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The Conduct of Drug Metabolism Studies Considered Good Practice (I): Analytical Systems and *In Vivo* Studies

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Abstract

This review serial outlines practical and scientifically-based approaches to conducting contemporary drug metabolism studies considered good practice for drug development and regulatory filing. The present part addresses analytical methods used in the drug metabolism studies and evaluates advantages and disadvantages of these methods as well as the related sample preparations. The methods described here cover from conventional radioactive labeling of drugs, which includes selection of a proper radioisotope, its labeling position, and modern radio-pharmacokinetics employed in microdosing by using a radionuclide to visualize drug distribution *in vivo*, to currently widely-used liquid chromatography (LC) in conjunction with mass spectrometry (MS), tandem mass spectrometry (MS/MS), and nuclear magnetic resonance (NMR) for quantitative detection of metabolites and characterization of their structures. Although the analytical tools have progressed sufficiently to allow determination of metabolites, proper *in vitro* models and *in vivo* studies have to be carefully designed in order to understand drug metabolism. Points for consideration when conducting *in vivo* drug metabolism studies include interspecies differences in systemic exposure and metabolism pathways, identification of the major metabolites and unique human metabolites that become the regulatory focus, local metabolism in addition to liver metabolism, time points for sampling, and synthesis of the authentic metabolites to confirm their formation. The next part of this serial article will focus on *in vitro* drug metabolism studies.

Keywords

Analytical techniques; drug regulations; *in vivo* drug metabolism; interspecies differences; major drug metabolites; metabolite identification; radiolabeled drug; pharmacokinetics

INTRODUCTION

The emerging modern bioanalytical technology along with automated genomic and proteomic sequence analyses has greatly facilitated the current drug metabolism studies. Drugs are typically subjected to a variety of biotransformation reactions, and the metabolites formed through these reactions must be considered when conducting drug safety studies for new chemical entities. A drug is eliminated either by excretion or by metabolism to one or more active or inactive metabolites. When elimination occurs primarily by metabolism, the routes of metabolism can significantly affect the drug's safety and efficacy and the directions for use. If elimination is primarily by metabolism, the principal metabolizing routes should be

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understood. When elimination occurs via a single metabolic pathway, individual differences in metabolic rates can lead to large differences in drug and metabolite concentrations in the blood and tissue [1].

The goals of conducting drug metabolism studies are to identify and characterize all of major metabolites of the test drug and specific enzymes responsible for its metabolism; to evaluate the impacts of the metabolites on safety and efficacy of the drug; and to utilize the drug's metabolism information to maximize its intellectual property. Before developing strategies to reach these goals, one must strike to a reasonable balance between the collection of data that will have a meaningful impact on the safety evaluation of a new drug and the practical consideration that characterizing every metabolite presented in humans at any level is an impossible task. To achieve these goals, specific, sensitive and efficient analytical methods have to be developed before using *in vitro* and *in vivo* models for further comprehensive investigations. Development of sensitive and specific methods is critical to the study of drug metabolism, and has long been a high priority for drug development programs. Efficient sample preparation and LC are often required to conduct analytical experiments in complex biological matrices to achieve superior specificity of the analysis. Technological advances during the past decade have greatly improved analytical capabilities to detect, identify, and characterize metabolites at previously unattainable levels [2].

The authors felt that a comprehensive analysis and review of current technology and best practices in conducting drug metabolism studies would contribute to the field timely. The intent of the article is to harmonize the related approaches used by various laboratories and define a minimal best practice for drug metabolism studies. The objectives of this article are to provide researchers in this field with an up-to-date, easy-to-use set of protocols to aid in their study designs whilst at the same time affording maximal consideration for drug regulatory requirements. The review serial is based on the peer-reviewed papers published by us and others, as well as our 'in-house' standard operation procedures established after many practices. It is hoped that the views presented herein will form a basis for continued dialog and considerations among scientists and regulatory agency representatives.

