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Commentary

An *in vitro* approach for comparative interspecies metabolism of agrochemicals

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ABSTRACT

The metabolism and elimination of a xenobiotic has a direct bearing on its potential to cause toxicity in an organism. The confidence with which data from safety studies can be extrapolated to humans depends, among other factors, upon knowing whether humans are systemically exposed to the same chemical entities (*i.e.* a parent compound and its metabolites) as the laboratory animals used to study toxicity. Ideally, to understand a metabolite in terms of safety, both the chemical structure and the systemic exposure would need to be determined. However, as systemic exposure data (*i.e.* blood concentration/time data of test material or metabolites) in humans will not be available for agrochemicals, an *in vitro* approach must be taken. This paper outlines an *in vitro* experimental approach for evaluating interspecies metabolic comparisons between humans and animal species used in safety studies. The aim is to ensure, where possible, that all potential human metabolites are also present in the species used in the safety studies. If a metabolite is only observed in human *in vitro* samples and is not present in a metabolic pathway defined in the toxicological species already, the toxicological relevance of this metabolite must be evaluated.

1. Introduction

The metabolism and elimination of a xenobiotic has a direct bearing on its potential to cause toxicity in humans. The confidence with which data from safety studies can be extrapolated to humans depends upon knowing whether humans are exposed to the same chemical entities (*i.e.* a parent compound and its metabolites) as the laboratory animals used to study toxicity. In the pharmaceutical industry, *in vitro* interspecies metabolic comparisons are made at an early stage in the drug development process. These studies provide a screen for qualitative similarities and differences in metabolism between humans and toxicology animal species. This comparison allows the selection of the most appropriate animal species, with a metabolic profile closest to that of humans, for use in future safety studies and also helps with the planning or interpretation of safety and clinical studies. Publication of the "Safety Testing of Drug Metabolites" in 2008 by the US FDA and the guidance for metabolite testing (Topic M3 (R2)) in 2009 by the ICH, has emphasised the need for an interspecies comparison of metabolites with human for safety evaluation (FDA, 2008; ICH, 2009). One of

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the key factors in these guidelines, for drugs, is that further testing of a human metabolite(s) is only warranted when that metabolite(s) is observed at systemic exposures greater than 10% of total drug-related systemic exposure or parent compound and at significantly greater levels in humans than the maximum systemic exposure seen in the safety studies.

The requirement for an interspecies comparison of metabolism for agrochemicals was introduced in the data requirements (Commission Regulation (EU) No 283/2013) for EU Regulation 1107/2009 (EU Commission, 2009; 2013a; 2013b). The aim is to ensure, where possible, that all potential human metabolites are also present in the species used in the pivotal safety studies. In Section 5.1.1 of the data requirements it states that these studies can be used to determine the relevance of the toxicological animal data, guide the interpretation of findings and in further definition of the testing strategy. Where a unique human metabolite, *i.e.* one detected only in human material *in vitro* and not in the tested animal species, is found, "*an explanation shall be given or further tests shall be carried out*" (EU Commission, 2013a).

Ideally, to understand a metabolite in terms of safety, both the chemical structure and the systemic exposure need to be determined. However, as systemic exposure data of test material and/or metabolites will not be available for agrochemicals in humans, due to the inherent safety concerns of human volunteer experiments, an *in vitro* approach must be taken. To meet this new data requirement, a tiered testing approach is proposed, which is described below and summarised in Fig. 1.

2. Approaches to *in vitro* testing

2.1. Test system

2.1.1. Species

The first step in this tiered approach is to generate and compare *in vitro* metabolite profiles from human with the animal species used in pivotal safety studies, *i.e.* those studies used to support human safety and/or set human reference doses. As the majority of relevant end-points (toxicity from acute to chronic, carcinogenicity, reproductive, developmental and neurotoxicity) are conducted in the rat, the initial interspecies comparison should be made between human and rat. If this comparison demonstrates that all *in vitro* human metabolites are found in the rat, no further testing should be required. However, mouse, rabbit or dog may be included or substituted on a case-by-case basis depending on whether they are a species used in pivotal safety studies, testing strategies and specific attributes of the molecule. If a metabolite identified in human (*in vitro*) is not observed in the pivotal toxicological species (*in vitro* or *in vivo*), then those additional species listed above, should be considered for *in vitro* testing. The implications of finding the metabolite in one or more of these other species, but not the one used in pivotal safety studies, will need to be assessed on a case-by-case basis. The strain of animal from which the *in vitro* model system is obtained, should where possible, mimic that used in the safety studies. However, where this is not always practical or feasible, another strain may be used; as many metabolic pathways are conserved, but some differences have been reported (Saito et al., 2004; Sakai et al., 2005; Imamura and Shimada, 2005; Ito et al., 2007, Chovan et al., 2009; De Graaf et al., 2002).

