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Special Section on Perspective on Drug Metabolism and Disposition, Part I—Minireview

Human Absorption, Distribution, Metabolism, and Excretion Studies: Origins, Innovations, and Importance[§]

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ABSTRACT

Human absorption, distribution, metabolism, and excretion (hADME) studies represent one of the most important clinical studies in terms of obtaining a comprehensive and quantitative overview of the total disposition of a drug. This article will provide background on the origins of hADME studies as well as provide an overview of technological innovations that have impacted how hADME studies are carried out and analyzed. An overview of the current state of the art for hADME studies will be provided, the impacts of advances in technology and instrumentation on the timing of and approaches to hADME studies will be discussed, and a summary of the parameters and information obtained from these studies will be offered. Additionally, aspects of the ongoing debate over the importance of animal absorption, distribution,

metabolism, and excretion studies versus a “human-first, human-only strategy” will be presented. Along with the information above, this manuscript will highlight how, for over 50 years, *Drug Metabolism and Disposition* has served as an important outlet for the reporting of hADME studies.

SIGNIFICANCE STATEMENT

Human absorption, distribution, metabolism, and excretion (hADME) studies have and will continue to be important to the understanding and development of drugs. This manuscript provides a historical perspective on the origins of hADME studies as well as advancements resulting in the current-state-of the art practice for these studies.

Introduction

Across human clinical studies, the characterization of the absorption, distribution, metabolism, and excretion (hADME) of a new drug is a necessary and important part of the suite of information submitted for regulatory review (Coppola et al., 2019). Sometimes, the term “human mass balance study” is used interchangeably for the hADME study. Strictly speaking, mass balance is a parameter itself derived from the study, and referring to it merely as a mass balance study provides a perfunctory description of the information obtained. Conversely, the nomenclature of “hADME” actually belies this important experimental parameter that is derived from the study, namely the mass balance.

The hADME study has two main objectives: 1) to identify and quantify circulating parent drug and metabolites and 2) to quantitatively determine routes of elimination for all drug-related material. An understanding of the biotransformation reactions that the drug undergoes as well

as assigning and quantitating the routes and extent of elimination provide important insights. Specifically, an understanding of the biotransformation of a drug and enzymes involved may provide perspective for drug-drug interactions as well as the possible impact of pharmacogenomic differences in patients on metabolism of the drug. Additionally, appreciation of the routes of elimination of a drug may also inform on any necessary dose adjustments, for example, in patients with renal or hepatic impairment.

The sections that follow will provide additional information relating to the origins of hADME studies, the current state of the art for their practice, and additional details regarding the importance and impact of hADME studies.

The Origins of Human ADME Studies

The origins of the current hADME study were likely borne out of the use of isotopes as tracers originally proposed and established by George de Hevesy, for which he earned the 1943 Nobel Prize in Chemistry. Early work by Hevesy used radioactive lead (²¹⁰Pb and ²¹²Pb) in both chemical and biologic studies, establishing the use of “radioelements as indicators” (Hevesy and Hofer, 1934). Incorporation of a radioisotope, generally tritium (³H) or carbon-14 (¹⁴C) or stable isotopes (²H, ¹³C, ¹⁵N, ¹⁸O, etc.) into a substrate or intermediate involved in a chemical

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ABBREVIATIONS: ADME, absorption, distribution, metabolism, and excretion; AMS, accelerator mass spectrometry; AUC, area under the curve; FDA, Food and Drug Administration; F-NMR, fluorine-NMR; hADME, human absorption, distribution, metabolism, and excretion; HPLC, high-performance liquid chromatography; LC, liquid chromatography; LSC, liquid scintillation counting; MIST, metabolites in safety testing; $t_{1/2}$, half-life; TFMSA, trifluoromethoxy salicylic acid.

reaction or biotransformation allows one to track or trace labeled intermediates or products. Use of radiolabeled compounds also allows one to quantitate intermediates and products via radiochemical detection. In later work, Hevesy further expanded his tracer work into biologic systems, including the use of a stable isotope (^2H) and another radioisotope (^{32}P) to determine the rate of elimination of water from the body and for the latter to determine the amounts of ^{32}P found in the organs and excreta of rats over time.

Hevesy's use of tracers was rapidly implemented by others and eventually extended to the study of intermediary metabolism and biochemistry. Early examples of these studies include exploration of photosynthesis using $^{14}\text{CO}_2$ and $^3\text{H}_2\text{O}$ (Wilson and Calvin, 1955; Moses and Calvin, 1959), confirmation of the Krebs cycle using both stable and radioisotope-labeled compounds (Tokumitsu and Michio, 1974), and establishing that DNA, not protein, was hereditary material using ^{32}P and ^{35}S , such as in the Hershey-Chase experiment (Hershey and Chase, 1952).

Although ^3H was first discovered in 1934 (Oliphant et al., 1934), its use in nuclear weapon development limited its availability and use for research in the 1940s and 1950s (Lappin, 2015). Early biochemical studies (Ruben et al., 1939; Evans and Slotin, 1940a, 1940b, 1941) employed the short half-life ($t_{1/2}$) ($t_{1/2}$, 20.4 minutes) radioactive carbon isotope, ^{11}C . However, the discovery of the long half-life carbon isotope ($t_{1/2}$, 5730 years), ^{14}C , in 1940 resulted in greater application of this radioisotope for biochemical studies. The availability of ^{14}C obtained from the Berkeley Radiation Laboratory allowed for the radiosynthesis of the carcinogen [^{14}C]dibenzanthracene (Heidelberger et al., 1947). The resulting [^{14}C]dibenzanthracene was used for the first published example of the use of ^{14}C in an absorption, distribution, metabolism, and excretion (ADME) study for a xenobiotic in animals reported in 1948 by Heidelberger and Jones (1948) (Lappin, 2015). This study bears resemblance to modern-day ADME studies in the collection and characterization of elimination of ^{14}C in excreta as well as bile.