1. ANALYTICAL SYSTEMS

1.1. Radioactive Analytical Methods

The classical method used for more than three decades in metabolite analysis is radioactive labeling of a parent drug and detection of its metabolites by LC with radioactivity detection. The method is especially useful when combined with on-line MS detection. Radioactivity detection provides localization of drug metabolites in a chromatogram, and mass spectrometry ensures structure specific identification of the metabolites. Parent drug contains one or more radionuclides at a selected position within the molecule under the assumption that the radionuclide will not be converted to a metabolite that could be incorporated into endogenous metabolism (e.g., CO_2, H_2O, acetic acid, etc.). Both ^{14}C and ^{3}H have long decay half-life of 5730 years and 12.3 years, respectively. This makes ^{14}C and ^{3}H the most appropriate radioactive labels for a mass balance study because there is no need for correcting decay for ^{14}C and ^{3}H. However, use of γ-emitter $^{191/193}Pt$ ($t_{1/2}$ = 2.9 days), ^{131}I (decay $t_{1/2}$ 8.0 days), and β-emitter ^{35}S ($t_{1/2}$ 87.5 days) ^{32}P ($t_{1/2}$ 14 days) requires correction for decay.

^{14}C is the most frequently used radionuclide due to the fact that there is typically a variety of positions in the tested drug into which ^{14}C can be incorporated. In addition, ^{14}C is a relatively safe, low-energy radiation emitter. ^{3}H is sometimes used, providing it is not readily exchangeable or metabolically labile. Other advantages of ^{14}C over ^{3}H used in the metabolism study [5] include 1. ^{14}C is about 8-fold more energetic than ^{3}H (0.156 Mev vs 0.019 Mev). Thus, ^{14}C can be detected easily with more sensitivity. If accelerator mass spectrometry (AMS)

is used in connection with ^{14}C to improve sensitivity, the amount of ^{14}C can be further reduced [6]; and 2. the kinetic isotope effect: replacement of a regular atom with its radioisotope often results in a minor kinetic isotope effect. The greater the difference in mass between the atom and its radioisotope, the stronger the replaced chemical bond is. This replacement can affect original reaction rate. The relative mass difference between ^{12}C and its radioisotope ^{14}C is about 17%. Whereas, the relative mass difference between ^{1}H and ^{3}H is 200%. Therefore, a drug labeled with ^{14}C produces impacts lesser than that labeled with ^{3}H on the original bond strength and kinetics of the drug. As a result, the use of ^{14}C as the radioisotope is preferable to the use of ^{3}H.

^{14}C is usually incorporated into aromatic or alicyclic ring systems instead of at a metabolically labile site that may result in a rapid loss of the tracer, and further metabolic products may become no longer traceable. If a substantial portion of the drug is expected to be cleaved into two significant portions, then the radionuclide should be incorporated on each side.

In the study of absorption, distribution, metabolism and elimination (ADME), humans or animals are administered a mixture of known amount of a drug labeled and non-labeled for the metabolism experiments to evaluate the mass balance and tissue distribution of the drug [5, 6]. The total dose used in the radiolabel studies is usually a pharmacologically relevant dose, which can be determined from results of the early efficacy studies. If a specific dose has not yet been established, a total dose of radioactivity could be 100 μCi, or less, providing that exposure of a specific tissue to the radiolabeled drug does not exceed radiation limits to avoid local radiation damage and/or local ‘over-hot’ consequence [7]. Before animals are dosed, the radiochemical purity needs to be evaluated, which can usually be conducted by the provider. The stability of the radiolabeled drug in physiological matrices must be known. It is necessary to verify that the pharmacological action and physical properties are not affected by the label, and that the bond of the label is stable *in vivo*, and the potential for label dissociation *in vivo* does not exist [7]. If the radiolabel is non-metabolically removed from the drug, the results from the drug metabolism experiments or other studies using the labeled drug will have little, if any, meaning or usefulness in the determination of the metabolic fate of the drug.

Recently, two ultra-sensitive analytical methods based on nuclear physics have been developed to measure drug and metabolite concentrations in the low picogram to femotgram range. They are AMS and positron emission tomography (PET) [8,9]. Both techniques rely on the analysis of radioisotopes incorporated into the drugs under study. In the case of AMS, ^{14}C is the most useful isotope for drug metabolism studies. Whereas, for PET, ^{11}C is proven to be the most useful. It is worth noting the huge difference in radioactive half-life of the two isotopes. ^{14}C has a half-life of 5730 years as mentioned above, whilst ^{11}C has a half-life of 20 min. In the latter case, the radiosynthesis laboratory must be in the very close proximity to the experiment site. In contrast, the stability of ^{14}C means that provided no radiolytic or chemical decomposition occurs, the synthesized labeled molecule is stable for many years. AMS is used for determining labeled drug or metabolite from body samples over the period of experiment time up to 100 days after drug administration. PET provides primarily pharmacodynamic data through real-time imaging and some limited pharmacokinetic data. By using PET technique, drug distribution data can be obtained for only some 2 hours after drug administration (i.e., six decay half-lives of ^{11}C).

