Biomedical accelerator mass spectrometry: recent applications in metabolism and pharmacokinetics

Graham Lappin & Lloyd Stevens

Xceleron Ltd, The Biocentre, Innovation Way, York, YO10 5NY, UK and Pharmaceutical Profiles Ltd, Mere Way, Ruddington Fields, Nottingham, NG11 6JS, UK

Background: Accelerator mass spectrometry (AMS) is a sensitive isotope ratio technique used in drug development that allows for small levels of \(^{14}\)C-drug to be administered to humans, thereby removing regulatory hurdles associated with radiotracer studies. AMS uses innovative study designs to obtain pharmacokinetic and metabolism data. Objective: This review addresses the metabolism and pharmacokinetics relevant to cases where therapeutic drug concentrations are achieved in humans. Methods: The review focuses on two study designs: i) administration of tracer \(^{14}\)C-drug intravenously with a simultaneous non-labelled extravascular therapeutic dose to obtain the pharmacokinetic parameters of clearance, volume of distribution and absolute bioavailability without extensive intravenous toxicity safety studies or formulation development; and ii) use of low levels of \(^{14}\)C-drug during Phase I studies to investigate the quantitative metabolism of the drug in humans early in drug development, as required by the new FDA guideline issued in February 2008. Results/conclusions: Early knowledge about a drug’s clearance, volume of distribution, absolute bioavailability and metabolism can affect the development of a new drug candidate.

Keywords: accelerator mass spectrometry, drug metabolites, intravenous pharmacokinetics


1. Introduction to AMS

Accelerator mass spectrometry (AMS) was first applied to drug development around 2000 [1], although the technique was used for archaeological carbon dating for at least 20 years before this time [2,3]. AMS is an extremely sensitive isotope ratio technique [4] and whereas it can be used to measure a number of isotopes, its principal application in biomedicine has been in the determination of the \(^{12}\)C:\(^{14}\)C ratio [5]. The first uses of AMS in drug development were associated with radiotracer drug metabolism studies in humans [6]. Radioactive doses administered to humans are strictly regulated, limiting the dose to not much > 1 mSv [7]. For a typical drug with a plasma half-life of a few hours and one that does not exhibit any significant tissue binding, 1 mSv typically equates to around 3.7 MBq (100 µCi) per human volunteer. For drugs with long plasma half-lifes or drugs that exhibit, for example, extensive melanin binding, the amount of radioactivity has to be reduced to ensure radioactive exposure is within prescribed limits. In addition, for some drugs, labelling the molecule with \(^{14}\)C leads to instability due to autoradiolysis and hence imposes a limit on its specific activity. For these reasons, the levels of radioactivity that can sometimes be given to human volunteers are too low to be reliably measured in plasma, excreta or other samples by standard methods such as scintillation counting. It was under these
conditions that the first drug metabolism studies with AMS were performed [8-13]. Before the advent of biomedical AMS, \(^{14}\)C and other radioisotopes used as tracers were measured by scintillation counting, whereby the energy of the ionising radiation is converted into photons of light, which are then counted with the aid of photomultipliers. The number of recorded photons is proportional to the number of atomic disintegrations occurring in a given time. The maximum specific activity of a radioisotope is defined by Equation 1 [14] in which the number of disintegrations per minute (dpm) per mole of radioisotope is equal to the natural log of 2 divided by \(t_{1/2}\) of the radioisotope multiplied by Avogadro’s number \(N_A\).

\[
dpm/mole = \frac{\ln 2}{t_{1/2}} \times N_A
\]

For \(^{14}\)C, with a \(t_{1/2}\) of 5760 years, the maximum specific activity achievable is 2.3 GBq/mmol (1.38 \(\times\) \(10^{11}\) dpm/mmol). Therefore, just 1 dpm will occur on average within a population of \(4.35 \times 10^9\) atoms of \(^{14}\)C. The sensitivity of AMS is, therefore, explained because unlike scintillation counting, which detects the number of atomic disintegrations, AMS counts individual atoms of \(^{14}\)C in relation to the abundance of \(^{12}\)C.

2. Background

This journal contained a review of biomedical AMS in 2005 [10] and the content will not be repeated here, and the current review provides an update on some of the more recent applications. AMS has been described as an enabling technology [5] meaning that the implications go wider than just considering it as a ‘highly sensitive scintillation counter’. The application of AMS facilitates certain innovative study designs, particularly in studies with human volunteers thereby enabling data to be acquired that is very difficult, if not impossible, using other techniques. This review does not concern itself with the AMS technology itself but rather on how the data are used to determine drug metabolism and pharmacokinetics and what impact this has on subsequent drug development.

AMS is perhaps best known for its application in microdosing in which preliminary metabolism and pharmacokinetic data can be obtained in human subjects at a very early stage by the administration of subpharmacologic doses, up to a maximum of 100 µg [15,16]. At such low doses, the regulatory authorities do not require the full safety toxicology package usually required for a Phase I clinical trial [17,18]. In the US the safety package can be as little as a single dose 14-day rat study [18]. Microdosing has been the subject of several reviews and will, therefore, not be considered further here [15,16,19-31]. There remains, however, the question of whether pharmacokinetic data from a microdose study will be predictive of the pharmacokinetics at a higher, therapeutic, dose. There is a growing body of evidence to support the utility of microdosing [32-35] although the area does remain somewhat controversial [36,37]. Perhaps the association of AMS technology and microdosing is so strong that there is a tendency to assume that they are one of the same but this is far from the truth. AMS has many applications relevant to therapeutic doses. Indeed, this review is not concerned with microdosing per se and all the applications covered here, unless stated otherwise, are relevant to situations in which the systemic concentrations of drug are at pharmacologically active concentrations and, therefore, issues of pharmacokinetic linearity across doses do not apply. Understanding this is of critical importance to this review as some of the concepts may be easily confused.

