Predicting hepatotoxicity: Reactive metabolite trapping using glutathione and freshly isolated hepatocytes

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Introduction
The formation of reactive electrophilic metabolites has been implicated as a potential mediator of cellular toxicity. The mechanism is believed to involve covalent binding of the reactive metabolite to cellular proteins and/or causing oxidative stress to cellular biochemical pathways. Various chemical agents (nucleophiles) have been used as ‘trapping agents’ to ‘trap’ the formation of stable and reactive intermediates which can be used to confirm the generation of reactive species from particular compounds (or class of compounds). Compounds used as ‘trapping agents’ include: semicarbazide, glutathione, potassium cyanide and N-acetyl-cysteine.

We are currently developing nucleophilic trapping assays to assess test compounds for potential reactive metabolite formation to complement our current suite of ADME optimisation assays available to our Clients. This poster presents our results to date using microsomes and hepatocytes and glutathione (GSH) as the nucleophilic trapping agent. Glutathione is a tri-peptide consisting of glutamic acid, cysteine and glycine sub-units and reacts with potentially harmful electrophilic toxins.

During mass spectrometric (MS) analysis, glutathione undergoes cleavage giving rise to a characteristic neutral loss of 129 (terminal l-glutamic acid). Therefore, constant neutral loss scanning (CNL-129) can be used to ‘screen’ samples for the presence of GSH-conjugates. Our aim is to use neutral loss scanning for presence of GSH-conjugates of metabolites (neutral loss of 129) and selective reaction monitoring for [M+H]+ → [M-H-129]+ (to detect direct GSH-conjugates of parent compounds).

Based upon the initial screen results, samples can then undergo further analysis by LC-MS/MS in an attempt to obtain structural information on the precise nature of any GSH-conjugates formed using full scan, product ion or precursor scan techniques as appropriate.

This poster presents our results to date using clozapine (a compound known to be associated with GSH-adduct formation) as substrate and using stable-isotope GSH (GSH-13C2,15N) to enhance specificity. In addition, all analyses have been conducted using an Waters Acquity UPLC-MS/MS. Results we have obtained in hepatocytes are compared against findings using human liver microsomes (HLM).

Materials
Freshly isolated Human hepatocytes were received from the United Kingdom Human Tissue Bank (UKHTB), Leicester, UK, under ethical approval. Non human hepatocytes were freshly isolated in the laboratories of Quotient Bioresearch (Rushden) Ltd. using standard isolation procedures. The initial viabilities of the hepatocytes was determined by Trypan blue exclusion and were >80%.

Experimental
Clozapine was incubated with human liver microsomes or freshly isolated hepatocytes using the conditions detailed below:

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Species</th>
<th>No. of GSH-conjugates of parent clozapine detected</th>
<th>No. of GSH-conjugates of clozapine metabolites detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomes</td>
<td>Human</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Dog</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Mini-pig</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Monkey</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

Representative chromatograms (CNL m/z - 129)

Results
The number and type of GSH-conjugates detected are presented in the Table below:

Human liver microsomes

Freshly isolated female rat hepatocytes

Conclusion
GSH-adducts of both parent clozapine and clozapine-metabolites can be detected using both liver microsomes and freshly isolated hepatocytes using UPLC-MS/MS operating in a CNL m/z-129 screening mode. Further work is being conducted at Quotient Bioresearch (Rushden) Ltd. to better understand the chemical nature of these adducts.

These studies used incubation medium supplemented with 13C-GSH to enhance specifically during the MS/MS analysis. It was interesting that 13C-GSH adduct formation was not readily detected during the hepatocyte analysis. This is likely to be due to the use of endogenous intra-cellular GSH for conjugation reactions within the hepatocyte or 13C-GSH not being transported across the hepatocyte cell membrane.