However, the conventional radioactive approach has several disadvantages. For example, synthesis and purification of radioactive drugs are expensive and time-consuming, radiation is a potential health risk for humans, and the requirements for handling radioactive material and wastes make the use of radiolabeled drugs costly. For these reasons and the fact that radioactive compounds are usually not available in early discovery phase, simpler and quicker MS techniques are increasingly used in metabolism studies.

1.2. Advanced Analytical Systems

Since the introduction of the electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) techniques, LC/MS has become an ideal and widely used method in the identification, structure characterization and quantitative analysis of drug metabolites owing to its superior specificity, sensitivity and efficiency over others such as radioimmunoassay (RIA), gas chromatography/mass spectrometry (GC/MS) and liquid chromatography (LC) with UV [10], fluorescence, radioactivity and mass spectrometry (MS). It needs to mention that the reversed-phase liquid chromatography is still commonly used in metabolite analysis owing to its universality [11,12] and good compatibility with APCI-MS and ESI-MS [13,14, 15]. Buffer systems made from 5–10 mM ammonium acetate and 0.1% acetic acid provide sufficient repeatability of the retention times and efficient ionization of basic compounds in positive ion ESI [16]. Ionization of many neutral and acidic compounds is also achieved, provided that the proton affinities are high enough. Analytical times < 5 min can easily be achieved.

Atmospheric pressure ionization techniques provide efficient ionization for various molecules, including polar, labile and high molecular mass drugs and metabolites. These techniques enable detection and quantitative determination of formed metabolites and depleted parent drug concomitantly in the same sample collected [17]. Mass spectral full-scan of an extracted sample is performed to compare the ion chromatograms of each sample extracted at different time points and/or under different conditions. ESI as a gentle ionization technique is suitable for labile conjugates, such as glucuronides and sulfates, and is therefore preferred in metabolite analysis. Two runs, one made in the negative ion mode and another in the positive ion mode, should be conducted to ensure detection of potential metabolite(s), and define the structure(s). The metabolite profile should be based on the detection of protonated, deprotonated or adduct ions but not on the fragment ions [2]. However, for molecules with weak polarity and strong lipophilicity, APCI may be more suitable than ESI in ionizing these molecules, and used as interface [18].

The MS full-scan may reveal all the ion chromatograms of the expected metabolites according to predicted gains and losses in molecular masses of the metabolites compared with the molecular mass of the parent drug. The peaks detected in the ion chromatograms correspond to the mass-to-charge ratio (m/z) of possible metabolites. Metabolite identification software screens all the ion chromatograms of the expected metabolites, and can reveal possible metabolic reactions and related changes in masses of the tested drugs (Table 1).

Relative metabolite quantitation can be made on the basis of percentage of peak areas of each metabolite as a function of incubation time compared to the total area of all chromatographic peaks if it is difficult and expensive to synthesize the authentic metabolites at the early stage of drug development although the data may not be reliable [6]. The lower limit of quantitation is defined by a lower limit of signal-to-noise ratio (3:1) rather than a lower limit defined by a standard curve (signal-to-noise, 10:1). Metabolite identification can be carried out by co-chromatography of the metabolite sample with its authentic standard to show their identical shapes, retention times and mass spectra [5]. To achieve acceptable reproducibility and reliability in quantitative LC/MS, an internal standard calibration method is often used to correct variations in mass spectrometric response and in extraction recovery to minimize possible matrix effects. Ideally, the internal standard should be either a stable isotopically labeled (^{13}C, ^{2}H, ^{15}N, ^{18}O) analog of the metabolite or a molecule with a closely similar structure to the analyte. The difference between the molecular masses of the analyte and its internal standard should be at least three mass units to avoid overlapping in the monitored mass peaks. The metabolite standards must be synthesized although in some cases this could be a challenge.

