Chemical Research in Toxicology

Review

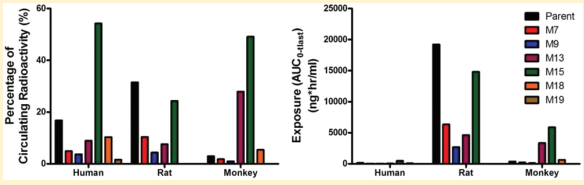
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Radiolabeled Absorption, Distribution, Metabolism, and Excretion Studies in Drug Development: Why, When, and How?

Natalia Penner, Lin Xu, and Chandra Prakash*

Department of Drug Metabolism and Preclinical Safety, Biogen Idec, Cambridge, Massachusetts 02142

ABSTRACT: Absorption, distribution, metabolism, and excretion (ADME) studies are an integral part of the comprehensive safety evaluation of a new molecular entity, and they represent a standard suite of studies included in the registration package for all new small molecule drugs. In vivo studies in preclinical toxicology species and humans using radiolabeled (^{3}H or ^{14}C) compound provide quantitative assessments of overall routes of excretion of drug-related material, pharmacokinetics of total drug-derived radioactivity in circulation, relative to parent compound and quantitation, and characterization of metabolites in excreta and circulation. These data serve as the starting point for metabolite in safety testing (MIST). These studies involve the administration of a radiolabeled drug to laboratory animals and humans followed by a quantitative collection of excreta and blood. Using appropriate plasma-pooling strategies, these studies could allow for modeling the metabolite exposure at the steady state. Information from the radiolabeled human study is used to design clinical drug−drug interaction (DDI) studies and to obtain a waiver for bioequivalence studies. This article describes the various aspects of conducting ADME studies and the use of radiolabeled analogues of drug candidates to investigate their metabolism and how to compare the exposures of metabolites in humans and toxicology species.



CONTENTS

1. Introduction 513
2. Conduct of Radiolabeled Absorption, Distribution, Metabolism, and Excretion (ADME) Studies 514
   2.1. Objectives of Radiolabeled ADME Studies 514
   2.2. Choice of Radioisotopes and Their Position in the Drug Molecule 515
   2.3. Standard or Typical Studies with Radiolabeled Compounds 515
   2.4. Sample Analysis 517
3. Utilities of Radiolabeled Studies in Development 517
   3.1. Characterization of Absorption and Routes of Excretion 517
   3.2. Metabolism/Clearance Mechanism 518
   3.3. Characterization of Unusual and Unexpected Metabolites 519
   3.4. Covalent Protein Binding (CPB)/Reactive Metabolites 519
   3.5. Distribution/Tissue Targeting 521
   3.6. Human MIST 522
   3.7. Microdosing/Microtracer Studies 524
   3.8. Bioequivalence Waiver 525
4. Limitations of Radiolabeled ADME Studies 525
5. Regulatory Requirements and Their Effect on the Timing of Radiolabeled Studies 525
6. Summary 527
Author Information 527
   Corresponding Author 527
   Notes 527
Acknowledgments 527
Abbreviations 527
References 527

1. INTRODUCTION

Drug discovery, lead candidate selection, and preclinical and clinical development are undergoing rapid changes, which are driven by scientific advances in areas such as combinatorial chemistry, molecular and cell biology, and high-throughput technology as well as by strong competition, intense regulatory scrutiny, and economic forces. In early discovery, in silico models,[1−4] in vitro studies using animal and human hepatocellular and subcellular fractions and/or recombinant enzymes,[5−8] and in vivo studies using a range of experimental animal models[9−11] are used to predict the disposition of new molecular entities (NMEs) in humans. In vitro studies provide the cross-species comparison of metabolic profiles, which may provide guidance for the selection of animal species used for safety evaluation to ensure that the selected animal species are exposed to all major human metabolites.[6,7] These in vitro systems (microsomes, S-9, and hepatocytes) can often provide a good correlation with in vivo metabolic profiles, but their capacity to do so is inevitably limited, as evidenced by recently published investigations of in vitro−in vivo correlations.[6,7]

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This is particularly true when a NME undergoes in vivo metabolism via multiple sequential transformations prior to excretion of the drug-related material.

Using in vivo/in vitro techniques, human pharmacokinetic (PK) predictions can now be achieved with reasonable accuracy (within a ±2-fold range for up to 80% of compounds).[11−13] However, such a success rate is often insufficient to discriminate between the performances of closely related drug candidates in one chemical series.[13] To address these issues, innovative and relatively inexpensive approaches such as "microdose" (or subpharmacologic dose) and low pharmacologically active dose studies (PADS) have been used for early characterization of the PK and PD of NMEs.[14,15] Human PK parameters such as clearance, volume of distribution, absorption, and absolute bioavailability of a NME have been determined after an intravenous (iv) administration of a radiolabeled tracer dose with an extravascular dose given at a therapeutic dose level.[16−19] These approaches allow smarter, faster, and more cost-effective selection of the drug candidates. However, such studies require highly sensitive analytical methods such as positron emission tomography (PET) or accelerator mass spectrometry (AMS).[16−20]

There is no doubt that modern analytical technologies and in vitro metabolism techniques have enabled detailed characterization of metabolites from studies with unlabeled material, which was not possible earlier.[21] However, such early metabolism studies remain semiquantitative and noncomprehensive in nature and can only be viewed as the first step in the overall metabolite characterization process. The recent regulatory guidance on metabolites in safety testing (MIST) has highlighted not only the importance of the identification of drug metabolites but also their quantitation to ensure that human metabolites have adequate coverage in toxicology species used for long-term safety assessment.[22,23] Accurate quantification of metabolites from in vitro or label-free clinical studies often is very challenging when authentic metabolite standards are not available, especially for highly metabolized or low-dose drugs. A simple peak area ratio comparison approach (UV or MS) is the most facile and applicable approach to make a first assessment of whether metabolite exposures in animals exceed that in humans. However, because of differences in UV absorbance and/or MS ionization efficiency of metabolites and parent drug, care must be exercised in quantitative assessment of metabolites. Plasma-pooling methods, combined with NMR (^{1}H or ^{19}F) and LC/UV/MS, high-resolution MS, and radiolabeled calibrants have also been used to estimate the exposure of circulating metabolites in preclinical toxicology and clinical studies. The use of radioactivity and radiochemical detection remains an excellent approach for generating quantitative data on metabolites, as well as detecting drug-related components in complex matrices.

In vivo studies in preclinical species and human volunteers employing a radiolabeled compound allow full elucidation of absorption and route(s) of elimination, PKs of the total drug-related material, and metabolic profiles.[24] Using appropriate plasma-pooling strategies, these studies could estimate the metabolite exposure at the steady state.[24] Such radiolabeled studies are critical in a drug development program and required by health authorities around the world for a new drug registration application. Therefore, no matter which way of obtaining metabolism information is chosen, the sponsor is still obligated to conduct radiolabeled studies in preclinical species and humans. Information from the radiolabeled human study can be used to design clinical drug−drug interaction (DDI) studies and to obtain a waiver for bioequivalence studies. However, there is no clear guidance for conducting these complex studies. In this article, we will describe various aspects of conducting such studies and the use of radiolabeled drugs to investigate their metabolism. In addition, this article will describe how data from these studies can provide information on the coverage of human metabolites in toxicology species.

2. CONDUCT OF RADIOLABELED ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION (ADME) STUDIES

2.1. Objectives of Radiolabeled ADME Studies. Radiolabeled studies also known as ADME studies are required by regulatory authorities for the registration of a new drug and, therefore, are an integral part of the majority of drug development programs. These studies are usually conducted in at least two preclinical species (rodent and nonrodent), which are used for long-term safety assessment of the drug and in most cases in humans as well. Quantitative whole body autoradiography/whole body autoluminography (QWBA/WBAL) studies in preclinical species must be performed before administration of a drug candidate to humans to ensure its safety and to calculate the maximum radioactive dose that can be administered to humans. QWBA/WBAL studies also provide information on the distribution, accumulation, and retention of drug material. In addition, metabolic profiles in mouse and female rabbit may be required to support carcinogenicity and reproductive toxicology studies, respectively.

The primary objectives of radiolabeled ADME studies are as follows:

- To determine mass balance, which is to compare the amount of administered radioactivity to the amount of radioactivity recovered in excreta
- To determine routes of elimination and the extent of absorption
- To identify circulatory and excretory metabolites
- To determine clearance mechanisms
- To characterize the distribution of the compound into tissues and organs (QWBA/WBAL)
- To determine the exposure of the parent compound and its metabolites
- To help validate the animal species used for toxicological testing
- To explore if metabolites contribute to the pharmacological/toxicological effects

The results from radiolabeled studies can be used to understand the behavior of a drug molecule. For example, understanding the clearance mechanism (renal, biliary, or metabolic) is important in providing recommendations to physicians treating hepatically or renally impaired patients. If the majority of a drug is excreted in urine, its exposure in patients with kidney disease could be altered significantly.

Comparison of PKs of unchanged drug and circulating drug-derived material provides the extent of metabolism and whether or not the parent compound is the major circulating component. Long-lived plasma radioactivity together with low levels of the parent drug indicate the presence of metabolite(s) with a long half-life. Such metabolites will accumulate upon

multiple dosing, which in turn may change the pharmacological and toxicological profile of a drug. Comparison of PK profiles of drug-derived material after oral and iv administration also could provide insight about absorption.

2.2. Choice of Radioisotopes and Their Position in the Drug Molecule. The choice of the radioisotope, its position in the drug molecule, the radiochemical purity, and the specific activity are very important parameters in designing the radiolabeled studies. There are several radioisotopes that can be used. However, labeling compounds with carbon-14 or H-3 (tritium) is by far the most widely used technique since carbon and hydrogen atoms are present in all drug molecules.[25] These isotopes are also long-lived; therefore, results of radioanalysis do not require correction for the isotope half-life.

The use of tritium label is attractive for many reasons; most importantly, a synthesis of tritium-labeled compounds is relatively simple and fast. Second, a tritium-labeled compound can be synthesized with a very high specific activity, which is useful for studying the highly potent compounds at a low dose. Unfortunately, the advantages of tritium use are outweighed by the drawbacks such as biological instability and isotopic effect (switching biotransformation pathway due to reaction kinetics).[26] When the tritium-labeled position is involved in biotransformation, tritium can be lost as the tritiated water.[27] Partial loss of the label can also complicate the quantitative analysis of metabolites. Therefore, unless the synthesis of ^{14}C-labeled compound is extremely difficult or impossible, the use of tritium is limited only to protein and ligand binding studies. Tritium-labeled compounds can be used to obtain biotransformation data in toxicology species before first-in-human (FIH) studies in late discovery/early development when investment in ^{14}C synthesis is usually not justified. In cases where the molecule splits into two large fragments by either oxidative cleavage or hydrolysis of the ester and amide bonds, it may be necessary to label the molecule at two different positions or use a mixture of two different radioisotopes. According to a recent informal survey of 15 companies of different size, ^{14}C-labeled compounds are used in the majority of radiolabeled ADME studies (80%), only two companies are using tritium, and one company uses both of the isotopes. Several companies use tritiated compounds in early ADME studies, but in most cases, these studies are repeated in development stage with a ^{14}C-labeled drug.