3. Intravenous pharmacokinetics

3.1 Why intravenous pharmacokinetics in humans is useful in drug development?

Most drugs, other than biologics, are administered by an extravascular route; typically orally. Although administering the drug to humans by the intravenous route would generate very useful pharmacokinetic data (see below), performing such a study would mean having to conduct a relatively expensive formulation development and safety toxicology package (two species, one of which is non-rodent) to support what may be only one subsequent intravenous administration. Furthermore, for some drugs, the development of an intravenous formulation for human use can be very challenging, especially for drugs with sparing solubility. Whereas data on the bioavailability of a drug is required for product registration there is no regulatory requirement to define the intravenous pharmacokinetics or absolute bioavailability. However, the regulatory authorities do sometimes ask for absolute bioavailability information for the extravascular route in cases in which the bioavailability is apparently low or variable and there is a proven relationship between the pharmacodynamics and the pharmacokinetics at therapeutic doses. In all such cases to conduct an absolute bioavailability study requires that the drug be given intravenously. Within the Guidance for Industry, Investigators and Reviewers on the use of a Biopharmaceutics Classification System as a waiver for conducting bioavailability and bioequivalence studies with immediate-release solid oral dosage forms, the FDA has suggested that ‘In the absence of evidence suggesting instability in the gastrointestinal tract, a drug substance is considered to be highly permeable when the extent of absorption in humans is determined to be 90% or more of an administered dose based on a mass balance determination or in comparison to an intravenous reference dose’. Pharmacokinetic data from the intravenous route in humans nevertheless can be extremely valuable as this provides information on the fundamental pharmacokinetic parameters of volume of distribution (\(V\)) and clearance (\(CL\)). Moreover,
intravenous pharmacokinetics along with those from the oral route for solid and liquid dosage forms allows determination, by appropriate modelling and deconvolution, of the in vivo absorption and dissolution rates and thereby establishes the pharmacokinetic platform of the in vitro/in vivo correlation. This is an essential component in the justification and selection of an appropriate modified delivery formulation. The intravenous pharmacokinetic data are also useful for simulation modelling of in vitro dissolution characteristics (input function) for potential modified release delivery of oral drugs. The lack of intravenous data, although understandable in terms of effort and cost (for the preclinical safety toxicology, intravenous formulation development plus clinical safety and tolerance), nevertheless leaves a worrying gap in the understanding of the drug’s absorption, distribution and systemic clearance from the body. Such data can be vital in identifying potential problem areas that may only become apparent in the late stages of drug development and in which the relative cost implications can be very significant indeed. For example, a given dose of drug will achieve a certain plasma concentration depending upon its volume of distribution. Lower than expected plasma concentrations following oral dosing, for example, could be owing to poor performance of the formulation, poor absorption or permeability (or first pass metabolism) or equally, to a higher than anticipated volume of distribution.

Drug clearance may be sensitive to, for example, hepatic blood flow. Changes in blood flow due to effects of food or other drugs, or indeed the age of the patient can, therefore, have profound implications for the drug’s clearance, which in turn may have consequences for the drug’s efficacy or toxicity. In situations in which a drug has a high hepatic extraction, low plasma binding and low renal clearance then total clearance of the drug is dominated by hepatic extraction and even relatively small changes in the hepatic blood flow can have affects on the plasma drug concentration. For example, zidovudine has a high hepatic extraction ratio with low affinity to blood plasma proteins and ~ 16% of the dose is excreted unchanged in urine. Food intake increases hepatic blood flow which causes up to 22% decrease in plasma concentrations independent of any absorption effects on bioavailability [38]. Under such circumstances with orally administered drugs, the situation can become quite complicated because as the blood flow changes so does the clearance and bioavailability in opposite directions. The above examples show how useful a knowledge of clearance, volume of distribution and absolute bioavailability are in drug development, although, until now, such data have been considered very difficult to obtain.

3.2 Pharmacokinetics behind absolute bioavailability
There are situations in which a regulatory authority may require information on the absolute bioavailability of a drug in humans [39]. Under these circumstances, the traditional absolute bioavailability study involves administration of the drug in a two-way cross over design, in which the extravascular dose is given on one dose occasion and an intravenous dose on a separate dose occasion. The respective AUCs to infinite time for the intravenous and extravascular doses are then used to calculate the absolute bioavailability (F) using Equation 2, in which F is the fraction of parent drug that enters the systemic circulation.

\[
F = \frac{AUC_{ev}}{AUC_{iv}} \left(\frac{dose_{ev}}{dose_{iv}}\right)
\]

where ‘ev’ is the extravascular dose.

