Reactive Metabolite Formation - Glutathione Screening Assay with the Addition of Stable Labelled Glutathione (Glutathione-$^{13}C_2$,^{15}N)

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Introduction

Adverse drug reactions (ADRs) are one of the major causes of death in the United States and Europe[1]. Idiosyncratic Drug Reactions (IDRs; also known as Type B ADRs) account for some 20% of all ADRs, and thus represent a major human health concern[2]. A direct link between toxicity and formation of reactive metabolites (RMs) has not been established but there is evidence to indicate that IDRs are due to the formation of RMs[3].

The likelihood of a compound to form RMs metabolically can be assessed through the use of *in vitro* incubations with the addition of trapping agents such as glutathione (GSH). GSH will react with a range of electrophiles including quinone imines, nitrenium ions, arene oxides, quinones, imine methides and Michael acceptors[4].

GSH can be detected using High Resolution Mass Spectrometry (HRMS) where post acquisition data mining for neutral losses confirms the presence of a reactive species. Neutral loss formation is dependent on analytical conditions and/or the fragmentation of the metabolites. Poor or absent neutral loss formation, particularly where potential reactive metabolites co-elute with endogenous material, often results in subjective interpretation of the data. Because of this, mining data for neutral losses can be time consuming and may result in false positives and false negatives.

The combined use of stable labelled glutathione (GSH-$^{13}C_2$,^{15}N) and unlabelled GSH provides an easily identifiable diagnostic splitting pattern with a mass difference of 3 Da[5], which may act as a more robust diagnostic tool. Here we describe the methods used and the results generated for the validation of the reactive metabolite glutathione screening assay using a combination of unlabelled GSH and GSH-$^{13}C_2$,^{15}N in human microsomes. Compounds will be assayed with combined stable label and unlabelled GSH, and with GSH only, and methods of data processing will be compared across both conditions to assess the reliability and efficiency of stable label as a primary diagnostic tool in the identification of RMs using the GSH trapping method, when compared to diagnostic neutral loss identification alone.

Methods

Incubation Conditions

Assay Condition	Condition selected for assays
Substrate concentration	50 µM
Protein concentration	1 mg/mL
Buffer type	0.1 M Phosphate buffer with 3 mM $MgCl_2$ pH 7.4
Cofactor	2 mM NADPH
Trapping Agent	Unlabelled GSH only: 1 mM GSH. With Stable Label GSH: 0.5 mM GSH and 0.5 mM GSH-$^{13}C_2$-^{15}N
Final solvent concentration	0.5% DMSO
Time points	60 minutes only

Table 1: Incubation Conditions

Microsomes, GSH and test compound were pre-incubated at 37 °C prior to the addition of cofactor (NADPH) to initiate the reaction. A minus cofactor control incubation was included for each compound tested where buffer was added instead of cofactor.

Each compound was incubated for 60 minutes for both active sample and minus cofactor control. The reactions were stopped by transferring incubate into an acetonitrile quench solution at 60 minutes in a 1:3 ratio. The termination plates were centrifuged at 3,000 rpm for 10 minutes at 4 °C to precipitate the protein.

Following protein precipitation samples are diluted 1:1 with water and then analysed by HRMS.

HPLC and Mass Spectrometric Conditions

Instrumentation	Waters Xevo Qtof G2-S, Acquity Binary Solvent Manager, Acquity Column Manager, 2,777 Autosampler				
Electrospray Voltage	0.3 kV				
Polarity	Positive Ion				
Cone Voltage	40 V				
Lockmass	Leucine Enkephalin (0.4 µg/mL @ 10 µL/min)				
MS	*m/z* 150 - 1200				
MS^E Collision energy ramp	10 to 30 V				
Column	Cortecs T3 (2.7 µM) 2.1 x 100 mm (Waters Ltd, Herts, UK)				
Mobile Phase A	10 mM Ammonium formate + 0.1% Formic Acid (aq)				
Mobile Phase B	Acetonitrile + 0.1% Formic Acid				
Temperature	60 °C				
Injection Volume	10 µL				
Gradient Profile	**Time (min)**	**Flow Rate (µL/min)**	**% Mobile Phase A**	**% Mobile Phase B**	**Gradient Profile**
	0.00	800	99.9	0.01	Initial
	0.10	800	99.9	0.01	6
	4.00	800	5	95	8
	4.60	800	99.9	95	11
	5.00	800	99.9	0.01	6

