Industry view: *In vitro* comparative metabolism studies to identify metabolites

Paul M. Whalley. Syngenta, Bracknell, UK on behalf of ECPA.

Regulatory Background for *In Vitro* Comparative Metabolism

Commission Regulation (EU) No 283/2013

5.1.1 ADME after exposure by oral route

- Comparative *in vitro* metabolism studies shall be performed on animal species to be used in pivotal studies and on human material … to determine the relevance of the toxicological animal data ….
- An explanation … or further tests shall be carried out where a metabolite is detected *in vitro* in human material and not in the tested animal species

5.5. Long term toxicity and carcinogenicity

- If comparative metabolism data indicate that either rat or mouse is an inappropriate model for human cancer risk assessment, an alternative species shall be considered

ECPA Project Team to Address Comparative Metabolism

- Remit was to develop a testing strategy to satisfy the requirement for comparative metabolism

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Commentary

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

Paul M. Whalley [a], Michael Bartels [b], Karin S. Bentley [c], Marco Corvaro [d], Dorothee Funk [e], Matthew W. Himmelstein [f], Birgit Neumann [g], Christian Strupp [h], Fagen Zhang [i], Jyotigna Mehta [d, *]

[a] *Syngenta, Jealott's Hill International Research Centre, Bracknell, RG42 6EY, UK*
[b] *ToxMetrics.com, LLC, Midland, MI USA*
[c] *DuPont Crop Protection, Stine-Haskell Research Center, Newark DE 19711, USA*
[d] *Dow AgroSciences, 3B Park Square, Milton, Milton Park, Abingdon, Oxfordshire, OX14 4RN, UK*
[e] *BASF SE Crop Protection, Global Consumer Safety, 67117 Limburgerhof, Germany*
[f] *DuPont Haskell Global Centers, USA*
[g] *Bayer CropScience AG, 40789 Monheim Am Rhein, Germany*
[h] *ADAMA MAH BV Amsterdam NL Schaffhausen Branch, 8200 Schaffhausen, Switzerland*
[i] *The Dow Chemical Company, Toxicology & Environmental Research and Consulting, Midland, MI 48674, USA*

Similarities between regulations and strategies employed by Pharmaceutical Industry

MIST (Metabolites in safety testing):

FDA Guidance for Industry. Safety Testing of Drug Metabolites, Nov 2016.

"We encourage the identification of any difference in drug metabolism between animals used in nonclinical safety assessments and humans as early as possible during the drug development process….

ICH Topic M 3 (R2) Toxicokinetic and Pharmacokinetic studies

EMA, Jun 2009

In vitro metabolic ….data for animals and humans and systemic exposure data (ICH S3A, Ref. 7) in the species used for repeated-dose toxicity studies generally should be evaluated before initiating human clinical trials

Similarities between regulations and strategies employed by Pharmaceutical Industry

Pharmaceutical

- Selecting &/or validating the most appropriate toxicological animal species for drug safety testing

- Selecting metabolites for:
  - testing of pharmacological or biological activity
  - monitoring in toxicology & clinical studies i.e. is metabolite active, major, of known toxicity, structural alert

- Determining if a human metabolite needs toxicological evaluation or if alternative toxicology species sought
  - if metabolite has a lower or no exposure in preclinical tox species, as toxicity not adequately assessed

When is a Metabolite of Concern?

- In pharma, qualitative differences in metabolism are extremely rare, i.e. unique human metabolites
- A more common situation is the formation of a circulating metabolite at disproportionately higher levels in humans than in the animal species
- However, if at least one animal test species forms this drug metabolite at adequate exposure levels (≥ than human exposure), as determined during toxicology testing of the parent drug, it can be assumed that the metabolite's contribution to the overall toxicity assessment has been established

When is a Metabolite of Concern

MIST (Metabolites In Safety Testing)

*Generally, metabolites id **only in human plasma** or those present at **disproportionately higher** levels in humans than in any of the animal test species should be considered for safety assessment. Human metabolites that can raise a safety concern are those formed at greater than **10 % of total drug-related exposure** at steady state*

