to follow the course of the metabolic process of that compound.

Specific Activity. A measure of the radioactivity per unit mass of a compound.

Autoradiolysis. Radiolysis of a radioactive material resulting directly or indirectly from its radioactive decay.

33.3 STUDY DESIGN

33.3.1 Timing of a Mass Balance Study

Performing a mass balance study with one or more animal species can be a costly and time-consuming investment. Hence, there needs to be evidence of activity at specified doses and dose schedules, initial toxicity studies must be satisfactory, and pharmacokinetics of unchanged drug should have been established. As will be argued, rational planning of a mass balance study requires knowledge of *in vitro* metabolic profiles of the compound in the species being used in preclinical safety studies and considered for the mass balance study. This will yield the most relevant information for clinical trials. Metabolic studies in animals may be performed in the late phase of the lead optimization [6]. However, even if the compound has already entered clinical trials, a preclinical mass balance can still be of use. If there are differences in (side) effects between humans and animals, these discrepancies may be explained by the *in vivo* metabolic profiles of the species.

33.3.2 Guidelines

Role of a Preclinical Mass Balance Study in Complying with Guidelines for Drug Development The U.S. FDA [7] and its European counterpart, EMEA [8], have a number of guidelines delineating information that is required to lead a compound through the drug development process. Although there is no guideline specifically for preclinical mass balance studies, a preclinical mass balance study can play a role in obtaining information crucial to drug development. The preclinical mass balance study provides a description of the metabolism of a drug and the contribution of metabolism to overall elimination. This information is crucial in the development of a drug [9]. In determining the maximum recommended starting dose (MRSD) for initial clinical trials from a human equivalent dose, as a default, the most sensitive animal species is used. However, based on differences in absorption, distribution, metabolism, and excretion between species, a different species may be selected, thus increasing the MRSD and reducing the time spent on initial clinical trials [10]. Furthermore, a mass balance study provides the information that is assessed by the (draft) guidance, *Safety Testing of Drug Metabolites* [5].

Performing a Mass Balance Study In performing a preclinical mass balance study, a number of guidelines and regulations must be followed. As regulations may vary by country, one should refer to the locally applicable institutional guidelines, but these guidelines will generally cover: IACUC, an institutional review board (IRB), working with radioactivity in general and in combination with animals specifically. As always in working with radioactivity, contamination must be minimized by working on absorbents, using disposable tools, and performing sweep tests after

every experiment. Surveys with detectors will not suffice because they are not sensitive enough to detect all radioisotopes (including those most commonly used—^{14}C and ^{3}H).

33.3.3 The Radioisotope and Its Position in the Molecule

The choice of radioisotope and its position in the molecule can have a major impact on chemical and metabolic stability and can thus determine the results of a mass balance study. Use of ^{14}C as the radioactive isotope is generally preferable to any other radioisotope. The labeled compound only serves its purpose if it behaves identically to the nonlabeled compound and if the radioisotope is not lost chemically by exchange or during metabolism. Prior knowledge of the metabolism of a compound (from microsomal incubation studies, known analogues, or *in silico* predictions) can assist in determining the optimal radioisotope and its position. Since ideally the same radiolabeled compound is used in both the preclinical and clinical mass balance studies, human metabolic data must be considered as well.

If the studied molecule is large and/or is expected to divide metabolically into two parts, dual labeling may be considered. By labeling one part of the molecule with ^{3}H and the other part with ^{14}C, both parts may be traced independently [11, 12]. Even triple labeling has been reported [13].

Isotopes Several different radioisotopes can be employed to trace the whereabouts of a drug and its metabolites. In choosing the isotope to be used, one should be aware of the type of decay, the decay half-life, and the energy of the emitted particle (see Table 33.1). The type of radiation that is easiest to handle is β^- radiation (electrons). Working with γ-emitters requires use of lead shielding, because the highly energetic γ-radiation easily penetrates solid matter and tissue. Contrary to γ-radiation, the surroundings of a β-source are easily shielded from radiation by a layer of plastic/Plexiglas, and the radiation of, for example, ^{14}C will only penetrate up to 50 cm of air [12]. In addition, detection of β-radiation is readily accomplished by liquid scintillation counting. If a radioisotope has a short half-life, care must be taken to minimize the time between drug preparation and usage in the study or the labeled drug will have degraded. The following formula can be used to calculate the percentage of radioisotope remaining after preparation of a radioisotopically labeled compound:

TABLE 33.1 Radioisotopes Employed in Preclinical Mass Balance Studies [12, 14]

Radioisotope	Type of Decay	Half-life	Energy (MeV)	Comments
^{3}H	β–	12.3 y	0.019	Easily lost, low energy
^{14}C	β–	5,730 y	0.156	Long half-life
^{32}P	β–	14.3 d	1.71	Correction for decay, complicated handling
^{35}S	β–	87.5 d	0.167	Correction for decay
^{36}Cl	β–	300,000 y	0.71	Long half-life
^{131}I	β–	8.0 d	0.80–2.16	Correction for decay
	γ		0.67, 0.77	

$$\%\ \text{Remaining} = 100 \cdot \frac{A_t}{A_0} = 100 \cdot (2)^{-(t/t_{1/2})}$$

where A_0 is radioactivity at time $t = 0$, A_t is radioactivity at time t, and $t_{1/2}$ is decay half-life. In addition, quantitated radioactivity levels must be corrected for the time expired between administration and quantitation. The following formula can be used to calculate the activity at $t = 0$ from the radioactivity quantitated at time t:

$$A_0 = A_t \cdot (2)^{(t/t_{1/2})}$$

Given the atomic composition of drug molecules, the most commonly used radioisotopes are ^{14}C (radiocarbon) and ^{3}H (tritium), although the use of other radioisotopes has been documented [12]. The radiation emitted by ^{14}C is about eightfold more energetic than that of ^{3}H (0.156 MeV versus 0.019 MeV). Thus, quantitation of ^{14}C is easier and suffers less from matrix effects [14]. Even so, the use of ^{3}H may be required when very small amounts of a compound need to be detected, since higher specific activities may be achieved with ^{3}H (29.1 Ci/milliatom) than with carbon (62.4 mCi/milliatom) [12].

Although positron emitters can have their use in investigating drug pharmacology, their generally very short half-life (<2 h) limits their use in mass balance studies [15].

On the rare occasion that a drug contains an element that occurs in the body only at trace levels, the use of a radioisotopically labeled compound may be avoided. For example, metal-containing compounds may be quantitated using element-specific techniques such as atomic absorption spectrometry (AAS), inductively coupled plasma atomic emission spectrometry (ICP-AES), or inductively coupled plasma mass spectrometry (ICP-MS) [16, 17], and fluoro compounds can be traced employing ^{19}F-NMR [18].

Although it would seem obvious that after labeling with the radioisotope the compound must have the same chemical formula, this is not always the case. Occasionally, the disposition of a compound is investigated by substituting with a foreign (radioactive) element [19]. This substitution results in a different compound, and consequently, the new compound likely displays different metabolic and dispositional characteristics.

Chemical Stability A major disadvantage of using ^{3}H, despite the ease and low costs, is its tendency to be exchanged with hydrogen in aqueous matrices, producing nonlabeled compound and tritiated water. Especially if ^{3}H bonded to heteroatoms or α-positioned relative to a double bonded heteroatom, it is likely to be exchanged [20, 21]. A ^{14}C atom is not likely to be chemically exchanged.

Metabolic Stability The use of a compound labeled at a chemically stable position does not guarantee that the detected radioactivity adequately represents its metabolism and disposition. The position needs to be metabolically stable as well. For example, positioning a radioisotope in an *N*-methyl moiety or the side chain part of an ester [22] (although easy to synthesize) will not result in a useful compound to trace metabolites. The radioisotope would quickly be lost by oxidative dealkylation and hydrolysis, respectively [23]. The isotope needs to be positioned in a relatively

central part of the compound to enable tracing of its primary metabolites. It is therefore not surprising that the radioisotope is preferentially positioned in an aromatic or alicyclic ring system. Computer-assisted metabolism prediction (CAMP) is available to predict the metabolic stability by means of databases of known metabolic transformations. The suitability of a label may be predicted by *in vitro* (liver microsomal incubation or hepatocytes) testing of the compound [24] or by reviewing the metabolism of analogues. For example, a review of the literature on the metabolism of pyrimidine antimetabolites shows the metabolic loss of the 2-carbon as carbon dioxide, while the 6-carbon is quite stable [25]. Thus, when deciding on the position of the ^{14}C in an investigational pyrimidine compound, the 6-position is preferred over the 2-position.

Kinetic Isotope Effect Although a radioisotope is in many regards equal to its stable-isotope counterpart, chemically it may still behave slightly differently. Heavier isotopes will form stronger chemical bonds, and thus reaction kinetics (like metabolic conversion) will be affected. This is called the *kinetic isotope effect* [21]. Thus, the radioisotopically labeled compound may well undergo a different metabolism and display a different disposition from the nonlabeled compound. The replacement of hydrogen (^{1}H) with tritium (^{3}H) is a tripling in mass, while the substitution of a carbon atom (^{12}C) with radiocarbon (^{14}C) is only a 17% increase. Therefore, the use of tritiated compounds is more likely to yield results biased by the kinetic isotope effect [21]. However, the kinetic isotope effect is relevant only if the rate-limiting step in the overall metabolic conversion is affected. Generally, cleavage of the carbon–hydrogen bond is the rate-limiting step in the oxidation of aliphatic hydrocarbons. Whether or not this is the case for an aromatic carbon–hydrogen bond depends on the substitution pattern [26]. An example illustrating the kinetic isotope effect is the reduced formation of the genotoxic α-hydroxy metabolite of tamoxifen in rats after exchange of hydrogen for deuterium [27].

