The use of radiolabelled compounds in the investigation of metabolism and drug-drug interactions *in vitro*

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INTRODUCTION

The investigation of the metabolic characteristics of a drug candidate using *in vitro* methods provides much valuable information and through human *in vitro in vivo* extrapolation (IVIVE) allows the prediction of the drug's metabolic behaviour and effect on other drugs in patients once administered clinically. The quality and hence utility of the data that are obtained from several *in vitro* studies is typically greatly enhanced by the use of a radiolabelled compound. Hence the initial financial outlay required to synthesise a radiolabelled analogue of the drug candidate should be seen as a judicious investment.

The advantages that the use of a radiolabelled form of the drug candidate brings are considered in this publication.

GENERAL CONSIDERATIONS

The Use of Radiolabelled Compounds in the Laboratory

Radiolabelled compounds are detected using scintillation counting, either in a static system (liquid scintillation counters) or in a dynamic flow-through system (radiodetector connected to a high-performance liquid chromatography (HPLC) system). Thus radio-laboratories need to be equipped with liquid scintillation counters for quantitative radiochemical analysis of samples – commonly referred to as 'counting of samples'. This type of analysis is used to quantify rapidly the amount of a specific type of radioactivity in a sample and is often used to check the concentration of radioactivity in dose solutions and tissue samples for distribution and profiling studies. DMPK-focussed laboratories also require the use of flow-through radio-detectors for analysis of biological samples by HPLC.

There are distinct analytical advantages in using radiolabelled compounds compared to non-radiolabelled compounds (where appropriate). Radioactive sample analysis (generally) does not suffer from matrix effects, ion suppression or inherent variability that analyses by mass spectrometry do. Assuming a suitable HPLC method has been developed to begin with – that is, a method that resolves all metabolites and parent compound and one that is suitable to use with mass spectrometry for later or concurrent metabolite identification investigations – multiple or repeat analyses of radiolabelled samples should not be necessary. Occasionally, chemical quenching/activation of the scintillant used to detect the radioactivity is observed but this can usually be overcome by switching between homo- and heterogeneous detection – liquid to solid scintillation in the radiodetector.

Custom Radiosynthesis

There are some key issues which should be considered when initiating the synthesis of a radiolabelled compound for use in metabolic *in vitro* studies:

Choice of Radiolabel

Typically for metabolism studies of 'small molecule drugs' or new chemical entities (NCEs), the radiolabel will be carbon-14 (^{14}C) because it has a suitably long half-life

(5730 years) and is less subject to 'exchange issues' – the exchange of a radioactive atom for a non-radioactive atom – which can be observed with tritium. However, the use of tritiated (^{3}H) compounds should not be overlooked, particularly for discovery and lead optimisation studies, where often lower amounts of compound are available and financial resources are more limited. Modern tritiation methods (site-directed catalysis) can now direct the location of insertion of the tritium atom, ensuring the molecule is radiolabelled in a metabolically stable position. Tritiated compounds can be used for a similar range of metabolic investigations as those labelled with ^{14}C.

Other radioisotopes may be used to label specific compounds, exploiting their unique chemical properties. Drug candidates labelled with iron-59, sulphur-35 and zinc-65, among others have been used for metabolism studies. Iodine-125 has been a popular radioisotope for labelling peptides and proteins, enabling the biodistribution of biological drugs to be followed. However there are disadvantages with working with a gamma emitter and other methods to radiolabel peptides and proteins have been developed, e.g. covalently binding a tritiated N-succinimidyl propionate 'tag' to the molecule (Quotient's 'Radiotag' method).

Position of Radioisotope Labelling

One of the important aspects of custom radiolabelling is to try and ensure the radioisotope is incorporated in the molecule in a metabolically stable position. That is, incorporate the radioactive atom in a position in which the atom is retained following metabolism so that it is present in the metabolites. The metabolites can then be monitored and quantified by radio-HPLC. To help assess the potential major metabolic liabilities of a compound prior to initiating custom radiosynthesis, initial *in vitro* metabolism studies using a non-radiolabelled form of the compound, with analysis by LC-MS/MS may be useful.

Specific Activity

Specific activity relates to the radioactive activity (measured in Curies, Ci or Megabequerels, MBq) per unit mass of the compound synthesised – via the incorporation of radioactive C or H atom. In order to make comparisons between compounds it is important to use molar specific activity. Generally, the higher the molar specific activity the better, assuming radio-instability is not an issue. This will provide greater analytical sensitivity during HPLC and LSC analysis and will make it easier to quantify minor metabolites.

