9 Mass Balance Studies in Animals and Humans

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9.1 SUMMARY

Mass balance studies play an important role in the development of new drugs. These studies give in-depth understanding of absorption, bioavailability, and routes (renal, biliary, hepatic, or gastrointestinal) and extent of excretion, metabolite profiling, metabolic pathways, and clearance mechanisms of a drug. In this chapter, fundamental scientific considerations for the successful design, conduct, and interpretation of conventional, basic mass balance and more comprehensive absorption, distribution, metabolism, and excretion (ADME) studies are reviewed and applied, practical case examples are provided.

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9.2 INTRODUCTION

Before first-in-human (FIH) studies with a new drug candidate, preclinical studies need to be conducted to fulfill regulatory requirements or to facilitate regulatory approval [1]. In the United States, investigational new drug (IND) applications with the Food and Drug Administration (FDA) may include IND enabling studies, such as (i) *in vitro* and *in vivo* pharmacology studies for intended use; (ii) toxicology studies supporting the starting dose and duration of an FIH study (such as single or multiple-dose studies in one rodent and nonrodent species with toxicokinetics, genotoxicity battery, and safety pharmacology battery); (iii) single-dose pharmacokinetics (PKs) in one rodent and nonrodent species; (iv) *in vitro* plasma protein binding in relevant species; and (v) *in vitro* cross species metabolism study in relevant species [2]. While not mandatory for IND submissions, comprehensive preclinical ADME studies, including biotransformation or metabolite profiling, conventional mass balance, or tissue distribution by quantitative whole-body autoradiography (QWBA) [3] can make a submission package more compelling for progression toward phase I clinical studies. Typically, preclinical ADME together with mass balance studies are recommended before conducting human ADME and mass balance studies [4,5], which are generally initiated after the successful synthesis of the radiolabeled drug. An example of the typical timings for conducting mass balance studies during the drug development process is shown in Fig. 9.1. For preclinical ADME species, the selection of doses is often based on pre-existing toxicity information and targeted therapeutic doses. For human ADME studies, which include the evaluation of mass balance, a relevant dose in the anticipated therapeutic range is typically chosen. Conventional mass balance studies only allow the calculation of mass recovery and PK parameters of the radiolabeled drug by quantification of the radioactivity present in excreta such as urine, feces, or bile. Nowadays, these mass balance studies are often conducted as part of more comprehensive ADME studies, where additional information is obtained, including (i) PK properties of the drug and its metabolite(s) in blood or plasma; (ii) absorption estimates via plasma/blood data, urinary data, or fecal data; (iii) elimination pathways and drug metabolism; and (iv) total drug exposure and its metabolites in different organs and tissues at different time intervals.

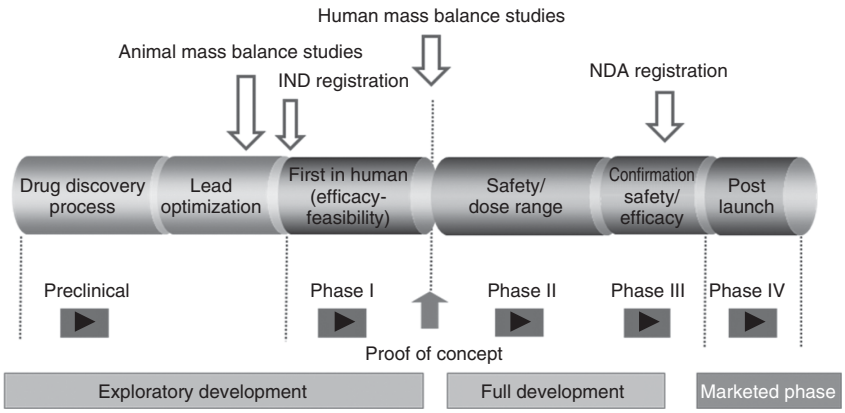

Figure 9.1 Representation of the timings for the conduct of animal and human mass balance studies parellely in the drug development process.

9.3 PURPOSE AND SPECIFIC AIMS

The purpose of mass balance studies along with ADME studies [6,7] can be summarized as follows:

1. To determine absorption of drug after extravascular administration.
2. To identify excretory pathways and recovery of drug-related radioactivity (usually urine and feces) in early stages of drug development.
3. To characterize the PKs of the intact parent drug and its metabolites as well as their contribution toward pharmacology/toxicology.
4. To determine clearance mechanisms, including metabolic and excretory fates and understand potential contributors of intersubject variability associated with drug–drug interactions (DDIs), supported by the identities of drug-related material in excreta.
5. To determine the organs/tissues distribution in preclinical species, which can be a cause for incomplete recovery of radioactivity in excreta, caused by significant retention in specific organs or tissues.
6. To provide all necessary information from preclinical studies in terms of design and potential risks, including dosimetry of radiation to support mandatory human mass balance studies.
7. To guide the use and choice of preclinical species in the toxicity studies based on the metabolite profiles.

Conventional mass balance studies in their simplest form allow the determination of excretory pathways of drug and its metabolites by determining the cumulative radioactivity recovered in excreta after an extended collection period, relative to the dosed amount of radiolabeled drug, most commonly via the oral (p.o.) or intravenous (i.v.) routes [7]. To study the mass recovery, the radiolabeled drug is typically administered to facilitate the identification and quantification of parent drug as well as its metabolites in different biological matrices. The data obtained from these studies are multifarious, as when a drug is administered to animals or humans, it undergoes a series of events such as absorption, distribution, and metabolism, which govern the drugs systemic exposure, and its pharmacological response [8] or pharmacodynamic effect, as well as its excretion routes [7,9]. For investigational drug candidates, ADME and mass balance properties can be crucial determinants in the selection process, leading to optimized clinical outcomes for drug candidates [10]. Essential elements for design and conduct of mass balance study have been described previously by Tse *et al*. [11] and Beumer *et al*. [4]. This chapter summarizes detailed factors to be considered when designing and conducting various types of ADME and mass balance studies, including how to (i) choose of the best dose, dosing route, in a suitable preclinical species; (ii) clearly identify major excretory pathways; (iii) identify and troubleshoot causes of low drug recovery; (iv) determine PK and ADME properties using total radioactivity or intact parent drug data; and (v) conduct dosimetric calculation to extrapolate from animal to human. A case example in rats is given to provide details on the conduct of a mass balance studies. Moreover, the purpose and conduct of human mass balance studies are reviewed.

9.4 EXPERIMENTAL DESIGN OF PRECLINICAL MASS BALANCE STUDIES

To identify metabolic routes of a drug candidate in early development stages, *in vitro* studies provide a basic direction for the preclinical studies in animals [12,13]. A carefully designed mass balance study, which includes metabolite identification in plasma, urine, bile, and feces can further aid in the identification of excretory and metabolic routes. Common challenges related for the successful study conduct include proper dose selection, bioanalytical factors, suitable formulations, and potential toxicities. Details for experimental design, along with study result interpretation are outlined. To aid in clarity, a preclinical rodent study can be divided into six groups. Two groups, one each for p.o. and i.v. administration, are used for the interpretation of mass balance data, absorption, and PK parameters such as fraction absorbed (F_{abs}), area under the time–concentration curve (AUC), and clearance (CL). Two additional groups, one each for p.o. and i.v. administration, are dedicated for biotransformation studies and metabolic profiling. The metabolite profiles from the i.v. dosed group, when compare to the p.o. dosed group, can provide the information on whether the drug is subjected to the first-pass metabolism in gastrointestinal tract. The remaining two groups, each separately for p.o. and i.v. dose administration, are used for biliary excretion studies, in which one continuous loop nonvascular catheter is surgically implanted. The i.v. dose in bile-duct cannulated (BDC) rats can further distinguish biliary excretion from the secretion of drug from the gastrointestinal tract, if any. Blood, urine, feces, and bile samples are collected at the designated time following the dosing. The carcasses are also collected for potential analysis in cases where the total recovery in excreta is low.

From the group where the mass balance is studied, a mass balance recovery $<80\%$ can possibly lead to challenges from regulatory agencies, as the mass balance study might not provide a complete picture of ADME parameters. Some publications put the recovery cutoff at 90% [4] and 85–95% or above as the boundary for the success or failure of a mass balance study [14]. Understanding the causes for incomplete mass recovery is typically required but can be challenging at the early development stage. Sometimes, incorporation of QWBA data for the analysis of tissues and organs distribution of radioactivity in mass balance studies can be used to understand the incomplete mass balance recoveries.

9.4.1 Choice of Preclinical Species

For ADME studies as a part of nonclinical safety testing, two animal species, one rodent (rat or mouse) and one nonrodent (dog or monkey), are usually chosen [15,16]. Rodents are the most common and suitable choice of species for preclinical mass balance studies. For species selection, the difference in strain, age, sex, genetic modification, and body weight can have a significant impact on the studies [4] and must be considered. More importantly, the interpretations of any ADME studies often require consistency for results comparisons between studies; thus, it is recommended to use the same rodent strain with a close match of age and weight as used in the toxicity studies. Male animals are normally used for the ADME studies unless the drug is developed for use in female patients only. Female animals may be used if there are significant differences in toxicokinetics parameters between genders are observed and *in-vitro* metabolism cannot explain the results. If the drug is being developed for an

IND application with Japanese regulatory agencies, studies in both male and female animals may need to be conducted. Disposition studies may be conducted in other species such as rabbits, which is used for reproduction or teratogenicity studies, or in the mouse, which is used for carcinogenicity studies. Dogs and monkeys are normally used as nonnative animals. Special studies in female rats such as placenta and milk transfer studies are required and are usually conducted at late stage of the drug development. Before the actual dose administration, animals are usually housed for at least 14 days for quarantining or conditioning [2] with controlled conditions (in a room with controlled temperature ranges typically at 72 ± 2 F), and a humidity range of $50 \pm 15\%$, and dietary conditions (fed or fasted) in accordance with Guidance for the Care and Use of Laboratory Animals. In this chapter, the focus is on the mass balance studies in rats in order to cover fundamental aspects of mass balance studies (including recovery from different matrices, e.g., excreta, tissue dissection, QWBA, carcass, and CO_2 exhalation) because rats are the most common and suitable choice of species. A schematic representation of the rat model used in a typical ADME study, including common routes of administration, absorption, metabolism, and excretion from different matrices, is illustrated in Fig. 9.2. A summary of animals commonly used for

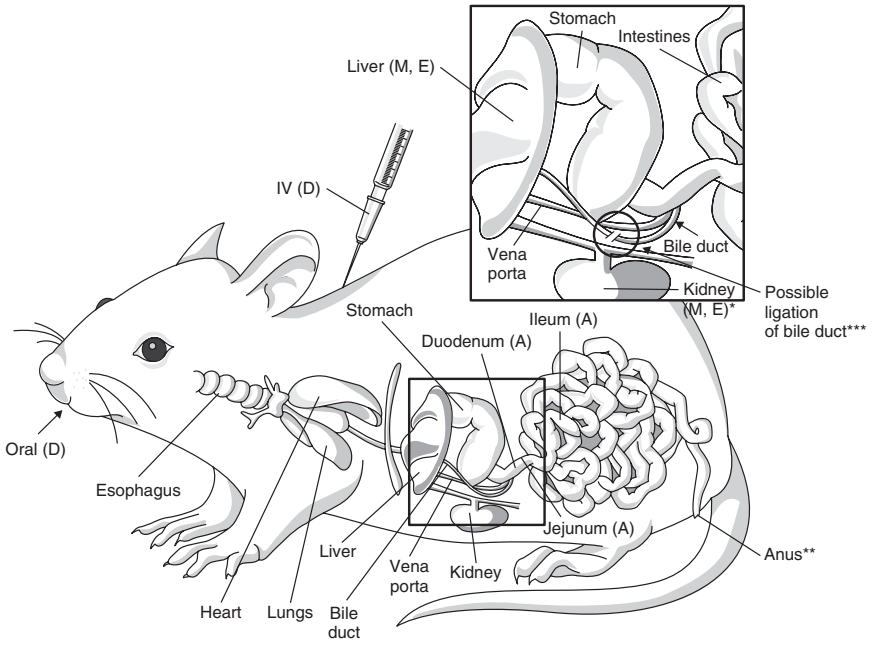

Figure 9.2 Schematic representations of various dosing, absorption, metabolism, and excretion routes. The most common dosing routes for mass balance studies are oral (p.o.) and intravenous (i.v.) administration. Urine and feces are separately collected after excretion. For biliary excretion studies, the bile duct that drains the bile from the liver to the upper part of intestine, usually duodenum, can be cannulated to collect bile. "D" represents p.o. or i.v. dosing routes, "A" represents various absorption sites, "M" represents metabolism sites, and "E" represents various excretion routes. *Urinary excretion site. **Fecal excretion site. ***Biliary excretion site. *Source:* The figure was drawn by Nicole Heimbach.