2.1.2. Metabolic system

The primary site of metabolism for many xenobiotics in mammals is the liver, therefore, sub-cellular fractions of liver (microsomes and S9) and hepatocytes are typically utilised to study metabolism *in vitro* (De Graaf et al., 2002; Brandon et al., 2003; Jia and Liu, 2007 Fasinu et al., 2012). In 2009, Dalvie et al. conducted a comprehensive assessment of the three commonly used *in vitro* systems, pooled human liver microsomes, liver S9 fraction and hepatocytes, to see if they adequately predicted *in vivo* metabolic profiles for drugs. The results suggested that all three systems adequately predicted human excretory and circulating metabolite profiles (33–54%), but for some compounds these metabolites were not generated *in vitro*. Furthermore, the success in predicting primary metabolites and metabolic pathways was high (>70%), but the predictability of secondary metabolites was less reliable in the three systems. The relatively low success in the prediction of secondary metabolites substantiates the observation that metabolic profiles *in vivo* could be more complex than those produced *in vitro*. Therefore, the *in vitro* assay will provide an initial comparison of metabolic pathways between species, but not perhaps final *in vivo* metabolites. A similar finding was reported by Pelkonen et al. (2009), where qualitative differences in metabolite profiles were relatively common between rat and human. In about a third of the 55 compounds tested there was a difference in the major metabolite(s) and in approximately half of the compounds some differences in minor metabolites. In general, these studies (Dalvie et al., 2009; Pelkonen et al., 2009) indicate that for a large number of compounds, the metabolite profile obtained *in vitro* quite accurately reflects the *in vivo* metabolite pattern, although it is limited to qualitative aspects. Therefore, *in vitro* systems alone cannot mitigate the risk of disproportionate circulating metabolites in humans, however they can indicate a potential for metabolite formation. As long as the limitations are recognized and appropriate cautions and considerations are taken in the design and interpretation of *in vitro* studies, they represent a viable tool for the comparative assessment of interspecies metabolism.

Of the three systems mentioned above, hepatocytes (isolated liver cells), contain the full complement of phase I and II enzymes (Hewitt et al., 2007; McGinnity et al., 2004). They have an advantage over the sub-cellular fractions in that additional co-factors are not required as these co-factors are already present in the hepatocytes (Jia and Liu, 2007). Primary hepatocytes in suspension are often used in drug metabolism and safety studies, because most of the activities of their metabolising enzymes are similar to those of intact liver (Hewitt et al., 2007; Soars et al., 2007). Cryopreserved hepatocytes also retain enzymatic activities similar to those of fresh hepatocytes and therefore offer convenience as an 'off the shelf' product (Brown et al., 2007; Griffin and Houston, 2004; Li, 2007; Jouin et al., 2006).

Microsomes are a preparation of the endoplasmic reticulum and contain the membrane proteins including cytochrome P450 (CYP), UDP-glucuronyltransferases (UGT) and flavin-containing monooxygenases (FMO). The benefits of microsomes are the ease of use, cost, reproducibility and ready accessibility, making microsomes an ideal choice, where there is indication of predominant phase I metabolism. However, the CYP and FMO reactions require the addition of co-factors such as NADPH. If microsomes are also being used to look at phase II metabolism, *i.e.* glucuronidation, the internalisation of enzyme requires addition of a detergent or pore forming agents such as alamethicin (Fisher et al., 2000), in addition to the co-factor UDPGA.

