Reactive Metabolite Trapping and Metabolite Identification using Multiple Reagents and Specific Survey Scans on a Rapid Scanning Linear Ion Trap

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ABSTRACT

Troglitazone (TGZ)—was incubated with three different reactive metabolite trapping reagents—glutathione (GSH), glutathione ethyl ester (GSH-EE), and N-acetyl cysteine (NAC). Five different scan functions were used to look for reactive metabolites in the incubated samples. Data was acquired using information-dependent acquisition (IDA) methods on a rapid scanning hybrid linear ion trap instrument, taking advantage of the ability to rapidly switch from specific triple quadrupole (TQ) mode MS/MS scans to sensitive linear ion trap (LIT) mode MS and MS/MS scans for confirmation.

INTRODUCTION

The formation of reactive metabolites through hepatic metabolism is considered a major liability for drug candidates. Accordingly, emphasis has been placed on sensitive, reliable, and high-throughput detection and identification of these metabolites early in the ADME process.

The most common trapping reagent for reactive metabolites is glutathione. However, other reagents such as GSH ethyl ester and N-acetylcysteine[1-3], among others, can provide additional coverage and different modes of trapping. Selective triple quadrupole scans such as neutral loss, precursor, and MRM can be used as survey scans to detect the trapped species. In this study, we evaluate the use of multiple survey scans on a fast scanning hybrid quadrupole linear ion trap system to detect reactive metabolites trapped by three different reagents.

The precursor and neutral loss scans included semi-targeted (based on the fragmentation of the parent drug) and more highly-targeted (based on the fragmentation pattern of the trapping reagent). In order to cover all of the possibilities, five different survey methods were created. The most specific was pMRM (predictive MRM), based on the fragmentation pattern of the parent drug and expected metabolites. In addition to the predictive MRMs automatically generated by LightSight® software, extra MRMs were added to account for the expected neutral loss (NL) of 129 from GSH and GSH-EE and loss of 42 from NAC. Many of the metabolites showed fragments from both the trapping reagent-specific NL and fragments expected from the fragmentation of the parent compound. Slightly less specific were precursor and neutral loss survey scans specific to each trapping reagent. For GSH, the specific precursor was m/z 272 in the negative ion mode and the neutral loss was 129 in positive ion mode. For GSH-EE, the negative pre-cursor was m/z 300 (the ethyl addition to the molecule is conserved in the fragment) while the neutral loss remained 129. For NAC, the negative precursor was m/z 128 and the positive neutral loss was 42, both of which can have interferences. The final two methods were precursor and neutral loss scans based on fragmentation of the parent drug, and therefore, not specific for the trapping reagents.

MATERIALS AND METHODS

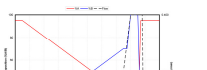
Incubations—Human liver microsomes (1 mg/mL) were incubated on ice with alamethicin (50 µg/mg of protein) for 15 minutes prior to adding to sample tubes containing 100 mM potassium phosphate buffer, pH 7.4, 5 mM saccharic acid 1,4-lactone, 1 mM magnesium chloride, and 1 mM trapping reagent (glutathione, N acetylcysteine, or glutathione ethyl ester) and the analytes (concentrations of 10 and 1 µM in the final reaction volume). Samples were pre-incubated for 10 minutes at 37 °C prior to initiation of the reaction by the addition of 5 mM UDPGA and 1 mM NADPH. Aliquots (250 µL) of each sample were removed at 0, 30, 60, and 120 minutes and quenched with 2 volumes of ice cold acetonitrile. The samples were centrifuged at 10,000×g for 30 minutes and the resulting supernatant was transferred to clean deep-well tubes and snap frozen. The samples were stored at -80 °C prior to analysis.

Mass Spectrometry—Data was acquired using the AB Sciex QTRAP® 5500 LC/MS/MS system and Analyst® 1.5 software (Applied Biosystems/MDS Analytical Technologies, Foster City, CA). The QTRAP 5500 system was operated in information-dependent acquisition (IDA) mode with TQ mode survey scans and LIT mode enhanced resolution and enhanced product ion scans for confirmation. Data was processed with LightSight and Analyst software packages.