The relationship between clearance, dose, AUC and F are shown in Equations 3 and 4.

\[
Dose_{iv} = CL \times AUC_{iv}
\]

\[
F \times dose_{ev} = CL \times AUC_{ev}
\]

The equations, therefore, assume that clearance is the same as that measured for the intravenous dose throughout the plasma drug concentration–time curve for the oral dose. Indeed Equation 2 only holds true if the values for clearance in Equations 3 and 4 are equal, which may not necessarily be true. Furthermore, the plasma drug concentrations attained from an intravenous dose and an extravascular dose in a crossover study might be very different depending upon the respective dose levels used. As an example, the mass of drug delivered by the intravenous route might be limited by its solubility. The extravascular dose may be many times higher and if highly bioavailable then the plasma concentrations attained from the extravascular dose might be very much higher than those of the intravenous dose. If the drug exhibits dose-dependent clearance (e.g., [40]) then this will lead to an error in the calculation of bioavailability as the clearance values in Equations 3 and 4 will be different. Although absolute bioavailability studies, when conducted, are commonly performed using a crossover design as described above, there is nevertheless the underlying assumption that clearance for the intravenous and extravascular doses are equal, a fact that often remains unrealised.

In an attempt to ameliorate this problem, a method was developed in the 1970s whereby the intravenous dose was isotopically labelled and administered simultaneously with a non-labelled extravascular dose (the technique was used overwhelmingly with the extravascular dose being oral) [39]. The isotopic label was typically a stable isotope such as \(^{13}\)C [41]. In systemic circulation the isotopic (intravenous) and non-isotopic (oral) drugs mixed and thus the isotopic drug acted as a tracer. Blood (plasma) samples were analysed by mass spectroscopy and the isotopically labelled drug was distinguished from the non-isotopically labelled drug by
virtue of their different molecular masses. Thus, the AUCs were determined for the intravenous and oral administrations from one set of plasma samples (Figure 1).

The intravenous dose could be given as a bolus dose at the same time as the extravascular dose. A better design, however, attempts to get the plasma drug concentration–time curve of the intravenous dose to mimic the shape of the curve for the extravascular dose during the absorption phase, thereby keeping the values for clearance between the two dose routes as equivalent as possible. This can be done in one of two ways. The intravenous dose can be given as a bolus dose at the estimated extravascular $T_{\text{max}}$ or better, by constant infusion from time zero to $T_{\text{max}}$ [42]. Notwithstanding the timing of the intravenous administration, by using a concomitant intravenous dose of isotopically labelled drug along with a non-labelled extravascular dose, the values for plasma clearance for both the intravenous and extravascular doses (at least during the elimination phase) were by definition the same. The simultaneous dosing technique had some merit in terms of equivalent clearance between the two dose routes but the limits of detection for stable isotope analysis are relatively poor because of the high natural abundance of such isotopes (e.g., 1.1% for $^{13}\text{C}$). The levels of isotopically labelled drug that had to be administered intravenously, therefore, were still in the same range as those that would have been administered in a traditional crossover design. It was, therefore, possible for the intravenous dose to perturb the pharmacokinetics of the extravascular dose, particularly in situations in which the relative plasma concentrations of the extravascular dose were low owing to poor bioavailability. Moreover, the above technique did nothing to alleviate the requirement for an extensive series of toxicology studies necessary to ensure the safety of the intravenous dose.

3.3 Intravenous pharmacokinetic studies and AMS

To ensure that the plasma concentrations attained from the isotopically labelled intravenous dose do not perturb the pharmacokinetics of the extravascular dose, then ideally, the intravenous dose should be at least 100–1000-fold lower than the extravascular dose. If such a low dose was administered then the method of measurement would have
to be very sensitive and this immediately precludes the use of relatively highly abundant stable isotopes. Radioactive isotopes, generally, have very low natural abundances simply because they decay away over time. $^{14}$C is a rare radioisotope of carbon (natural abundance about $10^{-11}$%). Some early absolute bioavailability studies were performed using $^{14}$C as the isotopic tracer in the intravenous dose but this technique was still restricted by the limits of detection of scintillation counting, given the limited amounts of radioactivity that could be administered to human volunteers [43]. As described in the introduction, AMS is many times more sensitive than scintillation counting because it measures individual atoms rather than relying upon relatively infrequent disintegration events. The use of AMS as the analytical method, therefore, enables the intravenous dose to be labelled with very small amounts of $^{14}$C. In terms of radioactivity, the amounts used are typically 3.7 – 7.4 kBq (100 – 200 nCi), which frequently results in radioactive exposures below those necessary for regulatory approval. The mass of $^{14}$C-drug administered intravenously can also be very small with doses as low as 1 µg, or even smaller, being feasible.

As an example, take a drug with a molecular weight of 400. Assume that the specific activity of the drug is 2.3 GBq/mmol (3.45 $\times$ 10$^8$ dpm/mg) (see Section 1). Assume the amount of radioactivity administered was 7.4 kBq (200 nCi), then at a specific activity of 2.3 GBq/mmol this would represent a mass of 1.3 µg. AMS can measure around 0.01 dpm/ml plasma by using HPLC separation. Thus, if an intravenous dose of 1.3 µg was administered, the limit of detection would be 290 ag/ml (2.9 $\times$ 10$^{-10}$ g/ml). The limit of detection, as with any isotopic method, is determined by the specific activity of the drug (notwithstanding any naturally-occurring background) [4]. Thus, if, for example, 500 ng of the above $^{14}$C-drug were administered, the limit of detection would remain at 290 ag/ml. Note that a plasma concentration of 290 ag/ml at $C_0$ for an intravenous dose of 1 µg would be the result of a huge volume of distribution of 3.4 $\times$ 10$^6$ litre! There is, therefore, a more than ample dynamic range in terms of the dose versus the required sensitivity of the assay.