S9 is the post-mitochondrial fraction obtained after the centrifugation (9000g) of the supernatant yielded from an initial centrifuged (1000g) liver homogenate. S9 fractions contain both microsomes and cytosol, expressing a wide range of metabolic enzymes (CYP, FMO, carboxylesterases and soluble phase II enzymes, *e.g.* dehydrogenases, N-acetyl-transferase, GST, SULT) (Fasinu et al., 2012). However, reactions must be supplemented with cofactors needed to support these enzymes (*e.g.* NADPH, UDPGA, SAM, PAPS, acetyl co-enzyme A). S9 has similar benefits to microsomes regarding ease of use, cost and accessibility. However,

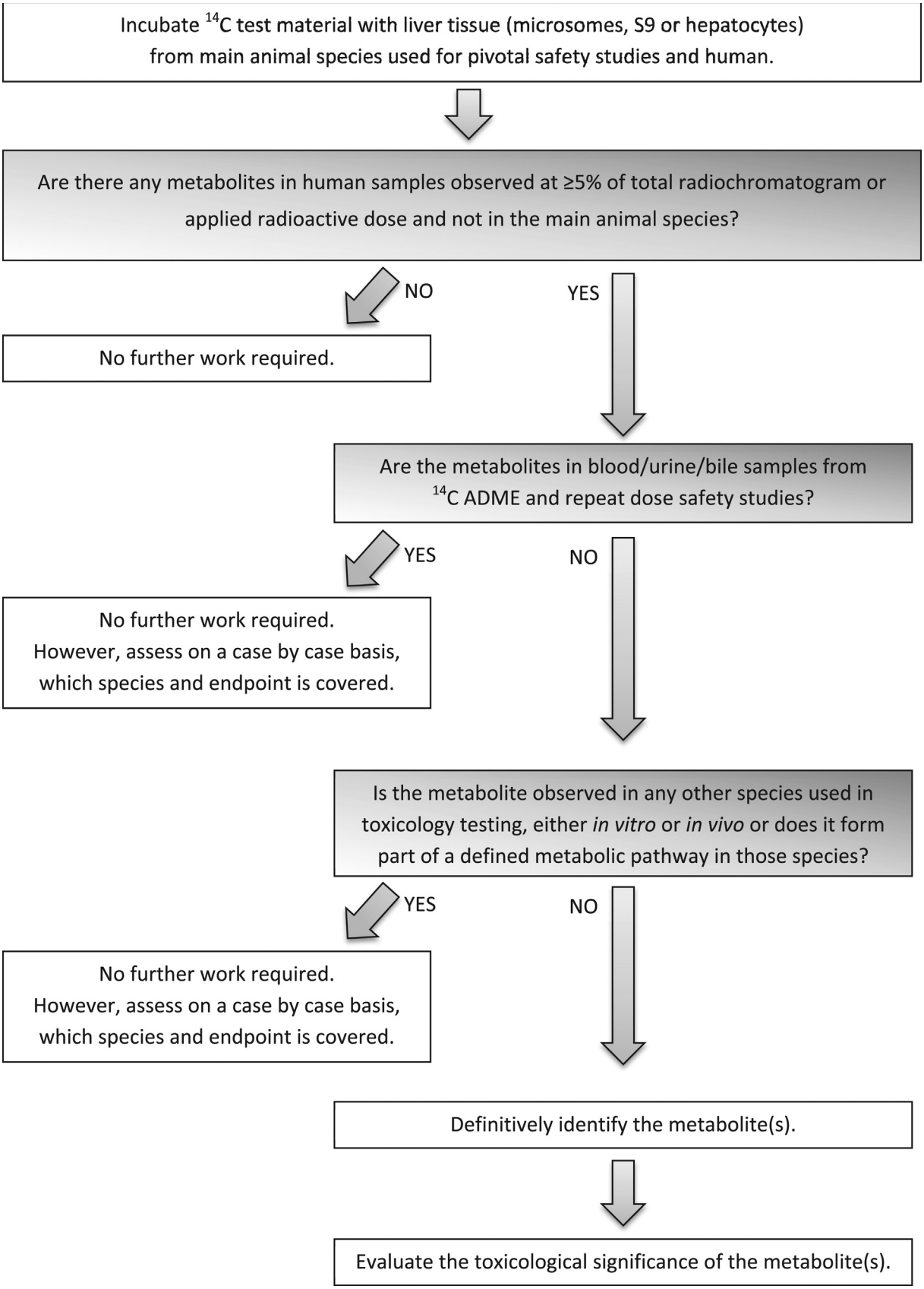

Fig. 1. Decision-tree outline for comparative metabolism study.