Liquid Chromatography—The HPLC system consisted of an CBM-20A system controller, a SIL-20AD HT autosampler, two LC-20AD pumps, and a CTO-20A column oven, all from Shimadzu (Columbia, MD).

Chromatographic Conditions

Column:	Imtakt Cadenza CD-C18 2.1 × 50 mm, 3 µm
Mobile Phase A:	95% Water, 5% acetonitrile, 0.1% formic acid 2 mM ammonium acetate
Mobile Phase B:	95% Acetonitrile, 5% water, 0.1% formic acid, 2 mM ammonium acetate
Flow rate:	0.3 to 0.4 mL/min
Injection Volumes:	5 & 10 µL
Gradient:	See graph



RESULTS

Figure 1. Structure of Troglitazone (TGZ)

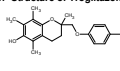


Table 1. TGZ Metabolites found in samples incubated with GSH by pMRM-triggered IDA experiments

#	Biotransformation	Mass Shift	Expected m/z	Q1 / Q3	R.T. (min)	1-Hour Peak Area	2-Hour Peak Area
1	(-CO + H_2)	-26.0	416.2	416.2 / 165.0	3.65	2.40E+04	**6.21E+04**
2	(-CO + H_2)	-26.0	416.2	416.2 / 165.0	3.94	3.02E+03	**1.70E+04**
3	(-CO + H_2)	-26.0	416.2	416.2 / 165.0	4.67	3.81E+04	3.44E+04
4	(-CO + H_2)	-26.0	416.2	416.2 / 165.0	6.53	1.26E+04	**8.23E+03**
5	(-C + H_2 + O)	-10.0	432.2	432.2 / 165.0	3.75	7.64E+03	6.90E+03
6	Adduct lost in-source?	0.0	442.2	442.2 / 165.0	4.04	5.40E+03	**2.66E+04**
7	Adduct lost in-source?	0.0	442.2	442.2 / 165.0	5.25	4.95E+04	**8.32E+04**
8	Parent	0.0	442.2	442.2 / 165.0	7.29	2.70E+04	**1.48E+04**
9	Oxidation	16.0	458.2	458.2 / 165.0	4.50	1.53E+04	**2.95E+04**
10	Oxidation	16.0	458.2	458.2 / 181.0	5.45	4.56E+03	**1.31E+04**
11	Oxidation	16.0	458.2	458.2 / 160.0	6.48	4.60E+03	9.33E+03
12	Tri-Oxidation	48.0	490.1	490.1 / 197.0	5.88	5.25E+04	**9.78E+04**
13	(-CO +H_2) + GSH-2H	279.1	721.3	721.3 / 592.2	4.80	8.63E+03	1.03E+04
14	(-CO + H_2 + O) + GSH-2H	295.1	737.3	737.3 / 608.2	4.38	7.50E+03	**1.68E+04**
15	GSH-2H + ?	297.1	739.3	739.3 / 610.2	3.57	3.20E+03	**1.25E+04**
16	GSH-2H	305.1	747.2	747.2 / 618.2	3.79	4.48E+04	8.63E+04
17	(H_2 + 2O) + GSH-2H	339.1	781.2	781.2 / 652.2	3.59	9.43E+03	**2.24E+04**

Note: Areas in the 2-hour peak area column of all tables are shown in boldface black if they increased and in boldface red if they decreased significantly from their corresponding 1-hour areas.

Table 1 lists metabolites found in the 1- and 2-hour incubations of TGZ at 1 µM with GSH using pMRM as the survey experiment with added MRMs for the NL of 129 Da. The list was limited to expected metabolites related to GSH adducts previously reported in the literature for TGZ[4]. The biotransformations listed in boldface (lines 13, 14, 16, & 17) were expected. In addition to the expected GSH-2H adducts, the list included their GSH (addition of 307 rather than 305 Da) counterparts.