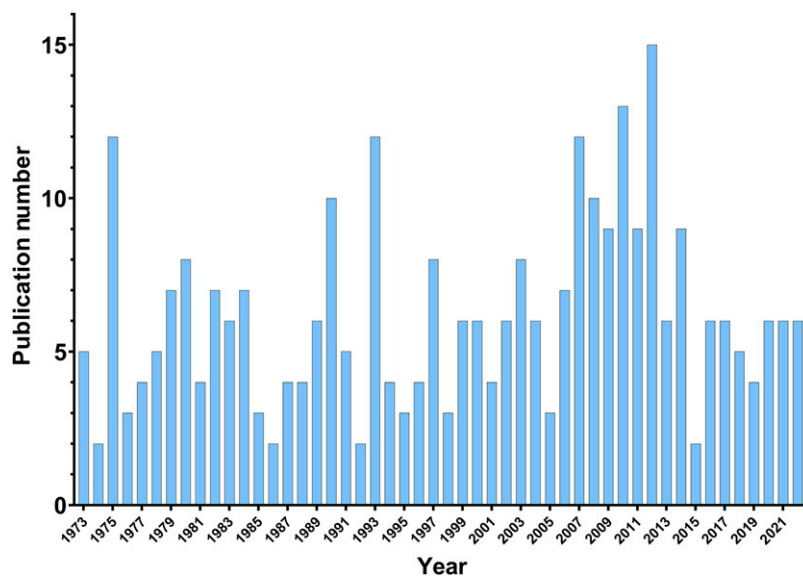
Throughout the 1950s, the availability of ^{14}C for medical research from the US Atomic Energy Commission (Oak Ridge, Tennessee) reactor resulted in an increase in the incorporation of ^{14}C into biologic molecules as well as xenobiotics (Maickel et al., 1971; Lappin, 2015). An early example of the use of ^{14}C in an ADME study in humans can be found in the study of the metabolism of [^{14}C]salicylic acid (Alpen et al., 1951). This study employed countercurrent distribution, a form of liquid-liquid extraction (Friesen et al., 2015), as well as paper chromatography to separate salicylic acid and metabolites from patient urine. This

report also describes the determination of total radioactivity and identification of salicylic acid and metabolites using various colorimetric assays and UV absorption. In addition to being one of the earliest reported hADME studies, this work exemplifies early hADME studies in a number of ways. Separation of parent drug and metabolites was limited to the above-described methods, such as thin-layer chromatography. Metabolite identification efforts were often limited to derivatization for functional group identification as well as spectral analyses. Lastly, early ADME studies were often limited to analyses of plasma or urinary metabolites, with little attention paid to parent drug and metabolites excreted in feces.

Although the specifics of when radiolabeled ADME studies became routine is difficult to pinpoint, Lappin (2015) suggests that "the use of radioisotopic tracers in ADME studies was certainly established by the early to mid-1950s." Furthermore, the execution and publication of the results of hADME studies, as in the pages of *Drug Metabolism and Disposition*, were commonplace by the early 1970s. Over the 50 years of *Drug Metabolism and Disposition*, some 310 hADME studies have been published in its pages (Fig. 1). A steady stream of hADME publications have appeared, with an average of more than six hADME studies published per year (range, 2–15). In addition to the historical perspective, the relatively large number of hADME studies included in Fig. 1 also provides evidence of the value of *Drug Metabolism and Disposition* as a repository for hADME studies.

Although many characteristics of the clinical aspects of an hADME study have not changed much since the early days of hADME studies, the analyses of samples and the structural determination of metabolites from these studies have benefitted from a number of advancements in analytical approaches and instrumentation. The development of gas chromatography mass spectrometry in the late 1950s (Gohlke and McLafferty, 1993), followed by the advent of high-performance liquid chromatography (HPLC) in the 1970s and 1980s and eventually ultrahigh-performance liquid chromatography in the 2000s (Arnaud, 2016), have led to these methods replacing thin-layer chromatography and other earlier separations methods. This has resulted in greater resolution of drugs and metabolites as well as more rapid analyses. Additionally, the coupling of these liquid chromatography (LC) separation methods to a thermospray interface by Vestal (1984) and an electrospray interface by Fenn et al. (1989), along with improvements in NMR instrumentation, have had a dramatic impact on the structure elucidation of metabolites (Murphy, 2008a; Murphy, 2008b).

Fig. 1. hADME studies published in *Drug Metabolism and Disposition* between 1973 and 2022. Search criteria and references used to generate this figure can be found in Supplemental Information.



Although metabolite separation and characterization have dramatically improved, the quantitation of drug levels using liquid scintillation counting (LSC) to determine mass balance has remained relatively unchanged over the years. Though LSC is commonplace for the determination of total radioactivity in plasma and excreta, the invention and introduction of accelerator mass spectrometry (AMS), as will be discussed below, has provided an alternative analytical method for the determination of total drug-related material. The coupling of LC with radiochemical detection using either liquid or solid scintillant has enabled in-line counting of radioactivity. Alternatively, the use of microplate scintillation counting after fractionation of LC eluants into solid scintillant-containing plates or after the addition of liquid scintillant has increased radiochemical detection sensitivity.

Together, the discoveries and advances described above have led to the standardization of various aspects of hADME studies and analyses. The current state of the art for hADME studies in terms of study design, sample analyses, and instrumentation will be discussed in the next section. Nonetheless, future advances in analytical methods and the introduction of new techniques and instrumentation may eventually lead to changes in how and when hADME studies are performed.

Current State of the Art of Human ADME Studies

Standard Study Designs. For many years, the design of a human ADME study has remained largely unchanged. Study volunteers are dosed with test a compound incorporated with ^{14}C at a metabolically stable position, i.e., a position resistant to metabolism so that the radiolabel will not be lost. Additionally, the site of the label is chosen so as not to yield hard-to-track metabolites, e.g., heteroatom demethylation reactions that can yield radiolabeled one-carbon molecules like formaldehyde, formic acid, or carbon dioxide. The dose of ^{14}C is high enough to permit reliable quantitation of all drug-related material by LSC and usually ranges between 40 and 100 μCi . The dose is administered using the same route as intended for therapeutic use (mostly oral). Since the ^{14}C -labeled material generally is a one-time administration of the drug, the formulation used in the study is not a final or commercial formulation but rather a solution or suspension generated specifically for this study. As such, the pharmacokinetics of the drug may not be an exact mimic of the pharmacokinetics that would be observed following administration of a tablet or capsule formulation.

Following administration to volunteers (usually 4–8), urine and fecal samples are collected over set intervals in as comprehensive a manner as possible. The duration of collection can be preset, based on estimates of when all drug-related material will be excreted, or in a manner in which samples are analyzed in real-time and release of individual volunteers from the study is data driven. When a predetermined recovery is achieved (typically 90%) or the rate of excretion of drug-related material drops below a predetermined threshold (such as 1% in a day), the volunteer can be released from the study. Unlike other quantitation methods described below, LSC can be done in short turnaround times to permit data-driven decisions on release of volunteers. Blood samples are also collected for determination of the pharmacokinetics of total drug-related material, which can be compared with the pharmacokinetics of parent drug.