TABLE 9.1 Factors in the Choice of Animals, Dose, and Formulations for Mass Balance or ADME Studies with Rationale and Comments

Factors	Design	Rationale and Comments
Dose levels	*Intravenous dose:* usually 20–100% of oral dose (based on bioavailability and solubility)	Ideally target similar exposure between oral and intravenous doses
	Oral dose: usually in the range of anticipated NOAEL dose studies	Support the toxicity study
Dose volume	*Intravenous:* $\leq$2 mL/kg	Ethical reasons and animal welfare considerations
	Oral: $\leq$10 mL/kg for rodents and $\leq$5 mL/kg for large animals; ideally the same as planned for toxicity studies	
		As in toxicity studies
Formulation	Ideally same formulation as in toxicity studies	After administration, an aliquot of formulation is frozen as a reference check in metabolic studies
Dose regimen	Single dose	—
Species[a]/weight (kg)	Dose for [^{14}C]-labeled drug[b] (μCi/kg)	—
Mouse (0.025–0.040)	100–300	For higher species, avoid high radioactive
Rat (0.15–0.30)	100–300	doses as animals are
Dog (8–13)	20–30	returned to colony
Monkey (2.5–4)	20–30	—
Rabbit (2–4)	30–50	—
Subject number	$N = 3$ for each route[c]	—
Feeding	Should be same as in toxicity studies	
Housing	Individually in metabolism or housing cages	

Abbreviation: NOAEL, no observed adverse effect level.

[a]Mouse and rat studies support the carcinogenicity study; rat, dog, and monkey studies support the general toxicity study; rat and rabbit studies support the teratogenicity study.

[b]For [^{3}H]-labeled drug, $\leq$500 μCi/kg for rodents and $\leq$100 μCi/kg for large animal.

[c]In mice, urine and feces may be pooled per time point. For PK and metabolite profiling in mouse plasma, due to small sample size, two to three animals per time point for each route may be used. In rats, additional three animals each will be needed for plasma metabolite identification and biliary excretion. In large animal, for intravenous dosing studies, often two animals are used due to more sample volume available and lower experimental variability compared to oral dosing.

the mass balance studies is listed in Table 9.1. All studies are designed to maximize quantitative aspects of the drug molecule, while minimizing radioactivity exposure.

9.4.2 Choice of Dose, Route of Administration, and Formulation

In mass balance studies, as most compounds undergo metabolic transformation before excretion, a radioisotopically labeled compound is commonly used so that both unchanged compound (parent drug) and its metabolites in different biological matrices can be accurately quantified. The total dose of the compound (radiolabeled and unlabeled) under investigation needs to be pharmacologically relevant and

comparable to toxicological doses [4]. Most of the time, the radiolabeled compound is diluted with nonradiolabeled compound to achieve the total targeted dose to be administered to the subjects for both preclinical (animal) and clinical (human) studies as the required radioactivity is typically only a trace amount. Moreover, the radiolabeled dose depends on the specific objectives of the study and sensitivity of the analytical method. The specific activity of the radioactivity in the dose must be chosen carefully, as the nonradiolabeled dose determines the PKs of the parent drug and mass balance studies for clinical practice, while the radiolabeled dose influences both the radioactive exposure in the animal/human and the analytical limits of quantification. For human studies, the route of administration preferred is the same as the intended route for the drug's clinical use, as the formation of metabolites and extent of metabolism may differ with the route of administration [7]. The selection of the radioactive dose amount is based on the balance between the radioactive exposure limits as outlined in 21 CFR 361.1 [1], other local radiation guidelines and regulations, and the safety/efficacy profiles of the drug molecule. The purity of the radioactive tracer used for the study plays an important role as impurities may result in different PK characteristics, metabolite profiles, and may result in toxicity concerns. The radioactivity purity must be $>98\%$ for mass balance studies unless there is a limit in purification technique. Various factors related to the choice of doses and formulations have been summarized in Table 9.1.

For p.o. administration, solutions, suspensions, or loose filled capsules prepared in certified labs are generally used for mass balance studies. A dosing vehicle also used in the toxicity studies is the preferred choice for preclinical mass balance studies. Solution formulations are a common choice with minimal difference in the physical properties (particle size) between radiolabeled and nonlabeled drug substance. For suspension or capsule formulations, care must be taken to ensure uniform distribution of radioactivity within the dose [7]. A premixed blend prepared by dissolving the radiolabeled and unlabeled drugs in appropriate solvent(s) followed by removal of solvent(s) can eliminate uniformity issues. Preparing the radiolabeled dosage form with commercially available large-scale manufacturing equipments will increase the risk of unnecessary radioactive contamination and is generally not preferred.

9.4.3 Choice of Matrices

The quantitative analysis of all mass balance studies is based on the cumulative amount of radioactivity collected in a given matrix at the given collection interval. Usually, blood, urine, and feces are routinely collected during a typical mass balance study. In order to understand the mechanism of clearance and potentially incomplete radioactivity recovery, more information can be obtained by sampling additional matrices, including bile, tissues, organs, carcass, and expired air [17]. The sample collection of blood and excreta begins before drug administration and continues until maximum recovery of radioactivity is achieved. The radioactivity of blood, plasma, urine, bile, feces, and cage wash samples is determined by liquid scintillation counting, while the parent drug is usually determined using the HPLC or liquid chromatography-tandem-mass spectrometry (LC-MS/MS) methods. The sample collection can be stopped when $<1\%$ of administered radioactivity is excreted for two consecutive days, as prolonged collection will not substantially increase recovery [4]. Moreover, the sample collection

time should also be practical for PK parameter calculations. The sample collection for different matrices is discussed in the following sections.

9.4.3.1 Blood and Plasma. Blood and plasma samples are generally used for the PK studies of both nonlabeled and radiolabeled compound. The choice of blood and plasma sample selection should be based on the blood-to-plasma distribution ratio, due to different PK behavior of unchanged drug and its corresponding metabolites in blood versus plasma. In the case of i.v. dosing, an early sample is imperative to determine the maximum plasma concentration (C_0). Thereafter, samples need to be collected at appropriate intervals in order to characterize the distribution and elimination phase separately. In the case of p.o. administered drugs, samples are frequently collected around the expected time of maximum plasma concentration (C_{max}). When designing the time points to take a sample, the volume of samples collected should be sufficient for at least one bioanalytical measurement. In the example of the rat ($n = 3$), blood (200 μL) can typically be collected at 0.083 (i.v. only), 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, 72, 96, and 168 h post dose. The blood samples are added preheparinized tubes in cooled (4°C) sample collecting carousals using automatic sample collecting systems such as a Culex® system. Saline (200 μL) is automatically injected after each sample is collected to clear the cannula and replace the volume of blood samples. The blood samples are further spun to collect the plasma at 4°C. The total blood volume collected should not exceed 1% of the animal's body weight. For metabolite profiling, 250 μL of blood samples are collected from each of three rats at each time point up to 48 h using the same procedures mentioned above. The samples are pooled, and then spun to separate the plasma. The total radioactivity of radiolabeled drugs can be measured using the liquid scintillation counting (LSC) method. The concentration of nonlabeled or parent drug in blood or plasma can be measured using different chromatographic instruments (e.g., HPLC and GC), mass spectrometers (e.g., LC-MS/MS), or other suitable analytical methods (e.g., ELISA). Sample pretreatment methods for the determination of radioactivity differ for blood and plasma. The plasma samples are transparent and therefore can be directly measured after mixing with liquid scintillation fluid for the quantification of radioactivity using LSC. Generally, decolorization is required for blood samples to reduce color quenching. This is achieved using a tissue solubilizer and oxidizer (such as 30% hydrogen peroxide). EDTA (50 μL of 100 mM) as an antifoaming agent can be added to the solubilized sample before addition of hydrogen peroxide. Alternatively, the combusting method can be used to collect $^{14}CO_2$ or $^{3}H_2O$ derived from the samples. A detailed list of various parameters needed to be considered in designing and handling of blood and plasma matrices following with rationale and comments is provided in Table 9.2.

If a tritium radiolabeled compound is used, then the samples to be analyzed for the radioactivity should be dried before analysis, as $^{3}H_2O$ has a long half-life and can interfere with the radioactivity of drug-related substances. Since the dried sample is hard to redissolve in the liquid scintillation cocktail, it is recommended that the dried sample be dissolved in solubilizer first, if the solubilization method instead of the combusting method is used.

9.4.3.2 Excreta and Cage Wash. Urine and feces are typically collected from each PK study groups at defined time intervals: pre dose, 0–24, 24–48, 48–72, 72–96, and 96–168 h post dose. Urine is a liquid matrix that can be collected separately from the

TABLE 9.2 List of Various Parameters Needed to be Considered in Designing and Handling of Blood, Plasma, and Excreta Matrices, with Rationale and Comments

Sample Collection	Design	Rationale and Comments
Blood samples		
Blood/plasma collection time points	*Intravenous and oral dose:* pre dose, 6–8 points on day 1. Then sampling at desired time points up to seven days	Ideal for PK calculations even if terminal half-life is long
Volume of blood sample	≤0.25 mL from rodents; 2 mL from rabbits, dogs, and monkey; 10 mL from human	Allows analytical measurements for PK, including radioassay and metabolic profiling
Number of measure-ments	One per time point of each individual in ADME studies	Small volume Pooled samples per time point may be analyzed if limited sample available
Excreta samples		
Urine, feces, and cage wash collection	Separate collection of urine and feces on ice (no cooling after 96 h); modified metabolic cage preferred	Mass balance and metabolite profiling (avoid degradation) Pooled collection per time point for urine and feces may be needed for mice
Time intervals	Daily up to 168 h post dose; Fixed time interval (0–24, 24–48, 48–72, 72–96, 96–168 h) or pooled for mass balance studies	To determine mass balance, metabolic, and excretory pathways
Cage wash	Extensive cage wash after last day, separate workup	Increase recovery and safety aspects (clean cages to be returned to storage)
Number of radioactivity measure-ments	*Urine:* One per time interval and for each species under study; *Feces:* Two per time interval and for each species under study; *Cage wash:* One per animal	Repeat if mean difference is >10% and total content >1% of dose

feces with a modification of a metabolic cage as represented in Fig. 9.3 for rodents. The urine samples are collected in defined time intervals in order to calculate the total excretion of the compound and its metabolites. The urine collection containers should be placed on ice bath or frozen conditions to reduce possible chemical degradation. The total volume and pH of the urine sample should be accurately measured. After collection, urine samples are quantitatively analyzed for radiolabeled drug, unlabeled drug, and/or their metabolites. The percentage recovery of radioactivity in urine is calculated from the ratio of concentration and volume recovered in the urine to the total concentration and volume dosed, where concentration is given in disintegrations per minutes (DPM) per milliliter and is represented by Equation 9.1:

$$\%\ \text{Recovery in urine} = \frac{\text{Volume recovered in urine (mL)} \times \text{Urine concentration (DPM/mL)}}{\text{Volume dosed (mL)} \times \text{Dose concentration (DPM/mL)}} \times 100 \quad (9.1)$$

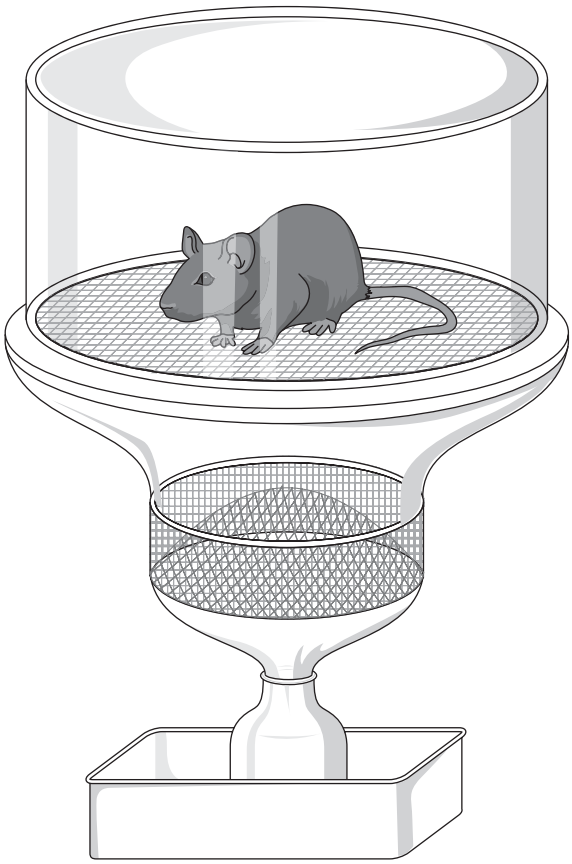

Figure 9.3 A representation of rat metabolic cage used for current mass balance studies for the separate collection of the urine (in bottle below) and feces (middle collection cone) for the rodents. For the metabolite profiling purpose, the samples (first 48 h) were collected over ice or frozen conditions.