In addition to the expected GSH-2H (305) adducts, MRMs for adducts resulting for their intact GSH (307) counterparts were included in the method—75 MRMs total—and found the mass shift of 297 Da (line 15). This metabolite at m/z 739 could not be the [M+2] isotope of the expected 737 Da addition as their retention times differ. It might, however, be due to in-source loss of 42 Da from the m/z 781 metabolite, as they have the same retention time, although this loss would be hard to rationalize. The product ion spectrum of this potential metabolite includes the expected m/z 165, 191, and 219 ions from the parent drug as well as the m/z 610 due to NL of 129 Da.

Table 2. TGZ Metabolites found in samples incubated with GSH by IDA experiments using other survey scans (Precursor and Neutral Loss)

#	Biotransformation	Mass Shift	Expected m/z	Survey	R.T. (min)	1-Hour Peak Area	2-Hour Peak Area
1	Adduct lost in-source?	0.0	442.2	Prec (parent)	5.23	1.00E+06	**7.65E+06**
2	Parent	0.0	442.2	Prec (parent)	7.26	6.89E+05	**1.50E+06**
3	Glutathione - 2H	305.1	747.2	Prec (parent)	4.50	1.00E+06	—
4	Glutathione - 2H	305.1	745.3	Prec (GSH)	4.77	2.64E+06	3.10E+06

Table 2 lists the parent and glutathione metabolites found in the 1- and 2-hour incubations of TGZ using the other four survey scans. Using a precursor scan based on the parent drug's fragmentation, the parent, one of the metabolites which is apparently an adduct lost in the source (see line 7, table 1) and the GSH adduct at m/z 747 were found. Using a precursor scan based on the negative m/z 272 precursor scan, only the GSH adduct was found (note the 2Da mass difference due addition of a proton in positive ion mode and subtraction in negative ion mode). The other adducts were formed at a lower level and may not have exceeded the intensity needed to be found by the precursor scan. Neither the neutral loss survey based on the parent drug's fragmentation nor the NL of 129 Da picked up any GSH metabolites.

Table 3. TGZ Metabolites found in samples incubated with GSH-EE by pMRM-triggered IDA experiments

#	Biotransformation	Mass Shift	Expected m/z	Q1 / Q3	R.T. (min)	1-Hour Peak Area	2-Hour Peak Area
1	(-CO + H_2)	-26.0	416.2	416.2 / 165.0	3.65	1.31E+04	**9.99E+03**
2	(-CO + H_2)	-26.0	416.2	416.2 / 165.0	3.90	3.48E+03	—
3	(-CO + H_2)	-26.0	416.2	416.2 / 165.0	4.65	3.19E+04	**1.91E+04**
4	(-CO + H_2)	-26.0	416.2	416.2 / 165.0	4.83	5.23E+03	—
5	(-CO + H_2)	-26.0	416.2	416.2 / 165.0	6.52	1.05E+04	**5.14E+03**
6	(-C + H_2 + O)	-10.0	432.2	432.2 / 165.0	3.75	4.70E+03	4.28E+03
7	(-C + H_2 + O)	-10.0	432.2	432.2 / 165.0	3.95	8.02E+03	6.89E+03
8	Adduct lost in-source?	0.0	442.2	442.2 / 165.0	4.24	2.01E+03	**2.95E+03**
9	Adduct lost in-source?	0.0	442.2	442.2 / 165.0	5.22	2.76E+04	—
10	Adduct lost in-source?	0.0	442.2	442.2 / 165.0	5.33	1.49E+03	**6.14E+04**
11	Parent	0.0	442.2	442.2 / 165.0	7.26	7.01E+04	**5.12E+04**
12	Oxidation	16.0	458.2	458.2 / 165.0	4.50	2.60E+04	**1.11E+04**
13	Oxidation	16.0	458.2	458.2 / 168.0	4.74	4.68E+03	**8.47E+03**
14	Oxidation	16.0	458.2	458.2 / 181.0	5.43	3.76E+03	—
15	**(-CO + H_2) + GSH EE-2H**	**307.1**	749.3	749.3 / 620.2	0.55	1.84E+04	—
16	**(-CO + H_2) + GSH EE-2H**	**307.1**	749.3	749.3 / 620.2	2.50	1.03E+05	1.11E+05
17	**(-CO + H_2) + GSH EE-2H**	**307.1**	749.3	749.3 / 620.2	5.19	1.30E+04	**6.67E+03**
18	**(-CO + H_2 + O) + GSH EE-2H**	**323.1**	765.3	765.3 / 636.2	4.76	6.43E+03	**1.08E+04**
19	**GSH EE-2H**	**333.1**	775.3	775.3 / 646.2	5.24	1.70E+05	**6.79E+04**
20	**Oxidation + GSH EE-2H**	**349.1**	791.3	791.3 / 662.2	4.11	1.37E+03	—
21	**(H_2 2O) + GSH EE-2H**	**367.1**	809.3	809.3 / 680.2	3.95	5.90E+04	**2.89E+04**