When measuring total drug-related material in a standard radiolabel ADME study, urine samples can be subjected to direct analysis by LSC. The total mass of urine excreted over each collection period is measured and small aliquots are withdrawn and analyzed using LSC. Following corrections for counting efficiency and multiplying the measured value by the ratio of total urine to the aliquot measured, the total radioactivity is calculated, and this value is divided by the total radioactivity administered to yield the percentage of the dose excreted over that

time interval (thus, measurement of the total radioactivity in the dose and assurance that the entire dose was administered is a critical component in study execution). Data from each interval are summed to yield the total percentage of dose excreted in urine. This is a straightforward procedure. For fecal samples, the laboratory manipulations are a bit more complex in that the samples must be diluted and homogenized before analysis. Weights and aliquots are dealt with in a similar manner; however, using LSC for fecal homogenates directly may not yield a complete reading of total radioactivity because ^{14}C within particulates may not be efficiently counted, and colored materials may also interfere by quenching scintillation. Thus, fecal samples are subject to combustion to $^{14}\text{CO}_2$, which is trapped and measured. The calculation of dose in each fecal sample is done the same way as for urine, and the urine and feces data are combined to yield total recovery. It is not typical to collect other samples from the volunteers, such as expired air or perspiration, but it is possible for drug-related material to exit the body via such routes, and in those rare instances, considerations should be given for the collection and analysis of those matrices.

Plasma, and sometimes whole blood, are also analyzed for total radioactivity. This is also done by subjecting aliquots to LSC. Plasma can be measured directly, but blood may require processing like fecal homogenates, or these can be subjected to bleaching prior to scintillation counting to prevent quenching. In the typical ADME study, the parent drug is also measured using a specific quantitative assay [usually high-performance liquid chromatography-mass spectrometry (HPLC-MS)], and the C_{max} , time to reach C_{max} , area under the curve (AUC), and $t_{1/2}$ of the parent drug can be compared with the corresponding parameters for total radioactivity.

Plasma, urine, and fecal homogenates are also evaluated for the quantitative metabolite profiles in each matrix. A limited sample-processing procedure is employed to make the samples suitable for injection onto HPLC while striving to not selectively lose metabolites in the process. Thus, simple miscible liquid extractions are typically employed to permit the removal of salts and proteins by centrifugation, and the supernatant containing the drug-related material is evaporated and reconstituted for HPLC analysis. In some cases, solid-phase extraction can be employed for this purpose. Recoveries of total radiolabel through the sample work-up process should be 90% or greater to offer a level of confidence that specific metabolites were not lost in the process. Chromatographic separation of metabolites into discrete peaks that can be quantified by LSC (either by fraction collection with off-line measurement or using an in-line radiometric flow detector) is done, with a portion of the HPLC eluent diverted to a mass spectrometer to gain structural information of the metabolites.

It is general practice to not generate a metabolite profile for every individual excreta and plasma sample. When using LSC as the quantitative method (as opposed to other methods—see below), urine collected from each volunteer is pooled across the sampling intervals to yield a single sample that contains at least 90% of the drug-related material that was excreted in urine. The same is done for fecal homogenate samples. The volumes/weights of each sample must be carefully considered in a proportional manner to generate a sample for analysis that is truly representative of the total excretion. For plasma, it is also typical practice to generate a single plasma pool for each individual volunteer that is constructed in such a way to represent the AUC of radioactivity over an interval that represents at least 90% of that AUC [practitioners in the field colloquially refer to this as generating a “Hamilton pool,” in reference to the first listed author of a publication that describes the underlying mathematics behind the pooling scheme to generate a time-averaged sample (Hamilton et al., 1981)]. These pooled samples are processed as discussed above, the reconstituted extracts are analyzed by radiometric HPLC-MS, and the percentage that each metabolite comprises of a

pooled excretory matrix sample or pooled plasma sample is calculated. For excreta, these percentages are converted to percentage of total dose; for plasma, the values represent the percentage that each metabolite comprises of total drug-related material. The excreta values are used to address the clearance pathways for the drug, whereas the plasma values are useful in identifying metabolites that may merit further evaluation in drug safety studies [i.e., the metabolites in safety testing (MIST) criteria; see below (Schadt et al., 2018)].

Accelerator Mass Spectrometry-Enabled Study Designs. The advent of the use of AMS to measure ^{14}C in human ADME studies has changed what these studies have the potential to include (Lappin et al., 2011; Spracklin et al., 2020). AMS as a technique has been around since the 1970s; however, the instrumentation has only become suitable in size and cost for small laboratories over the past 10 years (Young and Seymour, 2015). In application to ADME studies, AMS detects ^{14}C at levels that are orders of magnitude below levels detectable by LSC, and this enables doses in the 100–1000 nCi levels to be administered. In fact, the amount of ^{14}C in the plasma and excreta samples in an AMS-based ADME study are so low as to no longer be considered radioactive. The extremely low exposure to ionizing radiation poses no safety risk to study volunteers, and thus, quantitative whole-body autoradiography studies in animals used to make tissue dosimetry estimations are no longer a prerequisite for the conduct of a human ADME study. In addition to the advantage of using much lower amounts of ^{14}C , the application of AMS as the detection technique in ADME studies allows for enhanced study designs that deliver more information about the total disposition of a drug.

When discussing the use of AMS in human drug disposition studies, the difference between microdose and microtracer dose is an important distinction. A microdose is one in which the ^{14}C -labeled drug will be of a high specific activity, and AMS technology, through its high sensitivity, permits the administration of extremely low subtherapeutic total dose levels. This can be done to gain pharmacokinetic information in humans without requiring safety studies in animal species [also referred to as a “phase 0” study (Rowland, 2012; Bosgra et al., 2016)]. A microtracer dose is one wherein a standard pharmacologically relevant total dose is administered, but it contains a very small amount of ^{14}C -labeled material as a tracer. It is the microtracer dose approach that has found use in human ADME studies.

AMS technology has opened the door to the inclusion of an intravenous dose as part of the ADME study (without the prerequisite of intravenous animal toxicology studies or lengthy investigations into formulation development). This permits gathering important pharmacokinetic parameters (Table 1) that can only be gained from doing a combined intravenous/oral study, including systemic CL, VD_{ss}, VD_β, F, and Fa. In a sequential crossover design, study volunteers are first administered an oral microtracer dose (e.g., 100–1000 nCi ^{14}C material plus the pharmacologically relevant dose of unlabeled material), and blood and excreta are collected to obtain mass balance and metabolite

profiles in the same way as in a standard ADME study. In the second leg, following a suitable wash-out period, the same oral dose level is given of non- ^{14}C -labeled material, and at a time approximating the time to reach C_{max} , an intravenous dose of 100–1000 nCi ^{14}C material only is administered by short infusion. Blood and excreta are collected as before. By measurement of total ^{14}C in excreta and measurement of unlabeled and ^{14}C -labeled drug in plasma, multiple pharmacokinetic parameters can be measured (Table 2). Plasma, urine, and fecal homogenates can be subsequently analyzed for quantitative metabolite profiles using HPLC and collecting fractions for AMS analysis off-line [it should be noted that the coupling of HPLC directly to AMS instrumentation has been reported but is not a common practice at this time (Maden et al., 2019)]. The data can be reconstructed to yield a ^{14}C chromatogram from which each metabolite can be quantitated and converted to percentage of dose. Fractions containing ^{14}C can also be analyzed by HPLC-MS to gain information on the identities and chemical structures of the metabolites.