Most of the work in metabolite analysis is carried out by using triple-quadrupole mass spectrometers (QQQ) [6,16,17]. The main advantages of QQQ are their superior quantitative capabilities in the multiple reaction monitoring (MRM) mode and the fact that a family of metabolites can easily be identified using neutral-loss and precursor ion scans. MRM built in with the MS/MS has the advantage in reducing interference and enhancing sensitivity over the selected ion monitoring. However, the sensitivity of full-scan MS or MS/MS by QQQ may not suffice in the identification of metabolites and therefore the use of ion trap (IT)MS and time-of-flight (TOF)-MS has increased. Both of these techniques provide high full scan sensitivity. In addition, MS^n and the high-resolution capabilities provided by ITMS and TOF, respectively, are highly efficient in structure analysis of metabolites, and enhance confidence of structural characterization of metabolites. Even higher resolution and configuration of MS can surely be expected in the near future because of the constant development of MS technology.

Although LC/MS provides efficient technology for metabolite analysis, identification of all the possible metabolites of a certain drug is still a challenging task. The specificity of unit or even high-resolution MS is not always enough, neutral-loss and precursor ion scans may not suffice with any of the MS techniques to detect trace quantities of metabolites in complex biological matrices. Furthermore, the determination of the site of metabolic reaction in a drug molecule is not always possible by MS. To overcome the problems in metabolic analysis, new technologies are continuously being developed. The recently introduced LC/MS/nuclear magnetic resonance (NMR) technology, although expensive, provides unambiguous characterization of metabolite structure. Although the sensitivity of NMR does not suffice for the analysis of metabolites in trace quantities, the sensitivity of the technology is continuously being improved.

Microfluidic systems offer other possibilities to integrate all the experimental steps of metabolite analysis on one microchip, providing complete analysis cycles (e.g., sample pretreatment, chemical reactions, analytical separation, detection and data processing) on a single device with a high level of automation. It is reasonable to foresee that the metabolite analysis will be carried out by miniaturized lab-on-a-chip techniques integrated with miniaturized mass spectrometers in future.

1.3. Sample Collection, Preparation and Comparison

In vitro and *in vivo* samples are collected at different time points. The metabolites are identified by comparison of the ion chromatograms between samples possibly containing metabolites and samples without metabolites that are collected at the very beginning of the reaction or at low temperature where metabolites can not be appreciably produced. In sample preparation, it is essential to remove interfering materials, such as proteins, salts, and endogenous and background compounds to avoid clogging of columns and capillaries and improve the sensitivity, selectivity, and reliability of analysis. Common pretreatment methods include protein precipitation and centrifugation followed by liquid-liquid extraction, or solid-phase extraction. The latter has achieved the widest acceptance owing to the ease of automation and to the availability of a wide variety of commercial sorbent materials. It is worth noting that precipitation of proteins with acids may catalyze the hydrolysis of some conjugates such as glucuronides and sulfates. This can be avoided by using organic solvents in the precipitation. Also, care must be taken that the highly polar or ionic compounds with low retention factors are not lost in reversed-phase solid-phase extraction. The samples collected at various intervals are analyzed by LC/MS. If the following criteria are met, the unknown drug metabolite is well identified with great confidence: 1. shapes and retention time of the peak of the unknown metabolite collected from the samples are identical to those of the authentic metabolite synthesized on purpose to test the hypothesis of the metabolism; 2. the mass-to-charge ratio of the two peaks, one from the collected samples and another from the authentic metabolite, is

the same; and 3. the relative abundance ratio of the two peaks reflects the concentration ratio of the authentic metabolite to the metabolite collected from the samples.

New chemical entities can be classified into three categories based on stereoisometric purity: racemates, single-enantiomers and achiral [20]. Achiral drugs have no stereocenters and, therefore, only one stereoisomeric form of the molecule is possible. Racemates and single-enantiomers contain at least one stereoisomeric atom that can present itself into two possible three-dimensional configurations—R or S. Racemates are mixtures that contain equal amounts of each R and S enantiomer. Single-enantiomers are stereoisomerically pure molecules that contain only one stereoisomeric form of the molecule, R or S, and they are not mixtures. Stereoisomeric bio-inversion of racemates was shown in rat liver microsomes [21]. R- and S-enantiomer of a drug may show somewhat differences in their efficacy, pharmacokinetic and toxic profiles as demonstrated in the case of gossypol [17,22,23]. Therefore, it is intriguing to separate enantiomers of a drug in biosamples by using chiral chromatographic column filled with cyclodextrin stationary phases for chiral separations (www.astecusa.com), and characterize the differences in metabolism profile of the enantiomers.