2.3. Standard or Typical Studies with Radiolabeled Compounds. Study design and a sample collection schedule should be chosen to achieve the objectives of the study. These objectives usually include full characterization of routes of elimination, characterization of metabolic profiles, and identification and quantitation of circulating and excreted metabolites. Mass balance studies are generally conducted in two species (rodent and large animal) used for long-term toxicological assessment of the drug. In most cases, rats are used as the rodent species. Dogs are preferred as large animal species due to their availability and cost; monkeys are selected only when the use of dogs is not justified, for example, due to unsatisfactory exposure or difference in metabolic profile. Occasionally, minipigs are used as nonrodent toxicology species, although their use is more common in Europe.

If the drug intended for chronic administration progresses beyond phase I, information about its disposition and metabolism in additional preclinical species may be required. A radiolabeled study in mice is conducted to support carcinogenicity assessment, while information from studies in female rabbits is used in embryo fetal development evaluation. In both cases, the main goal of the study is to confirm that the circulating and, to a lesser extent, excretory metabolites in these species are representative of humans.

Dose administration and sample collection for typical radiolabeled studies conducted in various species including humans are presented in Table 1. For the majority of drugs, ADME studies are conducted as single-dose studies; however, a few examples of multiple dose administration of a radiolabeled compound are reported.[28] For example, the absorption and distribution of radioactivity were investigated following a 14 day period of daily oral administration of ^{14}C-pramipexole (0.5 mg/kg/day) to male rats. The level of radioactivity in plasma was increased with each dose and reached a steady state after 12−13 doses. The elimination of radioactivity from the

Table 1. Typical Radiolabeled Studies Objectives, Design, and Sample Collection[a]

species	study objectives	no. of subjects	sample collection
rat	mass balance/excretion routes; identification and quantitation of excreted metabolites	3/gender	urine and feces: 0−12, 12−24, and every 24 h interval up to 168 h
	biliary excretion and metabolite identification	3/gender	bile: 0−8, 0−24, or 0−48 h
	PKs of parent drug and total radioactivity	3/gender	blood: 6−8 time points
	identification and quantitation of circulating metabolites	2/gender/time point	"terminal" blood: 4−6 time points
	tissue distribution (QWBA)	1/gender/time point	blood and selected tissues at 6−8 time points
dog	mass balance/excretion routes; identification and quantitation of excreted metabolites	2−3/gender	urine and feces: 0−12, 12−24, and every 24 h interval up to 168 h
	biliary excretion and metabolite identification	2	bile: 0−8, 0−24, or 0−48 h
	PKs of parent drug and total radioactivity	NA	blood: 6−8 time points
	identification and quantitation of circulating metabolites	NA	additional blood at 4 time points
human	mass balance/excretion routes; identification and quantitation of excreted metabolites	4−6	urine and feces: 0−12, 12−24, and every 24 h interval up to 168 h
	PKs of parent drug and total radioactivity	NA	blood: 8−12 time points
	identification and quantitation of circulating metabolites	NA	additional blood at 4−6 time points
mouse	identification and quantitation of excreted metabolites	3−5/gender	urine and feces: 0−12, 12−24, and 24−48 h
	identification and quantitation of circulating metabolites	2/gender/time point	"terminal" blood: 4−6 time points
rabbit	identification and quantitation of circulating metabolites	2/gender/time point	blood: 4−6 time points

[a]NA, not applicable.

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plasma after the last dose was similar to that after a single administration.[29]

The duration of the in-life phase of a mass balance/biotransformation study is usually between 72 and 168 h (~70% of surveyed companies) but can be shortened or extended depending on the compound's disposition properties. In most cases, 168 h is sufficient to collect ~90% of the administered radioactivity. To fully characterize the excretion routes and the extent of absorption, bile samples are routinely collected from rats, in addition to urine and feces. The combined percent of dose excreted in bile and urine after oral administration is a good measure of drug absorption if an iv arm of the ADME study is not conducted. Bile duct cannulated (BDC) nonrodent species are often used in an ADME study when the majority of dose is excreted in the feces and the level of absorption is not clear. The collection interval for BDC animals rarely exceeds 48 h.

To fully characterize the absorption of an oral drug candidate, an iv arm is recommended for inclusion in the ADME study design. Oral absorption is then calculated as a ratio of either AUC of total radioactivity (TR) or the amounts of TR excreted in the urine after oral and iv doses as shown (eqs 1 and 2):

$$\text{absorption (Fa)} = \frac{A_{\text{total percent radioactivity in urine after po dose}}}{A_{\text{total percent radioactivity in urine after iv dose}}} \quad (1)$$

$$\text{absorption (Fa)} = \frac{\text{AUC}_{\text{total radioactivity after po dose}}}{\text{AUC}_{\text{total radioactivity after iv dose}}} \times \frac{\text{dose}_{\text{iv}}}{\text{dose}_{\text{po}}} \quad (2)$$

Shukla et al. and Barbhaiya et al. have demonstrated the differences in the extent of absorption of drug-related material and the systemic bioavailability of nefazodone in humans and dogs after oral and iv administration of [^{14}C]nefazodone.[30,31] Plasma was analyzed for nefazodone and its metabolites, and plasma, urine. and feces were analyzed for TR. The bioavailability of nefazodone was estimated as 14 and 15% in dogs[30] and humans,[31] respectively. The recovery of TR in urine and feces was similar after po and iv administration for both dogs and humans, indicating complete absorption of the drug after oral administration. This indicated that the low bioavailability in dogs and humans was due to first pass metabolism rather than poor absorption.

A complete plasma concentration−time profile is collected for up to five half-lives of the parent compound to characterize TR and the unchanged drug in blood/plasma or serum as well as major metabolites. Plasma collection for metabolite profiling depends on the pooling approach used and will be discussed below. Study features characteristic of each species used in radiolabeled studies are outlined below.

2.3.1. Mass Balance and Excretion. *2.3.1.1. Rats.* An ADME study in rats is usually the most comprehensive study, which can include po and iv arms, administration to both genders, collection of excreta for mass balance, and bile collection from the BDC animals. In most cases, plasma samples are collected terminally from 2 animals/gender/per time point. If serial blood samples are collected for PK analysis, additional animals are dosed to collect a sufficient amount of blood/plasma for metabolite profiling and occasionally to evaluate for blood/plasma partitioning.

2.3.1.2. Dog/Monkey. As mentioned above, dogs are the preferred large animal species due to their availability and cost, while monkeys are selected only when the use of dogs is not justified due to unsatisfactory exposure or metabolic profile. Differences in pharmacologic responsiveness are also important considerations; for example, if a species does not have the target pharmacologic receptor or biochemical pathway or shows hypersensitivity in early toxicology studies, this species is not appropriate for further toxicity assessment. For oral drugs, both males and females are usually dosed orally; an iv arm is optional but useful addition to the study. Serial blood samples are collected at several time points for PK analysis (parent and TR). Samples from selected time points are then used for metabolite profiling. The volume of blood collected for these time points can be increased to achieve the necessary detection limit for radioactivity profiling.

2.3.1.3. Mice. ADME studies in mice are conducted to ensure that all human metabolites are covered for carcinogenicity assessment. Only one route of administration, po or iv, depending on the intended route of administration in a clinical setting, is usually utilized. Multiple mice are housed together (~3−5 per cage), and combined excreta samples are analyzed to evaluate the excretion pattern. Terminal blood samples are usually collected from multiple mice for each time point; occasionally, bile from the gallbladder is also collected from these animals for biliary metabolic profile assessment.

2.3.1.4. Rabbits. The metabolism study in rabbits is conducted in females only to evaluate metabolic coverage for embryo fetal development assessment. A full mass balance characterization in rabbits is not required. Serial plasma sample collection from 2 to 4 rabbits with subsequent PK analysis for TR, the parent concentration, and metabolic profile is sufficient. Only one route of administration, po or iv, depending on the intended route of administration in a clinical setting, is usually utilized.

2.3.1.5. Humans. A radiolabeled study in humans is one of the core studies in drug development programs and still remains the gold standard for understanding the metabolism of a drug candidate. In human studies, the route of administration will usually be the same as that intended for the drug's clinical use, because the formation rate of metabolites and extent of metabolism may differ by administration route. Oral administration is used in the majority of radiolabeled human studies; guidelines to support dermal administration are being discussed with the FDA.

The study is usually conducted in healthy adult volunteers except for oncology programs where only patients should be used, especially in the case of cytotoxic drugs. A review focusing on mass balance studies of anticancer drugs was recently published.[32] Generally, only males are included in the study to obtain a homogeneous group of subjects. The study may be conducted in females of nonchild-bearing potential when the target population is predominately females. Four subjects are generally sufficient; however, up to 6−8 subjects may be used depending on the PK characteristics of the compound [e.g., those with high PK variability or a specific population such as smokers/nonsmokers; CYP2D6 extensive metabolizers (EMs)/poor metabolizers (PMs), etc.]. In the latter cases, the objective of increasing the number of study subjects is *not* to gain statistical differentiation between populations but rather to gain a better understanding of their potentially different excretory and metabolic profiles.

On the basis of a recent informal survey of 15 pharmaceutical companies, only ^{14}C-labeled test articles are used in radiolabeled human studies. A single dose of a radiolabeled drug is administered in the majority of human studies (~70%); only ~30% of sponsors occasionally use multiple dosing. The radiolabeled

dose of the drug can also be administered after multiple doses of unlabeled test article as in the case of tigecycline,[33] which has been shown to bind to the bone. To minimize the amount of radioactivity binding to the bone and to maximize the recovery of radioactivity, [^{14}C]tigecycline was administered after six doses of unlabeled tigecycline.