One disadvantage of current AMS technology relative to LSC is the length of time it takes to make the measurements and the cost of the equipment. LSC is simple—the sample to be analyzed is simply mixed with scintillation fluid, and, depending on the amount of radioactivity, the data for each sample is obtained in minutes. More challenging fecal homogenate samples can be combusted, and the trapped $^{14}\text{CO}_2$ is measured as described above. Excreta samples can be measured in real time, and thus, data can be used to determine when study volunteers have excreted enough dose to permit their release from the study site. Quantitative metabolite profiles in plasma and excreta are easily obtained by radiometric HPLC, either with radiometric flow detectors or 96-well fraction collection and off-line LSC. However, for AMS, all samples must either be graphitized or processed to trapped $^{14}\text{CO}_2$ (Getachew et al., 2006; Miyaoka et al., 2007; van Duijn et al., 2014). Although sample preparation for AMS is lengthier and more labor-intensive than for LSC, excretion data can still be provided in real time to dismiss volunteers from the study.

NMR Spectroscopy. Throughout time, the vast majority of ADME studies have been accomplished by dosing ^{14}C -labeled material because this offers both specificity (no interferences from endogenous materials) and universal quantifiability (drug and metabolites have the same response factor). NMR spectroscopy can offer the latter quality for quantitating drug-related materials. However, for the specificity aspect, proton NMR is lacking since biologic matrices are rife with proton-containing materials (Dear et al., 2008). But fluorine is present in many drugs, and in contrast to protons, there are no endogenous fluorine-containing interferences; thus, fluorine-NMR (F-NMR) can be used for ADME of fluorine-containing drugs. This offers the further advantage that special ^{14}C -labeled material, which can extend timelines by several months and cost several hundreds of thousands of dollars, does not need to be prepared; the study can be done with the drug itself.

TABLE 1
Data obtained from an AMS-enabled human ADME study with sequential oral and intravenous administration

Parameter	How Measured
Mass balance excretion	Total ^{14}C in urine and feces by AMS
Clearance (CL)	HPLC fractionation of plasma following intravenous dosing, with AMS analysis of the fraction(s) containing the parent drug
Volume of distribution (VD)	HPLC fractionation of plasma following intravenous dosing, with AMS analysis of the fraction(s) containing the parent drug
Oral bioavailability (F)	HPLC fractionation of plasma following intravenous dosing, with AMS analysis of the fraction(s) containing the parent drug and compared with HPLC-MS analysis of the parent drug following oral administration
Oral absorption (F _a)	Total ^{14}C in urine by AMS following intravenous and oral administration
Metabolite profile	HPLC fractionation of plasma and excreta following oral dosing, with AMS analysis of the fractions and HRMS analysis of metabolite peaks for structural information

HRMS, high-resolution mass spectrometry.

TABLE 2
Comparison of technical approaches to human ADME studies

	Standard Radiometric	Accelerator Mass Spectrometry (Microtracer)	¹⁹ F-NMR
Dose	40–100 μCi	<1 μCi	No radioactivity
Label	¹⁴ C or ³ H; requires radiosynthesis	¹⁴ C; requires radiosynthesis	Study drug must possess fluorine in its structure
Detection method and instrumentation	Liquid scintillation counting	AMS of ¹⁴ C/ ¹² C ratio	600 MHz NMR with fluorine cryo microprobe
Sensitivity	High	Extremely High	Low
Dose route	Intended for therapy	Intended for Therapy with Option for IV study leg	Intended for therapy
HPLC Metabolite profiling	Can be done with in-line flow detectors	Requires fraction collection and post-run analysis	Requires fraction collection and post-run analysis
Sample pooling	Pool of each matrix for each individual study volunteer	Pool of each matrix is combined across all volunteers	Pool of each matrix is combined across all volunteers

When using F-NMR for an ADME study, sample processing procedures are not as simple as LSC but are simpler than for AMS. The greater challenge is due to the low sensitivity of NMR as compared with LSC and AMS: large sample volumes require processing and concentration to reliably quantitate drug-related material, even when using high frequency instrumentation (>500 MHz). Proof of concept of F-NMR for ADME was first demonstrated in animal ADME studies (Mutlib et al., 2012), and a retrospective comparison was made between ¹⁴C and F-NMR for an hADME study (James et al., 2017). The use of F-NMR for an hADME study was first reported by Pearson et al. (2019) for the phosphatidylinositol-3-kinase δ inhibitor, leniolisib. More recently, F-NMR was employed for the hADME for nirmatrelvir, the first protease inhibitor for the treatment of COVID-19 (Singh et al., 2022). Analysis of samples by NMR requires lengthy data acquisition times, which obviates real-time sample analysis for the discharge of study volunteers and also requires that samples be pooled for metabolite profiling by HPLC. Also, analogous to ¹⁴C, success of the study depends upon the fluorine atom(s) not being lost through metabolism.

Comparison of ADME Detection and Quantitation Methods. ADME studies done using ¹⁴C-labeled materials with radiometric analysis have been the standard for decades. A comparison of the technical aspects of hADME studies is provided in Table 2. Theoretically, any quantitative detection system could supplant radiometric analysis, but these new technologies, such as AMS or NMR, need to prove that they give data of comparable quality. One aspect of data quality for hADME studies is overall mass balance. A meta-analysis of overall mass balance for hADME studies using LSC and AMS as the detection methods is listed in Table 3 and shown graphically in Fig. 2. Mass balance for published studies before 2007 that used LSC was reported by Roffey et al. (2007) and yielded a median value of 92.0% (range, 39.0%–113.0%; CV, 13.0%), and a subsequent analysis of studies available in *Summary Basis of Approval* documents from the US Food and Drug Administration (FDA) yielded similar results (median, 91.4%; range, 42.7%–110.1%; CV, 9.5%). Studies that have used AMS detection are much fewer. However,

comparable mass balance values have been observed, suggesting that mass balance obtained using AMS is identical to that obtained using traditional LSC (median, 92.2%; range, 63.3%–98.3%; CV, 22.2%; Table 3).