Typically, small molecules are custom synthesised with a specific activity of approximately 50 mCi/mmol for ^{14}C-labelled and up to approximately 20 Ci/mmol for ^{3}H-labelled compounds.

Requirements

This will generally depend on the intended applications but for typical *in vivo* ADME studies in animals 20-30 mCi is normally sufficient. *In vitro* studies will often only require very small amounts, typically 0.5-1 mCi.

Radiochemical Purity

Radiolabelled compounds are synthesised and then supplied purified to a particular minimum radiochemical purity (RCP). This refers to the percentage of total radioactivity that the synthesised product (parent compound) represents when compared to all of the radioactivity present in the sample/solution provided. Although absolute (100%) RCP can never be achieved, it is important to use a compound that is of sufficient RCP for the experiments being undertaken.

Generally, a RCP greater than 97% is acceptable for most DMPK-related studies. For certain *in vitro* metabolic studies, where only the metabolite profiles are being compared, it is possible to use lower than this if the radiolabelled breakdown product(s)/impurities are well resolved from the parent drug and main metabolite region during radio-HPLC analysis – that is, are 'non-interfering'. For other types of study a lower radioactive purity is generally not acceptable as the effect of the breakdown products on the *in vitro* test system cannot be readily evaluated.

Stability

All radiolabelled forms of a compound will be less stable than their non-labelled form due to the presence of the higher-energy radioactive atom(s) and therefore correct storage conditions are essential to help minimise self-decomposition. Generally this will involve storage in solution – particularly for ^{3}H-labelled compounds – and at sub-zero temperatures – either -20 or -80°C. Typically, however, even if a radiolabelled compound becomes <97% radiochemically pure, it is possible to repurify the remaining stock to an acceptable level, rather than resynthesise the compound.

THE USE OF RADIOLABELLED COMPOUNDS IN METABOLISM STUDIES

The decision to initiate the custom radiosynthesis of a new chemical entity is generally taken when there is the requirement to assess the detailed metabolic disposition of a compound in animals and humans. Following the publication of the Metabolites in Safety Testing (MIST) FDA Guidance for Industry in 2008 (1), the ability to clearly establish that the major metabolites formed by humans are also formed in the species of animal(s) used to assess the toxicity of the new drug – typically rat and dog, but can include mouse, rabbit, monkey and mini-pig, among others – is of critical importance.

The simplest and most efficient way of identifying major and minor circulating metabolites is through the use of a radiolabelled compound. Crucially, the use of the radiolabelled form of a compound means that the level of parent drug and each metabolite produced can be easily quantified, without the use of analytical standards and a validated analytical method. In addition, the recovery of the drug from the test system can also be readily assessed ensuring that the radiolabelled metabolic profiles obtained are representative of all drug-related material.

In addition to metabolic

Type of *in vitro* study	Form of compound		Usual *in vitro* test system
	Radiolabelled	Non-radiolabelled	
Species comparison of metabolism	✓✓	✓	Hepatocytes Liver microsomes
Reaction phenotyping (enzymology)	✓✓	✓	Human liver microsomes Recombinant enzymes
Covalent binding	✓✓	✗	Human liver microsomes Hepatocytes
Transporter assays - uptake	✓✓	✓	Cells (primary, transfected, differentiated)
Transporter assays - efflux	✓ ✗	✓ ✓✓	Cells (primary, transfected, differentiated) Vesicles
CYP450, UGT inhibition	✗	✓✓	Human liver microsomes
CYP450 induction	✗	✓✓	Human hepatocytes
Plasma protein binding	✓	✓	Plasma
Plasma: blood cell-partitioning	✓✓	✓	Whole blood Plasma

✓✓ assay best conducted using this form of compound
✓ information obtained may be limited
✗ not recommended to use

Table I – The application of radiolabelled and non-radiolabelled compounds in typical in vitro *metabolism and DDI studies.*

studies, radiolabelled compounds are also extensively used in the investigation of absorption, distribution and excretion of a drug candidate, in both human and pre-clinical species. Tissue distribution can be assessed using quantitative whole-body autoradiography (QWBA), as well as the more traditional dissection-combustion approach.