33.3.4 Animals

Nonclinical safety testing is usually performed in rats and mice, and a nonrodent mammal [28, 29]. The default rule for starting a first-in-human-study is two mammals, one of which is nonrodent. When dealing with biologicals, species selection will be linked to the presumed mechanism of action and the presence of the target in the available species. In the literature, preclinical mass balance studies are often reported in a rodent species, in dog, and in a primate. However, the choice of species must be a conscious one. Every animal has its own characteristics that influence the metabolic fate of a drug, and the costs and ease of performing the study. Even within species, different strains may have different properties. Inbred strains are the result of several generations of brother × sister mating. Consequently, inbred animals are homozygous at almost all loci, except sex. The genetic homogeneity of present and future generations ensures homogeneity in phenotype and thus more consistent experimental results. Conversely, outbred animals display a more variable genotype and phenotypic behavior.

Metabolic Differences Ideally, the animal species to use for the preclinical mass balance study is the one showing the highest degree of *in vitro* metabolic

concordance with humans. Early identification of human metabolic routes of elimination and metabolites by *in vitro* studies can provide a clear direction for preclinical studies in animals [30, 31]. *In vitro* incubations of the drug with liver slices, hepatocytes, and microsomal and nonmicrosomal fractions can provide useful information to assess this metabolic concordance. It should be noted that these *in vitro* incubations are unable to capture all aspects of the *in vivo* metabolism. First, gastrointestinal, kidney, and lung metabolism and biliary excretion are not assessed in these experiments. Second, phase II metabolism does not necessarily occur in microsomes without addition of the various conjugating moieties, and liver slices have limited enzymatic stability [30].

Obviously, known interspecies differences in metabolism should be taken into account. If the drug has an aromatic amine moiety, it is likely a substrate for *N*-acetyltransferase. Since dogs lack this enzyme [31], to perform a mass balance study with this drug in dogs is not expected to yield information that is relevant to human metabolism, disposition, and safety of this drug. Table 33.2 lists some reported metabolic differences of animal species relative to humans. In addition to drug-metabolizing enzymes being either present or absent in different species, the organ distribution can also vary widely [32]. With the advent of genetically altered animal strains, some of these differences can be diminished, if not eliminated. For example, esterase activity in rodents greatly exceeds esterase activity in humans. Currently, plasma-esterase-deficient mice have become available, providing a better metabolic model for compounds that are a likely substrate for esterases [33, 34].

Animal Size Animal size has many implications for the mass balance study. A large animal allows repeated blood sampling. It also produces more, more frequent, but likely less concentrated urine that can enable metabolite profiling. On the other hand, large animals are more costly to buy and to keep, are less easily handled,

TABLE 33.2 Some Metabolic Differences of Animal Species Relative to Humans

Species	Enzyme	Effect	Substrates	References
Mouse	Carbonyl reductase	Low activity	Anthracyclines	31
	Carboxylesterase	High activity	Esters, lactones	34
	Deoxycytidine kinase	Low activity (10-fold)	Cytidine analogues	87
Rat	Amidase	High activity	Amides	88
	Carboxylesterase	High activity	Esters, lactones	22, 34
	CYP2C8	Substrate mismatch	Paclitaxel	31
	Cytidine deaminase	Absent	Cytidine analogues	32
	N-acetyltransferase	High activity	Aromatic amines	31
Dog	Cytidine deaminase	Low	Cytidine analogues	89
	N-acetyltransferase	Absent	Aromatic amines	31
All	Microsomal epoxide hydroxylase	Low	Epoxides	90
	UGTs	Negligible	Various, SN38, AZT	31

and require a larger amount of the (radioisotopically labeled) investigational compound.

Age In general, young but mature adult animals are used for pharmacokinetic studies. Many enzyme activity levels are age dependent. In immature animals, enzyme activity may not have fully developed yet. In older animals, activity of some enzymes may be declining while others may increase. In addition, body composition changes, with an increasing fat percentage over time [35–37]. Most importantly, the results must be comparable with preclinical safety studies, and the age of the animals used in those studies can be used as a guideline.

Sex Sex can be an important determinant of pharmacokinetic and metabolic parameters in animals [38–42]. Sex-dependent differences may be predicted by *in vitro* studies [43]. However, sex-dependent metabolic differences in humans are less dramatic, even though they may be present [44]. Therefore, the sex of the animals to be used may be determined by the methodology of the preceding safety studies.

Sample Size The sample size of the study is dependent on the information that needs to be obtained, and it obviously influences costs and logistics. If only an excretory mass balance study is needed, it will suffice to use few animals (three or four should be a minimum), and to collect excreta for a specified time (e.g., until an acceptable portion of the dose has been recovered). However, if the plasma metabolic profile of the compound is to be investigated, serial sampling is required, which is often destructive in smaller animals. A minimum of three or four animals should be used per time point to allow determination of a standard deviation. The number of time points is dependent on whether the investigation of the metabolic profile is more qualitative in nature, or the complete plasma profile of the drug, total radioactivity, and/or metabolites need to be determined. Usually, larger animals are studied in a smaller sample size for both cost and ethical reasons.

Genetically Modified Species As mentioned earlier with regard to esterase-deficient mice, it is possible to use genetically modified strains of a species in order to better mimic the human metabolic system. However, there is another application for genetically modified strains. Various gene-knockout (KO) and gene-knockin (KI) models have been developed for the mouse, to illustrate the *in vivo* function of a variety of enzymes/efflux pumps, for example, the CYP3A4-KI [45], *mdr1a*(–/–) [46], *mdr1a/b*(–/–) [6, 47], *ABCG2*(–/–) [48], and *SLC22A1*(–/–) [49]. When a mass balance of an IND is performed in these genetically altered strains and their wild-type counterparts, basic information can be obtained pertaining to the excretory function of the efflux pumps. In addition, quantitation of unchanged drug or radioactivity in the organs (especially those known to express efflux pumps like brain, testis, and heart) [47, 50–53] of these animals shows the relevance of efflux pumps to organ disposition. These data can predict possible interactions with other drugs competing for or blocking the efflux pumps. To diminish interspecies differences in substrate specificity of CYP450 enzymes, mice with humanized enzymes are under development [54, 55].

33.3.5 Dosing

Pharmacological Dose Assuming that the compound and its radioisotopically labeled counterpart behave identically, the pharmacological dose consists of the sum of unlabeled and labeled compound. Usually, the contribution of the radioactive part is negligible relative to the unlabeled amount of drug, hence the use of the term "tracer." However, to obtain a sufficient specific activity (Bq/mg) of a potent (dosed in low amounts), high molecular weight drug, a large fraction of the molecules (e.g., 80% [56]) will actually have to be labeled. To be relevant, the pharmacological dose should be comparable to pharmacological or toxicological relevant doses as established in safety studies. Even if linear pharmacokinetics of the parent compound has been shown, the metabolic disposition may be nonlinear. Therefore, the pharmacological dose should not deviate too much from previously established doses.

Radioactive Dose The radioactive dose to be used depends on the specific objectives of the study and the sensitivity of the analytical methods. If the objective is to describe plasma and tissue profiles of the parent drug and metabolites (e.g., by HPLC with radioactivity detection), a higher radioactive dose may be required than for merely describing the excretory fate of a drug. The amount of radioactivity required is also dependent on the pharmacokinetic characteristics of the compound.

A literature search for mass balance studies with chemically related compounds and previous pharmacokinetic studies with the compound under investigation can give an impression of what results may be obtained with a certain dose. Next, a pilot study will provide valuable samples to test an analytical method and to assess the adequacy of the radioactive dose.

The radioactive dose is usually expressed in curies (Ci), as opposed to the SI unit of becquerel (Bq). The conversion of these units is 1 mCi = 37 MBq and 1 Bq = 60 DPM, where DPM means disintegrations per minute. Typical radioactive doses (^{14}C) are approximately 1–2 μCi/mouse, 5–20 μCi/rat, 5–100 μCi/dog, and 40–120 μCi/monkey, compared to 100 μCi/human. Recently, the use of accelerator mass spectrometry (AMS) was described in a mass balance study in dogs, requiring only 45 nCi of radioactivity [57]. AMS has received attention for its potential use in microdosing humans [58]. Given the complexity of the technique, its use in preclinical mass balance studies will likely be limited.

Dosing Schedule Most mass balance studies are performed as a single-dose study. However, when single-dose tissue distribution studies suggest that the apparent half-life of the test compound (and/or metabolites) in organs or tissues significantly exceeds the apparent half-life of the elimination phase in plasma and is also more than twice the dosing interval in the toxicity studies, repeated-dose tissue distribution studies may be appropriate [4, 59].

Route of Administration For the mass balance study to be relevant to clinical practice, the route of administration should mimic the anticipated clinical route of administration as much as possible. For comparative and/or scientific purposes, different routes of administration can be studied as well.

33.4 METHODOLOGY

33.4.1 Dose Administration

Preparation Parenteral dosing solutions should be prepared under aseptic conditions. Because radioisotopically labeled compounds may not be available as a sterile product, one should be aware of possible bacterial contamination of the dosing solution. Aseptic filtration may be used, but loss of compound by adsorption to the filter unit must be assessed. The dosing solution should be prepared in excess to enable qualitative and quantitative analysis after completion of the study.