Identification and quantification of metabolites of drugs in urine can be achieved using HPLC with off-line or on-line radioactivity detection and LC-MS/MS. For this purpose, urine samples can be pooled for analysis and may require to be concentrated in order to increase the detection sensitivity of the metabolites.

Feces are collected separately from urine with a modified metabolic cage for rodents, as represented in Fig. 9.3. Feces samples are subjected to homogenization, solubilization, and decolorization before radioactivity measurements. Homogenization can be done with water, buffer, bovine serum albumin solution [6], suspension agent solution, or water containing organic solvent(s). Similar to blood samples, after solubilization, decolorization is then performed with 30% hydrogen peroxide, if the solubilization method other than combusting method is used. As was the case for blood samples, EDTA (50 μL of 100 mM) can be added as an antifoaming agent to the solubilized sample before addition of hydrogen peroxide. Finally, scintillation fluid is added to each vial. Overnight storage in a dark place is recommended before quantification of radioactivity with liquid scintillation counting to reduce chemiluminescence. The

percentage of the radioactive recovery from the feces is calculated from the weight of feces recovered, and the homogenization factor is given by expression given in Equation 9.2:

$$\%\ \text{Recovery in feces} = \frac{\text{Weight recovered (g)} \times \text{dilution factor (g/g)} \times \text{homogenate concentration (DPM/g)}}{\text{volume dosed (mL)} \times \text{dose concentration (DPM/mL)}} \times 100 \quad (9.2)$$

To determine the concentration of unchanged drug and/or metabolites in feces, the homogenates are processed and cleaned up using an appropriate method (such as solid-phase extraction or liquid–liquid extraction) and then analyzed by LC-MS/MS. After the final sample collection is completed, each cage is rinsed with water and 50% ethanol (or another appropriate solvent), and the resulting cage wash is quantitatively analyzed using LSC. The percentage of radioactivity recovery from the cage wash can be calculated from the ratio of volume recovered after cage wash and the concentration to the volume dosed and the concentrations recovered in cage wash, using Equation 9.3:

$$\%\ \text{Recovery from cage wash} = \frac{\text{Volume recovered in cage wash (mL)} \times \text{sample concentration (DPM/mL)}}{\text{Volume dosed (mL)} \times \text{dose concentration (DPM/mL)}} \times 100 \quad (9.3)$$

The detailed design of collection of samples in different matrices at different time intervals with applicable methods for the measurement of total radioactive drug, parent drug, and metabolites, with rationale and recommendations have been listed in Table 9.2.

If tritium-labeled compound is used, then tritiated water formation can be estimated to assess the metabolic stability of the radiolabel using the following equations [2]:

$$C_0 = C_{\text{mid}} \times \frac{0.693}{t_{1/2}} t$$

$$f = \frac{C_0 \times V_{\text{bw}}}{\text{dose}}$$

where C_0 is the concentration of tritiated water at time 0; C_{mid} is the concentration of tritiated water in urine at the midpoint of the collection interval; $t_{1/2}$ is the average tritiated water half-life in the rat, 85 h; t is the midpoint of the collection interval; f is the fraction of dose converted to tritiated water; and V_{bw} is the exchangeable body water volume in the relevant species.

9.4.3.3 Tissues and Organs. As part of preclinical mass balance studies employing a radiolabeled drug, tissue distribution studies are usually conducted when the disposition kinetics, distribution, and potential for accumulation of the drug and/or its metabolites in various parts of the animal body needs to be assessed [6,18]. The concepts of tissue/organ distribution studies, following administration of radiolabeled drugs in rodent, are briefly discussed in this section. The main objectives of these studies are as follows [19,20]:

1. To determine the distribution of drug-related material in various tissues/organs in the body.
2. To predict the exposure of drug-related materials at the target site of pharmacological response.
3. To investigate the potential sites involved in the metabolism.
4. To investigate the quantitative transfer across the blood–brain barrier, reproductive system, and placenta barriers.

These studies can aid in identifications of reasons for incomplete recovery of radioactivity, which may be due to reversible or irreversible tissue binding or incorporation into different organs/tissues. The use of radiolabeled compounds is extensive and very valuable in tissue distribution studies, and it is the basic requirement from regulatory agencies that preclinical animal tissue distribution data are submitted as a prerequisite for estimating the potential radioactivity exposure in clinical studies unless a trace radioactive dose with accelerator mass spectrometry (AMS) is used. Tissue distribution studies are performed to quantify the distribution of drug-related radioactivity (unchanged drugs or metabolites) in the target tissues of animal species. Typically, these studies are limited to single doses by the intended route of administration. After the administration of radiolabeled drugs, designated tissues and organs are promptly dissected after animals were sacrificed at various sampling time intervals [10], and the radioactivity was quantified using LSC. This quantification of the radioactivity by LSC in various tissues/organs can be achieved either by analyzing the homogenized tissue/organ or through combustion of tissue/organ to [CO_2] that is further trapped in alkaline solution and quantified for ^{14}C radioactivity (for ^{3}H-labeled compound, $^{3}H_2O$ is trapped). The concentration of drug-related material (total radioactivity) in major tissues and organs reveals the degree of transient exposure of specific organs to the drug [20,21].

Another method to quantify tissue distribution of radioactivity into various organs/tissues is the QWBA technique [22]. QWBA is used to quantify the distribution of radioactivity in a larger extent to all body compartments, which helps target specific organs for further recovery of radioactivity as described before [3] or drug-related components, if needed. In QWBA studies, at least one animal per time interval is sacrificed at various intervals after dosing and then frozen in a "microtomb" at $-20^{\circ}C$. Before whole-body section collection, standards fortified with [^{14}C] radioactivity (as quality control) are placed into the frozen block for monitoring the uniformity of the section thickness. Then thin slides of the whole animal with a thickness of $\sim 40\,\mu m$, including representative of all major tissues, organs, and biological fluids, are prepared on adhesive tape. A section set from each animal is prepared by mounting a representative section from each level of interest. These mounted sections are exposed on phosphorimaging screens along with plastic embedded for subsequent calibration of the image analysis software. These screens are exposed for almost three days (or seven days for tritium) and then scanned using a phosphor imager to measure the radioactivity. On the basis of the body surface area and body weight, the exposure of radioactivity to each tissue and organ in rats can be scaled to human tissues for the calculation of human whole body exposure [2] and effective dose for human administration [23] (interested readers are advised to refer to Chapter 26,Volume V, for detailed explanation on quantitative whole-body

TABLE 9.3 Tissue Distribution Study Design: Quantitative Whole-Body Autoradiography with Various Rationale and Comments

Distribution (QWBA)[a]	Design	Rationale and Comments
Dose	Same as the mass balance study	Support toxicity studies Support human ADME study (dosimetry)
Time points	For example, 0.5, 1, 2, 4, 8, 24, 48, 72, and 168 h (or other time points according to pre-existing information)	Tissue distribution estimation in organs/tissues and spatial resolution
Number of animals (N)	$N = 1$ for each time point	Fewer animals necessary Pigmented rats with one albino at 168 h or v.v. (to investigate melanin binding in eyes or skin)

Abbreviation: v.v., vice versa.

[a]Usually small animals are preferred, that is, rat and mouse, unless larger species are required. Selected organs of interest can also be collected for further analyses for the parent and/or metabolite(s).

autoradiography). The numbers of animal per study group, doses, and time intervals for both dissection and QWBA with rationale and comments have been compiled in Table 9.3.

9.4.3.4 Bile. Biliary excretion is an important elimination route and many compounds that are excreted in bile can be reabsorbed in the intestine, to complete an enterohepatic circulation cycle. The cycle can influence the absorption and elimination rate constants and alter the plasma concentration–time profile [24,25]. Bile-duct cannulation provides a way to examine the biliary excretion and potential enterohepatic circulation [26,27] and thus contributes useful information on the rate and extent of excretion of drug and/or metabolites via biliary pathways. Biliary excretion studies can be conducted by surgically ligating the common bile duct and inserting a cannula for bile collection [28] (see also Fig. 9.2). A recovery period of at least 24 h should be allowed before administering the test drug in order to avoid possible delays in gastrointestinal transit associated with postoperative adynamic ileus [29]. Tse and Ballard [26] developed a method using a pair of bile duct–duodenum cannula-linked rats, which permits a realistic approximation of biliary excretion and reabsorption in intact animals. After the acclimation period of the surgery, animals are lightly anesthetized and the continuous loop is then cut into two sections, for the bile collection and synthetic bile replacement to maintain the bile flow. A 50-cm extension tube is then attached to both sections of the continuous loop in order to pass through protective tether and spring. The animal is then placed back to metabolic cage and externalized tubing is attached to a dual channel swivel. The bile flow needs to be closely monitored for 1–2 h before the studies [30], and if possible, liver function tests can be performed by monitoring the level of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and bilirubin [31]. Control bile is collected from the bile-duct cannula up to 0.5 h pre dose. After the administration of dose, synthetic bile is infused (via duodenal cannula in rodents) at a continuous rate of 1 mL/h for the duration of the study. Bile samples are collected at predefined time intervals (e.g., pre dose, 0–2, 2–4, 4–8, 8–12,

TABLE 9.4 List of Various Parameters Needed to be Considered in Designing and Handling of Bile-Duct Cannulated Animals with the Rationale and Comments

Bile-Duct Cannulation	Design	Rationale and Comments
Criteria	If >65% of radioactive dose is recovered in feces after intravenous dosing	If <65% of dose is recovered, study is conducted if there is strong interest in specific metabolites
Doses	Low for oral route (standard); *For intravenous route:* same as used in intact animals	Extent of biliary excretion/absorption/recycling
Bile substitution	Yes, method to be chosen	To avoid bile depletion
Time intervals	Bile in daily fractions up to 72 h (e.g., 0–2, 2–4, 4–8, 8–12, 12–24, 24–48, 48–72)	Maximum collection time feasible
Number of animals (N)	$N = 3$ for oral dosing; $N = 2$ for intravenous dosing	Means values are calculated
Volume of samples	Same as excretion studies	—
Number of measurements	Same as excretion studies	—

12–24, 24–48, and 48–72 h post dose). In addition, urine and feces are also collected in 24-h intervals for the duration of study. All excreta (urine, bile, and feces) are stored at approximately -20°C or lower until the time of analysis. After 72 h, the animals are sacrificed and the animal carcasses are retained at -20°C for further analysis as needed. To evaluate the enterohepatic circulation, the blood samples can be collected from BDC rats. The enterohepatic circulation can be estimated based on the blood or plasma exposures between BDC rats and intact rats. The enterohepatic circulation can also be accessed by introducing bile from the animal (donor) dosed with the drug to the intestine of another animal (receiver) without drug dosed. The design and handling of BDC animals are summarized in Table 9.4. The percentage of radioactivity recovered in bile is calculated from the ratio of the amount of radioactivity recovered in bile to the amount of radioactivity administered using Equation 9.4:

$$\%\ \text{Recovery from bile} = \frac{\text{Volume recovered in bile (mL)} \times \text{bile concentration (DPM/mL)}}{\text{Volume dosed (mL)} \times \text{dose concentration (DPM/mL)}} \times 100 \quad (9.4)$$