As well as *in vivo* studies, there are several crucial *in vitro* studies for which the use of a radiolabelled compound provides distinct advantages (Table I). For certain other types of *in vitro* studies, which do not require extensive measurement of the compound itself, non-radiolabelled compounds are best used. In terms of drug-drug interaction (DDI) assessment these are generally the perpetrator studies and therefore include cytochrome P450 (CYP450) and UDP-glucuronosyltransferase (UGT) inhibition and induction studies.

Fundamentally, with all *in vitro* systems, it is important that the physico-chemical behaviour of the compound being tested is known. For example, aqueous solubility and non-specific binding are both crucial parameters that need to be considered when using and interpreting data from *in vitro* systems – particularly the drug transporter models. The use of a radiolabelled form of a drug allows both of these crucial parameters to be assessed rapidly, accurately and without the use of mass spectrometry.

Comparative Metabolism

An *in vitro* comparative metabolism study is a highly efficient way of obtaining early human metabolic data and to identify potential major species differences in metabolism.

In this type of *in vitro* study the radiolabelled form of the drug is incubated with freshly isolated hepatocytes and/or liver microsomes – or other subcellular fraction – from human and the actual or proposed toxicity species.

The full quantitative human hepatic metabolic profile (*in vitro*) can be quickly obtained and compared with the non-human species. These studies generally have several aims, the most important of which is to assess the formation of any human-specific metabolites. Another aim is to assess the complexity of the metabolic profile and to highlight major and minor human metabolites with a view to assessing whether there are metabolites likely to have to undergo separate safety evaluation according to the MIST guidance.

The typical minimum concentration of test compound to use in these studies tends to be 1-10 μM, otherwise the radioactive sensitivity in the incubation samples may be too low for HPLC analysis with an on-line radiodetector. In the event that lower concentrations are required to be incubated, for example to minimise enzyme inhibition or to incubate a more clinically-relevant concentration, then an off-line analytical method such as fractionation in conjunction with off-line counting (eg. TopCount™ or Micro Beta Counter™) or a stop-flow system can be considered.

The findings from this type of study can also be used to help plan when the first time in human (FTIH) study should be conducted. If there are no major differences in the human metabolic profile when compared to the other animal species used, then the definitive human phase I study may be conducted later in the development programme. If however, differences in the profiles are obtained *in vitro*, then the ^{14}C human study should be conducted earlier in order to assess the definitive human metabolism to ensure the relevance of toxicological data obtained in other species.

It is important to appreciate that this kind of analysis requires that a suitable gradient HPLC method is designed to fully resolve all major metabolites and parent drug. The output from this HPLC analysis will (generally) consist of a radio-chromatogram produced by the on-line HPLC radiodetector. It is worth reflecting on just how important the radio-chromatogram is.

The radio-chromatogram shows pivotal information including the amount of parent drug present in a sample and also the number of metabolites present – assuming a good HPLC gradient method has been developed and used. But, in addition, and perhaps most importantly, the radio-chromatogram allows for the rapid quantification of metabolites present in the sample. From the examination of a radio-chromatogram it is evident how many metabolites are produced and how much of each has been produced.

It is not possible to easily obtain this crucial information any other way.

The use of a non-radiolabelled compound with analysis by LC-MS/MS requires the use of standards to quantify each metabolite and very often precise metabolite structure will not be known so these standards will not be available. Even when the structure is known, significant effort may be required to synthesise the standard to sufficient purity and quantity to enable its use analytically. Multiply this effort by the number of metabolites – which could easily be 10 or more – and the limitations of this approach to quantitative metabolite profiling are easy to see.

Furthermore, even with the use of LC-MS/MS, it is not possible to be certain that all drug-related material has been identified since the formation of specific metabolites needs to be assumed and then looked for in the incubation mixture. If not predicted, the formation of certain, potentially important metabolites may be overlooked. A radio-chromatogram, on the other hand, displays all the radiolabelled metabolites that are formed – provided that the parent compound is radiolabelled in a metabolically stable position.

Once the metabolic profile of a compound has been generated *in vitro*, metabolite identification studies can then be undertaken, generally using high-resolution, accurate mass MS/MS with further, definitive assignment by NMR.