The administration of very low intravenous doses also impacts on the toxicological requirements. In most cases (in which pharmacopeial intravenous vehicles and excipients are used) the need for a formal local tolerance assessment can be waived and with knowledge of drug exposure from the extravascular route and associated toxicity and pharmacology data, it is possible to justify the intravenous dose without any further safety testing. As a worse case, however, the safety toxicology studies prescribed for a microdose study (see Section 2) may have to be performed but this still represents only a fraction of that normally required to support a higher dose intravenous administration.

A common misunderstanding of the above approach is to consider the intravenous dose as a ‘microdose’. This is incorrect for two reasons. First, a microdose study is performed before a Phase I study (hence microdose studies are also known as human Phase 0 studies). Absolute bioavailability studies described here are performed in a Phase I setting, because the extravascular dose is administered at the therapeutic dose level and, therefore, the necessary toxicology studies are required to justify the safety of the dose for the extravascular route. Secondly, in a microdose study the resultant drug plasma concentrations are relevant to the microdose administered. In the absolute bioavailability studies described here, the plasma drug concentrations for the intravenous dose are overwhelmed by the systemic concentrations of the drug from the extravascular route and, therefore, the contribution (or interference) from the intravenous route will be negligible (assuming the intravenous dose is 100 – 1000-fold less than the extravascular dose, as suggested above). The shape of the plasma drug concentration–time curve obtained for the intravenous dose (and thus the values for clearance and volume of distribution) will be governed by the total drug concentration in the plasma. As the study design demands that the extravascular administration is given at a clinically relevant therapeutic dose the drug plasma concentrations are, therefore, by definition equivalent to those achieved by therapeutic dose administration.

Thus, in a study in which the intravenous $^{14}$C-drug is administered intravenously at very low doses (a few microgram) along with a non-labelled extravascular therapeutic dose, there is no issue of dose-dependent pharmacokinetics as the pharmacokinetics are governed by the total drug concentration in the plasma. This situation is often confused with microdosing in which dose-dependent pharmacokinetics is a possibility but the two situations are entirely separate. In order not to confuse the two study types, it is preferable to call the intravenous $^{14}$C-dose (administered with the extravascular therapeutic dose) a ‘tracer-dose’ rather than a microdose. It is also worth reiterating that the study design described above (in which a $^{14}$C-tracer intravenous dose is given along with a non-labelled extravascular dose) relies on AMS technology as; i) the intravenous tracer by definition has to be labelled (with $^{14}$C for AMS) to distinguish it from the extravascular dose; and ii) the plasma concentrations attained can be very small (typically low pg to fg/ml) and AMS is the only technology that is currently able to measure down to these levels (Figure 1).

In summary, the simultaneous intravenous administration of a tracer amount of $^{14}$C-drug with a therapeutic extravascular dose and analysis of plasma samples by AMS allows both the oral and intravenous pharmacokinetics of the drug (and hence absolute bioavailability) to be determined in humans without having to perform time-consuming and costly toxicology studies to support the intravenous route. The intravenous pharmacokinetics obtained in humans is relevant to the therapeutic dose of the drug. Because such small amounts of $^{14}$C-tracer drug are administered intravenously then AMS is the only technology with sufficient sensitivity to be able to measure the plasma
concentrations. Determination of drug systemic clearance after intravenous administration allows elucidation of the key drivers of low and variable bioavailability after extravascular dosing. The levels of radioactivity associated with the use of $^{14}$C in this way are so small that, in all probability, the study will not require specific regulatory approval with respect to the radioactive dose. Such studies can be performed in a Phase I setting and indeed can be performed in parallel to Phase I clinical trials for maximum efficiency of resources.

The above approach was used to determine the intravenous pharmacokinetics and absolute bioavailability of midazolam, erythromycin and an ex-Schering drug ZK253 [33]. The absolute bioavailability of neflavin was determined following simultaneous administration of 1250 mg oral neflavin and an intravenous infusion of $^{14}$C-neflavin mesilate on day 1 and at steady-state. Neflavin oral bioavailability decreased from 0.88 to 0.47 over the 11-day study period. The moderate bioavailability of neflavin was owing to significant first-pass metabolism rather than low absorption, limiting the potential of formulation improvement to decrease pill burden [44]. More recently, the intravenous pharmacokinetics and absolute bioavailability of fexofenadine were determined [45]. Fexofenadine has not been previously reported as being administered intravenously although this drug has been on the market for over a decade. The absolute bioavailability of fexofenadine was previously only estimated at around 11% based upon the fraction of unchanged drug excreted in urine [46]. The absolute bioavailability of fexofenadine, based on simultaneous intravenous dosing of the $^{14}$C-drug along with an oral dose of a non-labelled drug, is now known to be nearer to 30%, thereby implicating the role of biliary excretion to a much larger degree than previously realised. These data also add to the debate regarding the role of transporters in fexofenadine absorption and excretion, supporting the theory that the efflux transporter P-glycoprotein not only limits the gross movement of fexofenadine across the gastrointestinal tract but also transports the drug from the hepatocyte into the bile and thereby implying that fexofenadine is transported into the hepatocyte by organic anion-transporting polypeptide [47].