Administration The dose should be administered according to established practice. There are guidelines for maximum volumes that may be administered to animals, which should be followed. Oral administration is usually performed after an overnight fast to assure rapid and reproducible gastrointestinal transit. When dosing multiple groups of animals, stratifying all the animals according to body weight and using one mouse of every stratum per group may achieve a homogeneous weight distribution.

When an infusion device is used, this must be flushed after administration to account for any radioactivity that may have remained in the lines. An organic solvent like methanol may be best suited for this. The amount of radioactivity recovered from the flushing solvent must be subtracted from the calculated dose administered to obtain the actual administered dose [25].

Quality Control Preclinical studies are usually conducted with a nonstandardized formulation of the compound, if it is formulated at all. This is especially true for a mass balance study, because the isotopically labeled compound is often custom synthesized in one batch [60]. In addition, the dose consists of both the isotopically labeled part and the nonlabeled part.

First, for both nonlabeled drug and tracer, the content needs to be determined. This is essential to assure that a relevant pharmacological dose was administered, and to calculate eventually the recovery of the dose in the excreta. In case of a known, established specific activity, the dose of drug can be determined by taking an aliquot for scintillation counting. Using the specific activity of the compound (Bq/g), one can convert the radioactive content to total drug content.

Second, the purity of the dose needs to be established. Given the incidental production of most radioisotopically labeled compounds, it is likely that the purity is not as high as with standard reference compounds and may even be lower than the declaration of the manufacturer [21]. In addition, the radioisotopically labeled compound is likely to be less stable than the nonlabeled compound. Due to the inherent radioactive nature of the compound, energy is released, producing free radicals, which may increase degradation of the compound, a process called *autoradiolysis*. In solution, ethanol, serum albumin, and ascorbic acid have been reported to slow down autoradiolysis [61–63]. In the literature, the purity of the compound used is often not reported. Yet a purity of at least 95% is essential to perform a mass balance study. Impurities can have different pharmacokinetic characteristics and may be toxic. The radiochromatographic system used for metabolic profiling may be employed as a stability-indicating assay for the compound and, afterwards,

the dosing solution. In general (with compounds containing a chromophore), HPLC with UV absorbance or photodiode array UV detection will be able to assess the overall purity of both labeled and nonlabeled compound together.

33.4.2 Sample Collection and Analysis

A mass balance study can consist of merely collecting the excreta after administration of a radiolabeled drug. These studies do not characterize the metabolism, distribution, or disposition of the compound within the body. However, much more information can be obtained from a mass balance study by sampling various matrices in time, as is discussed later.

In general, no stability data are available for the compound and its metabolites in the different matrices (and organs may contain drug-metabolizing enzymes). Consequently, immediate storage at –20 °C or less is advisable, in order to prevent degradation products to be mistakenly identified as metabolites.

Schedule In order to reliably estimate pharmacokinetic parameters, sampling should be adequately planned. In the case of intravenous administration, an early plasma sample is imperative to determine the area under the plasma concentration versus time curve. Thereafter, sampling must be frequent enough to separately characterize the distribution and the elimination phases. The elimination phase is ideally sampled for at least 4 half-lives. In the case of orally administered drugs, frequent plasma sampling should be performed around the expected time of maximum plasma concentration. Sometimes there is no data available to help schedule the sampling. A pilot study in a couple of animals may then be performed to get a rough estimate of the plasma concentration versus time profile, which can also serve to set the required concentration range of the analytical method.

Ideally, excreta are collected until all radioactivity administered has been recovered. However, 100% recovery will usually not be achieved [25]. Under the assumption of instantaneous excretion, 93% of radioactivity will have been excreted after four terminal radioactivity plasma half-lives. Unfortunately, the elimination half-life of radioactivity from plasma is usually not known. Another reported approach uses radioactivity levels in urine and feces for drug and radioactivity [25]. When less than 1% of administered radioactivity is excreted for 2 consecutive days, collection can be stopped. Prolonged collection will not substantially increase recovery. Small animals may be housed together to collect pooled excreta.

Urine Urine is a liquid matrix; it is easily mixed, aliquoted, and quantitatively analyzed. Urine can be collected separately from feces with a metabolic cage (Fig. 33.1). Because urine is often used for qualitative analysis, it needs to be collected on ice in order to halt any degradation processes. To calculate the total excretion of a compound and its metabolites, the volume of urine excreted in the collection period must be accurately established. This can be accomplished by determining the volume with a graduated cylinder or by weighing, but the latter only if the relative density of the urine is near 1. At the end of the collection period, the collection part of the metabolic cage should be washed with a solvent to remove any adsorbed radioactivity from the device. The solvent used for this wash step must be chosen

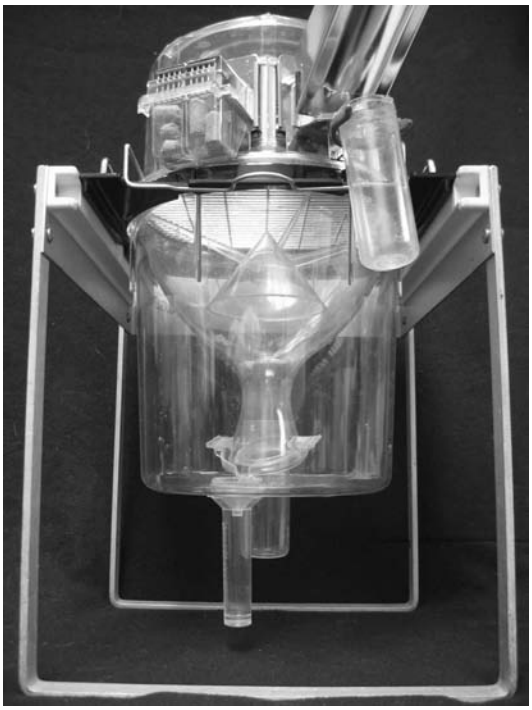


FIGURE 33.1 Metabolic cage for the separate collection of urine (lower left collection cone) and feces (middle collection cone) of rodents.

such that the compound is expected to dissolve easily. The radioactivity found in this wash fluid should be calculated by multiplying the volume of wash solvent that was used (not the volume that was collected) with the concentration of radioactivity. If the investigated compound is known, or suspected, to adsorb to container surfaces, addition of an organic solvent or bovine serum albumin may be considered before subsequent aliquoting and processing.

Feces Feces are collected separately from urine with metabolic cages. They are weighed and then homogenized with a homogenization device to obtain a pipettable, homogeneous matrix. Disposable homogenizer probes are available and may be preferable when processing radioactive samples. Depending on the eventual use of the homogenate (extraction of individual metabolites) and the properties of the compound and its metabolites (solubility, adsorption to container surfaces), homogenization may be done with water, buffer, or bovine serum albumin solution. In general, adsorption to container surfaces will not be an issue with fecal homogenate because the fecal particles will have a high surface area relative to the container surface area. The ratio with which feces are homogenized (varying from 2:1 to 10:1, v/w) will affect pipettability and sensitivity of subsequent analyses. A more dilute homogenate will result in a truly pipettable matrix, with proportionally reduced concentrations of the analytes. More concentrated homogenates may be pipettable, but not accurately so. When determining the radioactivity content, aliquots of

homogenate should be weighed. Because feces is a dark-colored matrix, decoloration prior to liquid scintillation counting may be warranted.

Bile Occasionally, bile is collected to assess the biliary excretion of radioactivity and analytes. Because reabsorption of bile salts is essential for normal bile production (both quantitatively and qualitatively), bile may not be sampled over extended periods of time, that is, longer than 1–2 hours [64, 65]. An elegant but intricate way to deal with this issue is to replenish with blank bile from undosed animals while sampling the bile from the treated animal. Similarly, the contribution of enterohepatic recirculation may be assessed [66].

Blood and Red Blood Cells Blood must be collected from a site distant from that of administration. This is done to prevent residual dosing solution at the administration site from biasing the concentration of the drawn sample. Next, one can determine total radioactivity in either whole blood or in red blood cells (RBCs) after centrifugation and aspiration of plasma. In combination with plasma radioactivity levels, this allows calculation of the blood/plasma ratio or the RBCs/plasma ratio, respectively. These parameters can be interconverted, but such a conversion requires information about the hematocrit [67].

Because red blood cells and blood can be somewhat viscous, an aliquot should be accurately weighted in a sample vial. Liquid scintillation counting of blood or red blood cells is often difficult due to the intense red color, which necessitates decoloration before liquid scintillation counting. A suitable protocol may be: weighting approximately 150 mg of the red blood cell pellet into a 20 mL scintillation vial; adding 500 µL of tissue solubilizer and 500 µL of water, followed by vortexing and incubating for 10 min at 50 °C; adding 100 µL of 0.1 M EDTA and 200 µL of 30% hydrogen peroxide, followed by vortexing of the sample and incubation at 50 °C for 10 min; finally, 20 mL of scintillation fluid may be added prior to scintillation counting.

Plasma Plasma can be aspirated after centrifugation of blood. Because it is a relatively colorless matrix, liquid scintillation counting is straightforward. It consists of mixing an aliquot with liquid scintillation fluid followed by counting.