Table 2: HPLC and Mass Spectrometric Conditions

Data Analysis

Potential RMs were identified by processing samples through the instrument manufacturers' software, Metabolynx XS. Potential conjugations were identified by searching against a list of expected phase I biotransformations including the addition of glutathione or an addition of CysGly (when pyroglutamic acid is lost from glutathione during incubation6). For the potential conjugation to be considered genuine the following criteria had to be met:

- Potential conjugations must have had a ratio of at least three to one when compared to a corresponding peak in the control samples
- The mass error must not have exceed 5 ppm
- For conditions using combined GSH and GSH-$^{13}C_2$,^{15}N, the characteristic splitting pattern must be observed. A reactive metabolite may be reported if the isotope splitting pattern is observed but no diagnostic neutral losses are observed.
- Data mining will also determine if diagnostic neutral losses (Table 3) have been observed

GSH Conjugation Diagnostic Neutral Losses	CysGly Conjugation Diagnostic Neutral Losses
Glycine, $C_2H_5NO_2$, 75.032 Da	Glycine, $C_2H_5NO_2$, 75.032 Da
Pyroglutamic Acid, $C_5H_7NO_3$, 129.0426 Da	Reduced CysGly, $C_5H_8N_2O_3S$, 176.0256 Da
Reduced Glutathione, $C_{10}H_{15}N_3O_6S$, 305.0682 Da	CysGly, $C_5H_{10}N_2O_3S$, 178.0412 Da
Glutathione, $C_{10}H_{17}N_3O_6S$, 307.0838 Da	–

Table 3: Diagnostic neutral losses from GSH and CysGly

Results

Test Compound	Reactive Metabolite Formed	Retention time (Min)	Diagnostic Losses Observed	Stable Label Observed
Ticlopidine	GSH + Reduction	2.69	305, 307	Yes
	GSH + Hydration	2.47	75, 129, 307	Yes
	GSH + Hydration	2.53	307	Yes
Amodiaquine	CysGly + Parent – $C_5H_{11}N$	2.64	None	Yes
	CysGly + Parent	2.45	None	Yes
	GSH + Parent – $C_5H_{11}N$	2.76	129	Yes
	GSH + Deethylation	2.50	129	Yes
	GSH + Parent	2.57	129	Yes
Clozapine	CysGly + Oxidation	3.00	None	Yes
	GSH + Parent	2.93	129	Yes
	GSH + Parent	3.03	75, 129	Yes
	GSH + Parent	3.20	129, 305, 307	Yes
	GSH + Oxidation	3.07	75, 129	Yes
Imipramine	CysGly + Oxidation + 2x Desaturation	3.33	75	Yes
	CysGly + Oxidation	2.92	None	Yes
	GSH + Oxidation	3.02	129	Yes
	GSH + Oxidation + Hydration	2.86	129	Yes
	GSH + Oxidation + Hydration	2.94	129, 307	Yes
Nefazodone	CysGly + Oxidative Dechlorination + 2x Desaturation	3.35	75	Yes
	CysGly + Oxidative Dechlorination	3.07	None	Yes
	CysGly + 2x Oxidation	2.99	75	Yes
	GSH + Oxidation	3.22	75, 129	Yes
	GSH + 2x Oxidation	3.06	75, 129	Yes
Paroxetine	GSH + Demethylation – Glu + Dehydration	3.26	75	Yes
	2x GSH + Demethylation – Glu + Dehydration	3.04	75, 129, 305	Yes
Pioglitazone	CysGly + Reduction + Decarbonylation	3.14	178	Yes
	CysGly + Reduction + Decabonylation + Oxidation - NH	2.91	176	Yes
	CysGly + Reduction + Decabonylation + Oxidation	2.67	176	Yes
	CysGly + Reduction	3.40	176, 178	Yes
	GSH + Reduction + Decarbonylation	2.89	75, 129, 305, 307	Yes
Rosiglitazone	CysGly + Reduction + Decarbonylation	2.58	176, 178	Yes
	CysGly + Oxidation + 2x Desaturation	3.36	75	Yes
	CysGly + 2x Oxidation + Desaturation	3.29	None	Yes
	GSH + Reduction + Decarbonylation + Demethylation	2.61	75, 129, 305	Yes
	GSH + Reduction + Decarbonylation	2.67	75, 129, 305, 307	Yes