The sample collection schedule should be chosen to fully characterize routes of elimination and metabolic profile and to identify the circulating and excreted metabolites. Specific details on how to design ^{14}C-human ADME studies have been described in the literature.[24] Typical sample collection schedules are shown in Table 1. The duration of subject confinement and sample collection can vary between 7 and 10 days until ~90% of administered radioactivity is excreted and/or excreta contain less <1% of the administered radioactivity for two consecutive sample collections. In terms of addressing the MIST requirements, the guiding principles for collecting blood samples from ^{14}C-human ADME studies in general follow the same rules as for the animal studies. The specific time points and sample volume requirements for metabolite profiling will depend on the pooling approach used.[24]

2.3.2. Quantitative Whole-Body Autoradiography (QWBA)/Whole-Body Autoradioluminography (WBAL). Drug regulatory agencies require that tissue distribution studies should be conducted in laboratory animals (rats) as a prerequisite for projecting tissue exposure to radioactivity before administration of radioactive drugs to humans. The objectives of this study are to determine the radioactivity in each organ of interest after administering the radiolabeled drug to animals. The concentration of drug-related material (TR) is determined by QWBA/WBAL or liquid scintillation counting in tissues of interest. The majority of companies (>90%) use rats, while only one company uses dogs for tissue distribution studies.

Table 1 shows a typical experimental design for the tissue distribution study. Each rat receives a single oral or iv dose of a radiolabeled drug of preferably high specific activity (~160 μCi/kg). There are inconsistencies among pharmaceutical companies in the design of these studies especially related to the number of time points used to determine the PK parameters for tissues. In general, time points are at T_{max}, two additional time points up to 24 h, then on days 3 and 7 and often on day 28 to check for residual radioactivity. For iv administration, additional early time points (e.g., 5 and 30 min and 1 h) are recommended. At each time point, one rat is anaesthetized, and a blood sample is obtained via cardiac puncture. The animal is then euthanized by overdose of CO_2, and the intact carcasses are frozen and cryosectioned. The whole body sections, which represent all tissue, are measured for radioactivity contents qualitatively and quantitatively using the phosphor imaging technique. Because it generally takes weeks for sample processing and measurement, long-lived radiolabels such as ^{14}C and ^{3}H are often chosen for the study. Whole blood and plasma are also analyzed for TR using liquid scintillation counting techniques. The selected tissues are processed for radioactivity concentration analysis by QWBA/WBAL.

2.4. Sample Analysis. *2.4.1. Quantitation of Radioactivity.* Scintillation counting is used for the analysis of plasma and excreta. Samples at each time point or time interval are usually counted in triplicate. Feces are first mixed with water in a ratio of 1:2 or 1:3 (w:v) and homogenized. Fecal homogenates are combusted in a biological sample oxidizer, and the liberated $^{14}CO_2$ is trapped in a suitable scintillation cocktail and counted. The radioactivity in urine and fecal homogenate is reported as the percentage of the administered radioactivity excreted at each time interval and as the total percent of dose excreted (cumulative recovery). The mass balance of administered radioactivity is determined by the summation of the "% of dose" excreted in both urine and feces during the study period.

2.4.2. Metabolite Profiling. Understanding of the metabolite profiles of a drug candidate in animals and humans is essential to ensure that animal species used for its toxicological evaluation are appropriate models of humans. HPLC coupled with online or off-line radioactivity detection and mass spectrometry have become the method of choice for profiling metabolites in radioactive samples. These samples usually require long (up to 90 min) HPLC methods to achieve separation of all major metabolites with peak widths sufficient for radiometric detection. HPLC-MS/radiometry can provide simultaneous acquisition of radiochromatographic profiles for quantitative analysis and mass spectra for structural elucidation. The effluent from a HPLC column, after UV detection (optional, but useful), is split to direct approximately 10−20% of the flow into a mass spectrometer, while the majority of the flow goes into a radiometric detector such as accurate radioisotope counting (ARC, AIM Research Co., Hockessin, DE) or β-RAM (LabLogic Systems, Inc., Brandon, FL) flow scintillation analyzer (FSA) or to a fraction collector. Microplate scintillation counting (MSC) is a viable alternative to online radiometric detection.[34,35] Fractions of the column effluent are collected into 96- or 384-well plates containing solid scintillant and counted using MSC. When using online radiometric detector, the radiochromatographic profile is available immediately after an analytical run is completed. Off-line methods require longer time for generating a radiochromatogram (due to fraction collection, plate drying, and scintillation counting) but offer several advantages such as higher sensitivity, ability to profile samples with low radioactivity, increased S/N ratio, and a possibility to reconstitute collected fractions and analyze them by a mass spectrometer.

The amount of radioactivity in a sample defines which method of radioprofiling should be used. The sensitivity of detection via online radiometry is relatively good when 10000−25000 disintegrations per minute (DPM) of sample are analyzed. However, if the amount of radioactivity in the sample is below 1000 DPM, a more sensitive detection method is required. Analysis by MSC may result in a well-defined chromatogram, which is acceptable for quantitative analysis and similar to one obtained with the use of FSA after injecting a much higher amount of radioactivity.

3. UTILITIES OF RADIOLABELED STUDIES IN DEVELOPMENT

3.1. Characterization of Absorption and Routes of Excretion. Mass balance studies are routinely used to characterize the excretion pattern, identify the mechanisms of clearance, and define the rate of elimination by renal or biliary/fecal routes. They can also be used to estimate the extent of absorption, even without an iv arm, which is especially important when urinary excretion of the compound is low.

The extent of absorption of lasofoxifene was investigated in rats, monkeys, and humans after administration of a single oral dose of radiolabeled compound.[36,37] The majority (66.1−95.6%) of the administered radioactivity was recovered in the feces, suggesting that fecal was the primary route of elimination of the radioactivity in these species. The low recovery (1.52−6.39%) in the urine suggests poor absorption. However, in a

separate BDC study in rats, 83% of the administered radioactivity was excreted in the bile following an oral administration. These data suggest that at least 85% of the dose (% recovery in urine and bile) was absorbed and that the fecal excretion in intact animals likely reflects excretion in the bile rather than incomplete absorption. The data obtained in rats were used to confirm that lasofoxifene is also well absorbed in monkeys and humans (Table 2).

Table 2. Percent of Dose Excreted in Urine and Feces or Bile of Rats, Monkeys, and Humans after Oral Administration of Radiolabeled Lasofoxifene

		% of radioactive dose			
animal	dose (mg/kg)	urine	bile	feces	total
male rats	1	1.92	NA	94.5	96.4
female rats	1	1.52	NA	95.6	97.1
male rats	1	ND	86.9	ND	86.9
female rats	1	ND	79.1	ND	79.1
male monkey	1	1.89	NA	95.2	97.1
female monkey	1	1.93	NA	89.7	91.6
human	0.28	6.39	NA	66.1	72.5

Mass balance studies are useful in the determination of differences in absorption, excretion, and metabolism between normal and PMs as demonstrated in the case of desloratadine (DL).[38] DL is well absorbed in five out of six subjects (T_{max} = 5−8 h) (Figure 1). Subject 5 showed slower absorption

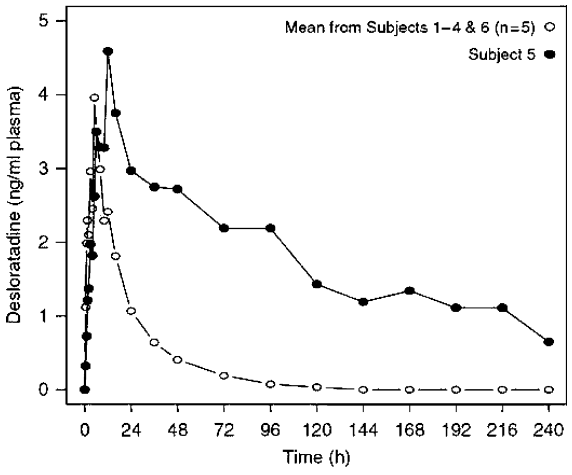


Figure 1. Plasma−concentration time curves for DL in humans. Reprinted with permission from ref 38. Copyright 2007 Informa Plc.

(T_{max} = 12 h) with a considerably slower elimination rate. The systemic exposure of DL in subject 5 was 5.6 times higher than for the rest of subjects. The elimination of radioactivity in urine and feces was also slower for subject 5, leading to a much lower cumulative recovery of radiocarbon as compared with each of the other five subjects. The plasma concentrations of DL and radioactivity reflect the slower rate of DL absorption and metabolism in this subject, which was confirmed by the metabolite profiling studies. In fact, DL was extensively metabolized in all subjects except subject 5, where the majority of profiled excreted radioactivity was represented by unchanged parent drug. On the basis of the results of the human radiolabeled study, additional safety assessment of slow metabolizers of DL was conducted. No safety issues were associated with this polymorphism of DL metabolism.

3.2. Metabolism/Clearance Mechanism. Metabolic profiling and metabolite identification of a NME in excreta and plasma are the critical steps of in vivo ADME studies. The information helps define the mechanism of elimination of the drug from the body. For example:

- Is the drug eliminated unchanged or as metabolites?
- If the drug is eliminated via metabolism, which drug-metabolizing enzymes are involved?
- What is the route of elimination from the body, that is, via urine, bile/feces, or expired air?

The presence of radiotracer in urine, bile, and fecal samples facilitates the detection and quantitation of metabolites (% of the dose). The contribution of each metabolite into excretion is calculated as follows:

$$\%\ \text{dose for metabolite} = \%\ \text{dose excreted in a given sample} \times \%\ \text{radioactivity for a metabolite}$$

On the basis of the abundance of metabolites and parent compound in excreta, the contribution of metabolism (% drug excreted as metabolites) to drug elimination can be calculated. The identified drug clearance pathways together with enzymes responsible for metabolic conversion aid in designing the clinical DDI studies. If the majority of a drug is excreted in the urine, PK in renally impaired subjects needs to be determined. The exposure of circulating metabolites in humans and animals can be compared to guide metabolite safety testing in preclinical species as described in detail in section 3.6.

For characterization of clearance pathways, it is important to profile a representative sample from each collected matrix. For metabolite profiling of urine and feces, samples collected at different time intervals for each subject should be pooled to account for 80−90% of the radioactivity excreted in a given matrix. Pooling is conducted in proportion to the amount (weight or volume) of excreta collected in each sampling period. If no obvious difference in PK or excretion rates among subjects is observed, a single pooled sample for each matrix can be prepared.

On the basis of % dose, metabolites can be categorized as major, minor, or trace levels. The abundance of a metabolite defines its importance in clearance of the drug and depth of structural elucidation required. On the basis of a recent survey, the majority of pharmaceutical companies conduct elucidation of excretory metabolites only if they exceed 1% of the dose, with 25% of sponsors characterizing metabolites >5% of the dose. A few companies (~17%) are looking into elucidation of metabolites representing <1% of the dose.

In general, the routes of elimination and clearance mechanism can be divided into three categories.