9.4.3.5 Carcass and CO_2 Exhalation. In some cases, the mass balance recovery is not complete, as determined from the total recovery in excreta, when compared with the administered dose. The most common reason is that the drug and/or its metabolites have a long half-life or are retained in certain tissue/organs, via, for example, irreversible binding to tissue/organ components or the incorporation of the radioisotope into endogenous components [6]. In such cases, as briefly discussed, the QWBA data may provide useful information. The radioactivity retained in the organs or carcass also can be analyzed to identify reasons for low recovery. In this method, animals are

sacrificed at the last time point, for example, 168 h, with an overdose of appropriate anesthesia and are stored at approximately -20°C pending potential analysis. Most organs can be easily homogenized and/or digested with a solubilizer. The carcass is often digested in organic alkaline solution followed by homogenization. For instance, the carcass may be digested in 30% methanolic potassium hydroxide solution (1-g sample weight per 1 mL) for approximately two days. Ethanol (two volumes of the sample weight) can be added to the solubilized sample and the net sample weight is measured for later calculation. The sample is further homogenized by a Polytron®. Owing to the high collagen content, a higher solvent volume to carcass weight ratio is generally required for homogenization on the large animal species, especially nonrodents, to prevent coalescing of the homogenate. An aliquot of homogenate ($\sim$200 mg) is taken in three to four replicates for the radioactivity determination using liquid scintillation counting to calculate the total remaining radioactivity. The percentage recovery in a carcass is calculated using Equation 9.5:

$$\%\ \text{of recovery in carcass} = \frac{\text{Total weight of soubilzed material (g)} \times \text{sample concentration (DPM/g aliquot)}}{\text{Volume dosed (mL)} \times \text{dose concentration (DPM/mL)}} \times 100 \quad (9.5)$$

TABLE 9.5 List of Various Factors to be Considered, While Addressing Radioactivity Recovery Issues, Including Recovery From Carcass and CO_2 Exhalation with Rationale and Comments

	Design	Rationale and Comments
Carcass		
Criteria	If mass balance in excreta <90% (consider residual radioactivity in tissues)	Only in intact animals; Small animals preferred but mainly depend on drug under study
Method of analysis	Solubilization or homogenization and analysis using liquid scitillation counting or HPLC with radiochromagraphy detector	
Number of animals	Animals with recovery <90%	To add information about incomplete recovery
Time intervals	At the end of the study, for example, 168 h	—
Expired CO_2		
Criteria	If mean mass balance inclusive carcass <90%	To add information about incomplete recovery
Number of animals	$N = 2$ (only select one representative route)	—
Collection	0–12, 12–24 h	To avoid saturation of absorber
	Optional: 24–168 h in intervals metabolic cage	To get mass balance recovery

Also see Fig. 9.6 for the basic considerations of troubleshooting.

If the mean mass balance including excreta and carcass is <90% for all routes and dosages and carbon dioxide is expelled as a metabolic product of the compound, one can monitor the radioactivity in the expired [$^{14}CO_2$] for more complete radioactivity recovery [32]. In a number of position of radioactive [^{14}C]-labeled compounds, the radioactivity of expired [$^{14}CO_2$] was successfully measured for the calculation of mass balance in the expired air [33]. Expired air can be collected by keeping the animals (rodents or nonrodents) in special metabolic cages in which air is drawn by vacuum pump with continuous air replacement. Exhaled air exiting the metabolic cage is passed through an appropriate trapping solution (usually alkaline solution), such as 2-methoxy-ethanol and 2-amino-ethanol (2:1) [34], and converted to nonvolatile carbonates. The trapping solution is replaced and assayed at designated times post dose so that the total radioactivity expired as labeled carbon dioxide can be determined [17,21,33]. The percentage recovery of expired radioactivity through exhalation is calculated from the concentration of radioactivity collected as carbonate using Equation 9.6:

$$\%\ \text{Recovery from } CO_2 = \frac{\text{Volume of trapping solution (mL)} \times \text{concentration (DPM/mL)}}{\text{Volume dosed (mL)} \times \text{dose concentration (DPM/mL)}} \times 100 \quad (9.6)$$

Various parameters of designing and handling of radioactivity recovery in carcass and CO_2 are listed in Table 9.5.

9.5 USE, SELECTION CRITERIA, AND LIMITATIONS OF RADIOLABEL COMPOUNDS

Radiolabeled drugs have been widely used in mass balance studies since the radioactivity can be easily detected and quantified using scintillation techniques, and the disposition fate of drugs can be simultaneously characterized. The radioisotope needs to be carefully selected or designed for a successful mass balance study, along with the position of radiolabel in the compound, radiochemical purity, and the specific activity, for they can have a significant impact on the formation of metabolites and detection and thus affect the recovery of total radioactivity. For example, radiolabel isotope should be at metabolically stable position of the parent molecule such that it can stay associated with the metabolites for identification and quantification. Failure to do so not only affects the mass balance results but can also cause difficulties in differentiating endogenous compounds from the true metabolites [21]. Various radioisotopes can be used in mass balance studies, including tritium-[^{3}H], carbon-[^{14}C], phosphorus-[^{32}P], sulfur-[^{35}S], and iodine-[^{131}I] [8,35–38]. The selected radiolabeled compound must have identical PK properties and metabolic fate as the unlabeled compound [39]. Prior knowledge of the metabolism of a compound (or known analogues) obtained from *in vitro* metabolism studies and/or *in silico* predictions can assist in determining the optimum radiolabeled positions and isotopes. Ideally, the same radiolabeled compound is used in both the preclinical and clinical mass balance studies, making human mass balance data more comparable to preclinical studies. [^{3}H] and [^{14}C] are the most commonly used isotopes in most of the mass balance studies. If heavier radioisotope are selected rather than [^{14}C] and [^{3}H], the corresponding kinetic isotope effect needs to be considered because metabolic conversion can be affected by change in reaction

kinetics, as a result of the formation of strong chemical bonds by heavier isotopes [40]. [^{14}C] is a preferred isotope due to its physiochemical stability, longer decay half-life, and higher radiation energy for better analytical sensitivity. Carbon [^{14}C] has eightfold more energetic β-radiations (electrons) than that of tritium [^{3}H] (0.156 vs 0.019 MeV), and thereby the detection sensitivity of [^{14}C] is higher than [^{3}H]. On the other hand, tritium-labeled isotope allows for a less expensive and more rapid chemical synthesis in most cases and a higher specific activity can be achieved (29.1 Ci/milliatom) than with [^{14}C] (62.4 mCi/milliatom) [7,40]. These advantages may be useful for the early development stage and studies with a need of very small amounts of a compound. However, the major disadvantage of [^{3}H] is its tendency to be exchanged with hydrogen in aqueous matrices, resulting in unlabeled compounds, especially when [^{3}H] is bonded to a heteroatom or α-positioned to a double-bonded heteroatom [40,41]. If the drug compound has a large molecular weight (>700 g/mol), it is likely the molecule will divide into two metabolic moieties. The molecule can be dually labeled with [^{3}H] and [^{14}C] atoms to the designated moiety, respectively, and, thereby each moiety can be traced independently [21]. For either [^{3}H]- or [^{14}C]-labeled drug at the metabolically stable position, radiochemical purity is preferred >98% and should not be <95%. The purity of the radiolabeled drug should be validated before the dose preparation, and stability of the radiolabeled drug under conditions of administered should be checked at predose and postdose time points [2].

9.6 DETERMINATION AND ANALYSIS OF MASS BALANCE AND PHARMACOKINETIC PARAMETERS

The main purpose for conducting the mass balance studies is to determine the mass recovery of administered radioactive dose, identify major metabolites and excretory pathways, and characterize the PK profile of the drug compound by means of measuring the concentration of parent drug/metabolites or total radioactivity in blood, plasma, urine, bile, and feces. In order to confirm the validity of mass balance results, these PK parameters obtained from the mass balance study can be compared with those from preclinical toxicity studies. All PK parameters for total radioactivity and parent drugs can be calculated with the software programs (e.g., WinNonlin), using noncompartmental or compartmental analysis method. For the p.o. dose, highest average plasma concentration ($C_{\max}$) and times to achieve this peak concentration ($T_{\max}$) are recorded directly from experimental observations for the drug and its metabolites. The radioactivity in blood and plasma is measured separately using LSC. These measured radioactivity parameters are also called *pseudo-pharmacokinetic parameters* and are calculated in the same manner as those of parent drug. The ratio of AUC of unchanged, intact parent drug versus the AUC of total radioactivity gives a measure of metabolites formation and their importance in drug disposition. The $AUC_{(0-last)}$ is calculated using the linear trapezoidal rule, whereas $AUC_{(0-\infty)}$ is calculated using the Equation 9.7 as

$$AUC_{(0-\infty)} = AUC_{(0-last)} + \frac{C_{(last)}}{\lambda_z} \tag{9.7}$$

Here, the slope (λ_z) is derived from the slope of the log-linear line from at least the last three data points. This is used to calculate the half-life ($t_{1/2}$). Distribution half-life and elimination half-life are calculated to predict the accumulation in different

compartments and elimination time of the drug. The elimination half-life ($t_{1/2}$) of radioactivity in the plasma is important in selecting the time interval and further aid in determining the study end point, maximize the radioactive recovery in mass balance studies, and also assess the risk of accumulation of metabolites after multiple dosing [4]. Comparing the half-life of parent drug with that of total radioactivity gives a better understanding of the accumulation of the metabolites (*note:* detection sensitivity also needs to be taken into consideration). For the i.v. dose, the concentration of parent compound at time zero $C_{(0)}$, is back-extrapolated, based on a log-linear regression. The apparent volume of distribution at steady state (V_{ss}) is calculated from the i.v. data using expression given by Equation 9.8:

$$\mathrm{Vss} = \frac{\mathrm{dose}_{(\mathrm{i.v.})} \times \mathrm{AUMC}_{(0-\infty)}}{[\mathrm{AUC}_{(0-\infty)}]^2} \tag{9.8}$$

where $\mathrm{AUC}_{(0-\infty)}$ and $\mathrm{AUMC}_{(0-\infty)}$ are the area under the concentration–time curve and area under the first moment concentration–time curve from time zero to infinity, respectively.

The total body clearance ($\mathrm{CL_t}$), renal clearance ($\mathrm{CL_r}$), biliary clearance ($\mathrm{CL_b}$), and hepatic clearance ($\mathrm{CL_h}$) can be calculated using following expressions.

The total systemic clearance ($\mathrm{CL_t}$) is estimated from the ratio of dose divided by $\mathrm{AUC}_{(0-\infty),(\mathrm{i.v.})}$ from the parent dose after i.v. dose and is given by Equation 9.9 [42]:

$$\mathrm{CL_t} = \frac{\mathrm{dose}_{(\mathrm{i.v.})}}{\mathrm{AUC}_{(0-\infty),(\mathrm{i.v.})}} \tag{9.9}$$

In most cases, drug concentrations are measured in plasma rather than in blood, because sample preparation and drug analysis in plasma are simpler than in blood and this total systemic clearance is defined as equal to the total systemic plasma clearance, unless otherwise indicated in the text.

Renal clearance ($\mathrm{CL_r}$) can be estimated from amount of unchanged parent dose excreted in urine over an extended period of time dividing by $\mathrm{AUC}_{(0-t)}$ of parent drug after i.v. dose and is given by Equation 9.10 [42]:

$$\mathrm{CL_r} = \frac{\text{Amount of parent drug excreted in urine up to time } t}{\mathrm{AUC}_{(0-t),(\mathrm{i.v.})}} \tag{9.10}$$

The urine collection time should be long enough to account for almost the entire parent drug excreted in urine in order to get a reliable estimate of the $\mathrm{CL_r}$. Additionally, it is desirable that $\mathrm{AUC}_{(0-t)}$ is greater than 90% of $\mathrm{AUC}_{(0-\infty)}$.

Biliary clearance ($\mathrm{CL_b}$) of a drug is experimentally determined from the ratio of the amount of parent drug excreted in the bile versus the $\mathrm{AUC}_{(0-t),(\mathrm{i.v.})}$ of parent drug after i.v. dose and is given by Equation 9.11 [42]:

$$\mathrm{CL_b} = \frac{\text{Amount of parent drug excreted in bile from time 0 to } t}{\mathrm{AUC}_{(0-t),(\mathrm{i.v.})}} \tag{9.11}$$

where $\mathrm{AUC}_{(0-t),(\mathrm{i.v.})}$ is the AUC in plasma from the time 0 (zero) to t hours, and t is usually more than 24 h for sufficient bile collection after i.v. administration of a drug.