Table 3 lists the metabolites found in the 1- and 2-hour incubations of TGZ at 1 µM with GSH-EE using pMRM as the survey experiment with added MRMs for the NL of 129 Da. The biotransformations listed in boldface (lines 15-21) were expected as analogous to the GSH adducts found in the literature (table 1). More investigation needs to be done to see if there really are three separate m/z 749 metabolites. Note that GSH-EE trapped the oxidative metabolite (line 20) whereas it was not detected in the samples incubated with GSH (table 1). In the other experiments, the GSH-EE specific precursor scan found the m/z 775 metabolite corresponding to the m/z 745 metabolite on line 4 in table 2.

Table 4. TGZ Metabolites found in samples incubated with NAC by pMRM-triggered IDA experiments

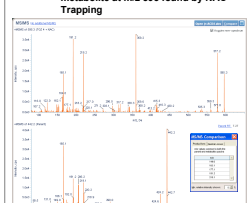
#	Biotransformation	Mass Shift	Expected m/z	Q1 / Q3	R.T. (min)	1-Hour Peak Area	2-Hour Peak Area
1	(-CO + H_2)	-26.0	416.2	416.2 / 165.0	3.62	1.00E+04	**1.84E+04**
2	(-CO + H_2)	-26.0	416.2	416.2 / 165.0	3.88	3.30E+03	**6.55E+03**
3	(-CO + H_2)	-26.0	416.2	416.2 / 165.0	4.66	2.32E+04	**1.72E+04**
4	(-CO + H_2)	-26.0	416.2	416.2 / 165.0	6.55	9.19E+03	7.40E+03
5	(-C + H_2 + O)	-10.0	432.2	432.2 / 165.0	3.71	1.31E+04	**6.38E+03**
6	(-C + H_2 + O)	-10.0	432.2	432.2 / 165.0	3.91	5.14E+03	5.18E+03
7	Adduct lost in-source?	0.0	442.2	442.2 / 165.0	4.01	1.28E+04	**3.31E+04**
8	Adduct lost in-source?	0.0	442.2	442.2 / 165.0	5.26	1.08E+05	**2.12E+05**
9	Parent	0.0	442.2	442.2 / 165.0	7.28	5.95E+04	6.32E+04
10	Oxidation	16.0	458.2	458.2 / 165.0	4.50	2.34E+04	**3.48E+04**
11	Oxidation	16.0	458.2	458.2 / 165.0	5.15	1.51E+04	1.59E+04
12	Oxidation	16.0	458.2	458.2 / 181.0	5.44	—	1.48E+04
13	Oxidation	16.0	458.2	458.2 / 160.0	6.50	—	3.85E+03
14	**(-CO + H_2) + NAC-2H**	**135.0**	577.2	577.2 / 165.0	4.28	1.95E+04	**2.46E+04**
15	**(-CO + H_2 + O) + NAC-2H**	**151.0**	593.2	593.2 / 165.0	5.20	9.31E+03	**1.36E+04**
16	NAC-2H + ?	153.0	595.3	595.3 / 165.0	4.21	2.56E+04	**3.76E+04**
17	**NAC-2H**	**161.0**	603.2	603.2 / 165.0	5.93	2.18E+04	2.11E+04
18	DI-Oxidation + NAC-2H	193.0	635.2	635.2 / 165.0	5.34	6.48E+05	**1.18E+06**
19	**(H_2 2O) + NAC-2H** (or isotope of #18?)	**195.0**	637.2	637.2 / 165.0	5.34	5.16E+04	**1.10E+05**