### 4. HPLC–AMS

It has been incorrectly assumed that AMS only measures total $^{14}$C in a sample and is not able to determine the concentration of specific analytes such as parent drug, which is essential for the calculation of any meaningful pharmacokinetics [48]. In fact, HPLC and other chromatographic separations can be applied to AMS analysis for both metabolite profiling (see below) and the quantification of parent drug or metabolites, provided the $^{14}$C label remains intact within the molecular structure (see Section 6, Note 1). There is no routinely available interface between HPLC and AMS but some progress is being made in this direction [49,50]. The eluate from the HPLC is, therefore, collected as a series of fractions which are then analysed by AMS ‘off-line’ [6]. There are difficulties with this technique, however, that are specific to AMS analysis and which have been recently addressed. The principle behind HPLC–AMS analysis is somewhat different to LC–MS analysis as AMS relies on the determination of an isotope ratio, which is unaffected by the ionisation efficiency in the instrument ion source. Moreover, the response of the gas ionisation detector is, in practice, always linear to the isotope ratio as saturation of the detector can cause damage (or at least requires time to recover) and hence great care is taken to avoid saturation. There is however, a source of possible error in the determination of the drug concentration by HPLC–AMS because if extraction of the analyte from plasma is incomplete or if the HPLC column recovery is incomplete then these losses are translated directly into an error in the calculation of the drug concentration. Such losses are accounted for in LC–MS with the use of an internal standard (see Section 6, Note 2) but as AMS necessitates the inclusion of an isotopic tracer in any molecule under analysis, then the internal standard, it would appear, would also have to be $^{14}$C-labelled. Such an internal standard is impractical as it would have to be specifically synthesised for the assay, which would be time consuming and relatively expensive.

However, a method for accounting for analytical losses has been developed recently in which a non-labelled drug is used as the internal standard [51]. The method follows a similar procedure familiar to HPLC analysis. A curve is first generated whereby a series of plasma samples are spiked with rising concentrations of $^{14}$C-drug along with a known and equal amount of non-radiolabelled analyte as an internal standard. The ‘true’ concentration of $^{14}$C-drug in each spiked calibrant is determined from the amount accurately dispensed. Each plasma sample is extracted, the extract run on HPLC and the fraction corresponding to the retention time of the analyte is collected and the $^{12}$C/$^{14}$C ratio (expressed as Modern – see Section 6, Note 3) measured by AMS. It is necessary to conduct an isotopic dilution of the fraction before AMS analysis by the addition of $^{12}$C but not $^{14}$C. Such isotope dilutors are available from immensely old petrochemical sources, in which the $^{14}$C has decayed away. In addition to AMS analysis, the UV response for the analyte peak (internal standard) is measured (UV is used as an example but any suitable measurement technique could be used). A curve is then constructed from the true concentration on the x-axis (i.e., the Modern value that would be achieved assuming 100% recovery) and the Modern value for the HPLC fraction divided by its UV response on the y-axis. A line is then fitted to the data by linear regression. It is important to understand that the curve described here differs from the usual HPLC calibration line in that it does not calibrate an instrument response. The slope of the line is instead related to the analytical recovery, statistically fitted by linear regression. Indeed, it is not actually necessary to construct the curve but rather the gradient can be calculated.
by linear regression alone. The AMS instrument is calibrated separately using standards with precise $\text{^{12}C:}^{14}\text{C}$ ratios such as Australian National University sugar or oxalic acid.

To each sample under analysis, an equal and known amount of non-radiolabelled analyte is added as an internal standard and as a chromatographic marker. The sample is extracted, run on HPLC and the fraction corresponding to the retention time of the analyte is collected and the Modern value determined by AMS. In the same way as described above, the UV response is also measured. The amount of analyte in the fraction of the eluate analysed is then given by Equation 5.

$$K = \left( \frac{R_A}{mU} + C \right) \phi$$

where $K$ is the amount of the analyte in the HPLC fraction, $R_A$ is the Modern value of the analyte fraction after isotope dilution, $\phi$ is the amount of carbon added as the isotope dilutor, $L_{\text{max}}$ is the mass specific activity of the analyte, $m$ is the slope of the curve as determined by linear regression, $U$ is the UV response (or detector response of any suitable detection method) and $C$ is the $y$-intercept (see [51] for the derivation of Equation 5).

The addition of non-radiolabelled analyte to the sample does not interfere with the $^{14}\text{C}$-analyte measurements as it is distinguished by the presence of $^{14}\text{C}$ alone. The small amount of $^{12}\text{C}$ added to the sample from the non-radiolabelled analyte is insignificant compared to the $^{12}\text{C}$ in the isotope dilutor (typically $\mu$g amounts of non-radiolabelled analyte are added to mg amounts of isotope dilutor). In addition, as the non-radiolabelled analyte is added as a constantly, equal and exactly known amount then in effect all the samples contain the same total concentration of analyte and, therefore, there are minimal concentration-dependent effects. It should be borne in mind that with AMS analysis the concentration of the analyte in the sample is often very small (see Section 3) and, therefore, losses owing to nonspecific binding can often be significant. The addition of excess non-radiolabelled analyte helps overcome these nonspecific binding effects thereby improving recovery (UK patent application 0714040.3).

