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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,