Animal ADME Studies

Although the focus of this article is on hADME studies, some mention of ADME studies in laboratory animal species is warranted. It has been common practice, and still is in many cases, to conduct at least one radiolabeled ADME study in a laboratory animal species prior to the conduct of the hADME study. However, there has been discussion in the literature regarding the value of animal studies to drug development (Pellegatti, 2014). Historically, the development path was initiated with an ADME study in rats, followed by an ADME study in the second toxicology species. This was followed by a quantitative whole-body autoradiography study, which would enable tissue dosimetry calculations to be made that would determine the limits on the radioactive dose that could be used for the subsequent hADME study. Through the conduct of the animal ADME study, some aspects of laboratory procedures could be worked out in preparation for the hADME study, such as matrix extraction techniques, chromatography systems to resolve metabolites, and metabolite structure elucidation. The potential for incomplete recovery can be assessed, and studies in animals can also be more invasive, such as collection of bile through surgical implantation of cannulae. However, this is all limited by the fact that these studies are in animals, and thus, the overall metabolism and disposition may not be entirely reflective of that which occurs in humans.

In 2012, Obach et al. (2012) touched off a debate asking whether radiolabeled mass balance and excretion studies in laboratory animals were still necessary. The crux of their argument was that early understanding of human metabolites, not exhaustive studies in animals, is most important, and an early hADME study (no later than phase 2A), enabled with modern technologies, will permit identification of the major human metabolites. Once identified, appropriate comparisons can be made between clinical samples and toxicological samples using nonradiolabeled methods to assess whether metabolites in humans are present in adequate abundance in animal species used in risk assessments (the MIST issue; see below). The authors acknowledge that there may be individual instances that call for a radiolabeled mass balance study in animals (e.g., to investigate a species-specific metabolite potentially causing toxicity in that species, which is not relevant to humans). In response, White et al. (2013) argued that a radiolabeled mass balance study in at least one species was critical to drug development because it had become an “expected” part of the regulatory submission package, and the studies provided knowledge of the compound that would be helpful in handling the subsequent precious human samples.

TABLE 3

Mass balance of human ADME studies conducted using scintillation counting and accelerator mass spectrometry
Values for mean, CV, median, and range are percentages.

	N	Mean	CV	Median	Range
Scintillation counting – Roffey et al. (2007)	169	88.0	13.1	92.0	39.0–113.0
Scintillation counting – FDA-approved drugs 2005–2022 (FDA.gov)	278	88.8	9.5	91.2	42.7–110.1
AMS	25	88.5	22.2	92.2	63.3–98.3

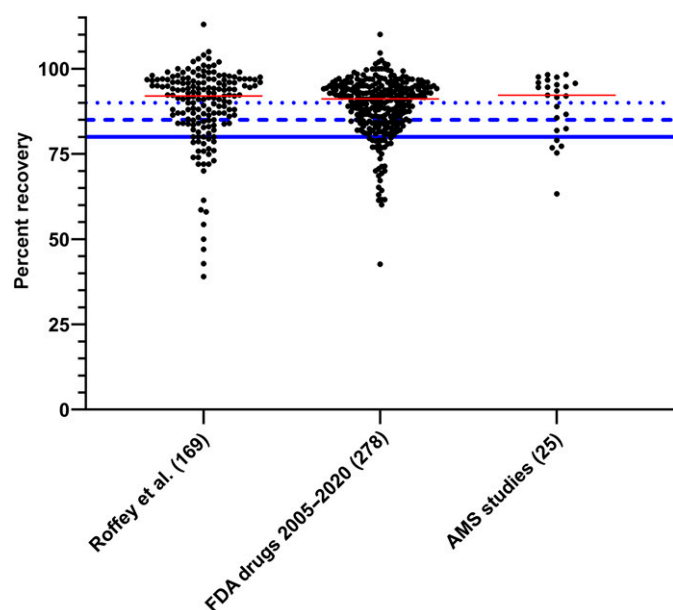


Fig. 2. Comparison of total radioactivity recoveries from LSC-based and AMS-based hADME studies. Radioactivity recovery data reported by Roffey et al. (2007) and from FDA-approved drugs 2005–2020 (www.fda.gov) were used to construct this figure. Red horizontal lines for each data set represent the median. Blue horizontal dotted, dashed, and solid lines represent recoveries of 90%, 85%, and 80%, respectively.

Currently, the debate continues. In an industry white paper published in 2022 (Young et al., 2023), it was acknowledged that there is a spectrum of views across pharmaceutical research and development

organizations on this issue, but there was general agreement that animal ADME studies should not be completed simply as regulatory check box but should be designed to address mechanistic ADME questions. Recently, the drug abrocitinib was approved for clinical use without conducting any radiolabeled excretion studies in animals: only the hADME (Bauman et al., 2022) and rat quantitative whole-body autoradiography (for the determination of tissue distribution) studies were done.

Importance of the Human ADME Study

One of the primary parameters obtained from excreta in an hADME study is the overall mass balance of recovered radioactivity in excreta. Although the mass balance parameter provides little information about the drug, it does provide some confidence or questions about the understanding of drug clearance and elimination. For example, low recovery may indicate that a sample was missed or incomplete, the drug is being sequestered in the body, or that the drug or metabolite is being eliminated in exhaled air. The question of acceptable recovery in hADME studies was addressed in the aforementioned analysis by Roffey et al. (2007), where a recovery of 80% or greater was suggested to be acceptable. However, a recent FDA draft guidance on radiolabeled mass balance studies (<https://www.fda.gov/regulatory-information/search-fda-guidance-documents/clinical-pharmacology-considerations-human-radiolabeled-mass-balance-studies>) suggested that recovery should be at least 90%. In light of the recent FDA draft guidance, reported hADME recovery data were compared with the proposed recovery of 90% (Fig. 2). This assessment indicates that a large number of hADME studies would fail to meet the criteria in this draft guidance. Therefore, sponsors would be required to provide “adequate justification” for failing to meet these criteria.