Table 4 lists the metabolites found in the 1- and 2-hour incubations of TGZ at 1 µM with NAC using pMRM as the survey experiment with added MRMs for the NL of 42 Da. The biotransformations listed in boldface (lines 14, 15, 17, & 19) were expected. In addition to the expected NAC-2H adducts, the list included their NAC (addition of 163 rather than 161 Da) counterparts. Once more, this found a new mass shift of 153 Da (line 16), which couldn't be the [M+2] isotope of the expected 151 Da addition as the retention times differ. This a of 153 Da is equivalent to the m/z 739 peak in the GSH incubation. However, this metabolite does not have the same RT as m/z 637 and so can not be loss of 42 Da from 637. Another unexpected mass shift is the 193 Da adduct on line 18 corresponding to di-oxidation and then NAC conjugation. The expected addition from the literature would be 195 Da (line 19), but the area for the addition of 195 and it's retention time suggest it may be an isotope of the 193 Da adduct.

Figure 2. Product ion spectrum of the potential TGZ metabolite at m/z 739 found by GSH Trapping

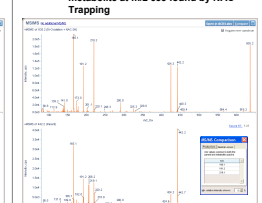
This is figure shows the product ion spectra of the metabolite at m/z 739 (top panel) and of TGZ (bottom panel). The inset shows the fragments common to both spectra. The m/z 610 ion in the metabolite's spectrum corresponds to the loss of 129 expected from a glutathione adduct.

Figure 3. Product ion spectrum of the TGZ metabolite at m/z 595 found by NAC Trapping



This is figure shows the product ion spectra of the metabolite at m/z 595 (top panel) and of TGZ (bottom panel). The inset shows the fragments common to both spectra.

Figure 4. Product ion spectrum of the TGZ metabolite at m/z 635 found by NAC Trapping



This is figure shows the product ion spectra of the metabolite at m/z 635 (top panel) and of TGZ (bottom panel). The inset shows the fragments common to both spectra.

Table 5. Summary of TGZ Metabolites found by the various trapping reagents

Metabolite #	Metabolites Found						Phase I Δ m
	GSH		GSH-EE		NAC		
	m/z	RT	m/z	RT	m/z	RT	
1*	721	4.80	749	5.19	577	4.28	-26
2*	737	4.38	765	4.76	593	5.20	-10
3*	747	4.79	775	5.24	603	5.93	0
4*	763	×	791	4.11	619	×	16
5*	781	3.59	809	3.95	637	5.34	34
6	739	3.57	767	×	595	4.21	8
7	779	×	807	×	635	5.34	32

*Metabolites 1 through 5 are well-documented in the literature

CONCLUSIONS

The table above correlates the metabolites found by each of the various trapping reagents. Metabolites M1-M5 are well-documented in the literature and all were found, although not all were found by each trapping reagent. M6 (unexplained as of yet) was found in samples incubated with GSH and in those incubated with NAC. M7 appears to be di-oxidation followed by NAC adduction and was found only in samples incubated with NAC.

The best mode for finding the trapped reactive metabolites was, as expected, the pMRM IDA. Addition of the extra MRM for the loss of 129 from GSH and GSHEE or 42 from NAC added to the ability to detect and confirm the metabolites.

Using the QTRAP 5500 System allows for rapid discovery at low incubation levels (1 µM) using multiple survey scans including MRMs with very low dwell (3-5 ms) and pause times (1-3 ms) and confirmation by enhanced product ion scans in the linear ion trap for comparing full scan product ion spectra of the metabolite to the parent drug.

FUTURE WORK

- spiking the *in vitro* incubations into plasma and bile.
- further structural analysis on the unexplained metabolites
- extending the study to other drugs
- running this type of analysis on samples obtained *in vivo*.

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