2. *IN VIVO* DRUG METABOLISM: REGULATORY CONSIDERATION

In vivo studies identify critical metabolic pathways for a new drug and its metabolites that are so formed by these pathways. The clinical significance of this information should generally be confirmed via *in vivo* studies. In many cases, *in vitro* studies, which are inexpensive and readily carried out, should serve as an adequate screening mechanism that can rule out the importance of a metabolic pathway and make *in vivo* testing unnecessary [1]. When a difference arises between *in vitro* and *in vivo* findings, the *in vivo* results should always take precedence over *in vitro* studies [1,24]. On the other hand, if there is some, but not a large effect of the metabolites observed in *in vitro* settings, predicting the effect of the metabolites on animals and humans by using *in vitro* data will be difficult. In reality, however, efforts have to be made to provide a comprehensive characterization of drug metabolism to meet regulatory requirements and acceptability.

Based on data obtained from *in vitro* and *in vivo* metabolism studies, when the metabolite profiling 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 investigated during nonclinical studies [25].

Recent FDA guidance made recommendations on when and how to identify, characterize, and evaluate the safety of unique human metabolites and major metabolites of small molecule drugs [25]. This guidance defines major metabolites primarily as those identified in human plasma that account for greater than 10% of drug related material (administered dose or systemic exposure whichever is less) and that are not present at sufficient levels to permit adequate evaluation during standard nonclinical studies. This guidance defines unique human metabolite as a metabolite produced only in humans. The rationale for setting the level at greater than 10% for characterization of metabolites reflects consistency with other FDA and Environmental Protection Agency (EPA) regulatory guidances [26,27] and is supported by actual cases in which it has been determined that the toxicity of a drug could be attributed to one or more metabolites present at greater than 10% of the administered dose. The US EPA recommended that metabolites which have been characterized in excreta as comprising 5% or greater of the administered dose should be identified. Identification of metabolites representing less than 5% of the administered dose might be requested if such data are needed for risk assessment of the test compound [27]. It is our view that there should be not a clear cut-off (i.e., 10%), and decisions should be made on a case-by-case basis. Some major metabolites present at less than 10% should also be tested for additional activities.

Generally, drugs with following characteristics are of particular concern and may warrant additional metabolism investigation [25]: narrow therapeutic indices, significant toxicity, significantly diverse metabolic profiles between human and nonclinical species, irreversible toxicity, and/or adverse effects not readily monitored in the clinic.

It is important to understand that it is not the general practice of the regulatory agency to request a complete drug metabolism profile once a major human metabolite has been identified. The decisions that determine the types of drug metabolism studies and the resulting non-clinical toxicity studies that need to be performed rely on scientific justification and comprehensive evaluation of the available data on both the parent drug and its major metabolite(s). In some cases, more extensive characterization of a major human metabolite may be necessary, whereas in other circumstances such as anti-cancer drugs, safety testing of metabolites may not be necessary. It is recognized that drugs being developed for life-threatening diseases may not be held to the same standards as drugs proposed for less serious indications.

3. *IN VIVO* DRUG METABOLISM STUDIES

In vivo drug metabolism studies include metabolite profiling in blood, selected tissues, urine, and bile to assess distribution and disposition of potentially important metabolites, such as the major metabolite and human unique metabolite mentioned above. Metabolite profiling requires a technique to separate the parent drug from metabolites and other endogenous components. Chromatography and electrophoresis are usually the method of choice. The former can be applied to both small and macro-molecules, while the latter is only suitable for macro-molecules.