CYP activities are approximately four to five times less than the microsomes (Jia and Liu, 2007). This is due to the CYP enzymes becoming concentrated in the microsomal pellet from the centrifugation process, which separates the microsomal fraction of S9 from the cytosol.

As the primary phase I metabolising enzymes are the CYPs, any of the three models may be used to study CYP-mediated metabolism. However, if it is considered that the test material may be metabolised by other enzymes than CYP, an *in vitro* model that incorporates the relevant metabolising systems should be considered, such as hepatocytes or S9. As the aim of these studies is to compare metabolite profiles across species and not inter-individual variability, hepatocytes and subcellular fractions should be prepared from at least 3 donors in a pooled batch. Unlike rats, where there are marked sex differences in CYP enzyme activities, sex differences in human hepatic P450-catalyzed drug metabolism are well documented, but are generally much less dramatic than in the rat (Parkinson et al., 2004; Waxman and Holloway, 2009). Parkinson et al. (2004) reviewed cytochrome P450 activity in nearly 150 samples of human liver microsomes and 64 samples of cryopreserved human hepatocytes. The data demonstrated that CYP enzyme activity in human liver microsomes and hepatocytes varied considerably from one sample to the next, irrespective of whether the samples were from males or females, young, middle-aged or elderly donors or from Caucasians, African Americans or Hispanics. Therefore, these variables (*i.e.*, gender, age, and ethnicity) provide no meaningful basis for selecting human-derived materials for routine studies of drug metabolism *in vitro*. Therefore, male, female or mixed gender human pools may be considered, but the recommendation would be to use a mixed gender pool.

2.2. Sample generation and analysis

The experimental details outlined here are provided as a guide to *in vitro* incubation conditions that should be considered, but are not prescriptive.

In vitro metabolism samples will be generated by incubating the test material with pooled hepatocytes, microsomes or S9 from the test species and from human. The aim of this study is an interspecies comparison of metabolites and not to study the rate of formation of metabolites. Therefore, incubation conditions will not be optimised for the rate of formation of individual metabolites, but chosen to maximise the chances of forming all possible *in vitro* metabolites. Substrate concentration should ideally be below the C_{max} seen at the NOAEL level in the toxicology species and at a substrate concentration that could represent circulating concentrations in human. However, this information is unlikely to be available for agrochemicals. Therefore, a starting concentration of 1–20 μM is recommended, based on likely circulating concentrations in the toxicological species and to allow for adequate analytical sensitivity. Concentrations in this range should also optimise the chances of keeping poorly soluble compounds in solution and reduce the chances for saturation of metabolic enzymes. This concentration may be increased if detection is limited by the specific activity of the radiolabelled compound and sufficient solubility is achieved. Higher concentrations up to 100 μM may be used to enhance detection and/or identification of metabolites or if considered relevant on a case-by-case basis. Radiolabeled test material is recommended.

It is recommended to incubate hepatocytes in suspension at 0.5–1 million cells/mL and microsomes and S9 at *ca.* 0.5–1 mg/mL protein. Higher concentrations can be incubated, but microsomal protein concentrations and number of hepatocytes are typically kept below 2 mg/mL or 2 million cells/mL, respectively, to prevent excessive non-specific binding in the assay (Jia and Liu, 2007; Di and Obach, 2015). To support phase I metabolism in microsomes and S9, the co-factor NADPH must be added to incubations. The NADPH may be added directly or as a NADPH regenerating system, at a final concentration of *ca.* 1 mM, so as not to become rate-limiting. Additional co-factors may also be used in S9 incubations, to support activity for other non-CYP phase I enzymes (*e.g.* NAD^+ for dehydrogenases). If microsomes are used to assess glucuronidation, alamethicin (50 mg alamethicin/mg microsomal protein) or detergent must be included in the incubations to disrupt the microsomal membrane. In addition the co-factor UDPGA at *ca.* 2 mM must be included to allow for UGT activity. For hepatocytes, no co-factors are required.