Expired Air For a number of positions of the radioactive ^{14}C label (e.g., 2-position of pyrimidines, *N*-methyl), the expiration of radioactivity as $^{14}CO_2$ is predictable [68]. However, it may not be possible to prevent expiration of radioactivity, especially when there is no information about the metabolic fate of a compound or any analogues. When mass balance is not achieved (i.e., a large part of the radioactive dose cannot be accounted for in the total of excreta, organs, and carcass), one must consider the possibility of loss through expiration as $^{14}CO_2$.

Expired air may be sampled by keeping the animals in an airtight chamber with continuous air replacement and leading the expired air through a basic solution, thus precipitating the $^{14}CO_2$ as the nonvolatile carbonate. A sodium hydroxide [12] or 2-methoxyethanol-ethanolamine (2:1) solution [69] can be used to capture the carbon dioxide. Before air is pumped into the chamber it may need to be led through a similar basic solution to eliminate any natural $^{14}CO_2$, thereby reducing the back-

ground radioactivity counts. Eventually, the precipitated carbonate is counted for its radioactive content.

Organs A preclinical mass balance study employing a radioisotopically labeled drug is usually conducted when the time course, persistence, and potential accumulation of the drug and/or its metabolites in various parts of the animal body needs to be assessed [4].

After dissection, organs should be weighed and frozen as soon as possible. To obtain a pipettable homogeneous matrix for further analysis, homogenization may be required. If no other measurements than total radioactivity determination are intended, direct tissue solubilization may be considered as a faster and easier sample processing method (see below).

A pipettable organ homogenate may be obtained by homogenizing the organ with 3 parts (v/w) of phosphate-buffered saline pH 7.4, water, or 4% bovine serum albumin. The choice of solvent may be dependent on solubility, stability, and adsorption characteristics of the compound under investigation. In addition, the solvent used may interfere with subsequent analytical or pharmacodynamic assays. A fixed ratio of liquid to organ weight is preferable to a fixed volume per organ because the former will assure identical concentrations of organ constituents like proteins, which may be relevant to solubility, stability, and adsorption.

If organ radioactivity concentrations are to be assessed, it is imperative to sample and homogenize the whole organ, and not to sample parts (of different lobes) of the organ. For example, analyte concentrations are reported to vary significantly within a lobe (52-fold) or between lobes of a single liver (25-fold), likely due to differences in regional structure and blood and lymph flows [70]. On the other hand, it may be necessary to determine intratissue distribution of radioactivity. For example, distribution of radioactivity of an anticancer drug to the viable part of a tumor is relevant, but this information will be lost during homogenization [71]. Tissue autoradiography may be employed to identify such a phenomenon.

The organs of the GI tract can be homogenized after removal of the content or as a whole. By removing the content followed by separate analysis, any radioactivity can be more exactly ascribed to GI content (unabsorbed or excreted radioactivity) and organ radioactivity, respectively. This may be especially relevant with orally administered compounds.

Carcass Oftentimes, the radioactivity in excreta does not account for the complete radioactive dose. A compound or its metabolites may exhibit a long half-life or may irreversibly bind to tissue components, or the radioisotope may get incorporated into endogenous compounds. Therefore, it may be required to assess the amount of residual radioactivity in the carcass. Individual organs are easily homogenized or digested and consequently analyzed for radioactivity. The carcass, however, is less easily processed. It contains remaining organs, muscle, connective tissue, and, most importantly, bone, skin, and fur. In order to achieve a homogenate with a high degree of homogeneity, the carcass (with an appropriate liquid phase) is first processed using a blender. Due to the high collagen content, a higher solvent volume to carcass weight ratio (from 4:1) may have to be used for homogenization to prevent coalescing of the homogenate. Next, a tissue solubilizer or a concentrated sodium hydroxide solution may be used to dissolve the tissues as much as possible (allowing it to stand

for several days or applying heating). The latter should be done in closed containers to avoid evaporation of solvents prior to taking an aliquot for scintillation counting.

Digestion, Decoloration, and Oxidation of Samples A number of problems may be encountered in the processing of samples for radioactivity counting. Homogenization is not suitable for all organs (e.g., bone, skin) and is labor intensive, yet it is less destructive (e.g., determination of proteins, DNA, or individual compounds remains a possibility). Digestion of organs (or homogenate aliquots) with a tissue solubilizer is easy and fast, reduces the color of the sample, but is totally destructive. Lastly, oxidation of samples is destructive and labor intensive, but may be essential for processing nonhomogenizable matrices or determination of ^{3}H in the presence of ^{14}C.

Digestion of organs or homogenate aliquots can be performed with so-called tissue solubilizers. Although destructive, this methodology can save a lot of time and effort because it may not be necessary to homogenize the sample beforehand. However, too much of these reagents may adversely affect the scintillation counting process, so that care must be taken to minimize the amount required. Manufacturers of these reagents can provide data on amounts required and the effects on the scintillation counting process.

Usually, the color interfering with scintillation counting is caused by iron-containing components of the sample like hemes. Treatment of the sample (homogenate or digested organ) with peroxide is the common method to reduce the color intensity. A suggested method would be taking a 100 µL aliquot of homogenate (3:1, v/w in water), adding 100 µL of 0.1 M EDTA (chelating metal ions, reducing excessive foaming of the hydrogen peroxide), followed by 200 µL of ~30% hydrogen peroxide. Incubation for 10 min at 50 °C will speed up the decoloration reaction. Next, 5–20 mL of liquid scintillation fluid may be added before counting. Variations of peroxides have been described, such as treatment with solubilizer followed by 0.4 mL of benzene saturated with benzoyl peroxide, or bleaching with glacial acetic acid and hydrogen peroxide [72, 73].

A sample oxidizer consists of a coiled platinum wire that can hold the sample. Additives may be applied to optimize the oxidation process. Subsequently, under a continuous oxygen flow, the sample is oxidized by heating the platinum wire using an electric current, and converting the carbon content to carbon dioxide and the hydrogen content to water, leaving nonvolatile components as ashes. The carbon dioxide containing the $^{14}CO_2$ and the water containing the $^{3}H_2O$ are trapped separately on adsorption columns and eluted into separate counting vials. Transfer efficiencies of over 95% are usually achieved with this technique. Especially in the case of double labeling of a compound with possible false-positive counts of ^{14}C in the ^{3}H counts, this technique is very suitable. Before bone is oxidized, it may be pulverized first to increase the surface area available for the oxidation process.

Irrespective of the eventual method used for sample processing, it is essential to determine the end efficiency of the procedure. Adding a known amount of radioactivity to a sample, processing it, and determining the liquid scintillation count easily achieves this. One should also prepare an identical sample without the organ component to establish the influence of the reagents used on the counting process. This may identify the need to use less of the reagents and instead increase the

duration of heating the sample. An overall efficiency/accuracy of at least 90% should be aimed for.

Liquid Scintillation Counting For the determination of total radioactivity in samples (the composite of parent drug and metabolites still containing the radioactive label), liquid scintillation counting is often employed [12]. The sample is processed in such a way that it is in a liquid form. An aliquot is mixed with liquid scintillation fluid, a mixture of compounds that are excited by β-radiation and convert the absorbed energy to multiple light pulses. The sample is analyzed by a liquid scintillation counter that converts the light pulses into a number of observed counts per minute (CPM). Because not all the disintegrating nuclei result in a count, the CPM need to be converted to the real number of disintegrations per minute (DPM). The ratio of CPM to DPM is the counting efficiency, which is higher for isotopes with more energetic radiation ($^{32}P > {}^{14}C > {}^{3}H$). Most liquid scintillation counters will be able to determine the counting efficiency of a sample and thus correct the measured count to the actual count. This correction is usually performed by either of two ways. The first is the external standard calibration. A radioactive (γ-) source is placed next to the sample, causing a known amount of additional excitation in the sample. The ratio of the actual counts to the expected counts is a measure of the counting efficiency. The second method is the internal standard calibration. With decreasing efficiency, the signal intensity versus energy distribution shifts to lower energies. Using a prerecorded quench curve, the counting efficiency can be determined from this shift. To maximize the counting efficiency, a sufficiently high scintillation fluid-to-sample ratio must be used (ideally more than 5:1). In addition, the mixture of sample and scintillation fluid must be one homogeneous phase. There are a variety of liquid scintillation fluids available that are suitable for organic, aqueous, and high salt content samples, respectively. Sometimes, especially when the sample is colored, counting efficiency drops, and the correction becomes flawed. This color quenching occurs, in particular, with red samples (blood, liver, kidney, etc.). A suitable sample preparation step involving digestion and decoloration may resolve this problem. Heating of the sample or exposure to sunlight may excite the scintillants (luminescence) and result in an erroneously high count. This can be avoided by letting the samples stand overnight in the dark before counting. The error in the radioactive count is inversely related to the root of the count and the counting time. Increasing the counting time will therefore result in more precise measurements, especially with lower concentrations of radioactivity. In general, a counting time of 5–10 min will suffice. When a double label is used (e.g., labeling a compound simultaneously with both ^{14}C and ^{3}H), the signal of one isotope can cross talk into the signal of the other isotope. The ^{3}H signal will also pick up part of the ^{14}C signal. Adjusting the settings of the liquid scintillation counter and using corrections may yield the separate counts for both isotopes.