Timing for blood collection should be exquisitely designed in order to catch the peaks of potential metabolites formed after systemic exposure of the parent drug. In theory, the production and accumulation of metabolites are in the proportion to the concomitant disappearance of the parent drug. A good chance to observe metabolites formed in animals or humans does not occur early, but a little late after the peak concentration of the parent drug in blood [17,19]. Metabolites are sometimes identified in other biological matrices such as urine, feces, or bile. In many instances, metabolites can be easily found in excreta, instead of blood [19]. If Phase II conjugation products of a drug are present in the excreta, one can assume that systemic exposure to the drug has occurred. Pooling of excreta to facilitate metabolite identification within a given dose group is acceptable [5,6,19,28]. Consecutive pooling and profiling of metabolites from each time period are recommended [27]. Appropriate qualitative and quantitative methods should be used to assay urine, feces, and expired air (if certain amount of the drug and its metabolites is expected to be exhaled into air) from treated animals. Reasonable efforts should be made to identify all metabolites and propose a comprehensive metabolic scheme for the test drug [2]. Structural confirmation of a metabolite along with methods validated should be provided whenever possible. Techniques such as mass spectrometry, nuclear magnetic resonance (^{3}H-NMR and ^{13}C-NMR are used for characterizing position and grouping of H and C, respectively), infrared spectroscopy (characterizing functional groups) and elemental analysis are used for this purpose. Metabolites that might elicit a pharmacological or toxicological response (especially those Phase I products) can be synthesized and tested in appropriate animal models. Some novel drugs have thus been discovered during metabolite characterization of drug candidates. These new compounds usually have attributes, such as better extent of delivery, disposition kinetics, clearance and/or less potential for accumulation, which make them better drug candidates in terms of safety and effectiveness than their parent compounds.

For metabolites detected in humans as well as in animal species, adequacy of systemic exposure should be assessed by measuring the concentration of the test drug in serum or plasma versus

time curve (AUC) [29–31]. The AUC is the most comprehensive pharmacokinetic endpoint, because this value includes both the plasma concentration of the drug candidate and the residence time *in vivo*. In the assessment of exposure, scientific judgment should be used to determine whether the AUC comparison should be based on data for the parent, parent and metabolites, or metabolites. Interspecies differences in clearance of a drug and its major metabolite are an important factor resulting in the interspecies differences in the AUC values of the drug. It is generally believed that the interspecies differences in renal clearance of any unbound free drug are related to interspecies differences in glomerular filtration rate (Table 2) [32,33]. The latter differences are mainly caused by the interspecies differences in the relative number of glomeruli (or nephrons) per kg body weight. Clearance rate varies in the direct proportion to the glomerular filtration rate of the unbound free drug. As a result, a small animal may show clearance of a free drug somewhat faster than a large species. Interspecies differences in protein binding should also be taken into consideration when relative exposure is estimated, and human pharmacokinetic data are obtained from studies encompassing the maximum recommended human daily dose.

The use of maximum feasible dose is often applicable to the studies of dietary drugs, amino acid derivatives [28] and energy boosters. In these cases, the maximum dose may be limited because of practicality, local tolerance, or saturation of oral absorption. Information that the absorption process has been saturated using the clinically intended route of administration is necessary. These data can usually be obtained during well-designed pharmacokinetic studies that evaluate linearity of absorption and dose proportionality using the route and frequency of dosing projected for human clinical studies. A turning point, which indicates no further linear increase in the AUC corresponding to the increasing doses of the test drug, can be defined as the lowest saturating dose [28]. When saturation of drug absorption occurs at a dose that produces no toxicity in animals, the lowest saturating dose, not the highest (non-toxic) dose should be used for calculating the human equivalent dose [34].

In vivo characterization of metabolic phenotyping of a test drug relies on its *in vitro* data and administration of CYP probes. Midazolam case is a good paradigm to follow [35]. Midazolam administered orally is metabolized exclusively via CYP3A and possesses all of the attributes of an ideal P450 probe substrate. An oral dose of 2 mg of midazolam was used to investigate *in vivo* inhibitory effect of the test drug, and changes in the plasma AUC of oral midazolam were used to measure the degree of inhibition of the test drug on the CYP3A activity (Table 3). Although the current classification system is based on effects of the test drugs on the plasma AUC of orally-administered midazolam, it may be possible in the future to measure, based on the similar system, the effects of the test drug on midazolam plasma concentration at a single time point, e.g., at 4 h post dose [35].