Clearance from different routes are additive and systemic or total clearance is given by the sum of individual organ clearances [43]. Assuming that the respiratory and extracorporeal clearances are negligible, hepatic clearance (CL_h) can be estimated by subtracting the sum of the renal and bile clearance ($CL_r + CL_b$) from the total clearance (CL_t) as expressed by Equation 9.12:

$$Cl_h = CL_t - (CL_r + CL_b) \tag{9.12}$$

Equation 9.13 assumes that nonrenal and nonbiliary clearances may be due to liver metabolism. The cumulative percentage of collected radioactivity recovery from different matrixes (urine, feces, bile, cage wash, carcass, and expired air) gives the estimate of total mass balance recovery for any compound when compared with the dose administered, that is:

Total % radioactivity recovered = % of radioactivity recovered in urine (Eq. 9.1) + % of radioactivity recovered in feces (Eq. 9.2) + % of radioactivity recovered from cage wash (Eq. 9.3) + % of radioactivity recovered from bile (Eq. 9.4 is only applicable for BDC rats) + % of radioactivity recovered from carcass (Eq. 9.5) + % of radioactivity recovered from expired air (Eq. 9.6).

Table 9.6 summarizes the different methods for the calculation of various parameters from the mass balance study design with rationale and comments.

In addition, the mass balance studies can also determine the following other parameters from intact and BDC animals:

1. percent of radioactivity retention in different organs at any time during the study or at the end of the study,

TABLE 9.6 List of Factors to be Considered for Pharmacokinetic Parameters Analysis in Mass Balance and ADME Studies

Design	Calculations	Rationale and Comments
Pharmacokinetics of radiolabeled in blood and plasma	C_0, C_{max}, T_{max} observed; $AUC_{(0–168h)}$ and $AUC_{(0–\infty)}$; $t_{1/2}$ terminal half-life %; absorption (fraction absorbed, F_{abs})	With i.v. dosing determine C_0; With oral dosing determine C_{max}, T_{max}; Estimation of exposure; Prediction of accumulation of the drug-related radioactivity; Estimation of fraction absorbed; Comparison to toxicity studies; Comparison to other species
PK of unchanged drug in blood or plasma	C_0, C_{max}, T_{max} observed; $AUC_{(0–168h)}$ and $AUC_{(0–\infty)}$; Distribution and/or elimination half-life; CL, V_{ss}, MRT	Prediction of drug behavior Oral bioavailability First-pass estimate
Radioactivity in urine, bile, feces, cage wash, and carcass in rodents, if recovery in excreta is low	Cumulative % of dose % mass balance recovery % absorption (fraction absorbed, F_{abs})	Contribution of excretion route; Estimation of fraction absorbed for permeability classification needed for potential FDA biowaivers

Abbreviation: MRT, mean residence time.

2. level of parent drug and its metabolites in the target organs,
3. ratio of radioactivity exposure in organs (AUC_{organ}) and in plasma (AUC_{plasma}), and
4. fraction absorbed (F_{abs}) across the gastrointestinal barrier.

Oral bioavailability ($F\%$) gives an estimate for the extent of absorption and first-pass metabolism and it can be calculated from the ratio of exposure of parent drug after an p.o. dose to the parent drug exposure after an i.v. dose. Some reports have stated that the area under the curve (AUC) obtained from plasma radioactivity [21] can be used to calculate bioavailability, but it is not valid for the compounds with high first-pass metabolism. Tse and Laplanche [44] had already demonstrated the differences in the amount of drug-related material absorbed and the systemic bioavailability of the parent drug using the radioactivity.

The bioavailability ($F\%$) is estimated from the calculated AUC after the p.o. and i.v. administrations of parent drug using the Equation 9.13:

$$F\% = \frac{AUC_{(0-\infty),(p.o.)} \times dose_{(i.v.)}}{AUC_{(0-\infty),(i.v.)} \times dose_{(p.o.)}} \times 100 \tag{9.13}$$

The fraction absorbed or absorption (F_{abs}) can be calculated from the ratio of $AUC_{(0-\infty)}$ after the p.o. and i.v. administrations of total blood/plasma radioactivity using the same equation as for $F\%$.

This maximum fraction absorbed can be used to distinguish the drug on basis of permeability classification, which can be served as an important tool for potential FDA biowaivers. In some cases, the radioactivity excreted in urine can be used to calculate F_{abs}. Various other methods that can be used for the calculations of fraction absorbed are listed in Table 9.7 along with the limitations and recommendation.

TABLE 9.7 Different Methods for Estimation of Absorption and Bioavailability from Calculated Parameters of the Intact Parent Drug or Radiolabeled Drug, Total Radioactivity

Estimation of Percent Absorption or Fraction Absorbed (F_{abs})	Equation	Comments and Limitations
F_{abs} calculated from total blood/plasma radioactivity amount	$F_{abs} = \frac{AUC_{(0-\infty),(p.o.)} \times dose_{(i.v.)}}{AUC_{(0-\infty),(i.v.)} \times dose_{(p.o.)}}$	Not valid for a compound with high first-pass metabolism
F_{abs} calculated from total cumulative urinary radioactivity recovery ratio (p.o. vs i.v.)	$F_{abs} = \frac{D_{(urine),(p.o.)}}{D_{(urine),(i.v.)}}$	Minimal absorption; best suited for compounds with renal excretion >10%
F_{abs} calculated from bile-duct cannulated animals	$F_{abs} = \frac{D_{(urine),(p.o.)} + D_{(bile),(p.o.)}}{D_{(uirne),(i.v.)} + D_{(bile),(i.v.)}}$	Value represents minimal absorption
F, oral bioavailability from intact parent drug, ideally in a crossover design	$F = \frac{AUC_{(0-\infty),(p.o.)} \times dose_{(i.v.)}}{AUC_{(0-\infty),(i.v.)} \times dose_{(p.o.)}}$	Assumes CL is independent of dosing routes and dose amount

D, fraction of the dose recovered from urine or bile.

9.7 CASE EXAMPLE FOR MASS BALANCE STUDY IN PRECLINICAL SPECIES

9.7.1 Compound A—Experimental Design

For compound A, preclinical mass balance studies were designed based on the methodology as described in Tables 9.1–9.5. The dose used was 5 mg/kg for the i.v. dose and 50 mg/kg for the p.o. dose for both the intact rat and BDC rat studies. The radiochemical purity was >99% for all study groups.

9.7.2 Compound A—Pharmacokinetic Parameters

All PK parameters for compound A were calculated based on the designs as discussed in Table 9.6. The time versus plasma concentration profile of unchanged compound A along with compound A-related total radioactivity after i.v. dosing and p.o. dosing are presented in Figs. 9.4 and 9.5, respectively. The maximum concentration (C_{max}) for parent compound A observed in plasma is 950 ng/mL, which is achieved at 2 h post p.o. dosing, indicating a moderate absorption rate. Other observed and calculated PK parameters in different matrices are summarized in Table 9.8 for both p.o. and i.v. doses. The ratio of $AUC_{(0-\infty)}$ of parent drug versus $AUC_{(0-\infty)}$ of radioactivity is ~55%, indicating that some amount of metabolite(s) were formed from compound A and present in the blood circulation.

9.7.3 Mass Balance Parameters and Interpretations

The percentage of dose recovered for unchanged compound A in different matrices is shown in Table 9.9: All mass balance parameters were calculated from the derived equations (Eqs. 9.1–9.6) and using the methodology discussed in Table 9.6. As shown, compound A is predominantly eliminated as unchanged drug (57–69% of the total dose) in urine and feces. The major portion of the drug is recovered in feces with a

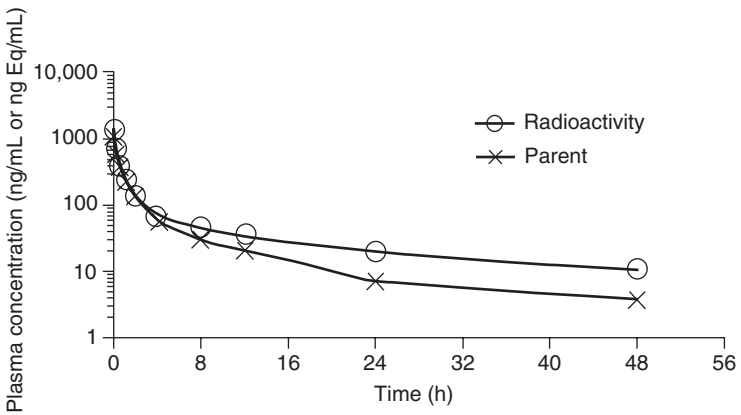

Figure 9.4 Plasma drug concentration versus time profile of parent (unlabeled) drug and radioactivity (labeled) drug after the intravenous dose. Concentration of unlabeled drug is given in units of ng/mL and concentration of labeled drug is given in units of ngEq/mL, respectively.

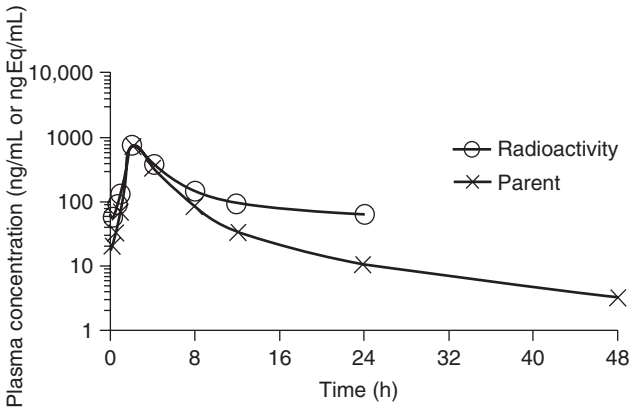

Figure 9.5 Plasma drug concentration versus time profile of parent (unlabeled) drug and radioactivity (labeled) drug after the oral dose. Concentrations of unlabeled drug is given in units of ng/mL and concentration of labeled drug is given in units of ngEq/mL, respectively.

TABLE 9.8 Pharmacokinetics Parameters of Unchanged Parent Compound A or Compound A-Related Radioactivity After Intravenous Dosing (5 mg/kg) and Oral Dosing (50 mg/kg) in Rats

PK Parameters	C_{max} (ng/mL or ngEq/mL)[a]		T_{max} (h)	$AUC_{(0-\infty)}$ (ng · h/mL or ngEq · h/mL)[a]		CL (L/h/kg)	V_{ss} (L/kg)	$F\%$ or F_{abs}	$t_{\frac{1}{2}}$ (h)	
	i.v.	p.o.	p.o.	i.v.	p.o.	i.v.	i.v.	p.o.	i.v.	p.o.
Plasma (parent)	1050	950	2	1500	3810	3.1	31	25	15	15
Plasma (radioactivity)	1350	1000	2	2700	6900	—	—	26	—	—
Blood (radioactivity)	1220	850	2	1680	4900	—	—	29	—	—

[a]Concentration units are ng/mL for parent and ngEq/mL for radioactivity. C_{max} after intravenous dosing is referred to as C_0.

TABLE 9.9 Unchanged Parent Compound A Recovered in Different Matrices

% Dose Recovery (Unchanged Drug)	Intact Rats		Bile-Duct Cannulated Rats	
	i.v.	p.o.	i.v.	p.o.
Excretion in urine	18	3.0	19	3.1
Excretion in feces	39	66	11	53
Excretion in bile	N/A	N/A	35	8.0
Total recovery (%)	57	69	65	64
CL_r (L/h/kg)	0.60	—	0.62	—
CL_t (L/h/kg)	3.1	—	—	—

Abbreviation: N/A, not applicable.

smaller amount in the urine following both routes of administration. The portion of unchanged parent compound A excreted in urine is approximately the same in intact and BDC rats after administration of both doses (19% for i.v. and ~3% for p.o.). As expected, the recovery in feces after i.v. dosing is lower in the cannulated compared to the intact rats (11% vs 39%), indicating biliary excretion of compound A, which was 35%. The renal clearance, CL_r, is comparable (~0.6 L/h/kg) between the intact and BDC rats.