33.4.3 Metabolic Profiling

The overview of the metabolism of a drug is often called the *metabolic profile* [25]. Recently, the FDA posted a draft guidance describing the need for safety testing of metabolites representing more than 10% of systemic exposure human [5]. Thus, early preclinical identification of metabolites of potential relevance to humans is

key. A mass balance study is well suited to elucidate the metabolic profile of a compound because it employs a radioisotopically labeled compound, enabling detection of the parent drug and its metabolites even if the latter are structurally still unknown. After structural elucidation, metabolites may be tested for their pharmacological and toxicological properties. This is important in drug development because the concentrations of the parent drug and/or its active metabolites may provide an important link between drug exposure and desirable and/or undesirable drug effects. For this reason, the development of sensitive and specific assays for a drug and its key metabolites is critical to the study of metabolism and drug–drug interactions [9]. Next, a discussion is provided of the various matrices, sample pretreatment, chromatographic separation, and detection.

Matrix Selection Ideally, metabolites are identified and quantified in plasma, urine, and feces. A matrix is especially interesting when a large proportion of the radioactivity is not explained by unchanged drug (this proportion consists of metabolites). This requires knowledge of the level of the total radioactivity and the unchanged drug in the matrix. Because the metabolites excreted in one matrix may be qualitatively different from those in another matrix, preferably, metabolic profiling is executed in all of these matrices. In addition, metabolites in a target organ (for activity or toxicity) may be relevant. However, quantification of metabolites can be complicated. Plasma may not be available in sufficient amounts to allow analysis of metabolites after analysis of total radioactivity and unchanged drug, especially when it concerns a small animal. Pooling of samples from individual animals may then be considered. It can be difficult to isolate compounds from feces, impeding metabolic profiling. Urine is a concentrated matrix that is suitable and most commonly used for metabolic profiling.

Sample Pretreatment Conventionally, sample pretreatment is aimed at selective extraction and concentration of one or more specific analytes. However, for metabolic profiling one needs to concentrate all metabolites with their heterogeneous physicochemical characteristics, while reducing matrix-derived interference. Consequently, sample pretreatment for metabolic profiling is usually nonspecific and consists of (1) addition of a water-miscible organic solvent, (2) centrifugation to precipitate salts, proteins, and insoluble matter, (3) concentration or evaporation of the supernatant, and (4) reconstitution in a suitable solvent. The efficiency of each of these steps can be monitored and optimized using radioactivity determinations [74]. Radioactivity lost during evaporation may be explained by volatile metabolites or tritiated water.

Chromatographic Separation The high resolution of high performance liquid chromatography (HPLC) makes it the method of choice over other chromatographic methods such as thin layer chromatography. By applying a shallow gradient elution (e.g., C18 column, 5–90% ACN linear, in 60 min), metabolites of a large polarity range can be separated. The use of a guard column can protect the column from high concentrations of matrix-derived constituents [12]. When subsequent mass spectrometric analysis is considered, mobile phase components should be chosen that are compatible with mass spectrometry (i.e., they should allow adequate

ionization of the compounds and must be volatile). In reverse phase chromatography, the parent compound (often less polar than its metabolites) elutes last. The gradient can be designed so that the parent compound elutes at the end of the chromatographic run. An initial run time of 60 min may be necessary to separate all the metabolites, but this may be shortened when the elution times of the metabolites are known and the assay is used more routinely [25].

Detection Techniques The compounds eluting from the HPLC system may be detected with various techniques, used in parallel or in series. Parallel detection techniques involve splitting the column effluent, thereby reducing sensitivity. However, some techniques are destructive in nature and thus cannot be employed in front of another technique. Because mass balance studies employ radioisotopically labeled compounds, radioactivity detection is the primary technique, unambiguously indicating the presence of metabolites at specific retention times. Ultraviolet absorption, preferably using a photodiode array, can be used to indicate changes in the chromophore. Mass spectrometry can be used to acquire additional structural information or to confirm the presence of a suspected metabolite. Finally, more complicated techniques like nuclear magnetic resonance (NMR) may be utilized, especially when investigating fluorinated compounds. Sometimes the radioactive samples may need to be diluted down to an acceptable level of radioactivity to permit their handling in different laboratory areas.

Radioactivity Detection Radioactivity detection may be performed either online or offline. Online radioactivity detection is rapid but destructive. It consists of admixing a scintillation fluid with the column effluent (usually in a 3:1, v/v ratio), passing it through a detection cell (a coiled, transparent tube), and recording the counts in time. The mixing of the column effluent with the scintillation fluid excludes any further analytical characterization of the effluent. Furthermore, the online radioactivity counting decreases sensitivity. When employing a flow rate of 1 mL/min, a 3:1 (v/v) ratio of scintillation fluid and a 500 μL detection cell, the residence time of any component in the detection cell is only 7.5 seconds. The offline detection method is more laborious, consisting of fractionation of the column effluent (every 15–60 s). Fractions can be counted for radioactivity, for example, for 5 min each (increasing sensitivity 40-fold relative to offline detection). Offline detection can be performed nondestructively by taking only an aliquot of each fraction for scintillation counting, reserving the residual sample of fractions with high levels of radioactivity for subsequent structural identification. A drawback of the offline radioactivity detection is its labor-intensive nature, precluding rapid sequential sample analysis.

When a more energetic radioisotope is being used (e.g., ^{32}P), it may be possible to detect the radioactivity without using scintillation fluid, by using Çerenkov detection. This allows collection of unadulterated effluent after radioactivity detection [12].

Photodiode Array Detection Absorption measurement using a photodiode array is a nondestructive detection technique. However, it is nonselective, necessitating comparison of the sample chromatogram with a blank. A shift in the spectrum

relative to that of the parent compound indicates a metabolic modification of the chromophore. When applying a gradient elution, the change in mobile phase composition may also cause a shift of the spectrum.

Mass Spectrometry More qualitative information may be derived from mass spectrometric analysis of selected fractions after offline profiling or after splitting the column effluent. Changes in the mass spectrum relative to the spectrum of the unchanged compound provide structural information. For example, a mass increase of +16 atomic mass units indicates hydroxylation, +42 atomic mass units indicates acetylation, –14 atomic mass units indicates demethylation, and so on. After tentative structural elucidation of a metabolite, the retention time and mass spectrum may be compared to that of a synthetic standard.

Nuclear Magnetic Resonance If there is a fluorine atom present in the compound, ^{19}F-NMR may be applied to detect, quantitate, and characterize parent compound and metabolites. A large signal for the fluorine ion indicates that the compound is extensively dehalogenated, and those metabolites are thereafter undetectable by ^{19}F-NMR [18]. Although at present quantitation with ^{19}F-NMR is hampered by matrix-related effects [75], in the future, more powerful NMR equipment is expected to improve quantitative application, even as to enable *in vivo* monitoring of drug and metabolite distribution [76].

Additional Characterization Besides chromatographic separation of a sample and detection of its metabolic constituents, there is more one can do with the samples. Collection of a particular fraction and repeated chromatographic separation may lead to isolation of a metabolite with sufficient purity to allow determination of the full mass spectrum, absorbance spectrum [77], NMR spectrum, and so on.

In addition, incubation with β-glucuronidase and sulfatase can indicate the presence of glucuronide and sulfate conjugates, respectively, of the parent compound or its phase I metabolites. A decrease in peak area indicates the presence of the conjugate, while an increase indicates the corresponding aglycone counterpart [78].

Finally, metabolites obtained from *in vitro* incubations of the compound of interest, that subsequently have been elucidated structurally, should be used as reference compounds.

33.5 RESULTS

There are a number of useful parameters that may be determined from a mass balance:

- Mass balance calculation
  - Recovery in urine, feces, air, carcass, and total recovery
  - Major excretory pathway
  - Ratio of unchanged drug/radioactivity

- Plasma pharmacokinetics (unchanged drug and radioactivity)
  - Half-life
  - AUC and the unchanged/radioactivity ratio
  - C_{max}, T_{max}
- Assessment of absorption
  - Unchanged drug AUC_{PO}/AUC_{IV}, bioavailability
  - Total radioactivity AUC_{PO}/AUC_{IV}, indication of fraction absorbed
- Organ distribution
  - Percentage of radioactivity in organs in time/at end of study
  - Drug and metabolite levels in target organs
  - Organ to plasma radioactivity levels in time
  - Organ AUC to plasma AUC ratios of radioactivity
  - Organ radioactivity ratios of genetically modified to wild-type strain
- Metabolic profiling
  - Number of metabolites (especially those accounting for >10% of radioactivity)
  - Structural information of previously unknown metabolites
  - Pharmacokinetic parameters of metabolites
  - Obtaining reference compounds
  - Concordance in metabolic profile between species

33.5.1 Mass Balance Calculation

The mass balance calculation consists of calculating the amount of radioactivity administered and calculating the amount of radioactivity recovered in urine, feces, and, if applicable, expired air:

$$\text{Recovery} = \frac{\begin{array}{ll}\text{Urine} & \text{volume (mL)}\cdot\text{concentration (DPM/mL)}\\ \text{Feces} & \text{weight (g)}\cdot\text{homogenization factor}\cdot\text{concentration (DPM/mL)}\\ \text{Organs} & \text{weight (g)}\cdot\text{homogenization factor}\cdot\text{concentration (DPM/mL)}\\ \text{Expired} & \text{DPM collected as } {}^{14}\text{C-carbonate}\end{array} +}{\text{Total}} \text{ divided by } \begin{array}{l}\text{Dosed}\end{array} \quad \begin{array}{l}\text{Volume dosed (mL)}\cdot\text{concentration (DPM/mL)}\end{array} \cdot 100\%$$

The total expired radioactivity can be calculated by integrating the ^{14}C-expiration rate versus time curve or collecting all the expired air. The relative contributions of feces and urine to the recovery of the dose determine the major excretory pathway.