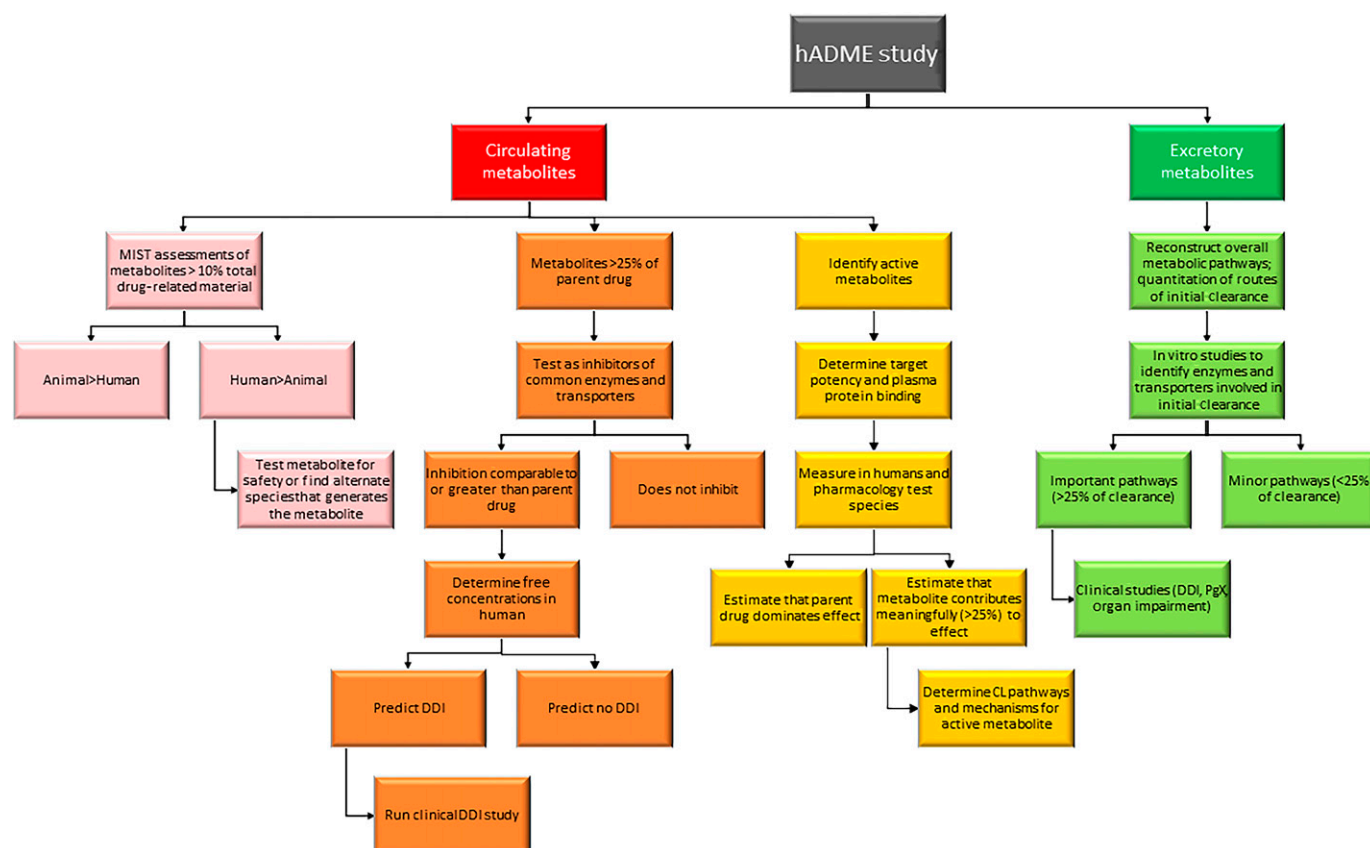


Fig. 3. The output from hADME studies triggers further mechanistic investigations.

Most drugs are eliminated by one or some combination of the following elimination mechanisms: 1) metabolism/transport in the small intestine, 2) metabolism/transport in the liver, or 3) glomerular filtration and tubular secretion by the kidneys. If the elimination pathway of a drug is somehow impaired, this can alter the pharmacokinetics of the drug to the extent that an adjustment in dosage may be considered. The decision to adjust dose for hepatic or renal impairment considers many factors, but one factor in this decision is knowing how much of the drug is eliminated via each pathway, and that is information derived from hADME studies.

The determination and quantitation of the metabolic profile of a drug in humans is important for a variety of reasons (Fig. 3) that can inform the strategy for further in vitro, animal, and human studies. Metabolites are identified from the hADME study, and their relative quantities are determined. Determination of the circulating profile of metabolites is required for the MIST assessment for a compound (<https://www.fda.gov/media/72279/download>; <https://www.ema.europa.eu/en/ich-m3-r2-non-clinical-safety-studies-conduct-human-clinical-trials-pharmaceuticals-scientific>; https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-investigation-drug-interactions-revision-1_en.pdf; https://www.ema.europa.eu/en/documents/other/international-conference-harmonisation-technical-requirements-registration-pharmaceuticals-human-use_en.pdf), which requires that metabolite levels for major human metabolites (i.e., >10% of total drug-related material in circulation) be compared across humans and animal species employed for risk assessment, and only those metabolites for which the animal-to-human ratio exceeds 0.5 are considered to have been qualified from a safety perspective. Profiling metabolites from the hADME study will also reveal any human-unique metabolites, which will require a different approach to qualify their safety. Also, the metabolite profile in circulation may reveal metabolites that could contribute to the effectiveness of the drug (i.e., active metabolites). This activity may be on or off target, and it is important to quantitate that contribution (https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-investigation-drug-interactions-revision-1_en.pdf; <https://www.fda.gov/files/drugs/published/In-Vitro-Metabolism-and-Transporter-Mediated-Drug-Drug-Interaction-Studies-Guidance-for-Industry.pdf>). Exposures to metabolites that contribute to pharmacological activity may be subject to interpatient variability, thus potentially affecting efficacy. As an off-target effect, metabolites in humans may also have effects on drug-metabolizing enzymes or transporters that are different from the parent drug, so determination of their concentrations and structures in the hADME study can inform the need for in vitro and/or clinical drug interaction studies (Callegari et al., 2013; Yu and Tweedie, 2013).

The excretory metabolite profile yields insights into the mechanisms of clearance of the parent drug. From the excretory profile, a metabolic scheme can be developed by inferring pathways based on the structures of the metabolites. The quantities of each of the metabolites along a single branch of the pathway are summed, and this represents the fraction of the dose of the parent drug that proceeds through that initial metabolic route. Routes deemed major should be characterized as to the identities of enzymes involved in the initial biotransformation reaction and their relative contributions using in vitro methods (Bohnert et al., 2016). The results from these investigations are used to determine if clinical drug interaction and/or pharmacogenetic studies should be conducted to understand interindividual variability.