1. Compounds Extensively Metabolized by Multiple Metabolic Pathways: The parent drug is extensively metabolized if no or a small amount of radioactive dose is recovered in excreta unchanged. For example, the majority of radioactivity for the recent development drug candidates, SB-649868, torcetrapib, and brivanib alaninate, in excreta was represented by the metabolites, suggesting that all are predominantly eliminated via metabolism.[39−41] From the identified structures of metabolites, SB-649868 was metabolized via oxidation of the benzofuran moiety and eliminated primarily in feces. Torcetrapib was also

metabolized via oxidation, but the metabolites were mostly eliminated via urine. Multiple metabolic pathways or enzymes are often responsible for the conversion of extensively metabolized drugs. The biotransformation of brivanib involves O-dealkylation, methyl oxidation, and sulfation. The involvement of multiple clearance mechanisms reduces the potential for a drug to become a victim of DDI. Sometimes, the exposure difference between the TR and the parent in plasma indicates a metabolically driven clearance. For SB-649868, the AUC of the parent was 22% of AUC of TR, suggesting that the rest of the circulating radioactivity was attributable to metabolites.[41]

2. Compounds Extensively Metabolized Predominantly by a Single Metabolic Pathway: Drugs that are extensively metabolized by a single pathway are susceptible to DDI. From human radiolabeled metabolism and disposition study, it was found that the anti-HIV integrase inhibitor, raltegravir, was extensively metabolized and solely eliminated as a glucuronide conjugate.[42] The UDP-glucuronosyltransferase 1A1 catalyzed this conjugation. Therefore, raltegravir was suggested to be a victim of DDI when coadministered with other anti-HIV drugs, which are known UGT1A1 inhibitors such as atazanavir. Similarly, PPAR activator, peliglitazar, and prostaglandin D2 receptor antagonist, MK-0524, possess a carboxylic acid moiety.[43,44] The radiolabeled mass balance studies showed that the major clearance pathway for both drug candidates was through biliary elimination of their acylglucuronides conjugates. However, multiple UGTs were found to catalyze the formation of the acylglucuronide conjugate of MK-0524;[45] thus, its potential for DDI with individual UGT inhibitors is less severe. Because the acylglucuronides of MK0524 and peliglitazar were also identified as the major circulating metabolites in humans, evaluation of the safety coverage of the metabolite in preclinical species was warranted.
3. Metabolically Stable Compounds: Metabolically stable compounds are eliminated primarily as an unchanged drug. The thrombin inhibitor, dabigatran, and antiretroviral agent, stavudine, were excreted intact,[46,47] and metabolism played a limited role in their elimination in humans. The outcome of radiolabeled studies suggested different types of follow-up clinical studies for assessment of safety and effectiveness of each drug. For example, the impact on exposure of stavudine is expected to be low when coadministered with inducers or inhibitors of drug metabolizing enzymes. However, stavudine was mainly excreted in urine, and as such, its exposure was expected to be influenced in patients with renal impairment but not in those with hepatic impairment. The follow-up clinical studies confirmed this expectation, and stavudine dosage was modified in patients with renal impairment.[48]

Radiolabeled human ADME studies are critical for evaluating the DDI potential of drugs. Only these studies can provide definitive information for determining the major biotransformation pathways for drug elimination. Once major clearance pathway(s) of a drug candidate in humans are known, it is important to identify which enzymes are responsible for each major biotransformation pathway. This evaluation includes two parameters:

1. How the drug can be affected by and affect other comedications;
2. Involvement of polymorphic enzymes (such as CYP2D6 and CYP2C19) in the biotransformation.

These data are critical in planning and designing the clinical DDI program. The identified drug clearance pathways together with corresponding enzymes will aid in the design of clinical DDI studies. There are a number of cases when results of human ADME study completely reshaped the clinical DDI program or helped to explain the apparent data disconnect. Ziprasidone, an antipsychotic drug, is a very good example. Early in vitro studies using P450 isoform-selective chemical inhibitors, correlation studies, and metabolism by recombinant human P450 isoforms suggested that ziprasidone is metabolized primarily by human liver CYP3A4.[49] However, phase II clinical studies showed only modest increases in exposure of ziprasidone with concomitant administration of ketoconazole, a potent inhibitor of CYP3A4, suggesting that the major clearance of ziprasidone in humans is due to metabolism by non-P450 enzymes. Human radiolabeled ADME studies indicated that the major clearance mechanism of ziprasidone (>60% of the administered dose) was due to the reductive cleavage of the benzisothiazole moiety.[50] Further in vitro studies using human cytosolic fraction suggested that the reductive cleavage of the benzisothiazole moiety is mediated by aldehyde oxidase, a non-P450 enzyme, and this pathway indeed was responsible for the clearance of ziprasidone.[51] This example supports the statement that biotransformation pathways of drugs in humans should be explored as early as reasonably possible in a clinical program to plan comprehensive clinical DDI studies.

3.3. Characterization of Unusual and Unexpected Metabolites. The current advances in LC-MS techniques such as the fast acquisition rate, high resolution, accurate mass and isotope abundance measurement combined with postanalysis processing software tools (background subtraction, noise reduction algorithm, mass defect, and isotope pattern filtering) have been very useful for the detection and identification of expected drug metabolites in complex samples. However, it still remains considerably challenging to detect unusual and unexpected metabolites. The presence of a radiotracer in biological samples allows the detection of such metabolites. For example, several novel and unusual quinoline metabolites and a sulfate conjugate of torcetrapib {(−)-[2*R*,4*S*]-4-[(3,5-*bis*-trifluoromethylbenzyl)methoxycarbonyl-amino]-2-ethyl-6-trifluoromethyl-3,4-dihydro-2*H*-quinoline-1-carboxylic acid ethyl ester}, a CETP inhibitor, were identified in animals after oral administration of ^{14}C-labeled torcetrapib labeled at two different sites.[52] These metabolites were formed by multiple pathways, including N-dealkylation, oxidation, aromatization, and sulfation (Figure 2). Recently, Yue et al. identified three metabolites of a potent and selective first-in-class small-molecule inhibitor of the Hedgehog signaling pathway, vismodegib (2-chloro-*N*-(4-chloro-3-(pyridin-2-yl)-phenyl)-4-(methylsulfonyl)-benzamide; GDC-0449), in rats and dogs after a single oral administration of [^{14}C]GDC-0449.[53] The metabolites resulted from an uncommon pyridine ring-opening pathway (Figure 3). Detection of these unexpected metabolites may not have been possible without the use of radiolabeled compound.

3.4. Covalent Protein Binding (CPB)/Reactive Metabolites. A majority of biotransformations convert drugs to more hydrophilic metabolites and, therefore, are considered a detoxification process. However, drug candidates can be metabolized

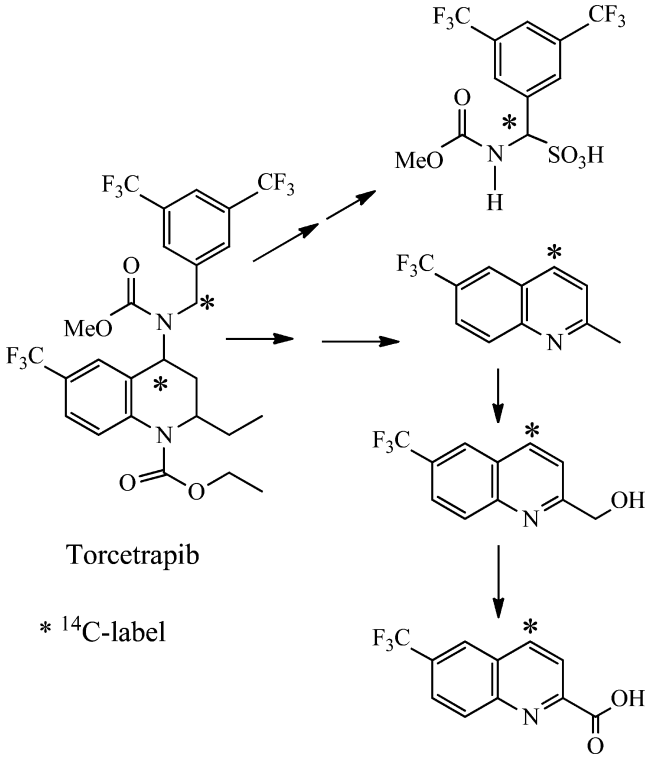


Figure 2. Unusual metabolites of torcetrapib in rats and monkeys.

to reactive intermediates, which may covalently bind to macromolecules, such as functional enzymes or DNA.[54,55] Consequences of covalent binding to macromolecules include enzyme inactivation such as mechanism-based inactivation of P450s.[56] Idiosyncratic drug reactions (IDR) can to some degree be explained by covalent binding.[57,58] Although the formation of reactive metabolites and CPB is not necessarily linked to toxicity, attempts are made to minimize the bioactivation of NMEs at the early stage of drug discovery. Discussions about association of CPB with toxicity have been published in recent reviews.[59−61]

Measurement of covalent binding using radiolabeled drug is considered a definitive approach because the amount of radioactivity covalently bound to proteins can be measured. Merck Research Laboratories[60] have provided protocols on how to assess the bioactivation potential of drug candidates via the CPB assay in vitro and in vivo. In vitro CPB studies are conducted by incubating radiolabeled drugs with liver microsomes or hepatocytes, depending on the primary mechanism of metabolism. Evans et al. introduced a conservative threshold value of 50 pmol drug equiv/mg protein as an upper limit for CPB evaluations. However, it should not be considered an absolute threshold for choosing drug candidates. Other factors such as significance of a metabolic pathway involved in the covalent binding, clinical relevance of the concentration, and dose should be considered during the evaluation of bioactivation risk.

In most cases, application of the in vitro CPB assay is more practical in the drug optimization stage. Measurement of CPB across multiple drug candidates provides a valuable tool in compound selection for further development. Because this assay does not provide information about the mechanism of bioactivation, it is often combined with glutathione or cyanide trapping studies. If the CPB is reduced by the addition of trapping agents such as glutathione or cyanide, it suggests the formation of reactive intermediates. The structures of conjugates often provide valuable information on the bioactivation pathways and guide further drug optimization. For example, a selective estrogen receptor modulator, compound **A**, showed high CPB of 1106 pmol equiv/mg protein in human liver microsomal incubation.[62] The CPB is reduced significantly by the addition of GSH and sodium cyanide to 350−460 pmol equiv/mg protein. The identified GSH or cyanide conjugates of compound **A** suggested at least three bioactivation pathways, namely, hydroquinone/*para*-benzoquinone, *ortho*-quinone, and iminium ion formations (Figure 4). After the structural modification, compound **B** did not show the formation of a reactive iminium ion, and its CPB was reduced to 461 pmol equiv/mg protein. The CPB of compound **B** was further reduced by GSH but not by sodium cyanide, indicating that bioactivation of compound **B** was only through the quinone intermediates. This example demonstrates how the CPB of the radiolabel drug candidates along with the trapping agents can facilitate an understanding of the bioactivation potential of a drug and can guide structural modifications of the NMEs.