After stopping collection of the excreta, the animals may be sacrificed, and the radioactivity still deposited in the carcass may (after suitable processing) be determined to increase the percentage of radioactive dose accounted for.

The fraction of radioactivity in urine and/or feces that is represented by unchanged drug is an indication of the importance of metabolism relative to unchanged excretion. However, this requires the availability of an analytical method suited to quantitate unchanged drug in excreta.

33.5.2 Plasma Pharmacokinetics

Although the validity of calculating (pseudo) pharmacokinetic parameters based on total or undiscriminated radioactivity levels has been debated, they may have some, if limited, use [79, 80]. Capturing as much of the area under the plasma radioactivity versus time curve increases the usefulness of pseudopharmacokinetic parameters. If a good fit of the terminal elimination phase is achieved and more than 90% of the $AUC_{0\text{-inf}}$ is captured in the $AUC_{0\text{-last}}$, the exposure to the total of the drug and its products is adequately described, even if theoretically the curve consists of multiple monoexponential functions.

Calculation of a half-life for total radioactivity may be useful when comparing such a value with the half-life of the unchanged drug. A much longer half-life for total radioactivity indicates a potential for underestimating the risk of accumulation of metabolites when solely focusing on parent drug. In addition, a pseudo-half-life for radioactivity may aid in determining the usefulness of continued collection of excreta to maximize recovery in the mass balance study.

The ratio of the AUC of unchanged drug over the AUC of total radioactivity is a measure of the importance of metabolism in the elimination of the drug. If this ratio in plasma is different from the ratio of unchanged drug to total radioactivity as determined in the excreta, this may indicate selective excretion or reabsorption of unchanged drug or metabolites, and degradation or metabolism of the drug after glomerular filtration or biliary excretion, respectively.

Finally, C_{max} and T_{max} should be reported, although they will generally coincide with those of the unchanged drug.

33.5.3 Assessment of Absorption

In the case of orally administered drugs that have an incomplete bioavailability, this may be due to incomplete absorption and/or first-pass metabolism by the intestine and the liver. The use of a radioactively labeled drug can provide a measure of the extent of absorption relative to first-pass. The (dose-corrected) ratio of the oral AUC versus the intravenous AUC of the unchanged drug in plasma yields the overall bioavailability. The ratio of the oral AUC versus the intravenous AUC of the total radioactivity in plasma (or the urinary recovery of radioactivity after oral versus intravenous administration) is an indication of the fraction absorbed [20, 81]. Although sometimes these ratios are used quantitatively, this is not correct. The radioactivity in plasma (or urine) after oral administration may consist of different metabolites compared to the radioactivity after intravenous administration. Therefore, distribution characteristics and excretion routes of the total radioactivity may differ between the administration routes and cannot be directly compared. Proper assessment of the contributions of gut and liver to bioavailability can be assessed by measuring plasma AUC of unchanged drug after oral, intraportal, and intravenous administration, respectively [82].

33.5.4 Organ Distribution

Documenting the organ distribution of radioactivity (and unchanged drug) can be informative.

After stopping the collection of excreta, radioactivity in the organs can complete the mass balance to 100%. Any radioactivity still unaccounted for is likely to have been lost through expiration ($^{14}CO_2$ or $^{3}H_2O$).

Following the radioactivity in organs during the study indicates how quickly drug-related material is taken up by an organ (e.g., target organs where it may exert its intended or side effect) and how quickly it is redistributed and excreted again. For example, the organ distribution of lovastatin radioactivity indicated preferential accumulation of radioactivity in liver, the target organ. Further speciation showed that the active hydroxy-acid metabolite constituted the majority of liver radioactivity [22].

Organ/plasma ratios of radioactivity may be a resultant of physicochemical characteristics (lipophilic drugs will accumulate in fat), the activity of drug transporters, and the retention by irreversible binding or uptake of the radioactive label into endogenous substances. Ratios of organ AUC to plasma AUC may be calculated as a measure of relative exposure. These data may also be used to extrapolate radioactivity dosimetry data for the human mass balance [83, 84]. Retention of radioactivity in organs may reflect tissue binding of metabolites, but it may also be caused by a delayed elimination of metabolites. Upon repeated exposure, this may result in accumulation of metabolites to possibly toxic levels.

Drug concentrations are not routinely measured in red blood cells. The ratio of radioactivity in red blood cells relative to plasma (if the ratio deviates from 1.0) may indicate (active) uptake of drug into red blood cells. Knowledge of drug partitioning into red blood cells is useful, so that drug clearances from blood by organs of elimination may be evaluated relative to organ blood flows [4].

Comparison of organ distribution data between wild-type animals and animals defective in a drug transporter can be informative to predict the effects of drugs when they are combined with known inhibitors of these transporters [52, 53].

33.5.5 Metabolic Profiling

Determining the metabolic profile is important given the frequent occurrence of clinically relevant active metabolites [85]. It also enables identification of interspecies differences in metabolic profiles [46]. Reference compounds of possible metabolites can be obtained from prior *in vitro* studies. The *in vivo* study can then confirm the presence of metabolites observed *in vitro*. Consequently, metabolites observed in the preclinical mass balance study are likely to be seen in the human mass balance study, and, therefore, metabolite reference compounds may be isolated from the urine or feces obtained in the preclinical mass balance study.

Metabolites can be quantitated even before the structure has been elucidated. The radioactivity-peak areas are simply converted to molar amounts using the specific radioactivity of the administered compound. If such quantification is possible with adequate precision and accuracy, even pharmacokinetic parameters of these metabolites can be calculated, as shown with formoterol in humans [86].

The presence of metabolites in urine while absent in plasma or the absence of parent drug in urine while present in plasma may indicate renal metabolism or reabsorption processes [69].

Human metabolites that constitute a quantitatively large part of total radioactivity in urine, feces, or plasma, and metabolites that (based on their chemical structure) are likely to have a pharmacological or toxic effect, should be investigated for safety testing [5]. Based on metabolites observed in the preclinical studies, such investigations may be initiated earlier.

REFERENCES

1. The European Agency for the Evaluation of Medicinal Products. *Note for Guidance on Toxicokinetics: A Guidance for Assessing Systemic Exposure in Toxicology Studies*. The European Agency for the Evaluation of Medicinal Products; 1995. Accessed 3 May 2004.
2. The European Agency for the Evaluation of Medicinal Products. *Note for Guidance on Non-clinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals*. The European Agency for the Evaluation of Medicinal Products; 2000. Accessed 3 May 2004.
3. Center for Drugs and Biologics, Food and Drug Administration, Department of Health and Human Services. *Guideline for the Format and Content of the Nonclinical Pharmacology/Toxicology Section of an Application*. Center for Drug Evaluation and Research; 1997. Accessed 3 May 2004.
4. Peck CC, Barr WH, Benet LZ, Collins J, Desjardins RE, Furst DE, Harter JG, Levy G, Ludden T, Rodman JH. Opportunities for integration of pharmacokinetics, pharmacodynamics, and toxicokinetics in rational drug development. *Clin Pharmacol Ther* 1992;51(4): 465–473.
5. US Department of Health and Human Services, Food and Drug Administration. *Guidance for Industry—Safety Testing of Drug Metabolites* (draft guidance). Center for Drug Evaluation and Research (CDER): 2005. www.fda.gov/cder/guidance/index.htm. Accessed 16 September 2005.
6. Balani SK, Miwa GT, Gan LS, Wu JT, Lee FW. Strategy of utilizing *in vitro* and *in vivo* ADME tools for lead optimization and drug candidate selection. *Curr Top Med Chem* 2005;5(11):1033–1038.
7. Department of Health and Human Services. US Food and Drug Administration-Center for Drug Evaluation and Research Home Page. http://www.fda.gov/cder/index.html. Accessed 7 January 2006.
8. EMEA. European Medicines Agency–Human Medicines Home Page. http://www.emea.eu.int/index/indexh1.htm. Accessed 8 January 2006.
9. US Department of Health and Human Services, Food and Drug Administration. *Guidance for Industry—In Vivo Drug Metabolism/Drug Interaction Studies—Study Design, Data Analysis, and Recommendations for Dosing and Labeling*. Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER); 1999. www.fda.gov/cder/guidance/index.htm. Accessed 16 September 2005.
10. US Department of Health and Human Services, Food and Drug Administration. *Guidance for Industry—Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers*. Center for Drug Evaluation and Research (CDER); 2005. www.fda.gov/cder/guidance/index.htm. Accessed 9 February 2006.