In summarizing the ADME studies, notable interspecies differences and similarities in pharmacokinetic and toxicokinetics can be appropriately interpreted by using the equivalent surface area dosage [2,28,36], not by the body weight dosage. The conversion factors used for changing a dose expressed in terms of mg/kg to an equivalent surface area dose mg/m^2 are 3 for mouse, 6 for rat, and 20 for dog, respectively, referring to the FDA guidance [34]. The useful conversion factors are summarized in Table 4. Interspecies differences in CYP enzyme induction or inhibition in relation to metabolite production should be discussed. Any references to observed species differences in toxicity and extrapolation of the findings to humans should be mentioned.

First Pass Drug Loss in Liver

Interspecies differences in liver metabolism of drugs result in marked dissimilarity in the blood levels of test drugs in different species [37]. These interspecies differences in the first pass in the liver have been implicated as a primary cause of bioavailability differences between humans

and dogs [38]. Uniqueness of each species' metabolic profiles must be considered when evaluating a compound's toxicity profile in liver. It was noted that [39] the overall concordance for human toxicity assessment was 17% when requiring that toxic events be observed in two or more animal species (rodent and non-rodent). In contrast, a 43% concordance was observed with rodents alone and a 63% concordance observed with non-rodent species alone. In 40–60% of the cases, animal models did not adequately predict human toxicity. Although it is desirable to identify a species which metabolizes the test drug like man, this ideal is often difficult to attain.

Intestinal Metabolism

The anatomical location of enzymes in the gut wall provides an important and highly sensitive site of metabolically based interactions with orally administered drugs [40]. The principle intestinal biotransformation enzymes responsible for Phase I reaction include CYP, esterases, epoxide hydrolase and alcohol dehydrogenase, and for Phase II reaction glucosyltransferases, sulfotransferases, N-acetyltransferases, glutathione S-transferase [40]. Intestinal gut wall enzymes exhibit highest activity at the villous tips. The activity decreases progressively towards the crypts. In addition, heterogeneity in enzyme concentration can be found between proximal to distal segments with enzyme concentrations tending to decrease from upper to lower small bowel [41,42]. Both large and small bowel segments contain multidrug resistance proteins [43]. As demonstrated in knockout mice and rats, p-glycoproteins (P-gp) are likely to be responsible for inhibiting the passage of many drugs across apical membrane surfaces [44,45]. These multidrug resistance proteins tend to be most prevalent at the brush border membrane of the villus tips, and can be found throughout the large and small intestine. In fact, the concentration of P-gp tends to increase from stomach to colon [43].

The action of efflux protein markedly affects the intestinal drug metabolism of a variety of drugs. Functioning in a complementary manner with the CYP family, these proteins maximize the opportunity for drug exposure to the metabolizing enzymes, pump drugs out of the mucosal cells and back into the intestinal lumen [46]. This interaction is facilitated by its co-localization at the apical membrane of the cells expressing CYP enzymes such as CYP3A. However, not all substrates for CYP3A act as substrates for P-gp [47].

Limited Luminal Degradation

In contrast to the oxidative and conjugative metabolism of drugs in the liver and intestinal mucosa, bacterial metabolic reactions are largely degradative, hydrolytic and reductive. As such, they are involved in the enterohepatic recirculation of many compounds. Drugs conjugated with polar groups in the liver prior to their secretion into the bile are hydrolyzed within the upper and lower intestine. β-Glucuronidase, sulfatase, and glycosidases are all bacterial enzymes found in the gut of human and domestic animal species [48,49]. Interspecies differences in the activity and location of β-glucuronidase have raised questions regarding interspecies extrapolation of bioavailability and toxicity data for certain compounds.

Although bacterial metabolism can have a critical role in both digestion and drug absorption, intestinal microflora have a relatively minor role in the metabolism of most drugs. In part, this is due to the limited number of microorganisms that can only exist within the low pH of the stomach, jejunum and upper ileum. Hill and Draser have provided the information about the bacterial species and organism concentrations found in the human gastrointestinal tract [50].