The percentage of dose recovered as total radioactivity in different matrices is shown in Table 9.10. In intact rats, the fecal excretion of the radioactivity is dominant after both dosing routes accounting for 74% of dose after i.v. administration and 93% of dose after p.o. administration. The urinary radioactivity excretion is minor, accounting for 20% of the i.v. dose and 4% of p.o. dose, and is consistent in both intact and BDC rats. On the basis of combined biliary (21%) and renal excretion (5%), the absorption after p.o. dosing is estimated to be at least 26% for compound A, which is comparable to the values estimated from total plasma or blood radioactivity data (26-29%). The mass balance of radioactive dose recovered in excreta, including the cage wash is calculated using Equations 9.1–9.6 and is completed (~100%) within 168 h post dose. From these obtained parameters of the mass balance recovery, the calculation for various other parameters such as bioavailability, fraction absorbed, total clearance, renal clearance, biliary clearance, and hepatic clearance can be calculated based on Equations 9.9–9.13 and are listed as follows:

- The estimation of p.o. bioavailability of compound A, calculated from the parent drug using Equation 9.13, is ~25%, which is low.
- The estimation of fraction absorbed, calculated from the total radioactivity using Equation 9.13, is 26% (plasma) to 29% (blood), indicating that the absorption of compound A in rats is low and incomplete.
- The estimation of total clearance from the total exposure of the parent drug with time curve and the total dose administered i.v. calculated using Equation 9.9 is 3.1 L/h/kg, which indicates a high plasma clearance for compound A.
- The renal clearance is calculated by dividing percentage of parent drug recovered in urine after i.v. administration by the area of concentration under the curve with time, using Equation 9.10, and is 0.6 L/h/kg, which is 19% of the total clearance.

TABLE 9.10 Mass Balance Parameters for Compound A, Radioactivity Recovery in Different Matrices

% Dose Recovered (Radiolabeled Drug)	Intact Rats		Bile-Duct Cannulated Rats	
	i.v.	p.o.	i.v.	p.o.
Excretion in urine	20	4.0	21	5.0
Excretion in feces	74	93	16	74
Excretion in bile	N/A	N/A	63	21
Cage wash recovery	2.0	1.0	1.0	1.0
Total recovery (%)	96	98	101	101

Abbreviation: N/A, not applicable.

- The biliary clearance can be estimated from percentage of parent drug excreted in bile after i.v. administration, divided by the area of concentration under the curve with time, from the BDC rats, using Equation 9.11, and is 1.2 L/h/kg, indicating that 39% of total clearance is through bile.
- The hepatic clearance is calculated by subtracting the cumulative renal and bile clearance from the total clearance using Equation 9.12 and is 1.3 L/h/kg, suggesting that ~42% of the total clearance is hepatic clearance, suggesting that the drug is not extensively metabolized.

9.8 COMMON ISSUES DURING CONDUCT OF PRECLINICAL MASS BALANCE STUDIES

The most common challenge in mass balance studies is a low mass recovery of radioactivity. Theoretically, complete mass balance studies would require that 100% of the radioactivity administered should be recovered from excreta and cage wash, which is rarely possible. The most common reasons are that the excreta is not completely collected during the study, contributing to the loss of radioactivity balance collection, especially if the loss occurs within a period of high excretion rate of drug-related material [14]. As a rule of thumb, excreta samples need to be collected until the recovery is <1% of administered radioactivity in urine and feces for two consecutive days [4]. In the case for compounds with a long elimination half-life, as indicated by a prolonged retention of the radioactivity in blood, the sample collection period requires to be extended. Some other issues can influence the recovery of mass balance. Position of the radionuclide within the drug molecule should be carefully considered in order to prevent a labeled fragment of the drug from entering endogenous compound

TABLE 9.11 Reasons for Low Radioactivity Recovery with Proposed Recommendations

Reason for Low Recovery	Proposed Recommendation
Artificial reasons	
Inaccurate dose	Careful consideration in dose preparation and administration
Incomplete collection of excreta	Use of modified cages; collection of excreta until 1% recovery for consecutively two days
Portion of dose lost in vomiting (without collection)	Careful observation is needed after the oral administration
Long half-life ($t_{1/2}$) of radioactivity, long collection period, or diluted sample with drug concentration below the LOQ	Use of sophisticated analytical tools is recommended to obtain the maximum recovery
Scientific reasons	
Position of radioisotope in molecule, which is lost in expired air	If the recovery is below 80%, it is recommended to measure the radioactivity in the expired air
Tissue binding and noncovalent sequestration	Must be carefully studied for the parent drug and its metabolites from the preclinical studies

Abbreviation: LOQ, limit of quantification.

metabolism and also to prevent its conversion to a volatile metabolite as $^{14}CO_2$ [14], resulting in incomplete mass balance recovery. For lipophilic drugs that have low recoveries in urine and feces, the compounds can suffer from significant nonspecific adsorption to the collection containers. Mass balance studies provide limited information regarding the covalent binding to tissue macromolecules or to the blood proteins, which may account for a low mass recovery compared to total drug administered. The potential causes for low mass balance recoveries from preclinical and human studies are summarized in Table 9.11, and the reader is referred to a review article by Roffey

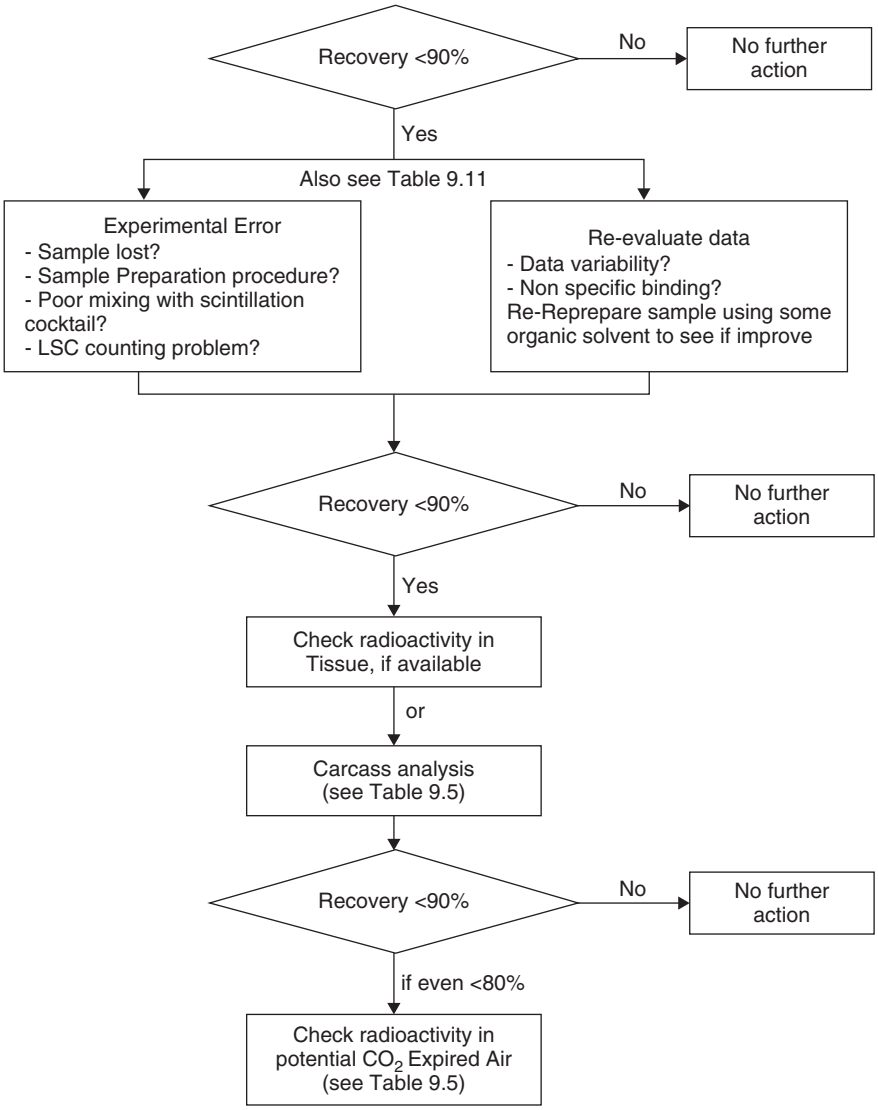

Figure 9.6 Basic considerations for the troubleshooting of low recovery issues in mass balance studies, usually conducted in rodents.

et al. [14]. Basic troubleshooting steps for mass balance studies with low recovery are listed in Fig. 9.6. In some BDC rat studies, the percent of radioactivity recovered in urine may be significantly higher when compared to that in intact rats even when the bile flow is in the normal range. This might be due to the alternation in the liver physiology (such as the changes in liver function, the expression of enzymes, and transporters) after bile-duct cannulation. In such cases, the data interpretation should be carried out with care and more analysis should focus on the results from the intact rats. As discussed, plasma samples may be taken for rat liver function tests before the bile study is conducted.

9.9 HUMAN MASS BALANCE STUDIES

After obtaining information of toxicity, PK, metabolite profiling, and organ/tissue radioactive exposure from preclinical mass balance studies, human mass balance studies can be conducted. These studies are required for small molecules by regulatory agencies before new drug application (NDA) filings and the mass balance data together with metabolite information is also important for the design of the clinical trails. The estimated timeline for conducting human mass balance studies is described in Fig. 9.1, which shows that the human mass balance studies are initiated once the phase 1 trials have been conducted and should be finished before the commencement of phase 2 trials. It is important to understand the metabolite profile of drugs in human and animal species as early as possible since both the ICH M3 (R2)[45] and the FDA [5] guidances highlight the need for providing safety data on human metabolites. Some metabolites are not much of toxicological concerns (e.g., most glutathione conjugates and glucuronides, except acylglucuronides). In general, ICH M3 (R2) recommends conducting the safety evaluation studies in preclinical species for metabolites that contribute more than 10% of total drug-related exposure and at significantly greater levels in human than the maximum exposure observed in the toxicity studies. The FDA guidance recommends that metabolites with an AUC exceeding 10% of the intact parent drug AUC will require toxicological evaluation in preclinical species. Because the use of radiolabeled drug in human mass balance studies adds more complexity, these studies are performed in accordance with the following codes and guidelines: Title 21, Part 56 CFR (Institutional Review Board Approval) [46]; Title 21, part 50 CFR (Protection of Human Subjects) [47]; the principles of the Declaration of Helsinki and its amendment; and Good Clinical Practice, in addition to standard International Conference on Harmonization (ICH) guidelines. For the studies to be conducted in the European Union (EU), European guidelines [48,49] are recommended to be followed. Before conduct all studies that include the use of radioisotope must be approved by the Administration of Radioactive Substances Advisory Committee (ARSAC). While these existing regulatory guidelines primarily focus on the safe and effective dose of radioactivity, in terms of exposure of ionizing radiations to various tissues/organs, to ensure "safe and effective" use of radioactive drugs [50], they do not state any acceptable criteria for total radioactivity recovery. Acceptable values for recovery, such as 85% [51] and 90% [4,14] have been proposed, but there is no widely accepted and defined cutoff value with a compelling underlying rationale [14]. The main objective of human mass balance studies is to establish the route of elimination of radioactive compounds and to compare the metabolites pattern in excreta, blood, and plasma with those obtained

from previously conducted preclinical mass balance studies for the safety exposure assessments. The nature of these studies is not intended for statistical evaluations, and ethics issues dictate the use of limited number of subjects [2]. Typically 3–8 subjects, aged 15–65 years, participate in human mass balance studies. It should be noted that conducting mass balance studies in children and pregnant women is virtually impossible due to the ethical issues, even though the drug disposition in these two groups differs significantly from the average population [52]. For anticancer drugs (mutagenic, carcinogenic, or teratogenic), healthy subjects are usually not used—patient subjects are needed for these mass balance studies. After at least an 8-h overnight fasting, in general, each subject receives a single clinically relevant dose of [^{14}C] drug containing 50–100 μCi of radioactivity. If [^{3}H] drug is administered the higher dose of radioactivity (50–1000 μCi) is needed to detect the low energy β-radiation of [^{3}H] [4]. The human radioactive dose must be evaluated for safety radiation exposure, using tissue distribution data from preclinical mass balance studies. Extrapolation of these data to humans allows the estimation of effective organ exposure to the radioactivity that is used for regulatory evaluation [50,53]. Mass balance studies in humans with the aid of the radiolabeled drug provide the total fate of drug-related material [7]. The projected human radiation exposure to selected radioisotope is estimated by direct extrapolation from the radioactivity exposure data obtained from the preclinical tissue distribution study (QWBA) based on body weight ratio [19] or drug exposure ratio (clinical plasma data compared to rat QWBA), with the assumption that drug-associated radioactivity is distributed and eliminated from the tissues in comparable manners between animal species and humans.