It is important to realise that failure to account for analytical losses as described above will result in an error in the determination of the plasma drug concentrations in that they will be artifically low, which can result in apparent absolute bioavailabilities of over 100%.

5. FDA guidance on safety testing of drug metabolites

5.1 Background

In February 2008, the FDA issued a guideline covering safety testing of drug metabolites [52]. Understanding how a drug is metabolised in humans is fundamentally important in its development and potential application, particularly if any formed metabolites are pharmacologically active or contribute to the adverse event profile for the drug. The new FDA ‘Guidance on Safety Testing of Drug Metabolites’ is particularly focused on situations in which metabolites are present in the human but are not present in sufficient concentrations in the plasma of the safety assessment animal species to represent adequate systemic exposure, thereby giving an incomplete safety profile. The guidance touches upon the complexities of such cases; for example when a toxicology test species does not produce a similar spectrum of metabolites from a qualitative or quantitative perspective to that generated in humans. Furthermore when plasma contains parent and one or two metabolites, this is a very different situation to one in which the drug is metabolised to many metabolites, perhaps over a prolonged period of time, any one of which constitutes only a small percentage of the total.

The agency states that if one or more human metabolites comprise $\geq 10\%$ of the parent drug AUC at steady-state in human that these need to be identified and characterised (they suggest using the $C_{\text{max}}$ concentration as an alternative). If animal toxicology models fail to produce this metabolite in equivalent concentrations to humans then: i) another animal model may need to be found which has a metabolite profile more similar to humans; or ii) the metabolite may need to be chemically synthesised and safety tested.

The definition of 10% of the AUC requires some explanation. It may be assumed that this means that 10% of the parent drug is metabolised to a given metabolite but this would not be correct. In broad terms, the AUC ratio is given by Equation 6 where $F_m^d$ is the fraction of drug metabolised to metabolite $m$ (i.e., if $F_m^d = 0.1$ then 10% of the parent drug is metabolised to the given metabolite), $d$ and $m$ represent the parent drug and metabolite, respectively (see Section 6, Note 4).

$$\frac{AUC_m^d}{AUC_m} = F_m^d \times \frac{CL_d}{CL_m}$$

If the clearance of the parent drug and metabolite are the same, then $F_m^d$ is the same as the ratio of the AUCs. For example, assume that the ratio of AUCs sits on the FDAs ‘trigger’ AUC ratio of 0.1 (i.e., 10%) and $CL_d$ and $CL_m$ are both equal to 10 ml/min/kg, then $F_m^d = 0.1$ (i.e., 10% of the parent drug is metabolised to metabolite $m$). If, however, the clearance of the metabolite is significantly lower than that of the parent drug (e.g., $CL_m = 2$ ml/min/kg and $CL_d = 10$ ml/min/kg and the AUC ratio is 0.1), then $F_m^d = 0.02$ (i.e., 2% of the drug metabolised to metabolite $m$). Thus, the differences in parent drug and metabolite clearance can lead to situations in which only a very small fraction of the drug needs to be metabolised to result in an AUC ratio of $> 10\%$. It is also important to realise that
there are drugs that are extensively metabolised but the metabolites do not appear to any great degree in the plasma. One such drug is tolbutamid, which is metabolised almost entirely to the hydroxyl and carboxy metabolites but these metabolites only represent about a hundredth of the parent because of their much higher clearance values [53]. Moreover, clearance, volume of distribution and $t_{1/2}$ are related by Equation 7.

$$t_{1/2} = \frac{0.693 \times V}{CL}$$

For example, continuing with the last case and assume that the volume of distribution of the drug and the metabolite are the same at 1500 ml/kg then $t_{1/2}$ of the parent drug ($CL = 10$ ml/min/kg) = 104 min whereas that of metabolite m ($CL = 2$ ml/min/kg) = 520 min. Time to reach steady-state is determined by the half-life (it takes just > 4.7 times $t_{1/2}$ for a drug’s plasma concentration to reach apparent steady-state after regular dosing is started). Thus, in this example, steady-state conditions for the parent drug are reached after ~ 8 h, whereas that of the metabolite are not reached until 41 h. Further to the above, the volume of distribution for the metabolite is probably different to that of the parent drug and, therefore, without knowing the actual values for the volume of distribution and clearance, the reason behind any difference in the half-life of the parent drug and its metabolite cannot be ascertained.

The above example underlines a deeper complexity in the FDA’s requirement to know the ratio of the AUCs of drug and metabolite at steady-state than may be immediately apparent. It also underlines the central importance of obtaining the fundamental pharmacokinetic parameters of clearance and volume of distribution, which can only be reliably obtained from an intravenous dose.