Information about the covalent binding of a drug metabolite to proteins can also be obtained from radiolabeled mass balance studies in vivo. When the plasma elimination half-life of radioactivity is longer than the parent and the extraction recovery in plasma is low (<80%), it often indicates the existence of circulating metabolites binding to plasma proteins. For example, traxoprodil is metabolized by CYP2D6 to form a catechol metabolite.[63] The circulating metabolite profiles in EMs and PMs dosed with [^{14}C]traxoprodil showed significant qualitative differences. Approximately 96, 95, and 88% of the radioactivity was recovered from the plasma of PMs at 2, 6, and 24 h after extraction. In contrast, radioactivity recovery from the plasma of EMs was 87, 61, and 25% at 2, 6, and 24 h, respectively (Figure 5). These data suggest that oxidative metabolism might have led to covalent binding to plasma proteins in EMs.

Metabolism-induced protein binding similarly impacted the thrombopoietin receptor agonist, eltrombopag, which was found to have 49 and 32 h half-life of radioactivity and parent, respectively, in human plasma.[64] The extraction recovery from plasma gradually decreased at later time points, and 20% radioactivity was detected in solubilized plasma protein pellets

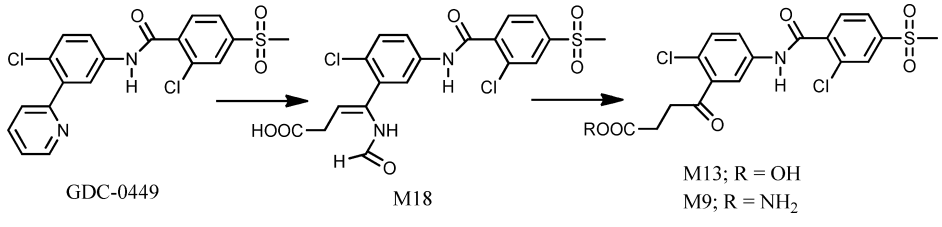


Figure 3. Pyridine-cleaved metabolites of GDC-0449 in rats and dogs.

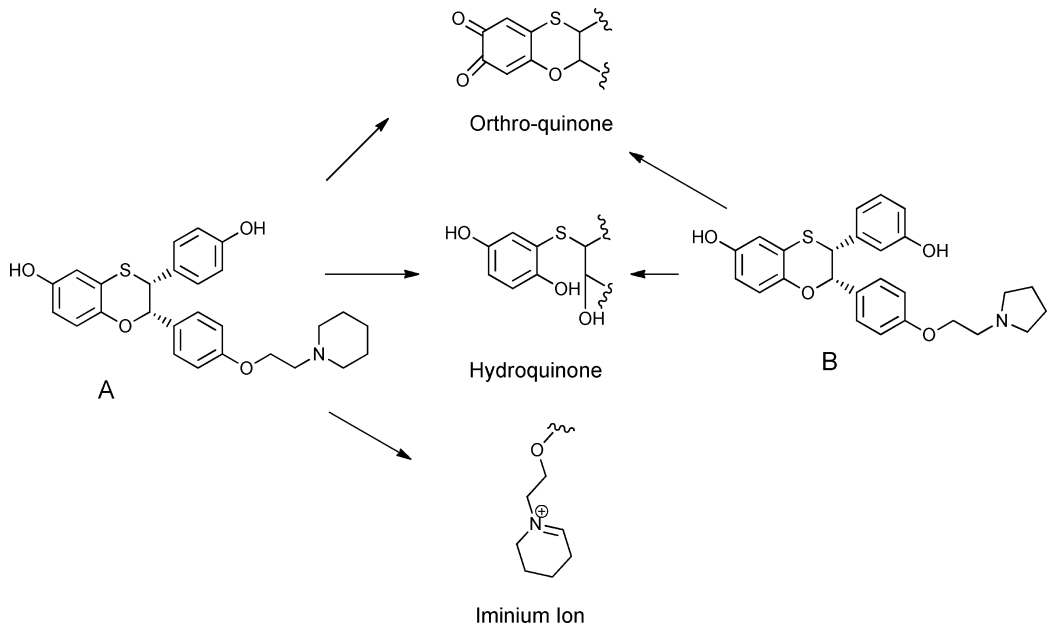


Figure 4. Bioactivation pathways of compounds A and B.

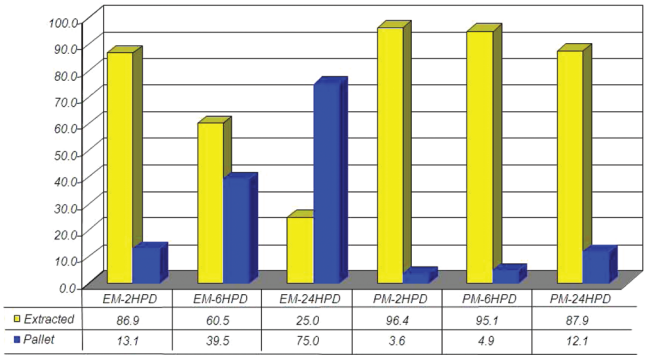


Figure 5. Percent extractable and nonextractable radioactivity from the plasma of CYP2D6 EMs and PMs after iv infusion of [^{14}C]traxoprodil.

in the 48 h sample. Further alkaline digestion of human plasma pellets released parent and oxidative metabolite M1. Therefore, reactive metabolites were proposed to be an acylglucuronide (M2) and an imine methide intermediate of M1 (Figure 6). Meanwhile, M1-related glutathione conjugates found in fecal samples confirmed the imine methide bioactivation.

Although the correlation of covalent binding in hepatotoxicity and idiosyncratic reactions has not been established, the radiolabeled covalent binding study provides an approach to determine the reactive chemical motif and to assess the associated risks.[59]

3.5. Distribution/Tissue Targeting. The purpose of distribution studies in vivo is to track how compounds of interest are distributed in the body. QWBA/WBAL (section 2.3.2) and PET are used to answer questions related to drug distribution and target binding.

At the discovery stage, QWBA is a good tool for determining NME localization at the target site or to identify potential toxicological tissues.[65,66] D'Souza et al.[67] utilized QWBA in tumor-bearing mice to confirm that ZD6126 related drug components were evenly distributed in tissues and tumors and had a longer half-life in the tumor in comparison to plasma. In a rat QWBA study, an immunomodulator, fingolimod, developed for the treatment of relapsing multiple sclerosis disease, was found to be specifically localized along myelin sheaths, indicating a novel mechanism. This drug and its active phosphorylated form not only modulate peripheral lymphocytes but also may have direct effects on glial or neuronal cells for protecting myelin.[68]

The QWBA image has a good resolution even for small tissues such as a fetus and provides valuable drug distribution information in developmental toxicity studies. The antimalarial drug, artesunate, damages erythroid cells leading to developmental toxicity. The QWBA study in pregnant rats demonstrated the highest concentrations of radioactivity were located in tissues involved in hemoglobin metabolism in both the mother and the fetus.[69] The radioactivity concentration in the fetal blood was 2−3-fold higher than in maternal bone marrow, which might explain the low-dose threshold for embryotoxicity. In a placental transfer study of antischizophrenic drug, olanzapine, using the QWBA technique, the radioactivity of drug was found in embryos throughout the time course, suggesting that olanzapine and/or its metabolites crossed the placenta.[70] Radioactivity found in breast milk also confirmed that the pup could receive the drug through milk ingestion. The radiolabel ^{125}I has often been used for protein and peptide distribution studies using the same concept.

Another important application of QWBA/WBAL is to measure melanin-associated binding in pigmented rats and to predict potential adverse effects to human volunteers participating in a human radiolabeled ADME study. Melanin is a pigment found in skin, the uveal tract, and hair. Melanin binding of radiolabel compound causes long exposure of radiation to pigment tissues, changes melanin electronic properties, and further damages tissue especially in eye.[65,71] The resolution of QWBA is sufficient to image drug-derived radioactivity in these small pigment tissues. Therefore, QWBA/WBAL studies in pigmented Long-Evans rats are recommended to measure tissue distribution and calculate human dosimetry for radiation safety of human radiolabel studies.

The tissue distribution of ^{14}C-lasofoxifene in Long-Evans rats demonstrates the importance of such studies. Rats were analyzed using WBAL following administration of a single oral

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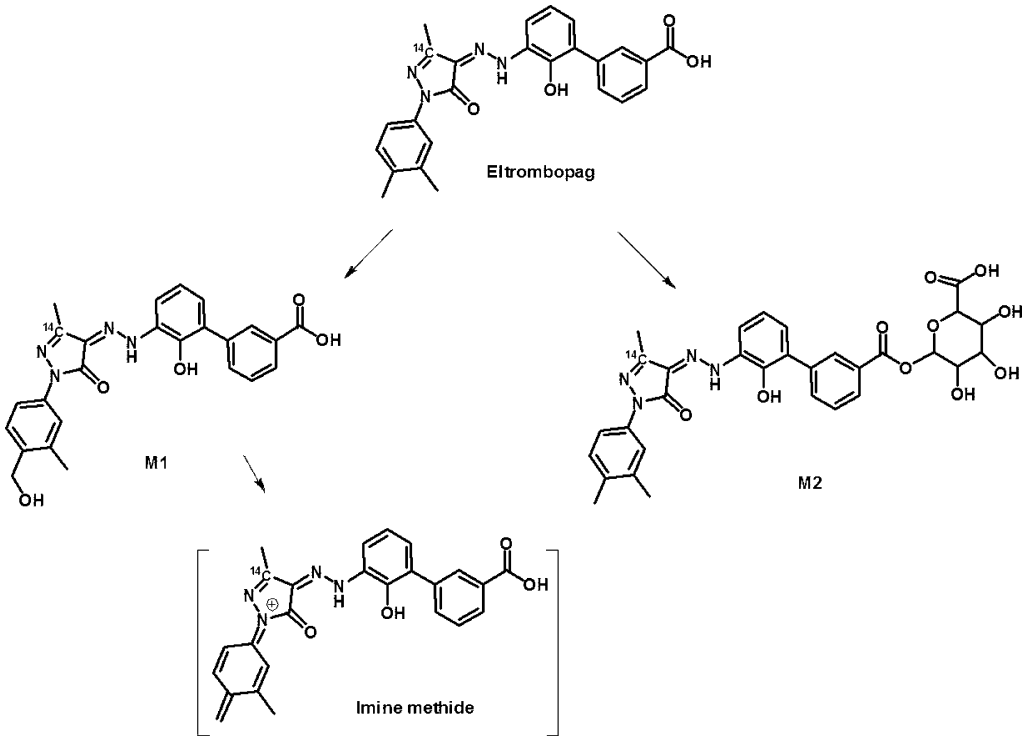