ICH Topic M 3 (R2) Toxicokinetic and Pharmacokinetic studies

*Nonclinical characterization of a human metabolite(s) is only warranted when that metabolite(s) is observed at exposures greater than **10% of total drug-related exposure** and at **significantly greater levels in humans than the maximum exposure seen in the toxicity studies**.*

Comparison between Pharma and Agrochemicals

Pharma

1. New potential drugs must be tested in a suitable rodent and non-rodent species before and throughout the clinical phases of drug development programmes to help assure their safe use in humans i.e. options to choose between rodents and between dog/monkey
2. Human metabolites that can raise a safety concern are those formed at greater than 10% percent of total drug-related exposure at steady state

AgChem

1. The pivotal toxicological studies and species are already established, at a global level i.e. unlikely that testing paradigm would change
2. Never know the concentrations of metabolites circulating in humans

Commission Regulation (EU) 283/2013

- Based on pivotal toxicological studies and species being already established and no knowledge of circulating human metabolites

- Our approach was to address the key question
  - **'is there a human specific metabolite(s) that has not been tested toxicologically?'**

- As *in vitro* techniques are the best option to address this, we considered the limitations, the conduct and the interpretation of these studies

Considerations for *In Vitro* Studies

- Typically, *in vitro* experiments provide guidance on species differences in metabolism, but are limited by incomplete enzyme composition or by viability

- They don't capture the distributional properties of metabolites, or their ability to be cleared via non-metabolic processes (or extra hepatic metabolism), which are important determinants of plasma concentrations

- Thus, whilst *in vitro* systems can often provide a good correlation with *in vivo* metabolic profiles, their capacity to do so is inevitably limited

- For some compounds solubility/lipophilicity may prohibit the assessment

Predictability of Circulating Metabolites from *In Vitro* Metabolism Studies

- For many xenobiotics, the liver is the primary site for mammalian metabolism, therefore, liver sub-cellular fractions and hepatocytes are typically used

Chem. Res. Toxicol. **2009,** *22,* 357–368

Assessment of Three Human in Vitro Systems in the Generation of Major Human Excretory and Circulating Metabolites

Deepak Dalvie,*,† R. Scott Obach,‡ Ping Kang,† Chandra Prakash,‡,|| Cho-Ming Loi,† Susan Hurst,‡ Angus Nedderman,§ Lance Goulet,† Evan Smith,† Hai-Zhi Bu,†,⊥ and Dennis A. Smith§

Pharmacokinetics, Dynamics and Metabolism, Pfizer Global Research and Development, San Diego California 92121, Pfizer Global Research and Development, Groton Connecticut 06340, and Pfizer Global Research and Development, Sandwich, Kent, United Kingdom

- Dalvie and co-workers (2009), showed that:
  - the three systems predicted 33-54% of human excretory and circulating metabolites
  - prediction of primary metabolites and metabolic pathways was >70%, but the predictability of secondary metabolites was much less reliable

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In Vitro Metabolism Studies

- Similarly, Pelkonen et al. (2009), found qualitative differences in metabolite profiles were relatively common between rat and human, but about a third of 55 compounds displayed a difference in major metabolite(s) and in about half of the compounds some different minor metabolites

- On a smaller scale Anderson et al (2009) detailed 12 cases, where *in vitro* data predicted in vivo adequately (41%), underpredicted (35%), overpredicted (24%)

Comparison of Metabolic Stability and Metabolite Identification of 55 ECVAM/ ICCVAM Validation Compounds between Human and Rat Liver Homogenates and Microsomes – a preliminary Analysis*

Olavi Pelkonen[1], Ari Tolonen[2], Timo Rousu[2], Larissa Tursas[1], Miia Turpeinen[1], Juho Hokkanen[2], Jouko Uusitalo[2], Michel Bouvier d'Yvoire[3] and Sandra Coecke[3]

[1]University of Oulu Department of Pharmacology and Toxicology, Oulu, Finland; [2]Novamass Ltd, Oulu, Finland; [3]EC Joint Research Centre ADD after EC Joint Research Centre, Institute for Health and Consumer Protection, European Centre for the Validation of Alternative Methods (ECVAM), Ispra, Italy

Chem. Res. Toxicol. **2009,** *22,* 243–256

spective

Predicting Circulating Human Metabolites: How Good Are We?

