A Representative Approach to Investigate *In Vitro* Comparative Animal Metabolism of Agrochemicals

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Introduction

The *In Vitro* Comparative Metabolism (IVCM) study requirement originates from Commission Regulation (EU) No 283/2013. The objective is to evaluate whether all metabolites formed in the human *in vitro* test system as a surrogate of the *in vivo* situation are also present at comparable levels in animal species tested in toxicological studies, and, therefore, if their potential toxicity has been appropriately covered by animal studies. EFSA proposes a 3x3x3 sampling matrix (concentrations, time points, and technical replicates) which uses primary hepatocytes from 5 different species (human, rat, mouse, dog, rabbit) to identify Unique Human Metabolites (UHM) and Disproportionate Human Metabolites (DHM). Flexibility is needed to consider chemical specific and existing or emerging *in vivo* data.

Objective

To explore whether a simpler study design can address concerns for UHM and DHM. The incubation conditions were selected to maximise the chances of forming all possible *in vitro* metabolites (Whalley et al. Reg Tox Pharma 88:2017).

Parameter	Representative Design
Matrix	Hepatocytes (primary cryopreserved, resuspended), 5 species (human, rat, mouse, dog, rabbit)
Test material	^{14}C (sufficient to track metabolism)
Incubation time	1-3 time points
Cell concentration	1 or 2 × 10^6 (viable cells/mL), Trypan blue
Concentration	1 or 2 concentrations (<20 µM)
Stability	Negative control, terminal time
Metabolic activity	Positive controls: e.g., ECOD (7-ethoxycoumarin O-demethylation), and/or testosterone (terminal time).
Data Analysis	^{14}C mass balance (recovery & extraction efficiency) HPLC-^{14}C, HPLC-MS

Example Study Results

Positive Control – ECOD Assay

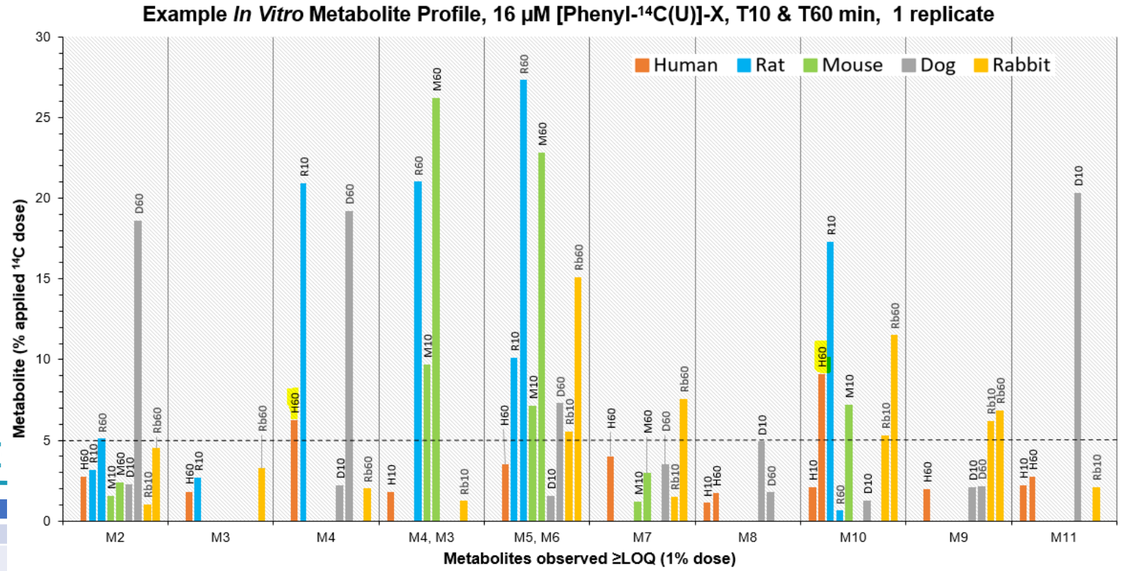
Radiolabel or Vendor [phenyl-^{14}C(U)]	Species	Rate(pmol/min/10^6 cells) 7-HC	7-HCG	7-HCS	ECOD
	Human	4.07	34.2	12.2	50.4
	Rat	2.85	18.8	137	158
	Mouse	3.62	298	93.7	396
	Dog	27.7	147	93.3	268
	Rabbit	3.30	328	95.5	427
Vendor (individual rates not provided)	Human	-	-	-	97.6
	Rat	-	-	-	145
	Mouse	-	-	-	181
	Dog	-	-	-	243
	Rabbit	-	-	-	590

In Vitro Metabolites from In Vivo ADME

Component	Description of Reaction
Test article(X)	Parent
M1	Ether bridge cleavage of parent (pyridine label not shown)
M2 (-O-gluc-a)	Hydroxylation & glucuronidation of parent phenyl ring
M3 (-O-gluc-b)	Hydroxylation & glucuronidation of parent pyridine ring
M4 (-gluc)	Glucuronidation of known metabolite not observed (pre-M4)
M5 (-gluc)	Glucuronidation of M10
M6 (HO-Pre-M4)	Hydroxylation of Pre-M4
M7	Series of reductive & oxidative steps on parent side chain (hydroxylation, O-demethylation, decarboxylation & ester hydrolysis)
M8 ([M+H]-402-a) M9 ([M+H]-402-b) (2 isomers)	Hydroxylation & hydrolysis of parent (definitive structures unknown)
M10	Ester hydrolysis of parent
M11 (HO-Parent)	Hydroxylation
M12 ([M+H]-326)	Isomer of known reference. Series of reductive & oxidative steps on parent side chain. Definitive structure not known.
Note	Metabolite identification customized to address specific project needs (*in vitro* vs *in vivo* comparison)

Aknowledgments

ML – Bayer; GN – FMC; MH – Corteva Agriscience, FK and TH – Adama; OD – BASF; KK - Syngenta



Results

- Initial cell viability 80-94%
- Positive control (ECOD) demonstrates Phase 1 & 2 enzyme activity, ECOD rates consistent with vendor rates
- [Phenyl-^{14}C(U)-X – recovery (98.8%) & extraction efficiency (89.6%) for human
- Parent turn over >20%
- *In vitro* metabolite profiles consistent *in vivo* rat ADME metabolism
- *In vitro* metabolism consistent across species
- No UHM or DHM observed

Conclusions

- A simple (e.g., 1x2x1) study design is sufficient to evaluate whether all metabolites formed in human are also present at comparable level in animal species used in toxicological studies.

Next steps:

- The Crop Life Europe Expert Group proposes a case study approach using existing data (Timing 2022).
- A more extensive ring trial and validation is needed before development of guideline.