Figure 6. Bioactivation of eltrombopag.

dose of the drug.[37] Distribution of radioactivity throughout the body was assessed at 1, 3, 8, 12, and 168 h postdose in individual animals. ^{14}C-Lasofoxifene-related radioactivity distributed rapidly in the rat with most tissues achieving maximal concentrations at 1 h. Tissue exposure was similar in male and female rats, with the greatest exposure in the uvea. The gastrointestinal contents had the highest concentrations of ^{14}C-lasofoxifene radioequivalents following oral administration. The half-life of radioactivity was longest in the uvea (124 h) and shortest in the spleen (~3 h). Radioactivity became undetectable in some tissues by 8 h postdose. Bone marrow radioactivity was 4- and 5-fold greater than myocardial blood concentrations at 1 h postdose and increased to 30-fold greater by 8 h postdose. At 168 h postdose, radioactivity could only be measured in the intestinal contents and uvea. Radioactivity was not observed in the uvea of albino rats, indicating that the uveal binding observed in this study is associated with binding to melanin present in the eye of pigmented rats. There was no evidence of accumulation or retention of ^{14}C-lasofoxifene radioequivalents in any tissue other than the uvea. The projected human exposure to ^{14}C-radioactivity is estimated by direct extrapolation from the exposure data obtained from the rat

A limitation of QWBA/WBAL studies is that they measure total drug-related radioactivity, which could contain mixtures of parent, metabolites, and degradants. Additionally, ^{3}H is relatively easy to dissociate from the parent molecule. The extent of dissociation needs to be investigated before interpreting the distribution results. Finally, QWBA is a terminal study, and thus, drug distribution could be obtained only in animals.

PET is the most sensitive noninvasive imaging technique using a radiolabeled tracer. In recent years, this technique has been increasingly used for screening NMEs with regard to their distribution, target occupancy, and pharmacological effects in preclinical and clinical research.[72−75] Positron-emitting radionuclides such as ^{11}C and ^{18}F are often introduced into molecules for PET image. Because of the short half-lives of ^{11}C (20.4 min) and ^{18}F (119.8 min), the preparation of radiolabeled compound is more challenging than that of the stable radionuclides. Distribution studies have to be completed within a couple of hours. Conversely, the short half-lives of these radionuclides do not require dosimetry in patients.

The most common and successful application of PET imaging is in the use of ^{18}F-labeled 2-fluoro-2-dexoy-D-glucose (FDG) for detecting unusual increased glucose metabolism in tumor tissues.[76,77] After penetration into cells, FDG is phosphorylated and trapped in the cytoplasmic area of cells. Because the tumor tissues exhibit heightened metabolism, ^{18}F-labeled components accumulate in these tissues and make them distinguishable in the PET images. Therefore, ^{18}F-FDG-based PET image is widely used in diagnostic cancer imaging. However, normal tissues or organs such as brain, heart, kidney, and muscle, which have high glucose consumptions, are also shown in the image. ^{18}F-FDG-based imaging is limited in its ability to detect tumors smaller than 5 mm such as cancerous lymph nodes. The recent ^{124}I-labeled humanized antibody CC49ΔCH2, which specifically recognized tumor-associated glycoprotein-72, demonstrated the improved specificity and resolution of PET imaging on cancer tissue diagnosis in animals.[78]

Because of its noninvasive nature, PET imaging technique has also been used to understand the influence of P-glycoprotein (P-gp) transporter on the penetration of drugs in the brain. The P-gp is highly expressed at the human blood−brain barrier and limits its substrates transport into the brain. The impact of P-gp inhibitors on brain penetration in humans is difficult to measure directly due to sampling challenge. However, ^{11}C-verapamil, a P-gp substrate, allows PET imaging to quantify verapamil concentration in the brain noninvasively with and without the P-gp inhibitor, cyclosporine.[79] Therefore, the influence of P-gp transporter on drug brain penetration in humans could be successfully investigated.

3.6. Human MIST. Metabolism plays an important role in the overall safety profile of a drug. Therefore, the

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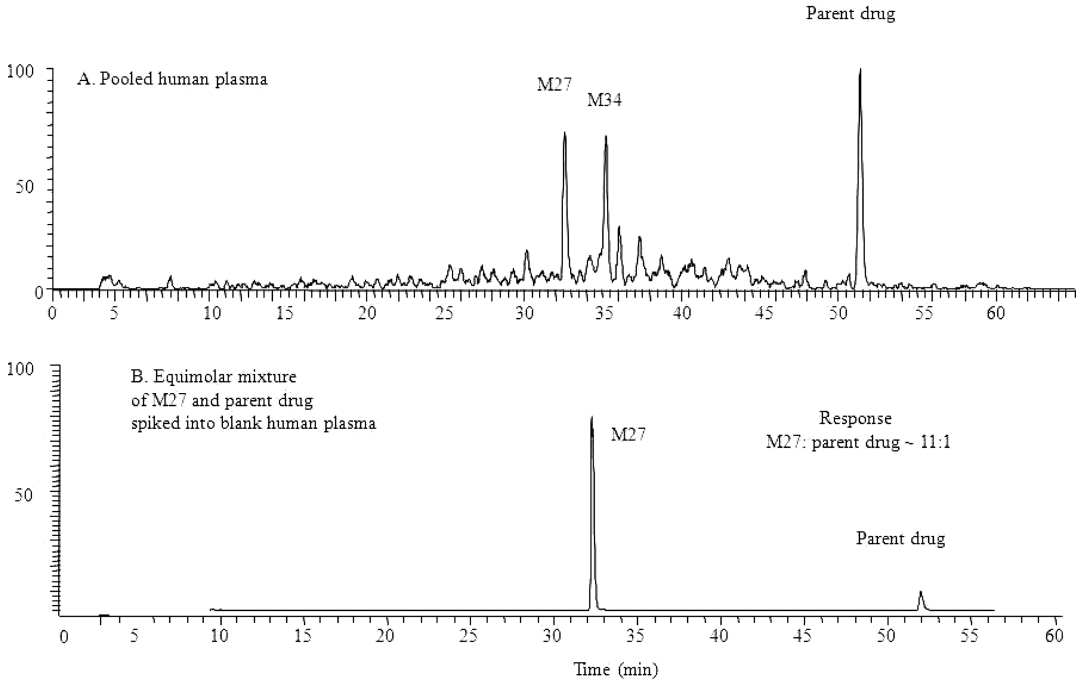


Figure 7. LC-extracted ion chromatograms of candidate, SCH 486757, and its M27 and M34 metabolites in pooled human plasma from subjects dosed with (SCH486757). Extracted ion chromatogram of parent drug and M27 metabolite in blank human plasma spiked with an equimolar mixture of M27 and SCH486757.

pharmaceutical industry has paid much attention to how and when to characterize the metabolites of NMEs. The FDA guidance on MIST encourages the identification of differences in drug metabolism between animals used in nonclinical safety assessments and humans as early as possible during the drug development process. Discovery of disproportionate drug metabolites late in drug development can potentially cause development and marketing delays as it is generally understood that all metabolism-related data should be available before initiation of large-scale clinical trials.[22,23] The identification of unique human metabolites is rare, but there are a number of examples when human metabolism was quantitatively different from preclinical species.[80−82]

Quantitation of drug metabolites in the plasma of preclinical toxicology and clinical studies is routinely carried out using the LC-MS signal. While this approach is convenient, differences in ionization efficiency can lead to significant errors.[83,84] For example, Ramanathan et al. observed that the levels of DL and its hydroxyl metabolites in human plasma estimated by LC-MS were ∼20 and 10 times higher, respectively than those obtained from the radioactivity response.[85] Similarly, two major metabolites, M27 and M34, of a drug candidate, SCH 486757, in human plasma yielded LC-MS signals that were ∼38% of the LC-MS response of the parent drug. However, analysis of the predose human plasma spiked with an equimolar mixture of reference standards for the parent and two metabolites (M27, M34) showed that the LC-MS signal for both metabolites in human plasma was approximately 11 times greater than the response of the parent drug. Taking into account the response ratio of M27 and M34 to parent drug, the actual amounts of M27 and M34 were less than 5% relative to the parent drug (Figure 7).[83] These findings clearly suggest that the LC-MS response alone cannot be used reliably to estimate metabolite levels without response correction. Recently, a number of other methods to estimate exposure to a metabolite using corrected responses in liquid chromatography−tandem mass spectrometry (LC-MS/MS)[86−91] or NMR-based methods[86−88] have been developed, but these methods are laborious, time-consuming, and still provide semiquantitative data.[86−91]

Definitive information on the mass balance and metabolite amounts can only be obtained after administration of a radiolabeled drug. Additionally, there is always a risk that the major drug-related entities were missed in cold studies, for example, because of poor ionization via mass spectrometry. A survey from >20 pharmaceutical companies showed that human radiolabeled AME studies are still used as the definitive data for triggering MIST-related activities. For quantitative profiling of metabolites in circulation, the plasma/serum collected at different time points can be pooled across time points for each subject according to the AUC pooling formula[92] and analyzed by LC with simultaneous radioactivity monitoring. The exposure (AUC_{0-t}) of each metabolite is estimated by multiplying AUC_{0-t} of TR by the percentage of metabolite in plasma (eq 3):

$$\text{metabolite AUC} = \%\ \text{of metabolite} \times \text{AUC of radioactivity} \quad (3)$$

The following example illustrates how data from radiolabeled ADME studies can be used to compare metabolite exposure for compound **C**. Radiolabeled ADME studies were conducted in rats, monkeys, and humans after oral administration of ^{14}C-compound **C**. In addition to parent, a total of six metabolites (M7, M9, M13, M15, M18, and M19) were identified in human plasma.[93] Metabolites M15 and M18 were the major circulating metabolites accounting for 54 and 10.3% of the circulating radioactivity, respectively, whereas M7, M9, and M13 accounted for only 4.95, 3.7, and 8.9% of the total circulating radioactivity, respectively (29.5, 22, and 53% of the parent,

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respectively). M19 was present at 1.6% of the TR (Table 3). In the monkey plasma, M15 and M18 constituted 49 and 5.4% of the radioactivity. The major circulating metabolite in rat plasma was M15 and represented 24.3% of the total circulating radioactivity, whereas other metabolites (M7, M9, M13, and M10) accounted for 4.4−10.4% of the circulating radioactivity. M18 was not detected in the rat (Table 3).