Shelby Anderson, Debra Luffer-Atlas, and Mary Pat Knadler*

Department of Drug Disposition, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285

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In Vitro Metabolism Studies

- In general, these studies indicate that for a large number of compounds, the metabolite profile obtained *in vitro* can reflect 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

- As long as the limitations are recognized and appropriate cautions and considerations are taken in the design and interpretation of *in vitro* studies, all 3 systems represent a viable tool for the comparative assessment of interspecies metabolism

Study Design to Address EU 283/2013

- The aim is a **qualitative** interspecies comparison of metabolites and not a rate of formation for 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.

Test Species

- Aim is to generate and compare *in vitro* metabolite profiles from human with the animal species used in pivotal toxicological studies i.e. those studies used to set human toxicological reference doses
  - rat, mouse, rabbit, dog

- Those pivotal toxicological studies and species, for agrochemicals, are already established, at a global level

- As the majority of relevant endpoints (toxicity from acute to chronic, carcinogenicity, reproductive, developmental and neurotoxicity) are derived from studies conducted in the rat, the initial interspecies comparison should be made between **human and rat**

Test Species

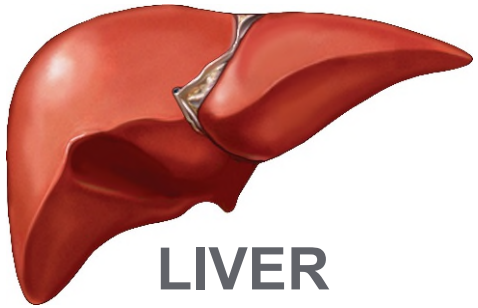
- **If all *in vitro* human metabolites are found in the rat, no further testing should be required**

- If a metabolite identified in human is not observed in the rat (*in vitro* or in vivo) then additional species mouse, rabbit or dog may be included

- If specific toxicological questions arise from a species other than rat, then include those species in testing

- If an end point has been set in a species, other than rat, any unique metabolite in that species would not necessarily be followed up – based on 'is there a human specific metabolite(s) that has not been tested toxicologically'

Which metabolic system: hepatocytes/microsomes or S9



LIVER

Hepatocytes

Phase I and Phase II

No co-factors required

Microsomes

CYP and FMOs- Phase I metabolism

UGTs – Phase II glucuronidation (+ detergent or alamethacin)

Co-Factors Required – NADPH, UDPGA

S9

Microsomes & NAT, SULTs and GSTs –

Phase II conjugations

Co-Factors Required – NADPH, UDPGA, SAM, PAPS, acetyl coenzyme A, etc

Number of Donors in Liver Fractions

- 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. In reality most commercially available pools now much larger

XENOTECH
A DIVISION OF SEKISUI

Human Liver Microsomes – I
Lot No. 1410013
Human Liver Microsomes
Mixed Gender, Pool of 50
Suspension medium: 250 mM sucrose

HEPATOSURE®

HepatoSure® is Sekisui XenoTech's 100-donor pool of cryopreserved human hepatocytes. As the largest pool that Sekisui XenoTech offers as a standard product, HepatoSure®

XENOTECH
A DIVISION OF SEKISUI

R1000 Lot No. 1310030
Sprague Dawley (SD) Rat Liver Microsomes
Untreated, Male, Pool of 405
0.5 mL at 20 mg protein / mL
Suspension medium: 250 mM sucrose

