QUOTIENT BIORESEARCH

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 compound). 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 ADMET 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 λ-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 $[M+H]^+ \rightarrow [M+H-129]^+$ (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$^{13}C_2$,^{15}N) 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:

	Microsomes	**Hepatocytes**
Concentration of Clozapine:	100 µM	100 µM
Concentration of GSH:	5 mM	1 mM
Concentration of protein/cells:	1 mg/mL	1 million viable cells/mL
Incubation time:	1 hour	2 hours

Incubations were stopped by the addition of an equal volume of acetonitrile. Incubation samples were vortex mixed for ca. 20 seconds and evaporated to dryness under nitrogen (room temperature) and reconstituted in 300 µL of methanol:water (1:1 v/v). Samples were vortex mixed for ca. 20 seconds and centrifuged 16,000 x g for 5 minutes at 8℃. The su pernatants were transferred into vials for UPLC-MS/MS analysis.

All microsomes were also incubated with ^{14}C-testosterone and all hepatocyte isolations were also incubated with ^{14}C-testosterone and ^{14}C-7-ethoxycoumarin to assess drug metabolising capacity. These data (not shown) demonstrated that the microsomes and hepatocytes used in these studies were of good drug metabolising capacity.

Mass spectrometry conditions

Mass spectrometer:	Water TQD
Autosampler:	Acquity Sample Manager
UPLC Pump:	Acquity Binary Solvent Manager
Analytical Column:	Acquity UPLC BEH C18 100 x 2.1 mm, 1.7 µm
Mobile phase A:	5 mM Ammonium Acetate
Mobile phase B:	5 mM Ammonium Acetate in 90:10 Methanol:water
Flow Rate:	400 µL/min
Run time:	10 minutes
Column Temperature:	40℃
Injection Volume:	10 - 15 µL
Sample Temperature:	4℃

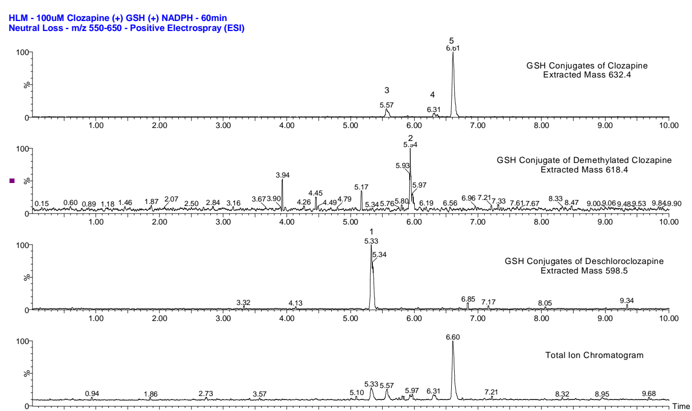
Results

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

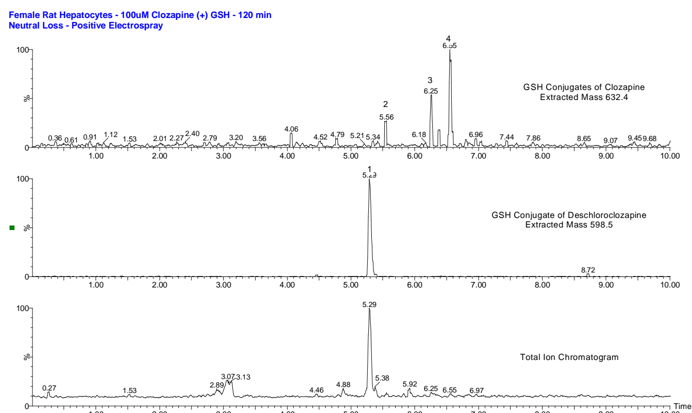
Matrix	Species	No. of GSH-conjugates of parent clozapine detected	No. of GSH-conjugates of clozapine metabolites detected
Microsomes	Human	3	2
Hepatocytes	Human	3	1
	Rat	3	1
	Dog	2	1
	Mini-pig	3	1
	Monkey	3	1

Representative chromatograms (CNL m/z - 129)

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 ^{13}C-GSH to enhance specificity during the MS/MS analysis. It was interesting that ^{13}C-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 ^{13}C-GSH not being transported across the hepatocyte cell membrane.