All microsomal/S9 incubations should be performed in a buffer such as Tris or phosphate at 0.1 M and maintained at pH 7.4 at 37 °C. Hepatocyte incubations will be performed in a suitable medium or buffer, for example Krebs-Henseleit with glucose (10 mM) and HEPES (25 mM) or Williams E buffer with HEPES (25 mM).

It may be useful to establish that solubility is achieved at the desired substrate concentrations under the intended incubation conditions. An assessment of non-specific binding to the *in vitro* protein matrix may be useful to establish the free fraction of substrate available for metabolism. At the time of incubation it is advised to include several control samples. A positive control, *e.g.* ethoxycoumarin or testosterone should be included. This is to confirm the viability of the test system and provide a metabolic profile comparable with literature, vendor characterisation information or historical control data from the testing laboratory. A stability control (*i.e.* no hepatocytes or microsomes) taken at t_0 and at termination may be included to show that any loss of parent compound or formation of metabolite is enzyme related.

It may be useful to perform an experimental recovery check, by comparing the total radiolabelled material quantified on the radiochromatogram with that incubated. The purpose of this would be to indicate whether compound was lost or not available during the incubation or whether losses occurred during HPLC analysis. Recovery is typically inclusive of test material recovered in the extraction solvent, pellet residue, and appropriate vial washes. Overall recovery should ideally be quantitative for applied radioactivity (*e.g.* ≥90%). However, if there is a difference, with substantially lower recoveries from human samples, or alternately if overall recoveries are low (ie: <90%), then further investigation may be required to ascertain the cause of the loss, whether through, for example, an error in dosing, non-specific binding to vessels or proteins, volatility or losses within the HPLC system.

Incubations should generally be terminated after 2–4 hours for hepatocytes and after *ca.* 1 hour for microsomal and S9 incubations, as beyond these time-points decreases in cell viability and/or enzyme activity may occur, in certain experimental conditions (Jones and Houston, 2004; Di and Obach, 2015). However, longer incubation times may be used dependent on the model system (*e.g.* plated hepatocytes) or testing laboratory protocol. Incubations should be terminated by the addition of an organic solvent, although other terminating agents such as acid may be used. Following termination, samples will be centrifuged to precipitate protein and the resulting supernatant will be removed for analysis.

Analysis of incubates will be with radiochemical or other suitable detection method. If a metabolite is suspected as being unique to human then definitive identification of the metabolite is required, for example by use of reference standards, MS and/or NMR, in line with metabolite identification described in the OECD 417 Toxicokinetics test guideline (OECD, 2010).

2.3. Interpretation

The objective of these studies is to ensure that metabolites of

plant protection products generated in humans have been assessed during the course of the animal toxicology studies. This is accomplished by a comparison of human and animal metabolite profiles (*e.g.* radiochromatograms) generated *in vitro*. If all human metabolites are observed in one or more of the animal species used in the toxicology studies or there is general concordance between metabolic pathways in the human and animal test species, no further evaluation is necessary. Only if metabolite peaks are identified in human material that are not seen in animal material may further evaluation be needed. *In vitro* metabolite profiling is considered to be semi-quantitative at best, and in the absence of human systemic exposure data a quantitative end-point cannot be applied to the *in vitro* studies. Therefore, it is proposed, as guidance, that any metabolite formed *in vitro*, representing $\geq$5% of the radiochromatogram or applied dose, whichever is considered appropriate, should be considered for evaluation. A metabolite(s) representing more than 5% shall be considered for further evaluation, if it is only present in human and not in animal samples tested (*i.e.* a qualitative difference between species profiles). To determine if this metabolite or its metabolic pathway is present in any other test species, existing metabolism data should be examined. If neither the metabolite nor a supporting pathway is observed in existing data, then further *in vitro* (and/or *in vivo)* experiments should be conducted.