Table 4: Top 5 Reactive Metabolites observed for each drug compound and their associated diagnostic losses

In literature, Paroxetine is known to form a reactive metabolite which cyclises and loses pyroglutamic acid (Glu) and water[7], amendments to the processing software parameters allowed detection of RMs, which go through this further cyclisation. Metabolites which form these cyclised RMs are still able to demonstrate the diagnostic loss of glycine, $C_2H_5NO_2$, m/z 75.0320. The stable label method was able to identify 35 GSH or CysGly conjugations when reporting only up to the top five conjugations for each compound. Of these, neutral loss identification was observable for 29 of the conjugations. Figure 1 shows the stable label splitting pattern for Pioglitazone GSH + Reduction + Decarbonylation, including a neutral loss of 129 where the labelled portion of GSH has been retained, and neutral loss of 305, where the labelled portion has been lost.

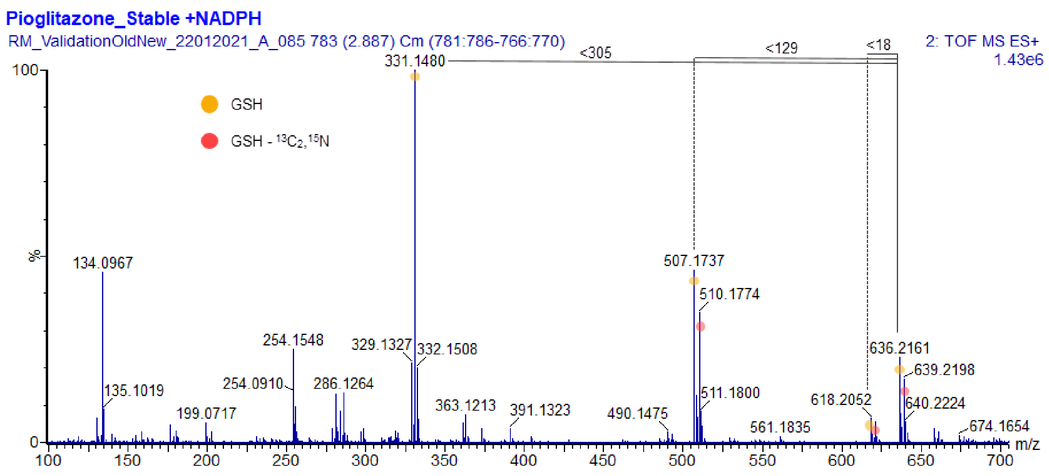

Figure 1: Mass Spectra of GSH + Reduction + Decarbonylation in Pioglitazone showing the stable labelled and unlabeled GSH splitting pattern

All reactive metabolites reported by the presence of diagnostic neutral loss alone were also observed with the diagnostic splitting pattern in the stable label GSH assay conditions. Additional metabolites displaying the diagnostic splitting pattern were found and reported from the combined stable label GSH assay where no diagnostic neutral loss were visible. Data processed by observation of the diagnostic splitting pattern produced results more efficiently than data processed by observation of a diagnostic neutral loss as false positives were able to be eliminated without the need for excessive data mining. Further work will aim to investigate the mechanism of CysGly conjugation formation and confirm this is formed during incubation as a result of metabolic activity.

Summary/Conclusions

Use of stable labelled GSH and unlabelled GSH in combination produced a characteristic splitting pattern that was able to clearly identify genuine conjugations for all eight validation compounds, and allowed more GSH or CysGly conjugations to be reported due to the presence of a diagnostic splitting pattern. The ease of identification allowed processing to quickly eliminate false positives greatly reducing the burden on data mining and therefore reduced the time taken to process.

References

1. Lazarou et al (1998) JAMA 279:1200-5
2. Kalgutkar et al (2002) Curr Drug Metab 3:379:424
3. Castro-Perez et al (2005) Rapid Commun. Mass Spectrom. 19:798-804
4. Shuguang et al (2009) Chemico-Biological Interactions 179:25-37
5. Yan & Caldwell (2004) Anal. Chem. 76:6835-6847
6. Wang et al (2019) J Mass Spectrom. 54:158-166
7. Lee et al (2014) Handbook of Metabolic Pathways, Compound Articles - Paroxetine