Table 3. Comparison of Exposure of Human Metabolites of a Drug Candidate with Preclinical Species

	% of circulating radioactivity			exposure (AUC_{0-24})		
analyte	human	rat	monkey	human	rat	monkey
parent	16.8	31.5	3.0	153	19200	361
M7	4.95	10.4	1.8	45.1	6340	216
M9	3.7	4.4	0.96	33.7	2680	115
M13	8.9	7.6	27.9	81.2	4640	3350
M15	54.2	24.3	49.1	494	14800	5900
M18	10.3		5.4	93.9		649
M19	1.6			14.6		

Using the eq 3, the exposures ($AUC_{0-tlast}$) of M15 and M18 were 494 and 94 ng equiv h/mL, respectively, in humans, and the estimated corresponding exposures in the monkey were 5900 and 649 ng equiv h/mL, respectively. Metabolite M18 was not present in the rat; however, the estimated $AUC_{(0-tlast)}$ of M15 in the rat was 14800 ng equiv h/mL. Thus, even though the relative percentage of M18 in monkeys is slightly lower than that in humans, the exposure in the monkey is actually greater. Using a similar approach, Deepak et al. have recently compared the exposure of human metabolites of a highly selective inhibitor of the sodium−hydrogen exchanger, zoniporide, in toxicology species.[82] Two metabolites (M1 and M3) accounting for 60 and 6.4% of the radioactivity in human plasma and 46 and 9.0% of the circulating radioactivity in the rat plasma were identified. The exposures ($AUC_{0-tlast}$) of M1 and M3 were 1760 and 187 ng equiv h/mL, respectively, in humans, and the estimated exposures of M1 and M3 in the rat were 1640 and 321 ng equiv h/mL, respectively. Metabolite M1 was not present in the dog; however, the estimated $AUC_{(0-tlast)}$ of M3 in the dog was 73 ng equiv h/mL.

In addition, we also performed a retrospective analysis of circulating metabolites from 25 in-house and literature human ADME studies using this approach. In these studies, a total of 123 circulating metabolites were identified in humans. Eighty of them were present at ≥10% parent AUC (Table 4). All of the human metabolites that have exposures ≥10% of the TR exhibited higher exposures in one or both animal species and, therefore, did not require additional assessment. Overall, it would be rare when a metabolite needs to be administered directly to animal species to address its safety in humans.

3.7. Microdosing/Microtracer Studies. The microdosing concept was introduced to address the failure of NMEs due to unsatisfactory PK or ADME properties. European Medicines Agency (EMEA), U.S. Food and Drug Administration, and the Ministry of Health, Labour, and Welfare, Japan, have recently issued a guidance on nonclinical safety studies to support clinical trials with a single microdose, defined as ≤1% of the expected pharmacologically effective dose up to 100 μg containing a ^{14}C-nanotracer.[94−96] For microdosing, the requirement for some safety-related studies in animals can be exempted before administration of the candidate compound to humans

Table 4. Coverage of Human Metabolites of 25 Drugs in Animal Species

NME, compd	total metabolites (#)	>10% parent (#)	>10% TRA (#)	coverage of metabolites
1	3	3	3	rat and dog (2); rat (1)
2	3	3	1	rat and dog (2); rat (1)
3	3	0	0	NA
4	12	11	5	monkey (5)
5	2	2	1	dog (1); rat (2)
6	2	2	2	dog (2); rat (1)
7	10	10	4	rat (4; NOAEL dose)
8	2	0	0	NA
9	5	5	2	rat and dog
10	4	3	1	rat and dog (1)
11	2	1	1	rat (1)
12	8	1	0	NA
13	4	0	0	NA
14	9	2	2	dog (1); rat (2)
15	4	4	2	rat and dog (1), rat (1)
16	3	3	1	rat (1; NOAEL dose)
17	4	1	1	rat and dog (1), rat (1)
18	4	4	3	rat (2)
19	2	0	0	NA
20	4	3	1	rat (1; NOAEL dose)
21	6	5	2	rat and monkey (1); monkey (1)
22	12	6	3	dog (1); rat (2)
23	9	9	3	rat and dog (3)
24	2	2	1	monkey (1)
25	4	0	0	NA

for the first time, thereby saving time and cutting costs. Therefore, traditional phase 1 studies might hypothetically be preceded by clinical studies ("phase 0") with single subpharmacological doses of one or several lead candidates containing a minute amount of radiotracer whereby human and metabolic profiles could be assessed prior to phase 1 investment. Human microdosing may offer improved candidate selection, reduced failure rates in drug development, and a reduction in the use of laboratory animals in early drug development, factors that will help to speed up drug development and also reduce the costs. In these studies, a low amount of radioactivity is determined by a highly sensitive technique, AMS.

Microdose strategies to assess the PK profiles of drug candidates in animals and humans have been reported.[97−99] Results from the Consortium for Resourcing and Evaluating AMS Microdosing (CREAM) and European Microdosing AMS Partnership Programme (EUMAPP) trials suggest that a microdose could provide useful insights into a compound's metabolism and disposition prior to a standard clinical development commitment.[17,19] In an EUMAPP clinical trial, PK of clarithromycin, Sumatriptan, propafenone, paracetamol, and phenobarbital in humans was compared between a microdose and a therapeutic dose. Where 100 μg iv ^{14}C doses were given alone and with an oral nonlabeled therapeutic dose, excellent accord between the PK parameters was observed, indicating that the disposition kinetics of the drugs tested was unaffected by the presence of therapeutic concentrations. Oral dose data, however, did not scale as well as the iv dose, but the authors concluded that the data obtained would have been useful in the selection of drug candidates for further development

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(or dropped from the development pipeline).[19] In addition, the absolute bioavailability has been determined in humans by simultaneous iv microdosing of ^{14}C-labeled drug with oral dosing of nonlabeled drug. Plasma concentrations of unlabeled drug are measured by LC-MS/MS and those of ^{14}C drug are measured by AMS.[32,100,101]

Measurement of ^{14}C by AMS involves the conversion of ^{14}C carbon to graphite, which requires manual operation that is labor intensive, time-consuming, and not readily amenable to automation, resulting in low-throughput sample preparation and high analysis cost. However, an alternative approach has recently been described to analyze ^{14}C in biological specimens that eliminates graphitization, thereby greatly reducing the total analysis time and cost.[102] This approach to sample processing has successfully been used to quantitate low amounts of ^{14}C in a wide variety of samples, such as urine, plasma, microsomal incubations, and tissues.[102,103]

3.8. Bioequivalence Waiver. Biowaiver or a possibility not to conduct a human bioequivalence study for different drug products is generally granted to compounds of BCS class I, which are characterized by high solubility and high permeability. It is possible to extend biowaiver to other classes of compounds, for example, class III, if high permeability is proven. If bioavailability of a drug exceeds 90% or if >90% of the administered dose is excreted in urine, this drug is considered highly permeable. However, high urinary excretion of the drug is relatively rare. Absolute bioavailability studies require substantial investment and are usually conducted as microdosing studies (section 3.7).

In humans, mass balance studies can be used to document the extent of absorption in the absence of iv data even if the majority of the dose is excreted through bile and feces. This information can later be used for application for bioequivalence waiver if the radiolabeled human study utilizes a sufficient number of subjects to provide a reliable estimate of extent of absorption. Additional experiments such as evaluation of drug stability in the feces are also useful to support a high absorption claim. For example, a radiolabeled compound **E** was dosed to six human subjects to determine routes of excretion, clearance, and major metabolic pathways. The majority of the administered dose was excreted (40.2% of the administered dose within 168 h) in feces with minor urinary excretion (4.5% of the dose). Profiling of pooled fecal (0−168 h) suggested that it is extensively metabolized and unchanged drug accounted for <2% of the administered dose. Stability experiments (incubation of compound **E** with blank fecal sample at 37 °C) showed that the parent compound was stable in the GI tract. Therefore, unabsorbed drug accounted at most for <2% of the administered dose, and absorption by far exceeds 90%.

Low recovery observed during a collection period specified in a given example does not preclude the molecule from high absorption status since any unabsorbed parent compound may be excreted following the first week postdose. Any parent compound excreted in feces beyond 168 h represents absorbed material, which was first secreted into bile.

4. LIMITATIONS OF RADIOLABELED ADME STUDIES

It is widely accepted that the main limitation of radiolabeled studies is the inability to obtain metabolic profiles of circulating metabolites in steady state, as required by MIST guidance. This fact alone has created doubts about the necessity of such studies especially when the cost of conducting them is taken into consideration. However, a well-planned plasma sample collection protocol can resolve this limitation. Comprehensive characterization of circulating human metabolites can be achieved by collecting plasma samples at multiple time points up to 5 half-lives and then pooling plasma across subjects for each time point. The amount of parent drug and metabolites in ng equiv/mL can be calculated for each time point based on metabolic profiles.

The second known limitation of radiolabeled studies is a lack of recovery of the dose, which can compromise data analysis. Recovery of the radioactivity in mass balance studies, especially in humans, can vary widely.[71] Upon comparison of 20 compounds, the mean recovery of radioactivity achieved in human radiolabeled studies ranged from relatively low (61%) to quantitative (101%). A number of reasons can be responsible for low recovery such as errors or inaccuracy in dose preparation, delivery, dose analysis, or combination of these. Errors or noncompliance in sample collection may include incomplete collection of excreta and missed samples including uncollected vomits/spits. Long half-life of circulating radioactivity and errors in sample analysis, such as quenching of radioactivity measurements in excreta, may contribute to low recovery. Errors in study design, such as radiolabeling of a metabolically labile position, lead to loss in expired air or the endogenous pool. Finally, binding to tissues, both covalent and noncovalent, can also result in low recovery.

According to Roffey et al., for relatively short-lived compounds ($t_{1/2}$ < 50 h), 80% recovery of radioactivity is normally achieved. If radioactivity $t_{1/2}$ is longer than 75 h, only two out of seven drugs were recovered at 85% or higher. A long half-life of radioactivity can occur if a drug is distributed into tissues, has high affinity to phospholipids, or binds to melanin or specific proteins. Mass balance studies were shown not to be a reliable indicator of covalent binding occurrence. For a number of drugs with known or suspected covalent binding, recovery of radioactivity was above 85%.[71] Overall, low recovery alone does not compromise the study if all major objectives were achieved.