GIBCO®

Quality Certificate

Liver Microsomes: Pooled
Species: Rat (Sprague-Dawley)
Number of Donors: 68

Catalog Number: RTMCPL
Lot Number: RT051

INVITROCYP 150-Donor Human Liver Microsomes
Subcellular Fractions
Microsomes

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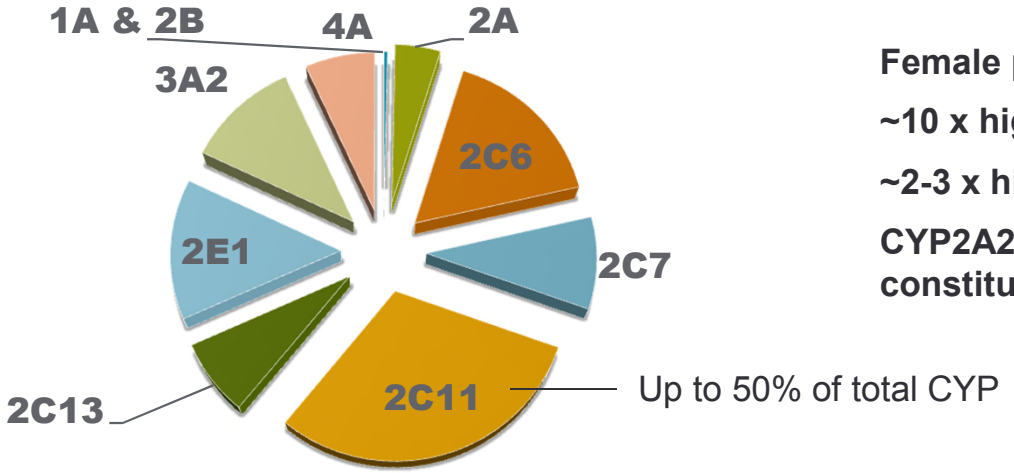
Sex of Donors in Liver Fractions

- Marked sex differences in CYP activities in rat, therefore recommend use of mixed sex pool of microsomes/ hepatocytes or include both male and female pools



Male SD rat immunoquantified CYP

Female predominant CYP:

~10 x higher levels of CYP2C12

~2-3 x higher levels of CYP2C7, 2A1 & 2E1

CYP2A2, CYP2C11 & CYP3A2 are constitutive male-specific hepatic isoforms

Ohishi et al 1994 Xenobiotica, 24, 873-880
Agrawal and Shapiro 2003 DMD 31, 612-619

- Sex differences in human hepatic CYP-catalysed drug metabolism are well documented, but much less dramatic. However, the recommendation would be for a **mixed sex pool of microsomes/ hepatocytes**

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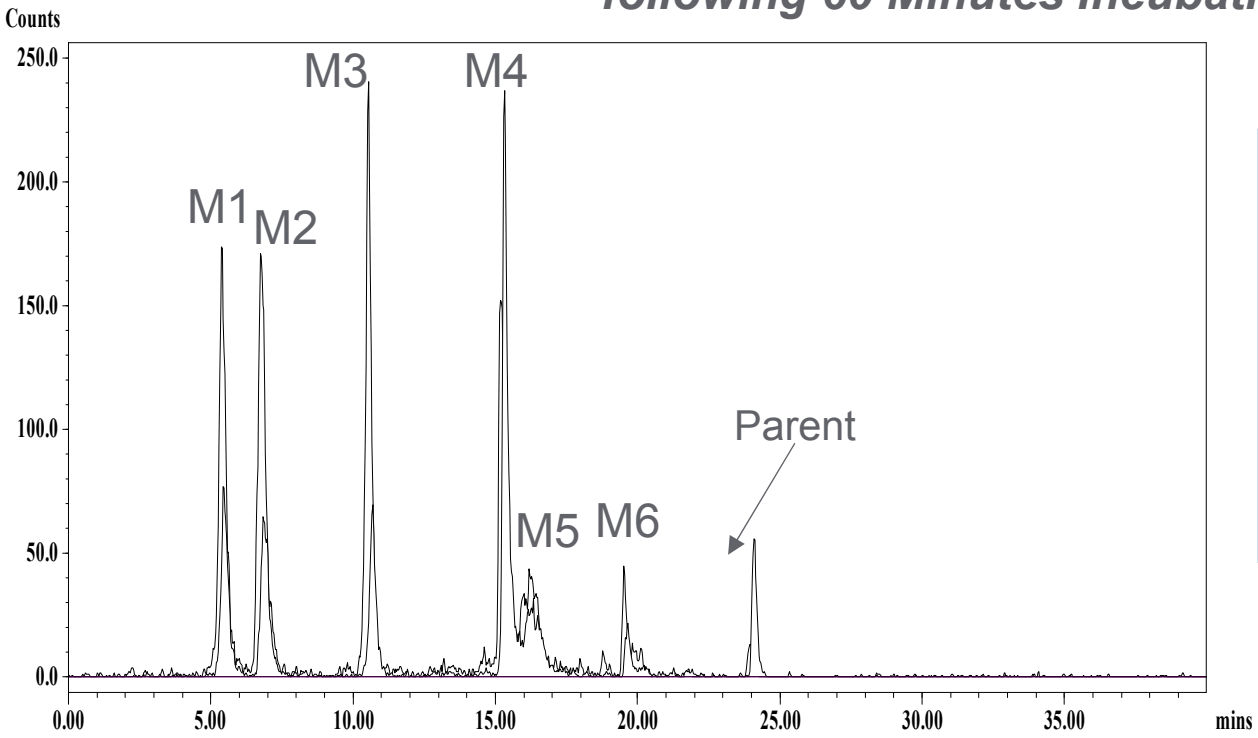
Typical Incubation Conditions

