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

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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
Similarities between regulations and strategies employed by Pharmaceutical Industry

**MIST (Metabolites in safety testing):**

“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
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.

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

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- 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
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%).
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

**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
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.
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.

- 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.

  **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
## Typical Incubation Conditions

<table>
<thead>
<tr>
<th>Test Compound</th>
<th>Microsomes</th>
<th>Hepatocytes (suspension)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Test Compound</strong></td>
<td>$^{14}\text{C} @ 10 \mu\text{M}$ in buffer or solvent (keep to below 1% v/v)</td>
<td>HEPES (25mM) with either: 0.1 M Krebs + glucose (10 mM) or Williams E (25 mM)</td>
</tr>
<tr>
<td><strong>Buffer (pH 7.4)</strong></td>
<td>0.1 M phosphate or Tris</td>
<td>None</td>
</tr>
<tr>
<td><strong>[Protein]/No cells</strong></td>
<td>Typically 0.5 mg/mL</td>
<td>Typically 0.5 million cells/mL</td>
</tr>
<tr>
<td><strong>Co-Factors</strong></td>
<td>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)</td>
<td>None</td>
</tr>
<tr>
<td><strong>Temperature</strong></td>
<td>Pre-warm to 37°C</td>
<td></td>
</tr>
<tr>
<td><strong>Incubation Time</strong></td>
<td>1 hour</td>
<td>3 hour</td>
</tr>
<tr>
<td><strong>Controls</strong></td>
<td>Positive control e.g. ethoxycoumarin or testosterone to confirm test system viability and provide a known metabolic profile</td>
<td>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</td>
</tr>
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<td></td>
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<td>Blank (i.e. no substrate) to aid in analysis</td>
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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. fingerprint approach.

Representative Comparison Radio-chromatogram following Incubation of [Label 1-\textsuperscript{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.
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

1. **Incubate** \(^{14}C\) test material with main animal species used for pivotal toxicology studies and human liver tissue (microsomes, S9 or hepatocytes).

2. Are there any metabolites in human samples observed at ≥5% of total radiochromatogram and not in the main animal species?
   - **YES**
     - Are the metabolites in blood/urine/bile samples from \(^{14}C\) ADME and repeat dose toxicology studies?
       - **YES**
         - Is the metabolite observed in any other relevant species either *in vitro* or *in vivo* or forms part of a defined metabolic pathway in these species?
           - **YES**
             - Definitively id the metabolite(s)
           - **NO**
             - Further Discussion Needed
       - **NO**
         - No further work required
   - **NO**
     - No further work required

3. Produce qualitative profile of rat v human.