5. REGULATORY REQUIREMENTS AND THEIR EFFECT ON THE TIMING OF RADIOLABELED STUDIES

Approaches on when and how to conduct radiolabeled ADME studies differ significantly among major pharmaceutical companies. A general scheme is outlined in Figure 8. Perhaps the only element of the drug discovery process on which there is consensus in the pharmaceutical industry is the importance of early assessment of metabolism in vitro. In most cases, this assessment is conducted in liver microsomes (or sometimes in S9, depending on compound structure) during lead compound optimization to assist chemists in improving metabolic stability. Similar experiment with cofactors, GSH, or cyanide can elucidate the potential of a given molecule to form reactive metabolites or conjugates.[104] The next widely accepted step in an in vitro study of metabolism is a cross-species comparison of metabolic profiles in hepatocytes. Results of this experiment are used to guide selection of an appropriate preclinical species for toxicity studies. It primarily defines the choice between monkeys and dogs as a large animal model. Metabolic profiles in hepatocytes from animals are compared to the profile obtained with pooled human hepatocytes. If all three profiles are qualitatively similar and PK parameters allow the choice between two species, then dogs are selected for GLP toxicity study due to their availability. Monkeys are selected in a

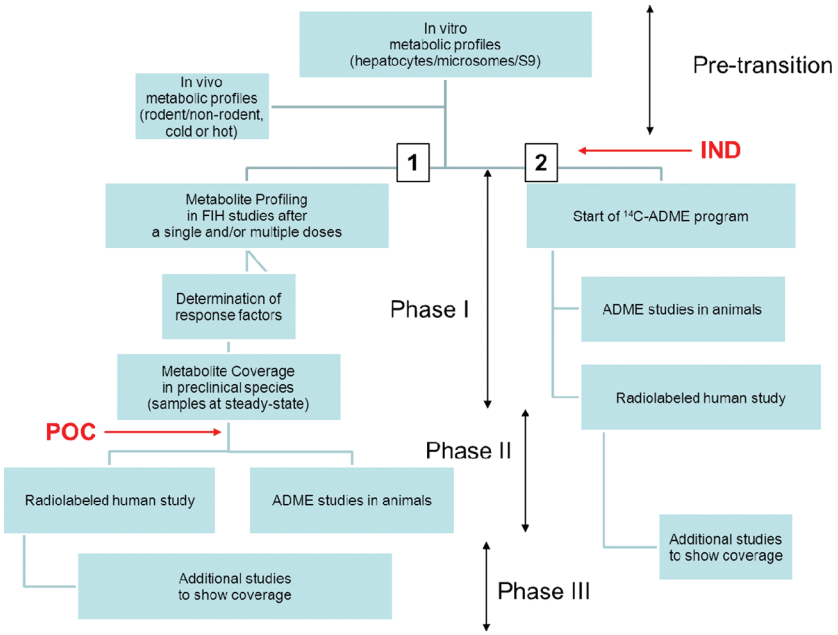


Figure 8. Flow chart outlining general approaches used in the pharmaceutical industry to address the biotransformation-related issues.

limited number of cases when either PK or metabolic profiles do not favor using dogs.

Even at the discovery stage, the amount of biotransformation information can vary from purely in vitro profiles in liver microsomes and hepatocytes to comprehensive in vivo studies with radiolabeled compound administered to multiple species. In the latter case, quantitative information about excretion of the compound as well as profiles of circulating, biliary, and urinary metabolites is known before transition to development.

A recent informal survey showed that timing of radiolabeled ADME studies varies greatly among pharmaceutical companies. A quarter of surveyed sponsors conduct such studies very early, before transition to the development, while the majority of companies conduct such studies together with IND-enabling toxicology or in phase 1, depending on the situation. Only one of the surveyed companies delayed ADME studies in animals until phase 3.

After the IND is filed, drug disposition and metabolism-related activities used in the drug development can be roughly divided into two major approaches.

1. Metabolite profiling of samples from FIH studies (plasma and urine), using label-free compound;
2. Metabolite profiling in human during ADME studies with radiolabeled compound.

Each approach has pros and cons in terms of timing to get information, the price that is paid to obtain this information, and how critical this information is for decision making.

The FDA clearly encourages sponsors to obtain human metabolism data as early as possible in a drug development program. FIH safety and tolerance studies in human volunteers are used by many companies to get the first read-out on human metabolism. Because analysis is conducted by LC-MS methods, quantitative information about metabolite abundance cannot be directly drawn from these analyses due to a bias in MS responses for the parent and metabolites. Given the current regulatory situation with more stringent requirements for metabolite coverage since the introduction of MIST guidelines, more and more pharmaceutical companies chose to follow route #2 at least to some extent.

There is no question that looking at FIH studies and profiling samples from toxicology studies provide valuable information but also put a significant burden on the sponsors. Complying with the FDA's new MIST guidance has also tremendously increased the burden on bioanalytical groups. Accurate quantification is especially challenging in cases of highly metabolized drugs or when the drug is administered at low doses. Additionally, there is always a risk that the major drug-related entities were missed in cold studies, for example, due to poor ionization.

In our opinion, the following approach is regarded as the most efficient and resource-conscious for addressing metabolite safety in humans. An early assessment of metabolism in vitro is unchanged in comparison to the approach in Figure 8. The need for in vivo metabolite profiling data at the discovery stage is determined by reliability of in vitro metabolite identification data for predicting in vivo profiles. For the majority of compounds, in vitro metabolite profiling data are sufficient for transition a compound from research to development. After transition to development, the drug candidate is subjected to up to 28 days of IND-enabling toxicology assessment to initiate human dosage. Because of the high cost of radiolabeled human ADME studies and also the high attrition of compounds in development, a growing number of pharmaceutical companies obtain preliminary data on circulating in vivo human metabolites from FIH studies and delay the studies until proof-of-concept/proof-of activity is shown. In our opinion, bringing radiolabeled study in humans forward, optimally conducting is immediately after multiple ascending dose study is a much more efficient approach. Radiolabeled studies in humans are essential to the drug development program and still remain the gold standard for understanding the metabolism of drug candidates. As discussed above, a well-thought radiolabeled study in humans conducted early can achieve all objectives and answer the majority of regulatory questions with minimum use of time and resources.

ADME studies in two animal species used for toxicological evaluation are optimally conducted in the same time frame with

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radiolabeled human study. In this case, if specific questions about metabolite coverage arise, they can be easily and quickly answered by reanalyzing samples from animals. Additional studies are done as needed.

The concept of "human-only ADME approach" was recently introduced by Obach et al.[105] The authors suggest that reliance on animals in drug metabolism studies is excessive and should be reduced by not conducting the radiolabeled studies in animals. Metabolite identification can be performed by mass spectrometric methods, which greatly advanced in the past decade. We agree that there is usually no need to conduct metabolism studies in every animal species. Two species, rat and dog/monkey, used for long-term safety assessment, are sufficient in most cases. Metabolite coverage in mice and rabbits can be demonstrated after a nonradiolabeled compound administration. The other reason for conducting radiolabeled ADME studies in toxicology species is to understand the species-dependent metabolism. For example, efavirenz, a potent nonnucleoside reverse transcriptase inhibitor widely prescribed for the treatment of HIV infection, produces renal tubular epithelial cell necrosis in rats but not in monkeys or humans. The radiolabeled ADME studies provide compelling evidence that a species-specific formation of glutathione conjugate(s) from efavirenz was responsible for the nephrotoxicity in rats.[106] Metabolite identification is the most expensive and time-consuming part of ADME study. If profiling radiolabeled samples is relatively straightforward, cold sample analysis takes much longer. Additionally, radiolabeled studies provide definitive answers about metabolite abundance quickly while doing the same with cold material can be tricky or impossible. If performed at the right time, radiolabeled studies provide extremely valuable information and do not constitute a mere "package". In our opinion, to fully utilize information from radiolabeled studies, they should be conducted early in the drug development as suggested in Figure 8.

6. SUMMARY

Radiolabeled studies are a valuable and irreplaceable tool to study drug ADME in drug discovery and development. Following administration of the radioactive drug, samples are analyzed to determine the amount of radioactivity, structure of metabolites, and PK of parent and metabolites. The radiolabel mass balance together with metabolic profiles in excreta make it possible to determine clearance mechanisms of drug candidates. The results will aid in predicting the intersubject variability of PK and potential drug−drug interactions. The qualitative and quantitative data on circulating metabolites in human and preclinical species help define whether human metabolites have adequate exposure in toxicology species and if any metabolite contributes significantly to its pharmacological activity.

Noninvasive imaging, as is possible with radiotracers, enables us to understand the time course of delivery, uptake, and retention of drugs in various tissues. The extent of drug distribution and accumulation can guide our understanding of the safety of novel drug candidates. Such imaging techniques have widely been used in receptor binding and recognition studies, disease diagnosis, and DDI in inaccessible tissues such as the brain. Although the approach of using radiolabeled drug faces many limitations, the noninvasive analysis and definite quantitative information provided uniquely aid our understanding of the fate of drugs in the body during drug development.

Regulatory policy dictates that exposure of administered radioactivity should be kept as low as possible in humans, which makes it imperative for radioactivity detectors and methods to provide the utmost sensitivity in radioisotope measurement. While the LC-ARC and TopCount (PerkinElmer, Waltham, MA) provide up to 20-fold higher sensitivity in detecting ^{14}C peaks as compared to commercially available flow-through radioactivity detectors, they are not sensitive enough for microtracer studies. AMS is the most sensitive technique, capable to detect down to <1 amol ^{14}C/mg carbon on milligram-sized samples.[107,108] Precision and accuracy in AMS analysis are as good as that that can be obtained in LC-MS/MS assays and meets the requirements of regulatory guidance; therefore, it will be routinely used for the determination absolute bioavailability in humans.[18,19,109] Excretion, mass balance, and metabolite profiles can also be obtained simultaneously for compounds that have long plasma half-lives or poor absorption using microtracer and AMS.[101] Microdosing can also help to establish the human relevance of animal models because the metabolism of NMEs can be studied directly in humans.

AUTHOR INFORMATION

Corresponding Author

*Tel: 617-679-3725. Fax: 617-679-3463. E-mail: Chandra.Prakash@biogenidec.com.

Notes

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ABBREVIATIONS

ADME, absorption, distribution, metabolism, and excretion; AMS, accelerator mass spectrometry; IV, intravenous; ARC, accurate radioisotope counting; BDC, bile duct cannulated; CPB, covalent protein binding; DL, desloratadine; FDG, 2-fluoro-2-dexoy-D-glucose; FIH, first-in-human; FSA, flow scintillation analyzer; IDR, idiosyncratic drug reactions; MIST, metabolites in safety testing; MSC, microplate scintillation counting; NME, new molecular entities; PADS, pharmacologically active dose studies; PET, positron emission tomography; QWBA/WBAL, quantitative whole body autoradiography/whole body autoluminography; TR, total radioactivity; EM, extensive metabolizer; PM, poor metabolizer

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