5.2 Drug metabolites and AMS study design

The description of how intravenous pharmacokinetics can be obtained in humans and plasma concentrations relevant to the therapeutic dose but without having to conduct the intravenous toxicology safety studies is described in Section 3.3. Such data will provide information on the drug’s clearance and volume of distribution. In addition to obtaining the pharmacokinetics from an intravenous dose of parent drug, there may be merit in having the metabolite synthesised, 14C-labelled and administering this as a tracer intravenous dose along with an extravascular therapeutic dose of non-labelled parent drug (see Section 3). The 14C-synthesis requires a little effort but because the intravenous dose is kept very low then there may not be any need for toxicity testing of the metabolite and by applying the rules of microdosing, the metabolite does not have to be synthesised according to good manufacturing practices. The extravascular dose of parent drug will be absorbed and metabolised to the metabolite(s). The plasma concentration of the non-labelled metabolite is measured (by, for example, LC–MS) and AMS measures the intravenous 14C-tracer over time. The plasma metabolite concentration–time curve for the extravascular dose will, therefore, follow a particular course, typically different to that of the parent drug but nevertheless, it will follow a phase whereby the curve is dominated by the introduction of the metabolite into circulation (by metabolism rather than absorption) followed by a phase in which its elimination is the dominant factor. Administering the intravenous 14C-metabolite to reflect the phase in which it is introduced into circulation is in practice difficult but notwithstanding this, a reasonable estimate of the intravenous pharmacokinetics of the metabolite could be obtained.

How do the above theoretical arguments help ascertain in practice whether a particular drug under development may form a metabolite that triggers the FDA’s 10% rule? Firstly, the complexities of making such determinations at steady-state are illustrated. Moreover, the drug developer will want an indication of such metabolites at the earliest possible stage as the consequences can range from doing nothing (other than making an argument for the lack of action to the regulatory authority) through to the possibility of having to synthesise quantities of the metabolite for toxicological testing. In addition, even if further toxicology is not required, a robust knowledge of the pharmacokinetic behaviour of the drug and perhaps key metabolite(s), will provide early indications of possible regulatory hurdles in the future, such as age effects due to changes in hepatic blood flow or the impact of hepatic or renal impairment (see Section 3) and the potential impact of probable drug–drug interactions.

As early an indication as possible of the presence of metabolites of concern would, therefore, seem prudent for future planning; in fact the FDA guidance encourages such an approach. Ideally, data should be obtained from the first-in-man studies (single ascending dose) albeit that these studies may not necessarily be relevant to steady-state pharmacokinetics. One method that has been used to examine samples taken from Phase I clinical trials for the presence of metabolites is mass defect filtering using high-resolution chromatography and high-resolution mass spectrometry together with data processing using mass defect filtering algorithms [54]. Although such technology is excellent at metabolite identification, the quantification of metabolites is more challenging. In the first instance, a quantitative metabolite profile is probably more useful and if human metabolites of concern are observed, structural elucidation can then follow.

By far the most reliable method of obtaining a quantitative metabolite profile is with the use of an isotopic tracer such as 13C. In the past, studies involving the administration of 13C-drugs to human volunteers have required the acquisition of dosimetry data to support an application to the relevant regulatory authority to allow the administration of radioactivity. As explained in the introduction however, by
utilising AMS as the analytical method it is possible to administer such low levels of $^{14}$C that the study is deemed non-radioactive (see Section 6, Note 5). Therefore, this opens the possibility of including small amounts (∼ 200 nCi or less) of $^{14}$C-drug into Phase I studies. The use of AMS enables very low levels of $^{14}$C to be administered, which will probably negate the need for regulatory approval in terms of the radioactivity. The $^{14}$C-drug is formulated with GMP-grade non-labelled drug to the appropriate therapeutic dose level and, therefore, all the data obtained are relevant to the therapeutic dose. Indeed this has been done during a first-in-man study [55]. The use of AMS in the case of metabolite profiling enables the data to be obtained at the earliest possible stage and avoids a number of regulatory hurdles.

Analysis of plasma samples collected from such studies then follows a series of steps, with each step revealing increasing levels of information. It is only necessary to proceed from one step to the next, if the data triggers the need (Figures 2 and 3). Step 1 involves the analysis of plasma samples by AMS for total $^{14}$C (i.e., no HPLC separation) and analysis by LC–MS (or the relevant assay) for parent drug and any for any suspected metabolites for which a suitable analytical method exists (potential metabolites are identified from in vitro and animal studies). The sum of parent and any metabolites analysed by LC–MS is compared with the plasma drug–concentration time curve based upon the total $^{14}$C. If the two curves (parent + metabolites by LC–MS and total $^{14}$C by AMS) are superimposable the plasma contains only parent drug. The example shown here only plots parent drug $\bullet$ for the sake of clarity, but the plot could equally include suspected metabolites in which assays exist. Units on the y-axis are arbitrary and are for illustration only.

In step 2, plasma samples are pooled across subjects at time points chosen from the curves generated in step 1. These plasma pools are then analysed by HPLC and AMS to generate a full metabolic profile, which will reveal metabolites not accounted for in the LC–MS analysis described above (Figure 3). Depending upon the results, unknown metabolites may then be structurally elucidated. The next step will depend upon the results of step 2. For example, if the unknown metabolites turn out to be conjugates of known metabolites, then no further action may be deemed necessary (depending upon the specific circumstances of the drug under development).