11. Dalvie D. Recent advances in the applications of radioisotopes in drug metabolism, toxicology and pharmacokinetics. *Curr Pharm Des* 2000;6(10):1009–1028.
12. *Principles and Methods of Toxicology*. Philadelphia: Taylor&Francis; 2001.
13. Fagerholm U, Breuer O, Swedmark S, Hoogstraate J. Pre-clinical pharmacokinetics of the cyclooxygenase-inhibiting nitric oxide donor (CINOD) AZD3582. *J Pharm Pharmacol* 2005;57(5):587–597.
14. Budavari S. *The Merck Index*. Whitehouse Station, NJ: Merck&Co; 1996.
15. Hutchinson OC, Collingridge DR, Barthel H, Price PM, Aboagye EO. Pharmacokinetics of radiolabelled anticancer drugs for positron emission tomography. *Curr Pharm Des* 2003;9(11):917–929.
16. Minami T, Ichii M, Okazaki Y. Comparison of three different methods for measurement of tissue platinum level. *Biol Trace Element Res* 1995;48(1):37–44.
17. Hughes MF, Devesa V, Adair BM, Styblo M, Kenyon EM, Thomas DJ. Tissue dosimetry, metabolism and excretion of pentavalent and trivalent monomethylated arsenic in mice after oral administration. *Toxicol Appl Pharmacol* 2005;208(2):186–197.
18. Desmoulin F, Gilard V, Malet-Martino M, Martino R. Metabolism of capecitabine, an oral fluorouracil prodrug: (19)F NMR studies in animal models and human urine. *Drug Metab Dispos* 2002;30(11):1221–1229.
19. Ferraiolo BL, Moore JA, Crase D, Gribling P, Wilking H, Baughman RA. Pharmacokinetics and tissue distribution of recombinant human tumor necrosis factor-alpha in mice. *Drug Metab Dispos* 1988;16(2):270–275.
20. Pleiss U. [Radioactive-labeled pharmaceutical agents.] Werkzeuge bei der Arzneimittelentwicklung. Radioaktiv markierte pharmazeutische Wirkstoffe. *Pharmazie Zeit* 2005; 34(6):514–519.
21. Dueker SR, Jones AD, Clifford AJ. Protocol development for biological tracer studies. *Adv Exp Med Biol* 1998;445:363–378.
22. Duggan DE, Chen IW, Bayne WF, Halpin RA, Duncan CA, Schwartz MS, Stubbs RJ, Vickers S. The physiological disposition of lovastatin. *Drug Metab Dispos* 1989;17(2): 166–173.
23. Horsmans Y, Saliez A, van d BV, Desager JP, Geubel AP, Pauwels S, Lambotte L. ^{14}C-Propoxyphene demethylation in the rat. An example of differences between liver and intestinal drug-presystemic metabolism. *Drug Metab Dispos* 1997;25(11):1257–1259.
24. Dalvie D. Recent advances in the applications of radioisotopes in drug metabolism, toxicology and pharmacokinetics. *Curr Pharm Des* 2000;6(10):1009–1028.
25. Beumer JH, Beijnen JH, Schellens JH. Mass balance studies, with a focus on anticancer drugs. *Clin Pharmacokinet* 2006;45(1):33–58.
26. Browne TR, Van Langenhove A, Costello CE, Biemann K, Greenblatt DJ. Pharmacokinetic equivalence of stable-isotope-labeled and unlabeled drugs. Phenobarbital in man. *J Clin Pharmacol* 1982;22(7):309–315.
27. Phillips DH, Potter GA, Horton MN, Hewer A, Crofton-Sleigh C, Jarman M, Venitt S. Reduced genotoxicity of [D5-ethyl]-tamoxifen implicates alpha-hydroxylation of the ethyl group as a major pathway of tamoxifen activation to a liver carcinogen. *Carcinogenesis* 1994;15(8):1487–1492.
28. Toxicokinetics: the assessment of systemic exposure in toxicity studies-ICH topic S3A. ICH; 1995. *CPMP/ICH/385/95*. Accessed 9 February 2006.
29. US Department of Health and Human Services, Food and Drug Administration. *Guidance for Industry—M3 Nonclinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals*. Center for Drug Evaluation and Research (CDER); 1997. www.fda.gov/cder/guidance/index.htm. Accessed 16 September 2005.

30. US Department of Health and Human Services, Food and Drug Administration. *Guidance for Industry—Drug Metabolism/Drug Interaction Studies in the Drug Development Process: Studies In Vitro*. Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research; 1997. www.fda.gov/cder/guidance.htm. Accessed 16 September 2005.
31. Collins JM. Inter-species differences in drug properties. *Chem-Biol Interact* 2001;134(3): 237–242.
32. Camiener GW, Smith CG. Studies of the enzymatic deamination of cytosine arabinoside. I. Enzyme distribution and species specificity. *Biochem Pharmacol* 1965;14(10):1405–1416.
33. Sharkey EM, ONeill HB, Kavarana MJ, Wang H, Creighton DJ, Sentz DL, Eiseman JL. Pharmacokinetics and antitumor properties in tumor-bearing mice of an enediol analogue inhibitor of glyoxalase I. *Cancer Chemother Pharmacol* 2000;46(2):156–166.
34. Morton CL, Iacono L, Hyatt JL, Taylor KR, Cheshire PJ, Houghton PJ, Danks MK, Stewart CF, Potter PM. Activation and antitumor activity of CPT-11 in plasma esterase-deficient mice. *Cancer Chemother Pharmacol* 2005;56(6):629–636.
35. van Bezooijen CF. Influence of age-related changes in rodent liver morphology and physiology on drug metabolism—a review. *Mech Ageing Dev* 1984;25(1–2):1–22.
36. Leakey JE, Cunny HC, Bazare J Jr, Webb PJ, Feuers RJ, Duffy PH, Hart RW. Effects of aging and caloric restriction on hepatic drug metabolizing enzymes in the Fischer 344 rat. I: The cytochrome P-450 dependent monooxygenase system. *Mech Ageing Dev* 1989; 48(2):145–155.
37. Leakey JA, Cunny HC, Bazare J Jr, Webb PJ, Lipscomb JC, Slikker W Jr, Feuers RJ, Duffy PH, Hart RW. Effects of aging and caloric restriction on hepatic drug metabolizing enzymes in the Fischer 344 rat. II: Effects on conjugating enzymes. *Mech Ageing Dev* 1989;48(2):157–166.
38. Czerniak R. Gender-based differences in pharmacokinetics in laboratory animal models. *Int J Toxicol* 2001;20(3):161–163.
39. Witkamp RF, Yun HI, van't Klooster GA, van Mosel JF, van Mosel M, Ensink JM, Noordhoek J, Van Miert AS. Comparative aspects and sex differentiation of plasma sulfamethazine elimination and metabolite formation in rats, rabbits, dwarf goats, and cattle. *Am J Vet Res* 1992;53(10):1830–1835.
40. Witkamp RF, Lohuis JA, Nijmeijer SM, Kolker HJ, Noordhoek J, van Miert AS. Species- and sex-related differences in the plasma clearance and metabolite formation of antipyrine. A comparative study in four animal species: cattle, goat, rat and rabbit. *Xenobiotica* 1991;21(11):1483–1492.
41. Griffin RJ, Godfrey VB, Kim YC, Burka LT. Sex-dependent differences in the disposition of 2,4-dichlorophenoxyacetic acid in Sprague–Dawley rats, B6C3F1 mice, and Syrian hamsters. *Drug Metab Dispos* 1997;25(9):1065–1071.
42. Kato R, Yamazoe Y. Sex-specific cytochrome P450 as a cause of sex- and species-related differences in drug toxicity. *Toxicol Lett* 1992;64–65(Spec No):661–667.
43. Klecker RW, Cysyk RL, Collins JM. Zebularine metabolism by aldehyde oxidase in hepatic cytosol from humans, monkeys, dogs, rats, and mice: influence of sex and inhibitors. *Bioorg Med Chem* 2006;14(1);62–66.
44. Schwartz JB. The influence of sex on pharmacokinetics. *Clin Pharmacokinet* 2003;42(2): 107–121.
45. van Herwaarden AE, Smit JW, Sparidans RW, Wagenaar E, van der Kruijssen CM, Schellens JH, Beijnen JH, Schinkel AH. Midazolam and cyclosporin a metabolism in

transgenic mice with liver-specific expression of human CYP3A4. *Drug Metab Dispos* 2005;33(7):892–895.