AMS can deliver analytical sensitivity far in excess of most other tools and has been applied to the preclinical and clinical mass balance studies [4,54]. The radiation risk, therefore, is not a concern for the study using AMS due to its low radioactive dose (tracer) and dosimetry data are not required. The high cost and low throughput are major disadvantages. The structure of metabolites should still be characterized by other analytical techniques unless the authentic reference compounds are available for coelution.

9.10 HUMAN DOSIMETRY CALCULATIONS

The purpose of the following section is to describe the various methods for the estimation of the human radioactive dose from preclinical mass balance studies using various calculation methods, which are also required for ARSAC approval (EU) to ensure the safety and efficacy after a human radioactive dose. The use of radiolabeled compounds in humans is also required to comply with the principles of As Low As Reasonably Acceptable (ALARA). International Commission on Radiological Protection (ICRP) [55] have modified the World Health Organization (WHO) exposure categories and made required the calculation of effective dose (E) [56]. This extrapolation of parameters obtained from preclinical mass balance studies to human organs is further based on the following factors:

1. the known human plasma level of the intact parent compound,
2. the observed ratio of radioactive compound relative to the parent compound in animals, and

3. the ratio of radioactivity in individual organs relative to the radioactivity in plasma for preclinical species.

The revised recommendations for radiological protection in ICRP publication-103 formally replace the previous ICRP publication-60 recommendations, and the reader is advised to follow the new guidelines to control the exposure from the radiation sources. The guidelines, provided by the U.S. Nuclear Regulatory Commission (USNRC), Part-20 of Standards for Protection against Radiation [56,57], which specify the list of different tissues/organs that are more susceptible to radiation damage and are required to be considered. Because of their susceptibility to damage, each organ is given a different weighing factor (W_T), by which the equivalent dose in various tissues/organs (T) is calculated. W_T represents the relative contribution of radioactivity in each defined tissue/organ and considers this information when calculating the total body radioactivity exposure, probability of fatal and nonfatal cancer, severe hereditary effects, and relative length of life lost. The ICRP publication-103 [58] and the U.S. Nuclear Regulatory Commission (USNRC), Part-20 of Standards for Protection against Radiation [56,57] have updated the radiation and tissue weighing factors based on the latest scientific information available and it is advised to follow these recommendations in both academia and industry. The list of specified tissues and their recommended weighting factor from ICRP-60, ICRP-103, and USNRC, Part-20 are listed in Table 9.12. For tissues/organs containing radioactivity, the exposure is calculated as the area under the radioactivity time curve (AUC) of the radiolabeled compound in the tissues/organs. Exposure in excretory organs, for example, gastrointestinal tract and bladder, is determined by the total radiation amounts in excreta (urine, bile, and intestinal contents) and the mean organ resistance time in humans [20].

9.10.1 Dosimetry Calculations

The calculations for the human radiolabeled dose of [^{14}C] drug are carried out using the equations given below. The equations are derived based on the recommendations of the ICRP (Limits for intakes of Radionuclide by Workers, Radiation Dose to patients from Radiopharmaceuticals) [56,60,61]. The units of curies (Ci), as opposed to the International System of Units, Becquerel (Bq), usually express the radioactive dose. The conversion of these units is 1 mCi = 37 MBq and 1 Bq = 60 DPM, where DPM means disintegrations per minutes. The fundamental equations derived for the calculation of the equivalent dose [59,62], which can be taken up in a given organ or tissue for [^{14}C] drug, are given as follows:

$$H_t = E \times f \times \text{AUC}$$

where

H_t = equivalent dose (mSv)

E = mean particle energy (MeV), and calculated for [^{14}C], MeV = 0.050

f = conversion factor = $\frac{\text{mSv}\times\text{g}}{\mu\text{Ci}\times\text{d}\times\text{MeV}}$ and calculated for [^{14}C], $f = 512$

AUC = time (d = day) integral of [^{14}C] drug concentration in target organ (μCi d/g).

TABLE 9.12 List of Specified Tissues and Their Weighting Factors by ICRP Publication 60, ICRP Publication 103, and the US Nuclear Regulatory Commission Part-20[a]

Name of the Tissue	Weighting Factor (W_T)		
	ICRP-60, Tables 2 and S-2	ICRP-103, Tables B.2 and B.3.5	USNRC Part-20, Sec. 20.1003
Gonads	0.20	0.08	0.25
Lower large intestine (colon)	0.12	0.12	NA
Lung	0.12	0.12	0.12
Red bone marrow	0.12	0.12	0.12
Stomach	0.12	0.12	NA
Bladder	0.05	0.04	NA
Breast	0.05	0.12	0.15
Liver	0.05	0.04	NA
Esophagus	0.05	0.04	NA
Thyroid	0.05	0.04	0.03
Bone surface	0.01	0.01	0.03
Skin	0.01	0.01	NA
Brain	NA	0.01	NA
Salivary glands	NA	0.01	NA
Kidney	NA	NA	NA
Remainder	0.05[b]	0.12[c]	0.30[d]

Abbreviation: NA, not applicable.
The weighting factors are expressed as a fraction. The sum of the weighting factors is equal to 1.
[a]Ref. 57–59.
[b]The remainder is composed of the following additional tissues and organs: adrenals, brain, upper large intestine, small intestine, kidney, muscle, pancreas, spleen, thymus, and uterus.
[c]The remainder is composed of the following additional tissues and organs: adipose tissue, adrenals, connective tissues, extrathoracic airways, gallbladder, heart wall, kidney, lymphatic nodes, muscle, pancreas, prostate, small intestine wall, spleen, thymus, and uterus.
[d]The remainder 0.30 results from 0.06 for each of the five remainder organs that receive the highest dose (excluding the skin and lens of the eye).

After inclusion of the radiation energy, for practical use, the formula for the equivalent dose H_t for any organ or tissue is

$$H_t = k \times \mathrm{AUC}_{(0-\infty)}$$

where k is the dose constant, and calculated for [^{14}C], $k = \frac{\mathrm{mSv}\times\mathrm{g}}{\mu\mathrm{Ci}\times\mathrm{d}} = 25.6$.

The integration of the [^{14}C] drug concentration–time function for the period zero to infinity is equal to the area under the [^{14}C] drug concentration equivalent–time curve, $\mathrm{AUC}_{(0-\infty)}$ (μgEq d/g), in the organ multiplied by the specific radioactivity (s):

$$\mathrm{AUC}_{(0-\infty)} = s \times \mathrm{AUC}_{(0-\infty)(\mathrm{organ})}$$

where s is the specific activity of radioactivity (μCi/μg).

Thus, the equivalent dose in any human organ or tissue (H_t) is calculated from the product of dose constant, specific radioactivity, and $\mathrm{AUC}_{(0-\infty)(\mathrm{organ})}$ as expressed in Equation 9.14:

$$H_t = k \times s \times \mathrm{AUC}_{(0-\infty)(\mathrm{organ})} \tag{9.14}$$

9.10.2 Dose Calculation for the Gastrointestinal Tract

The organs/tissues of the gastrointestinal tract are mostly exposed to the radioactivity present in the luminal contents of the organs and irradiating the organ wall. With the geometry factor of 0.5 and a defined organ residence time (τ) in the gastrointestinal tract segments, the organ equivalent dose, H_t (wall dose) is calculated by a modified formula from ICRP publication 53 [61] and expressed as Equation 9.15:

$$H_t = A \times F \times \frac{kt}{2m} \tag{9.15}$$

where

A = administering radioactivity dose (μCi)

F = fraction of the administered dose passing through the considered segment of the gastrointestinal tract

k = dose constant, and calculated for [^{14}C], $k = \frac{\text{mSv}\times\text{g}}{\mu\text{Ci}\times\text{d}} = 25.6$

τ = residence time given in days (d)

m = organ content weight given in grams (g).

As recommended by ICRP guidelines [60,61], the organ content weights and organ residence times in different organs for the human are summarized in Table 9.13. In the small intestine, the radioactivity exposure decreases due to absorption, and if absorption is a mono-exponential process, the decrease in radioactivity exposure in the small intestine is described by

$$A(t) = A^* e^{-\lambda t}$$

where A = radioactivity entering the small intestine (μCi) $A(t)$ = radioactivity remaining unabsorbed after time t, and passing to large intestine (μCi) λ = rate constant.

In that case, the equivalent dose of the small intestine is proportional to the time integral of the radioactivity present in the small intestine during the residence time τ and can be expressed as

$$H_t = A \times F \times \frac{k}{2m} \times \int_0^{\tau} e^{-\lambda t}\, dt$$

TABLE 9.13 List of Organ Content Weight and Organ Resistance Time in Human

Organ	Organ Content Weight (g)	Organ Residence Time (h)	(d)	$k \cdot \tau/2m^a$ (mSv/μCi)
Stomach	250	1	0.0417	0.00213
Small intestine	1040	4	0.167	0.00205
Upper large intestine	220	13	0.542	0.03152
Lower large intestine	135	24	1.0	0.09481
Renal tract	1400	24	1.0	0.00914

[a] m is organ content weight given in grams (g), τ is in units of days (d), k is 25.6 for ^{14}C.

From the limit 0 to τ after integration, this can be expressed as

$$H_t = A \times F \times \frac{k}{2m} \times \frac{1 - e^{-\lambda t}}{\lambda}$$

The rate constant, λ, is calculated by the following expression:

$$\lambda = \frac{\ln(A) - \ln(A\tau)}{\lambda}$$

9.10.3 Dose Calculation for Other Organs and Tissues

The equivalent dose (H_t) for any organ or tissue is calculated according to the general Equation 9.14. For the reference plasma, the equivalent dose H_{plasma} is

$$H_{plasma} = k^* s^* AUC_{(0-\infty)(plasma)}$$

For individual organs and tissues in humans, the equivalent doses H_t are calculated based on the equivalent dose in plasma multiplied with the organ factor R_t, which is the ratio of AUC_{tissue} to AUC_{plasma} of radiolabeled drug from animal data. The factor R_t can be determined from the animal organ/tissue distribution data. For a detailed explanation, the R_t values from a rat tissue distribution study are summarized in Table 9.14, which are used to estimate tissue exposure in human, assuming comparable organ distribution patterns in rat and human. Thus, equivalent dose for human can be calculated from the modified Equation 9.16 and expressed as

$$H_{t(human)} = H_{plasma(human)} \times R_t \tag{9.16}$$

9.10.4 Effective Dose

According to the ICRP publication-60 and ICRP publication-103 [Recommendations of the ICRP, Annals of the ICRP (1991 and 2007)] [23,56,58], the whole-body effective dose (E, in units of mSv) is the sum of the weighted equivalent doses of individual tissue/organs. The effective dose is determined from the individual organs and tissues equivalent doses, H_t, using Equation 9.17. It is recommended to be <1 mSv to fall in the minor to intermediate exposure effect category set by WHO. The comparisons of weighing factors for different tissues/organs recommended by ICRP publication-60 and revised ICRP publication-103 and USNRC Part-20 is listed in Table 9.12. The ICRP publication-60 specifies 12 tissues/organs (Table 9.12) that must be taken into consideration because of their susceptibilities to radiation damage and the remainder is composed of adrenals, brain, upper large intestine, small intestine, kidney, muscle, pancreas, spleen, thymus, and uterus. In a more recent ICRP publication-103, brain and salivary glands were added to the mandatory organs (Table 9.12), thus making them a total of 14 and the remainder is composed of adipose tissue, adrenals, connective tissue, extrathoracic airways, gallbladder, heart wall, kidney, lymphatic nodes, muscle, pancreas, prostate, small intestine wall, spleen, thymus, and uterus. On the other hand, USNRC Part-20 only specifies six tissues required for consideration and an additional five others that receive the highest dose excluding skin and lens of the eye. Based on the principle of ALARA, it is recommended to use the weighting factor recommended

TABLE 9.14 Determination of Human Equivalent Dose in Tissues and Calculation of Whole Body, Committed Effective Dose

Organ/Tissue	R_t for Organs[a]	Equation Used[b]	Human Equivalent Dose (H_t) (mSv)	Weighting Factor (W_T)	Individual Organ Effective Dose ($W_T \cdot H_t$) (mSv)
Blood (reference organ)	5.05	14	0.00592	NA	NA
Gonads (testis)	1.8	16	0.01069	0.20	0.00214
Bone marrow	6.93	16	0.04103	0.12	0.00492
Lung	4.56	16	0.02698	0.12	0.00324
Stomach	—	15	0.107	0.12	0.0128
Lower large intestine (colon)[c]	NA	15	4.266	0.12	0.5122
Liver	87.16	16	0.516	0.05	0.0258
Esophagus	14.1	16	0.0831	0.05	0.00416
Fat (white)[d]	4.36	16	0.00112	0.05	0.0000558
Skin	37.47	16	0.2218	0.01	0.0022
Thyroid gland	24.53	16	0.1452	0.05	0.00726
Bladder/renal tract[c]	—	15	0.2743	0.05	0.0137
Bone mineral	6.67	16	0.00171	0.01	0.0000171
Eye (membrane)[e]	1518	16	8.987	0.025	0.225
Average of remainder	—	16	0.03271	0.025	0.00818
Sum of weighting factors = 1.0					
Committed effective dose, E (mSv)		17	—		0.822

Abbreviation: NA, not applicable.