When conducting the metabolite-profiling part of a human ADME study, there can frequently be the observation of metabolites that had not been observed before in either animals or in vitro systems. Animals can yield different arrays of metabolites than humans, and in vitro systems may be limited to systems derived from single organs (e.g., liver). Additionally, in vitro systems do not recapitulate the metabolite profile if the drug undergoes several sequential transformations on its path to

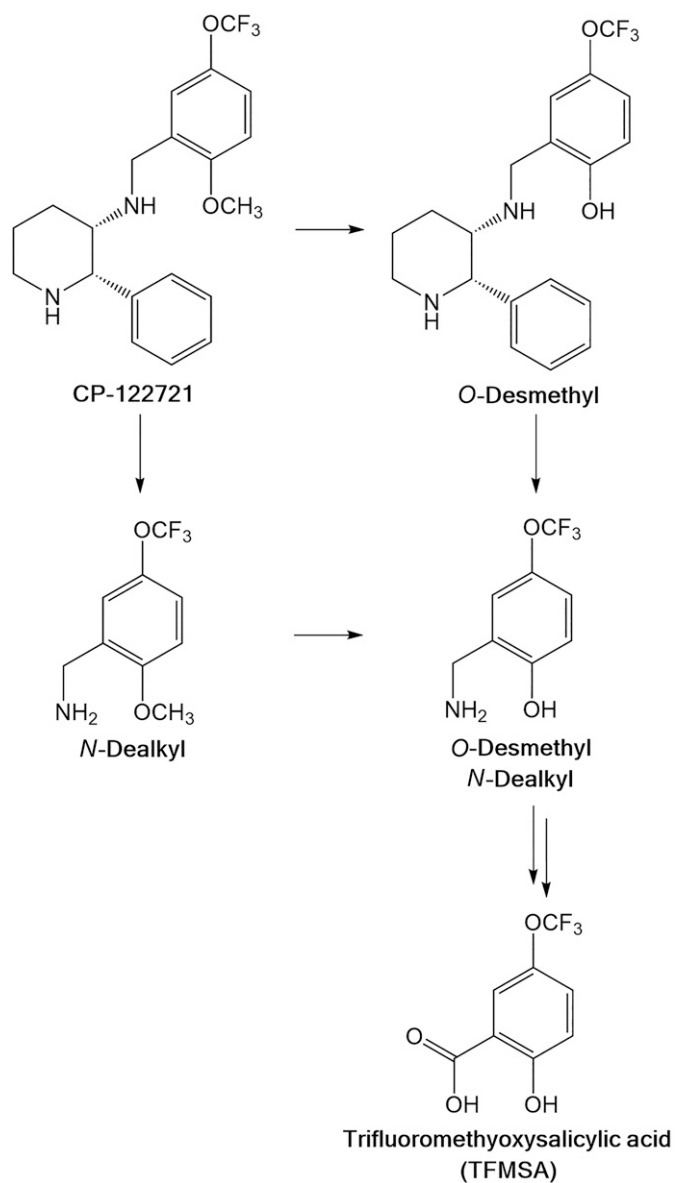
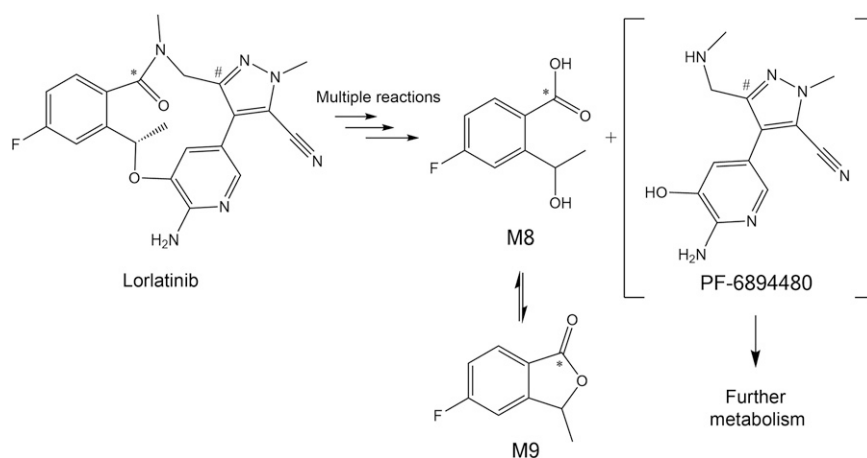


Fig. 4. Sequential biotransformation reactions CP-122721, resulting in TFMSA as a major metabolite in circulation.

becoming an excretable metabolite (Dalvie et al., 2009). Metabolite profiles in human circulation may not reflect the metabolite profile observed in vitro because the metabolite itself may not distribute from the plasma compartment. This is exemplified in the case of an NK-1 antagonist CP-122721, wherein a metabolite [trifluoromethoxy salicylic acid (TFMSA); Fig. 4] that required four sequential transformation reactions, and thus was not observed in vitro, was shown to be a major drug-related entity in circulation (Colizza et al., 2007). This was only first observed in the human ADME study, and the TFMSA metabolite was >50% of the total drug-related radioactivity, whereas the parent drug was 0.5%. This observation triggered a cascade of activities to demonstrate whether animal species that had been previously used in risk assessment studies were exposed to TFMSA and why this metabolite was observed at such great levels in humans. Although a minor metabolite in animal species, it was observed in dogs (Kamel et al., 2007), and because of the high dose used in safety studies, exposures to TFMSA were high enough as compared with humans at a pharmacologically relevant dose level. Demonstration of this metabolism in vitro required a

Fig. 5. Metabolic pathways of lorlatinib cleavage. Metabolism of lorlatinib in humans, showing the products arising from two cleavage reactions of the cyclic structure in the drug. The asterisk (*) indicates the position of the carbon-14 label used in the first study, and the hashmark (#) indicates the position of the carbon-14 label in the follow-up study. PF-6894480 was not itself observed, but subsequent metabolites of this portion were observed.



retrospective approach wherein the pathway was broken into its components to recapitulate the generation of TFMSA (Obach et al., 2007).

Timing of hADME Studies

Some still consider the hADME study as an afterthought for drug development and that its timing is to be delayed as long as possible. That perception may persist from some time ago when the study was often carried out as a “check-box exercise”; however, with additional safety aspects such as MIST to be considered, and with the application of technologies such as AMS and F-NMR, new study designs are possible that yield information from the hADME study that can proactively inform on compound safety and subsequent clinical development. Furthermore, there is a regulatory expectation that the data will be available before beginning large-scale clinical trials (phase 3 <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/clinical-pharmacology-considerations-human-radiolabeled-mass-balance-studies>), but the reason for companies’ delay is to save resources to counter the relatively high rate of attrition experienced during phase 2.

A recent white paper on hADME studies (Young et al., 2023) was the output from a consortium of pharmaceutical companies, sponsored by the European Federation of Pharmaceutical Industries and Associations drug metabolism and pharmacokinetics network, whose purpose was to consider shifts in the overall hADME strategy in light of emergent technologies such as AMS and the experience gained in the application of ^{14}C -microtracer studies. As with the use of animal studies mentioned above, there was a range of views among the companies in regard to the timing of the hADME. Often, companies will wait for a positive proof-of-concept signal to be obtained before the hADME is initiated due to the attrition risk associated with lack of efficacy. There is but one

example where a ^{14}C -labeled drug was used in phase 1 (Jensen et al., 2017). In this case, the early hADME data were useful to support MIST understanding but were also critical to show that the unexpectedly low exposure for the compound was due to first-pass metabolism and not due to poor absorption. The consortium did not offer a consensus recommendation on the timing of the hADME, only considerations for when it is appropriate.