Reaction Phenotyping

It is perhaps not widely appreciated but the greater risk of drug withdrawal (in terms of DDIs) is as a victim drug rather than a perpetrator drug. That is, if the test compound is a substrate for only a single enzyme, then there is a greater possibility of withdrawal than if it is found to be a CYP450 inhibitor for example. Conversely, a drug that is metabolised by several CYP450 enzymes has a lower potential for marked drug interactions *in vivo* (as a victim drug). Furthermore, it is important to know if a new drug is metabolised by CYP450 enzyme(s) which are polymorphically expressed in the population (for example CYP2C19, CYP2D6) or inducible (for example, CYP1A2, CYP2B6 and CYP3A4), as both of these phenomena will impact upon inter-individual variability in drug efficacy.

In view of this, both the FDA (2) and EMA (3) have recommended that the potential for CYP450-mediated oxidative metabolism is assessed as an integral part of safety assessment and subsequent product labelling. The FDA and EMA recommend that companies should determine which CYP450 enzymes – currently 1A2, 2B6, 2C8, 2C9, 2C19, 2D6 and 3A4 – contribute to the clearance of a drug.

Reaction phenotyping studies – for CYP450-mediated metabolism – are best conducted using radiolabelled ($^{14}C/^{3}H$) compounds so that the complete oxidative metabolic profile of the compound can be determined and the metabolites readily quantified. This allows for a more comprehensive metabolic analysis to be undertaken than if a non-radiolabelled compound was used. With a non-radiolabelled compound, generally only the parent compound can be fully quantified. This can be adequate if the parent compound is metabolised by a single CYP450 enzyme. However, most likely, multiple CYP450 enzymes will be involved – to a lesser or greater extent – and these situations are best unravelled using a radiolabelled compound, as each metabolite formation can then be

quantified and assessed in addition to just parent compound depletion. This information is then used to identify which CYP450s are contributing to the metabolism of a drug, using correlation analysis in microsomes prepared from individual human donors, incubation with CYP450-selective chemical inhibitors and human liver microsomes and incubation with recombinant cDNA expressed individual CYP450 enzymes.

Similar arguments apply to the UGT-catalysed glucuronidation of a drug candidate. A suitable chemical group on the molecule to which glucuronic acid can be conjugated may exist on the parent compound or it may be introduced through Phase I oxidative metabolism. Reaction phenotyping of UGT activity can be undertaken using correlation analysis and incubation with recombinant cDNA expressed individual UGT enzymes. The UGT inhibitors that are presently available commercially are generally not sufficiently selective to be useful in this type of analysis (although a few selective chemical inhibitors have been identified and can be used for some of the UGTs). Several of the cDNA expressed UGT enzymes are not found in the liver and therefore the incubation of the drug candidate with them can be used to distinguish between hepatic and extrahepatic glucuronidation.

Covalent Binding

Covalent binding to macromolecules – proteins, nucleic acids – by the parent drug and/or its metabolites is being increasingly associated with both direct toxicity and idiosyncratic (immune-related) toxicity, both of which are leading causes of drug withdrawal post-market. If a radiolabelled form of the drug is available then the assessment of irreversible/covalent binding of the compound to human hepatic protein is a relatively straightforward process.

A typical study involves incubating the radiolabelled drug with pooled human liver microsomes for a set period of time – the 'activation' period, where enzymes activate/metabolise the drug into metabolites – followed by an exhaustive extraction process to recover the radioactivity from the incubation mixture. An incomplete recovery of radioactivity can indicate covalent – hence irreversible – binding. The effect of adding other components such as UDPGA – cofactor for glucuronidation – and glutathione (GSH) and/or potassium cyanide (KCN) – as nucleophilic trapping agents – essentially to see if the addition of these components reduces the extent of binding can also be useful in rationalising the results obtained. It is also advisable to use a control compound, such as troglitazone which undergoes high covalent binding in human liver microsomes but which is also associated with hepatotoxicity.

The degree of covalent binding – pmol equiv. of drug/mg microsomal protein – can be calculated and either used in-house to discriminate compounds or to compare with other compounds which are associated with irreversible binding to human hepatic protein – troglitazone, clozapine, phenytoin for example.

Hepatic Uptake

Following the publication of a transporter concept paper by the International Transporter Consortium (ITC) in 2010 (4), an assessment of a drug's potential to interact with drug transporters is strongly recommended by both the FDA (2) and EMA (3). The regulatory authorities recommend an initial *in vitro* testing approach is adopted and the results used to assess whether clinical interaction studies *in vivo* will be required during clinical development. Assessment of hepatic uptake is increasingly being investigated, given that the widely-prescribed statins are substrates for the OATP1B1 and OATP1B3 drug transporters and therefore uptake via these transporters represents a significant possibility of a future DDI risk.