46. Marathe PH, Shyu WC, Humphreys WG. The use of radiolabeled compounds for ADME studies in discovery and exploratory development. *Curr Pharm Des* 2004;10(24):2991–3008.
47. Jonker JW, Wagenaar E, van Deemter L, Gottschlich R, Bender HM, Dasenbrock J, Schinkel AH. Role of blood–brain barrier P-glycoprotein in limiting brain accumulation and sedative side-effects of asimadoline, a peripherally acting analgaesic drug. *Br J Pharmacol* 1999;127(1):43–50.
48. Merino G, Jonker JW, Wagenaar E, van Herwaarden AE, Schinkel AH. The breast cancer resistance protein (BCRP/ABCG2) affects pharmacokinetics, hepatobiliary excretion, and milk secretion of the antibiotic nitrofurantoin. *Mol Pharmacol* 2005;67(5):1758–1764.
49. Jonker JW, Wagenaar E, Mol CA, Buitelaar M, Koepsell H, Smit JW, Schinkel AH. Reduced hepatic uptake and intestinal excretion of organic cations in mice with a targeted disruption of the organic cation transporter 1 (Oct1 [Slc22a1]) gene. *Mol Cell Biol* 2001;21(16):5471–5477.
50. Breedveld P, Pluim D, Cipriani G, Wielinga P, van Tellingen O, Schinkel AH, Schellens JH. The effect of Bcrp1 (Abcg2) on the *in vivo* pharmacokinetics and brain penetration of imatinib mesylate (Gleevec): implications for the use of breast cancer resistance protein and P-glycoprotein inhibitors to enable the brain penetration of imatinib in patients. *Cancer Res* 2005;65(7):2577–2582.
51. Schinkel AH, Wagenaar E, Mol CA, van Deemter L. P-glycoprotein in the blood–brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. *J Clin Invest* 1996;97(11):2517–2524.
52. Schinkel AH, Wagenaar E, van Deemter L, Mol CA, Borst P. Absence of the mdr1a P-glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. *J Clin Invest* 1995;96(4):1698–1705.
53. Schinkel AH, Smit JJ, van Tellingen O, Beijnen JH, Wagenaar E, Van DeemTer L, Mol CA, van der Valk MA, Robanus-Maandag EC, te Riele HP. Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood–brain barrier and to increased sensitivity to drugs. *Cell* 1994;77(4):491–502.
54. Gonzalez FJ, Yu AM. Cytochrome P450 and xenobiotic receptor humanized mice. *Annu Rev Pharmacol Toxicol* 2006;46:41–64.
55. Gonzalez FJ. Cytochrome P450 humanised mice. *Hum Genomics* 2004;1(4):300–306.
56. Beumer JH, Rademaker-Lakhai JM, Rosing H, Lopez-Lazaro L, Beijnen JH, Schellens JH. Trabectedin (Yondelis, formerly ET-743), a mass balance study in patients with advanced cancer. *Invest New Drugs* 2005;23(5):429–436.
57. Rickert DE, Dingley K, Ubick E, Dix KJ, Molina L. Determination of the tissue distribution and excretion by accelerator mass spectrometry of the nonadecapeptide ^{14}C-Moli1901 in beagle dogs after intratracheal instillation. *Chem-Biol Interact* 2005;155(1–2):55–61.
58. Lappin G, Garner RC. Big physics, small doses: the use of AMS and PET in human microdosing of development drugs. *Nat Rev Drug Discov* 2003;2(3):233–240.
59. *Pharmacokinetics: Guidance for Repeated Dose Tissue Distribution Studies—ICH Topic S3B*. ICH; 2005. *CPMP/ICH/385/95*.
60. Dent NJ. The inspection of drug metabolism and pharmacokinetic studies. *Qual Assur* 1992;1(3):230–236.

61. *Safe and Secure—A Guide to Working Safely with Radiolabelled Compounds*. Amersham; 2005. http://www1.amershambiosciences.com/applic/upp00738.nsf/vLookupDoc/196245642-A125/$file/Safe_Secure%202003.pdf. Accessed 2 November 2005.

62. Kishore R, Eary JF, Krohn KA, Nelp WB, Menard TW, Beaumier PL, Hellstrom KE, Hellstrom I. Autoradiolysis of iodinated monoclonal antibody preparations. *Int J Radiat Appl Instrum B* 1986;13(4):457–459.

63. Kinuya S, Yokoyama K, Tega H, Hiramatsu T, Konishi S, Yamamoto W, Shuke N, Aburano T, Watanabe N, Takayama T, Michigishi T, Tonami N. Rhenium-186-mercaptoacetyltriglycine-labeled monoclonal antibody for radioimmunotherapy: *in vitro* assessment, *in vivo* kinetics and dosimetry in tumor-bearing nude mice. *Japanese J Cancer Res* 1998;89(8):870–878.

64. Smit MJ, Temmerman AM, Havinga R, Kuipers F, Vonk RJ. Short- and long-term effects of biliary drainage on hepatic cholesterol metabolism in the rat. *Biochem J* 1990;269(3):781–788.

65. Accatino L, Hono J, Koenig C, Pizarro M, Rodriguez L. Adaptive changes of hepatic bile salt transport in a model of reversible interruption of the enterohepatic circulation in the rat. *J Hepatol* 1993;19(1):95–104.

66. Nezasa K, Takao A, Kimura K, Takaichi M, Inazawa K, Koike M. Pharmacokinetics and disposition of rosuvastatin, a new 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, in rat. *Xenobiotica* 2002;32(8):715–727.

67. Rowland M, Tozer TN, *Clinical Pharmacokinetics—Concepts and Applications*. Baltimore: Williams & Wilkins; 1995.

68. Mukherjee KL, Heidelberger C. Studies on fluorinated pyrimidines. IX. The degradation of 5-fluorouracil-6-C14. *J Biol Chem* 1960;235:433–437.

69. Melgar MD, Zuleski FR, Malbica JO. Metabolism, disposition, and pharmacokinetics of tracazolate in rat and dog. *Drug Metab Dispos* 1984;12(4):396–402.

70. Lee HJ, Chiou WL. Marked heterogeneity in the intrahepatic distribution of quinidine in rats: implications in pharmacokinetics. *J Pharm Sci* 1990;79(9):778–781.

71. Ings RM, Breen M, Devereux K, Gray AJ, Edwards FE, Lucas C, Briggs M, Robinson BV, Campbell DB. The comparative disposition of [^{14}C]-fotemustine in non-tumourous and tumourous mice. *Cancer Chemother Pharmacol* 1990;27(2):106–110.

72. Tomiyama Y, Brian JE Jr, Todd MM. Cerebral blood flow during hemodilution and hypoxia in rats: role of ATP-sensitive potassium channels. *Stroke* 1999;30(9):1942–1947.

73. Berger H, Sandow J, Heinrich N, Albrecht E, Kertscher U, Oehlke J. Disposition of the ^{3}H-labeled gonadotropin-releasing hormone analog buserelin in rats. *Drug Metab Dispos* 1993;21(5):818–822.

74. Slatter JG, Schaaf LJ, Sams JP, Feenstra KL, Johnson MG, Bombardt PA, Cathcart KS, Verburg MT, Pearson LK, Compton LD, Miller LL, Baker DS, Pesheck CV, Lord RS III. Pharmacokinetics, metabolism, and excretion of irinotecan (CPT-11) following I.V. infusion of [(^{14})C]CPT-11 in cancer patients. *Drug Metab Dispos* 2000;28(4):423–433.

75. Lenz EM, Wilson ID, Wright B, Partridge EA, Rodgers CT, Haycock PR, Lindon JC, Nicholson JK. A comparison of quantitative NMR and radiolabelling studies of the metabolism and excretion of Statil (3-(4-bromo-2-fluorobenzyl)-4-oxo-3*H*-phthalazin-1-ylacetic acid) in the rat. *J Pharm Biomed Anal* 2002;28(1):31–43.

76. Griffiths JR, Glickson JD. Monitoring pharmacokinetics of anticancer drugs: non-invasive investigation using magnetic resonance spectroscopy. *Adv Drug Deliv Rev* 2000;41(1):75–89.

77. Sparreboom A, Huizing MT, Boesen JJ, Nooijen WJ, van Tellingen O, Beijnen JH. Isolation, purification, and biological activity of mono- and dihydroxylated paclitaxel metabolites from human feces. *Cancer Chemother Pharmacol* 1995;36(4):299–304.
78. Gilbert PJ, Hartley TE, Troke JA, Turcan RG, Vose CW, Watson KV. Application of ^{19}F-n.m.r. spectroscopy to the identification of dog urinary metabolites of imirestat, a spirohydantoin aldose reductase inhibitor. *Xenobiotica* 1992;22(7):775–787.
79. Jansen AB. Total radioactivity half-lives. *Drug Metab Dispos* 1979;7(5):350.
80. Di Carlo FJ. Undifferentiated radioactivity revisited. *Drug Metab Dispos* 1980;8(4): 287–288.
81. Lemaire M, Bodoky A, Heberer M, Niederberger W. Quantification of absorption, intestinal and hepatic first pass effect in a chronic dog model. *Eur J Drug Metab Pharmacokinet* 1991;Spec No 3:132–135.
82. Vachharajani NN, Shyu WC, Shah VR, Barbhaiya RH. Pharmacokinetic assessment of the sites of first-pass metabolism of BMS-181101, an antidepressant agent, in rats. *J Pharm Pharmacol* 1998;50(3):275–278.
83. Dain JG, Collins JM, Robinson WT. A regulatory and industrial perspective of the use of carbon-14 and tritium isotopes in human ADME studies. *Pharm Res* 1994;11(6): 925–928.
84. *Code of Federal Regulations*. Prescription drugs for human use generally recognized as safe and effective and not misbranded: drugs used in research. FDA; 2002. Accessed 3 May 2004.
85. Garattini S. Active drug metabolites. An overview of their relevance in clinical pharmacokinetics. *Clin Pharmacokinet* 1985;10(3):216–227.
86. Rosenborg J, Larsson P, Tegner K, Hallstrom G. Mass balance and metabolism of [(3)H]Formoterol in healthy men after combined i.v. and oral administration-mimicking inhalation. *Drug Metab Dispos* 1999;27(10):1104–1116.
87. Collins JM, Grieshaber CK, Chabner BA. Pharmacologically guided phase I clinical trials based upon preclinical drug development. *J Nat Cancer Inst* 1990;82(16):1321–1326.
88. Uchida T, O'Brien RD. Dimethoate degradation by human liver and its significance for acute toxicity. *Toxicol Appl Pharmacol* 1967;10(1):89–94.
89. Dareer SM, Mulligan LT Jr, White V, Tillery K, Mellett LB, Hill DL. Distribution of [^{3}H]cytosine arabinoside and its products in mice, dogs, and monkeys and effect of tetrahydrouridine. *Cancer Treat Rep* 1977;61(3):395–407.
90. Kitteringham NR, Davis C, Howard N, Pirmohamed M, Park BK. Interindividual and interspecies variation in hepatic microsomal epoxide hydrolase activity: studies with *cis*-stilbene oxide, carbamazepine 10, 11-epoxide and naphthalene. *J Pharmacol Exp Ther* 1996;278(3):1018–1027.