[a] R_t in Equation 9.16 is the ratio of tissue AUC relative to plasma AUC of ^{14}C, usually from the rat. Human plasma data used in the calculation are not shown.

[b] For excretory organs (stomach, lower large intestine, and bladder), the H_t was also calculated by Equation 9.16 for the tissues. As Equation 9.15 for organ content provided a higher, more conservative H_t, this higher H_t value is reported.

[c] Dose calculation is based on radioactivity expected to be present in the lumen of the organ.

[d] Breast is not sectioned; the fat tissue is alternatively used with the weighting factor for the breast.

[e] A weighting factor of 0.025 is applied to ocular uveal tract as its equivalent dose is in excess of the highest dose in any of the twelve organs for which a weighting factor is specified.

in ICRP publication-103 in Tables B-2 and B-3.5. The average radioactive dose for these tissues/organs of the body is used for calculations of effective dose (E) [56] and is given by the following expression:

$$E = \sum W_T \times H_{t(human)} \tag{9.17}$$

where

W_T is the weighting factor for tissue or organ, T

$H_{t(human)}$ is the human equivalent dose in tissue or organ, T.

The calculation of effective dose gives a prospective dose assessment for the planning and optimization of radiation safety and allows a retrospective dose assessment to demonstrate compliance with dose limits or dose constraints in radiological protection.

9.11 EXPERIMENTAL DESIGN OF HUMAN MASS BALANCE STUDIES

With the knowledge on the study designs and the results of the preclinical mass balance studies, the design for human mass balance studies can be appropriately established. Before conducting these studies, an independent institutional review board must approve the study protocol and the written informed consent documents should be obtained from all subjects before the enrollment into the study. The person preparing the dose must be well trained in pharmaceutical handling of the radionuclide, and both nonlabeled drug and radioactively labeled drug should be formulated together in the final dosage form. Both the dosage form and route of administration are recommended to be relevant to the intended use in clinical practice, as determined from the other previous studies. As mentioned previously in preclinical studies, the therapeutic efficacy/purity of the administered dose, including unlabeled and radiolabeled tracers must be determined exactly for the estimation of PK parameters and mass balance recovery parameters. Administration of a radioactive substance to humans should be performed in separate dedicated rooms, and only the qualified study personnel should be allowed with the subject [4]. Coadministration of other supplements such as high fiber diet, mild laxative, or regular fluid intake during the study period to allow sufficient defecation and adequate urination are recommended. In addition, it is advisable to rinse the container after the p.o. administration (if the dosage form is a solution or a suspension and is administered through a container), with suitable solvent to quantify the residual radioactivity [4]. The collection of radioactive biological samples (blood, urine, and feces) for the human studies should be long enough and optimum to allow the estimation of maximum recovery of the radiolabeled compound and its PKs without excessively burdening the patients. The collection interval of blood and urine samples can be designed based on the preclinical and clinical information, such as terminal half-life of the compound; otherwise, it is recommended to take more frequent samples during the distribution phase but less frequent during the terminal elimination phase. The date, time, and weight of the collection container should be recorded, before and after the collection period. Ideally, the collection of excreta should be stopped when all administered radioactivity has been recovered, which is not always the case with human mass balance studies. Assuming the elimination of the drug-related materials (metabolites) is similar to the parent drug, then plasma samples must be taken for at least four terminal half-lives [63], which can be established from previously conducted phase 1 studies. Under this assumption, 93% of the total radioactivity should be excreted after four half-lives. However, if it is taking more than the expected time to meet the criteria of the radioactivity recovery, then the study completion time may need to extend for more than seven days, sometimes up to 10 days or longer. Under certain circumstances (e.g., too long to hold them in the study center), the subjects under study can be allowed to go back to their homes and asked to bring the excreta (urine and feces) samples to the designated study center to increase the recovery of radioactive dose. For this, it is recommended to determine the levels of radioactivity recovery simultaneously in the collected samples, to get evidence-based extension of collection period. Usually, if there is no stability data available for the study compound and its metabolites in the urine and feces, it is recommended to store all samples at -20°C or less; otherwise, degradation products can mistakenly be identified as metabolites [4]. The sample analysis and the calculation of mass balance and PK parameters are usually performed in the same manner as for preclinical studies

discussed earlier and are summarized in Tables 9.6 and 9.7. It is recommended to calculate these PK parameters for the unchanged drug and its metabolites in human plasma matrix, in place of drug-related total radioactivity, as it composes of unchanged drug and various radioactive metabolites, which will result in pseudo-PK parameters. These total radioactivity-based PK parameters may be used to estimate the absorption in the human body, and after the metabolite profiling, the elimination pathways can be estimated based on the amount of radioactivity recovery in excreta and metabolite profile in each excreta. The contributions of each circulating component (including the parent) are estimated from the ratio of AUC of each component to the AUC of total radioactivity. These metabolites further characterize their contribution toward desired pharmacological and/or toxicological effects as needed.

9.12 CASE EXAMPLE FOR HUMAN MASS BALANCE

9.12.1 Drug B—Mass Balance Study Design

Three healthy, nonsmoking males were chosen from age 20 to 40 years, and a single p.o. dose of 100 mg containing labeled [^{14}C] plus unlabelled drug compound was administered p.o. The p.o. route was chosen because this was the proposed route of administration for human use in the clinical setting. The PKs and disposition of radioactivity in humans were cautiously predicted and the parameters obtained from the preclinical studies were used to calculate the radiation dose for human administration. The radiation doses (equivalent doses) for the individual organs following a single p.o. dose were calculated as described in Section 9.10 and listed in Table 9.14. All the calculations used in this example were based on the ICRP publication-60, and for future studies, as mentioned earlier, readers are advised to follow the revised recommendation from ICRP publication-103 for the calculation of committed effective dose for humans to get a minimum human radioactivity exposure. As shown in Table 9.14, the committed effective dose (human whole-body dose H_{wb}) was calculated to be 0.822 mSv for the radioactive dose of 47 μCi at a drug dose of 100 mg. This predicted effective dose is below the ICRP recommended dose (1 mSv); thus, the radiation health risk of the study volunteers was extremely low.

The radioactive dose given per subject was 47 μCi (1.85 MBq) for the 100-mg dose after calculation. Blood was collected at 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 16, 24, 36, 48, 72, 96, 120, 144, and 168 h post dose by either direct venipuncture or an indwelling cannula inserted in a forearm vein. Eighteen milliliters of venous blood was collected at each time point in heparinized tubes. Plasma was separated from whole blood by centrifugation, transferred to a screw-top polypropylene tube, and frozen immediately. Urine samples were collected at pre dose and at 0–4, 4–8, 8–12, 12–16, 16–24, 24–36, 36–48, 48–72, 72–96, 96–120, 120–144, and 144–168 h post dose. Feces were collected as passed from time of dosing until at least of 168 h postdose. All these samples were stored at −20°C or less until analysis.

The radioactivity was measured in plasma, blood, urine, and feces by LSC with the same methodology discussed earlier in preclinical studies. The parent drug was measured using a validated LC/MS/MS assay and metabolites were measured semi-quantitatively using HPLC radiodetection with off-line microplate solid scintillation counting and structural characterization by LC/MS.

TABLE 9.15 Pharmacokinetic Parameters After a Single Oral Dose of 100 mg of Drug B in Humans

PK Parameters	Plasma Parent Drug	Plasma Radioactivity	Blood Radioactivity
C_{max} (ng/mL or ngEq/mL)[a]	397 ± 92	594 ± 153	470 ± 87
T_{max} (h)	1.1 ± 0.6	2.1 ± 1.3	2.0 ± 1.4
$AUC_{0-\infty}$ (ng · h/mL or ng Eq · h/mL)[a]	1610 ± 460	6000 ± 1610	3850 ± 1580
Apparent $t_{1/2}$ (h)	2.8 ± 1.0	4.6 ± 0.3	5.1 ± 2.5
CL/F (L/h)	65.2 ± 15.5	—	—
V_z/F (L)	269 ± 125	—	—

[a]Concentration units are in ng/mL for the intact parent drug and ngEq h/mL for drug-related radioactivity. All values are presented as mean ± SD.

9.12.2 Pharmacokinetics Parameters for Drug B

The PK parameters of drug B were determined by conducting noncompartmental analysis using WinNonlin. The highest concentration in plasma (C_{max}) was achieved at 2.1 h post dose with the mean value of 594 ng Eq/mL (total radioactivity) and 1.1 h post dose with mean value of 397 ng/mL for the intact, unchanged parent drug in all subjects, suggesting rapid gastrointestinal absorption of the drug. The mean plasma concentration–time profiles and other PK parameters of total radioactivity and unchanged drug compound after a single p.o. dose of [^{14}C] drug are summarized in Table 9.15. The average terminal elimination half-life ($t_{1/2}$) of radioactivity and parent drug was calculated to be 4.6 and 2.8 h, respectively.

9.12.3 Mass Balance Parameters and Interpretations

After a single p.o. administration of 100-mg dose of [^{14}C] drug B, radioactivity was excreted predominantly in urine (Table 9.16). After 168-h postdose, the excretion in urine and feces averaged 85.4 ± 4.4% and 14.8 ± 3.5% of the administered dose, respectively. The cumulative excretion of radioactivity was complete after 168 h postdose in all subjects averaging 100.2 ± 1.1%, as summarized in Table 9.16. The

TABLE 9.16 Cumulative Excretion of [^{14}C] Radioactivity in Urine and Feces after Single p.o. dose of 100 mg of Drug B in Humans (Mean ± SD)

Time Period (h)	Urine (% Dose Excreted)	Feces (% Dose Excreted)
0–24	72.7 ± 4.8	1.37 ± 2.0
0–48	81.6 ± 4.2	9.93 ± 8.0
0–72	83.8 ± 4.4	13.2 ± 4.8
0–96	84.7 ± 4.4	14.3 ± 3.7
0–168	85.4 ± 4.4	14.8 ± 3.5
Total (% radioactive dose excreted in urine and feces)	100.2 ± 1.1	

elimination of radioactivity was rapid as more than 90% of the radioactivity was recovered in first 48 h after the dose was administered. Approximately 27% of the drug dosed was excreted as intact, unchanged parent drug (22.5% in urine and 4.54% in feces). The extent of absorption is estimated to be higher than 85.4% (amount of dose recovered in urine) and likely to be even higher since only 4.54% of dose was recovered in feces as unchanged drug. These results were in agreement with the absolute p.o. bioavailability (85%) observed in another clinical study with a single i.v. dose. Since ~22.6% of the dose in urine was recovered as unchanged compound, the renal clearance was estimated to be ~15 L/h.

9.13 CONCLUSIONS

Mass balance studies play an important role in the development of new drugs. These studies give in-depth understanding of absorption, bioavailability, routes (renal, biliary, hepatic, or gastrointestinal), and extent of excretion, metabolite profiling, metabolic pathways, and clearance mechanisms of a drug. The results from these studies aid in designing and conducting renal and hepatic impairment studies, DDI studies, and monitoring the metabolite(s) that may relate to pharmacology or toxicological concerns. The mass balance information will also help in understanding the mechanisms of intersubject variability of the drug exposure and potential DDI in terms of metabolism or transport. Moreover, across species mass balance studies can provide important information on whether a metabolite observed in human is required for separate safety testing in preclinical studies to fulfill the regulatory requirement as stated in the FDA and ICH guidances.

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