Hepatic uptake experiments are best conducted using radiolabelled compound for a variety of reasons not least ease of analysis (LSC), ability to clearly distinguish partitioning – between cells and media – and the assessment of non-specific binding.

In uptake experiments, the radiolabelled form of the drug is incubated with isolated hepatocytes for a relatively short time period – generally up to 10 minutes. At regular intervals over this time period an aliquot of sample is removed and the medium separated from the hepatocytes by centrifugation through silicone oil. The amount of radioactivity associated within the hepatocytes is then compared with the amount of radioactivity present in the medium. The experiment is often conducted at both 37°C and 4°C on the premise that active processes – that is, in this case, OATP-mediated uptake – are much reduced at lower, non-physiological temperatures. By comparing the uptake at both temperatures an estimate of the fraction of drug that is actively taken up into the hepatocytes can be made.

Another approach to assess whether a compound is subject to active hepatic uptake is to measure the uptake using increasing concentrations of drug, so that a point is reached where the uptake becomes saturated – saturation being indicative of an active process that requires energy.

In addition to DDI assessment, hepatic uptake should also be determined when making quantitative predictions of IVIVE (as clearance is generally dependent upon a combination of both drug transport and metabolism).

Plasma Protein Binding

Plasma protein binding information is required to aid in the evaluation of pharmacological, pharmacokinetic and toxicological data, including establishing a safety margin. Radiolabelled compounds are often used for plasma protein binding studies *in vitro* as they offer several advantages. For example, non-specific binding to the *in vitro* model used – dialysis apparatus or ultrafiltration units – can be easily assessed and there is no requirement for 'matrix matching' as is often required with non-radiolabelled compounds and MS analysis.

However, if a radiolabelled compound is to be used for plasma protein binding studies, care should be taken when interpreting the results, particularly for compounds of low radiochemical purity but high protein binding. This is because the binding is calculated using total radioactivity and if the binding value is close to the radiochemical purity value then the binding of parent drug may be over-estimated – as the radiochemical impurities may contribute to the binding. However, the use of radiolabelled compound does allow the stability of the compound to be easily assessed in the plasma – by radio-HPLC – and therefore an educated assessment can be made about the impact of any radiolabelled impurities that may contribute to the binding results.

For highly bound compounds, levels of radioactivity in the dialysate may be below limits of detection using LSC and therefore quantification of parent compound by LC-MS/MS may also be advisable.

Plasma: Blood Cell Partitioning (PBCP)

The partitioning of drugs between whole-blood and plasma – generally conducted in human only – needs to be assessed to assist the development of suitable, robust, long term bioanalytical procedures and for pharmacokinetic analysis. Radiolabelled compounds are recommended for PBCP measurement because whole-blood can be a problematic matrix to use for bioanalytical measurements by MS.

In this type of study, whole-blood samples are spiked at one or two concentrations of radiolabelled compound and incubated at 37°C on a rotary mixer for up to 2 hours – with intermediate time-points. After incubation, aliquots of the blood are analysed using LSC for total radioactivity. Plasma is prepared from the remaining blood and also analysed for

levels of radioactivity and the ratio of radioactivity between the two matrices derived.

These data can then be used to show if there is any association of the compound to red blood cells and if not – as is generally the case – to demonstrate that plasma – which is more suitable as a bioanalytical matrix – can be used for the pharmacokinetic analysis of the compound.

CONCLUSIONS

Radiolabelling of a drug candidate is sometimes regarded as an expensive luxury, especially at the lead candidate selection and earlier pre-Phase I stages of development. However the quality and hence utility of the data that are obtained from several *in vitro* studies is often greatly enhanced by the use of a radiolabelled compound. Given that the cost of radiosynthesis need not be prohibitively expensive – tritiation, for example is a relatively low-cost option – and that the *in vitro* methods are well established and recognised by regulatory authorities, it is an option that should be given serious consideration. Once synthesised it is advisable to maximise the use of a radiolabelled compound and therefore the benefit that derives from its availability. This requires that the *in vitro* – and other – metabolism and DDI studies that will be conducted using the compound are agreed in advance of the radiosynthesis, and a work schedule established so that the compound can be utilised efficiently immediately it becomes available. By these means the human IVIVE can be optimised, with knock-on benefits to the conduct of clinical studies.

References

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