Colonic bacterial degradation may be the critical step in drug absorption. The colon serves as the primary site of absorption for active metabolites. An excellent example of this is sulphasalazine, which is metabolized by colonic microflora to sulphapyridine prior to absorption [51]. The colon contains the largest populations of microorganisms in the

monogastric gastrointestinal tract and is the major site of production and absorption of volatile fatty acids in sheep, pig, rabbit, rat, dog, and human [52]. It is also a major absorption site for drug absorption in horse. Owing to the dietary similarities between man and dog, it is not surprising that the intestines of these two species are populated by similar colonic flora [53, 54].

CONCLUSION

We present herein a number of practical, theoretical and regulatory consideration for those who design, review and conduct *in vivo* drug metabolism studies for late drug development using various advanced analytical methods. Although sensitivity, scanning, and data acquisition speed of analytical technology have increased exponentially over the past years, a good understanding of systems biology and regulatory requirements in conjunction with logical study designs including proper utilization of analytical instruments, sample timing and handling, as well as final data integration and comprehensive explanations could ensure a correct scheme of metabolism pathways for a test drug.

ABBREVIATIONS

ADME
: Absorption distribution metabolism elimination

APCI
: Atmospheric pressure chemical ionization

AMS
: Accelerator mass spectrometry

AUC
: Area under curve

CYPs
: Cytochrome P450 enzymes

DMSO
: Dimethyl sulfoxide

ESI
: Electrospray ionization

EPA
: Environmental Protection Agency

FDA
: Food and Drug Administration

IT-TOF
: Ion trap and time-of-flight

LC
: Liquid chromatography

MS
: Mass spectrometry

MRM
: Multiple reaction monitoring

MS/MS
Tandem mass spectrometry

NMR
Nuclear magnetic resonance

PET
Positron emission tomography

P-gp
P-glycoprotein

QQQ
Triple-quadrupole mass spectrometers

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Table 1

Possible Metabolic Reactions and Related Changes in Mass [2,6,19]

Metabolic reactions	Change in mass (u)
Demethylation	−14
Oxidative desulfuration	−32
Carbon hydroxylation	+16
Epoxidation	+16
N-hydroxylation	+16
N-oxidation	+16
S- or N-nitrosylation	+30
Phosphorus oxidation	+16/17
Glucuronidation	+176
Sulfation	+80
Methylation	+14
Acetylation	+42
Amino acid conjugation:	
Glycine	+57
Glutamine	+145
Taurine	+107

Table 2

Interspecies Comparison of Glomerular Filtration Rate (GFR) [32]

Species	Body weight (kg)	GFR (ml/min/kg)	No. of glomeruli/kg body wt.
Mouse	0.02	10.0	5.9×10^5
Rat	0.265	8.7	2.9×10^5
Rabbit	2.5	4.8	1.6×10^5
Dog	10	4.0	0.9×10^5
Monkey	5	2.0	-
Human	70	1.8	0.29×10^5

Table 3

Classification of Test Drugs for their *In Vivo* Inhibitory Effect on CYP3A [35]

Inhibitory categories of test drugs	Fold increase in plasma AUC of midazolam*
Strong inhibition	≥ 5
Moderate inhibition	>2 but <5
Weak inhibition	≤ 2
No inhibition	~1

* Midazolam, a typical substrate of CYP3A, is given orally at 2 mg.

Table 4

Conversion of Animal Doses to Human Equivalent Doses (HED) Based on Body Surface Area [34]*

Species and reference body weight	To convert animal dose in mg/kg to dose in mg/m^2 multiply by km**
Human, Adult (60 kg)	37
Child (20 kg)	25
Mouse (0.02 kg)	3
Hamster (0.08 kg)	5
Rat (0.15 kg)	6
Ferret (0.3 kg)	7
Guinea pig (0.4 kg)	8
Rabbit (1.8 kg)	12
Dog (10 kg)	20
Primates:	
Monkeys (3 kg)	12
Marmoset (350 kg)	6
Squirrel monkey (600 kg)	7
Baboon (12 kg)	20
Micro-pig (20 kg)	27
Mini-pig (40 kg)	35

*For example, in adult human of 60 kg, 100 mg/kg is equivalent to 100 mg/kg × 37 kg/m^2 = 3700 mg/m^2. For species not listed or for weights outside the standard ranges, human equivalent dose can be calculated from the formula: HED= animal dose in mg/kg × (animal weight in kg/human weight in kg)$^{0.33}$

**Km stands for surface area to weight ratios.