Step 3 involves the quantification of individual plasma samples for the unknown metabolite(s) by HPLC–AMS to generate their respective AUCs, to be compared with those of the parent drug. It should be borne in mind that because an isotopic tracer is used, analytes could be quantified without even necessarily knowing the molecular structure, although ideally a non-labelled standard is helpful (see Section 4). Step 3, therefore, provides a complete picture of the pharmacokinetics of parent drug relevant to the therapeutic route of administration, known metabolites and previously unknown metabolites, albeit following a single dose and, therefore, in the absence of steady-state conditions.

Reiterating the information above, given that the time to reach steady-state is dependent upon the half-life, then the only metabolites of real concern in terms of their steady-state AUC are those with half-lifes longer than the parent drug. Indeed probable AUC ratios ($\text{AUC}_{\text{met}}/\text{AUC}_{\text{d}}$) of metabolites at steady-state can be estimated from the single dose pharmacokinetics of the parent drug and metabolite(s). Only under specific circumstances, in which it is considered that there is unacceptably high exposure of a metabolite in the human at steady-state, compared to the exposure of the metabolite in the toxicology species, will specific studies need to be conducted in the human at steady-state conditions. Even under these circumstances, it should not be forgotten that the need for these investigations would have been
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identified at the earliest possible stage of drug development thus giving time to instigate, design and perform any additional preclinical safety studies in good time (or indeed abandon the drug at an earlier stage). Moreover, the knowledge gained from the single dose studies are very useful in the design of steady-state experiments, which by their nature are more challenging to design. There remains the possibility that metabolites may be detected after repeat administration that are not seen in the single dose studies (e.g., due to enzyme induction). Although this cannot be ruled out, the likelihood of only detecting metabolite after multiple dosing is not high. The possibility of administering repeat doses of $^{14}$C-drug under such circumstances is a possibility, providing each dose contained sufficiently low amounts of $^{14}$C so that the total amount administered did not exceed about 7.4 kBq (200 nCi), for example. Pharmacokinetics have been obtained from doses as low as 1.1 kBq (3 nCi) per volunteer [12] but a repeat dose study using such low levels of $^{14}$C has not, to the author’s knowledge, been previously performed.

6. Supplementary information

1. The assumption is that the $^{14}$C label is in the ‘core chemical scaffold’ of the molecule. There are occasions in which more than one radiolabelled form of the drug is necessary (i.e., the $^{14}$C placed in two separate positions within the molecule). Also see Note 6 below.

2. The internal standard in LC-MS analysis also compensates for ionisation efficiencies that may differ from compound to compound and, to some degree, from day to day. These issues do not apply to AMS analysis as it relies on the determination of an isotope ratio.
3. The use of ‘Modern’ to express a $^{12}$C:$^{14}$C isotope ratio reflects the origins of AMS in carbon dating. Modern is defined as 98 attomole $^{14}$C/mg carbon.

4. The situation is more complicated when some of the metabolite is formed during first-pass effects but for the sake of clarity, this has not been considered here.

5. Although a formal application may not be required it is nevertheless prudent to liaise with the regulatory authority to ensure the assumptions made in terms of radioactive dose are acceptable. It should be pointed out, however, that in the experience of the authors such studies have been acceptable to the regulatory authorities without formal application thus far.

6. The example assumes that a single radiolabelled form of the drug will provide data on all possible metabolites. In some cases, this is not possible and more than one radiolabelled form of the drug is necessary. This issue is routine in drug metabolism and, therefore, is not considered here. An example of a drug requiring two radiolabelled forms (meloxicam) is shown in [56].

7. Expert opinion

The application of AMS to drug development has enabled new approaches to be taken in gathering pivotal information on the pharmacokinetics and metabolism of drugs in the very early stages of development. It is possible to obtain the fundamental pharmacokinetic parameters of clearance, volume of distribution and absolute bioavailability for parent drug and metabolites, which necessitates the administration of the drug intravenously but without the requirement for lengthy and costly preclinical, formulation development and clinical studies to support this route of dosing. The metabolism of the drug can be quantitatively assessed at the time of the first-in-man studies thereby highlighting the production of possible metabolites that may require additional safety testing under the new FDA guidelines. Nevertheless, it is important to understand and apply good scientific principles in determining the need, design and interpretation of such pharmacokinetic and metabolism studies. By so doing the data produced will provide a more robust understanding of the mechanisms by which the body handles the drug and its metabolites.

**Declaration of interest**

G. Lappin is employed by and owns shares in Xceleron Ltd, a commercial company specialising in the use of AMS in drug development. L. Stevens is employed by Pharmaceutical Profiles, a commercial company specialising in the clinical evaluation of drug delivery systems and associated pharmacokinetics.
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** The principles behind obtaining intravenous pharmacokinetic data in humans.


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Affiliation
Graham Lappin1 & Lloyd Stevens2
1Author for correspondence
1Xceleron Ltd,
The Biocentre,
Innovation Way,
York, YO10 5NY, UK
Tel: +44 0 1904 561567; Fax: +44 0 1904 561560; E-mail: graham.lappin@xceleron.com
2Pharmaceutical Profiles Ltd,
Mere Way, Ruddington Fields,
Nottingham, NG11 6JS, UK