Irrespective of the usefulness of the knowledge that can be obtained on the total disposition of a new drug candidate, conducting the human ADME as part of the first-in-human studies is an aspirational goal that is difficult to meet because of the high upfront investment needed to be made in preparing GMP-quality radiolabeled material for administration to humans. Thus, it is seldom done, and any work done to understand the metabolism of the compound uses HPLC-high resolution mass spectrometry approaches to gain a qualitative sense of the metabolite profile, observing only those metabolites detected using this technology. However, if the new drug candidate possesses fluorine, then F-NMR can be employed to gain quantitative excretion and metabolite profile data. Although previously demonstrated to be feasible in a retrospective manner (James et al., 2017), this was recently accomplished in support of a drug candidate during the phase 1 first-in-human study for nirmatrelvir, the active antiviral agent of Paxlovid for the treatment of COVID-19 (Singh et al., 2022). The results showed that nirmatrelvir itself was the main drug-related entity in urine, feces, and plasma and that the most abundant metabolite at ~12% of dose arose via a hydrolysis reaction that is most likely generated by gut microflora. The data were valuable in supporting physiological based pharmacokinetic modeling used to predict drug interactions and pharmacokinetics in special populations. Some limitations of and challenges with NMR as an approach include the fact that the compound must possess fluorine at nonmetabolized

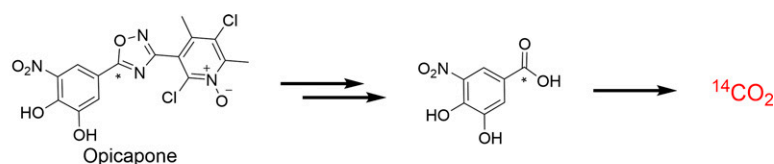
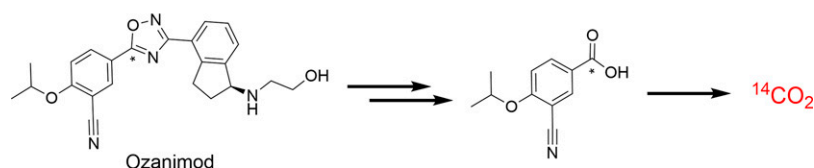


Fig. 6. Metabolic transformations resulting in loss of radiolabel for opicapone and ozanimod as $^{14}\text{CO}_2$.



sites, the dose cannot be too low (i.e., at least 100 mg or more), and sample work-up volumes need to be much larger than those used in LSC or AMS analysis. $^1\text{H-NMR}$ has also been reported to be used in generating a quantitative profile of metabolites in plasma from early phase I studies (Dear et al., 2008). Again, large matrix volumes are needed to be extracted for analysis due to sensitivity limitations, and, unlike F-NMR, background interferences in biologic matrices are massive for $^1\text{H-NMR}$, which precludes generation of mass balance data. The drug candidate requires downfield proton resonances that are distinct from endogenous materials.

The importance of the human ADME study and the benefits for conducting the study early in the clinical development program can be exemplified by the studies done to investigate the disposition of the anaplastic lymphoma kinase inhibitor lorlatinib (Stypinski et al., 2020). In a study wherein the carbon label was placed at a benzylic carbonyl carbon (for ease of radiosynthesis), a cleavage pathway that had not been previously observed in animal species or in vitro incubations was observed, and the metabolite arising from these transformations, M8, was shown to be a major metabolite that surpassed the MIST threshold (Fig. 5). This observation led to the conduct of a second human ADME study with lorlatinib labeled at a different position, which permitted following the other major portion arising from the cleavage reactions. Had the first study been conducted late in development, there would not have been enough time to complete the second study nor would there have been time to do follow-up evaluations of M8 in laboratory animal species used in risk assessment.

Two additional drugs, opicapone and ozanimod, serve as additional examples of where data from an early hADME may have been beneficial. Both the catechol *O*-methyltransferase inhibitor, opicapone, and the sphingosine 1-phosphate (S1P) receptor modulator, ozanimod, contain a central oxadiazole ring bearing the radiolabel. As shown in Fig. 6, metabolism, most likely involving gut microbes, results in scission of the oxadiazole, release of ^{14}C -benzoic acid metabolites, and subsequent decarboxylation releasing the radiolabel as $^{14}\text{CO}_2$. Although expired air was captured in one of the hADME studies run for opicapone, accounting for 20% of dose (Loureiro et al., 2022b), the hADME study for ozanimod did not include the capture of expired air, which likely contributed to the low recovery (63%) reported for this study (Surapaneni et al., 2021). Interestingly, in an ADME study run in rats for opicapone, only 1.5–2.2% of dose was recovered in expired air (Loureiro et al., 2022a), indicating that gut microbial metabolism of opicapone likely differs between rats and humans. For ozanimod, in addition to the low recovery, the hADME study, which was run concurrent with phase 3 studies, was further complicated by the identification of a major (~90% of circulating drug-related material), long-lived, disproportionate metabolite in plasma with similar activity and selectivity to ozanimod. The low recovery of radioactivity and the complicated metabolism and occurrence of a major, long-lived, and disproportionate human metabolite were likely exacerbated by the late execution of ozanimod's hADME study, and, although the number of complications encountered in the case of ozanimod is unusual, it provides a number of situations where early execution of an hADME study may be beneficial.

Conclusion

hADME studies represent one of the most important clinical studies in terms of obtaining a comprehensive and quantitative overview of the total disposition of a drug candidate. From their origins in the use of radioisotopes as tracers in biochemical studies, hADME studies have become a routine part of the characterization of a drug candidate and are regularly included in filing documents to regulatory agencies to aid in the understanding of safety and efficacy. Although the design of these studies has changed little over the years until recently, advances in the

technologies used to analyze samples from hADME studies have changed considerably. These advances have made dramatic improvements to sample analyses and expanded the quality and quantity of information obtained in these studies. Though issues such as the necessity of animal-based ADME studies, optimal timing of hADME studies, and the acceptable radioactivity recovery in an hADME study still remain to be settled, the importance of hADME studies to our understanding of a drug candidate's disposition is undeniable.

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Authorship Contributions

Performed data analysis: Cerny.

Wrote or contributed to the writing of the manuscript: Cerny, Spracklin, Obach.

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