	Microsomes	Hepatocytes (suspension)
Test Compound	^{14}C @ 10 µM in buffer or solvent (keep to below 1% v/v)	
Buffer (pH 7.4)	0.1 M phosphate or Tris	HEPES (25mM) with either: 0.1 M Krebs + glucose (10 mM) or Williams E (25 mM)
[Protein]/No cells	Typically 0.5 mg/mL	Typically 0.5 million cells /mL
Co-Factors	NADPH (at 1 mM, so as not to become rate limiting), Phase II UDPGA (ca. 2 mM) + alamethacin (ca. 50 mg per mg microsomal protein)	None
Temperature	Pre -warm to 37°C	
Incubation Time	1 hour	3 hour
Controls	**Positive control** e.g. ethoxycoumarin or testosterone to confirm test system viability and provide a known metabolic profile **Stability control** (i.e. no hepatocytes or microsomes) at t0 and termination to show that any loss of parent compound or formation of metabolite is enzyme related **Blank** (i.e. no substrate) to aid in analysis	
Terminate incubations, centrifuge to precipitate protein and remove the supernatant for analysis		

Analysis and Interpretation

- Analyse the incubates with radiochemical detection and compare the radiochromatograms, qualitatively, i.e. finger print approach

Representative Comparison Radio-chromatogram following Incubation of [Label 1-^{14}C]-SYN123456 (10 µM) between Han Wistar Rat and Human Liver Microsomes following 60 Minutes Incubation



In this example there is no obvious difference in the metabolite profile between species.

No further work would be required

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Analysis and Interpretation

- *In vitro* met profiling is considered semi-quantitative at best, but, in the absence of human systemic exposure data, it was considered that a quantitative end-point should be applied to the *in vitro* studies

- Therefore, it is proposed that any ***in vitro* metabolite ≥5% of the radiochromatogram** should be considered for evaluation (based on the OECD 417)

- A unique human metabolite shall be considered if it
  - represents ≥5% radiochromatogram
  - is only present in human and not detected in animal samples (i.e. a qualitative difference between species profiles)

Interpretation

- If a human metabolite is not observed in any of the toxicological species *in vitro* or questions arise from the chromatography:
  - Confirm identity of peak in human incubate by use of reference standards, MS and/or NMR
  - Look for the metabolite in existing in vivo data
    - e.g. single and repeated dose toxicology or ^{14}C ADME studies
    - Is the metabolite observed?
    - If it's not observed, does the metabolite form part of a defined pathway?

Interpretation

- Is there an *in vitro* : *in vivo* correlation (IVIVC) i.e. does the *in vitro* metabolic profile accurately reflect the in vivo metabolic pathway, qualitatively and to an extent, quantitatively?