If a metabolite is observed in the human *in vitro* incubations only, then the *in vivo* metabolite profile from the toxicological species used in safety assessment should be considered (if available). Firstly, this is to assess whether the human *in vitro* metabolite (or a respective metabolite along an equivalent pathway) occurs in the toxicological species *in vivo*. Secondly, it enables an *in vitro* to *in vivo* correlation to be made in the toxicological species, *i.e.* does the *in vitro* metabolic profile generated accurately reflect the *in vivo* metabolic pathway, qualitatively and to an extent, quantitatively. If such concordance exists, this gives more confidence that the *in vitro* data from other species tested may reflect the metabolism observed *in vivo*. However, if the profiles appear dissimilar, this may suggest that the human *in vitro* profile is not wholly representative of the *in vivo* situation and care must be taken in interpreting these data, with each metabolite assessed on a case-by-case basis. The occurrence of metabolites from the major toxicological species can be checked from *in vivo* study samples obtained from both single and repeat dose toxicology and from ^{14}C ADME studies. From single and repeat dose toxicology studies, plasma and urine (if available) can be analysed by LC-MS(-MS) to provide a qualitative, and to some degree, a quantitative, understanding of the metabolites systemically available. The ^{14}C ADME studies (though these are usually limited to the rat) will enable a quantitative and qualitative assessment of systemically available metabolites in plasma, urine and bile. In these cases, analysis of the samples will be with radiochemical or other suitable detection method with structural information derived by spectroscopic techniques (*e.g.* MS/MS or NMR). A metabolite will not be considered unique to human, if it is observed in animal *in vivo* studies or expected to be transient in the metabolic pathway observed in animal species. Subsequent evaluation of the toxicological significance of metabolite of concern, should be considered on a case-by-case basis.

In addition to the semi-quantitative nature of these *in vitro* studies, certain other caveats must be considered that could affect the model system's ability to produce metabolites at a concentration that allows reliable identification. These include, but are not limited to, solubility and lipophilicity and low intrinsic clearance compounds. It is not unusual for xenobiotics in the agrochemical industry to have a Log P > 3 and have limited aqueous solubility. Therefore, it must be considered that for these compounds *in vitro* incubations may only be carried out effectively at low test material concentrations. The incubations should be performed in an aqueous buffer at pH 7.4 and, although the compound dissolved in an organic solvent can be added, the concentration of solvent in these incubations should generally be $\leq$ 1%. At organic concentrations greater than 1% v/v, commonly used organic solvents such as DMSO, methanol, ethanol, acetonitrile and PEG 400 have all been reported to inhibit activity of microsomal CYP (Busby et al., 1999; Chauret et al., 1998). However, in hepatocytes, Easterbrook et al. (2001) reported that acetonitrile, at concentrations up to 2% had no apparent effects on any of the CYP, UGT or sulphotransferase activities measured. The lipophilic nature of many of these test materials also presents the problem of non-specific binding to the incubation vessels and/or non-specific binding to protein in microsomes, S9 or hepatocytes, therefore limiting the concentration of available substrate for metabolism. For low intrinsic clearance compounds, it is often assumed that no metabolism is occurring. However, *in vivo* studies may show this not to be the case. Alternative techniques are reported that may enable this to be addressed, such as the hepatocyte relay method (Di et al., 2012, 2013) and hepatocyte models such as HepatoPac, 3D InSight™ human liver microtissues and Reinnervate's Alvetex®Scaffold. However, the pursuit of these metabolites must be put into context with the *in vivo* data and considered on a case-by-case basis.

For highly insoluble and or highly lipophilic compounds, it is recommended that this data requirement be waived, if after assessment the experimental results are questionable, due to these physicochemical properties.

In summary, a metabolite will not be considered unique to human, if it is also formed from *in vitro* animal liver tissue, observed in animal *in vivo* studies or expected to be transient in the metabolic pathway observed in animal species. After the above assessment, if a metabolite is only observed in human *in vitro* samples and is not present in a metabolic pathway defined in the toxicological species already, further evaluation of the toxicological significance of this metabolite may be warranted.

3. Conclusion

The requirement for comparative metabolism was introduced in the data requirements for EU Regulation 1107/2009. The primary aim is to ensure, where possible, that all potential human metabolites have been tested in toxicological animal species. In the absence of metabolism data from human volunteer studies, an alternative *in vitro* approach must be taken using tissues or subcellular fractions from animal species used in pivotal toxicology studies and human. This paper has outlined a strategy to conduct and assess a comparative interspecies metabolism study for agrochemicals. The strategy for how to utilise these data in a risk assessment needs to be further defined.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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