- A poor rat IVIVC may suggest that the human IVIVC may be also poor. Therefore, care must be taken in interpreting the data, with each metabolite assessed on a case-by-case basis

- After the above assessment, if a metabolite is only observed in human *in vitro* samples and is not present in a defined metabolic pathway in the toxicological animal species, **the safety of this metabolite must be evaluated**

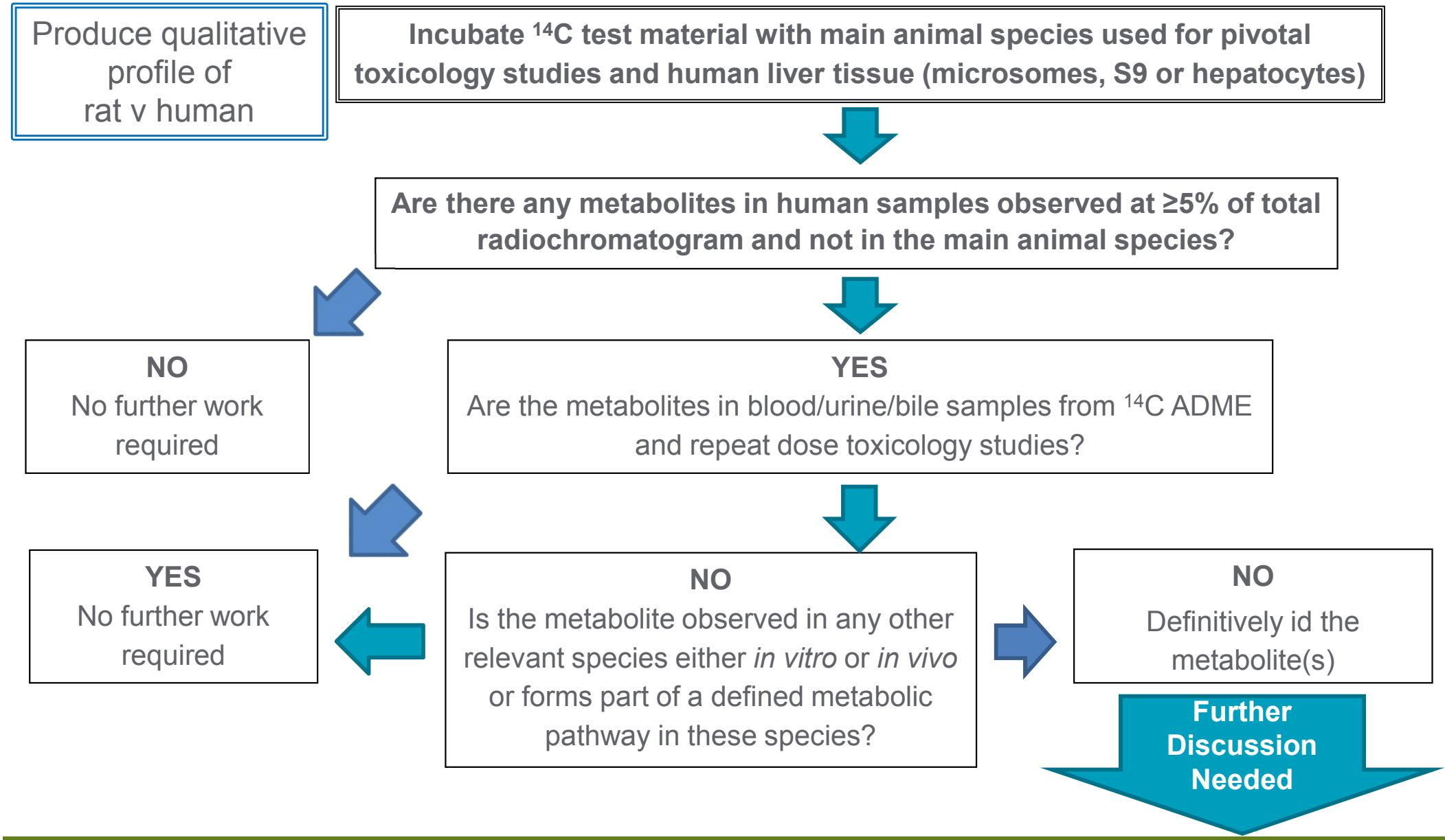
Conclusion

- Based on **preferred species being already established for pivotal toxicological studies,** it is highly unlikely we would change test species

- Our key question for these studies was: **'is there a human specific metabolite(s) that has not been tested toxicologically?'**

- To determine whether humans generate a unique metabolite of toxicological concern conduct **a qualitative interspecies comparison of metabolites** in line with the following Flow Diagram

ECPA Proposed Study Conduct



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