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The development of a higher throughput reactive intermediate screening assay incorporating micro-bore liquid chromatography–micro-electrospray ionization–tandem mass spectrometry and glutathione ethyl ester as an in vitro conjugating agent

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Abstract

An in vitro reactive intermediate screening assay, incorporating the use of the close analog of glutathione, glutathione ethyl ester (GSH-EE) as a conjugating agent, was developed to identify compounds that form reactive intermediates in an in vitro metabolite generating system. The biological assay consisted of substrate [s] = 10 μM, human liver microsomes, an NADPH generating system and glutathione ethyl ester. Conjugates were extracted from the biological matrix using a combination of protein precipitation and a semi-automated 96-well plate solid phase extraction (SPE) procedure. A micro-bore liquid chromatography–micro-electrospray ionization–tandem mass spectrometry (μLC–μESI–MS/MS) method detected glutathione ethyl ester conjugates using selected reaction monitoring (SRM) to simultaneously monitor for multiple MH^+ to $[MH - 129]^+$ transitions, where the 129 mass unit (Da) represents the neutral loss of the pyroglutamate moiety from GSH-EE. The multiple MH^+ to $[MH - 129]^+$ transitions (SRM mass table) were generated for potential reactive intermediates of each compound. Glutathione (GSH) and GSH-EE conjugate standards were used to evaluate MS detection sensitivity. Based on direct comparison of standard curve data, an approximate 10-fold increase in sensitivity was observed for conjugates containing GSH-EE moiety versus GSH. In vitro experiments were conducted using literature substrates acetaminophen, rosiglitazone, clozapine, diclofenac and either GSH-EE or GSH as a reactive intermediate conjugating agent. An increase in detection sensitivity was observed for each GSH-EE conjugate and in the case of acetaminophen–GSH-EE the peak area increase was approximately 80-fold. Twelve drug compounds, each having known biotransformation mechanisms, were used to further test the detection capabilities of the assay and establish a concordance to literature data. When GSH was used in the assay, conjugates were detected for 4 out of the 12 test compounds (33%). When GSH-EE was used in the assay, conjugates were detected for 10 out of the 12 test compounds (83%).

Keywords: Reactive drug intermediates; Higher throughput *in vitro* screening assay; Micro-bore liquid chromatography; Micro-electrospray ionization; Tandem mass spectrometry; Glutathione ethyl ester

1. Introduction

Drug induced toxicity is a common reason for drug candidates fail during development. Identifying toxicity potentials earlier in drug discovery avoids the higher costs and resource use associated with drug development and progresses better candidates with higher chances of survival. To date, in vivo toxicological endpoints have been identified that are indicative of various forms of organ and genetic toxicity, e.g. [1,2]. The occurrence of readily identifiable and detectable toxicological markers has prompted

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the development of in vitro toxicology assays. Current efforts in this area involve the use of these in vitro assay(s), or variation(s) thereof during early drug discovery to help prioritize compounds based on their potential to cause in vivo toxicity. However, while useful in predicting drug toxicity potentials, many of the current in vitro toxicological assays lack the sensitivity, selectivity and sample throughput necessary for early drug discovery screening.

Literature suggests inadequate detoxification of chemically reactive metabolites formed as a result of drug bioactivation is a pathogenic mechanism for tissue necrosis, carcinogenicity, teratogenicity and immune mediated toxicity [3–9]. Reactive intermediate formation is also implicated in the mechanism of several idiosyncratic drug reactions [10]. Screening compounds for reactive intermediate formation during early stages of discovery could provide a means to prioritize compounds based on their potential to cause idiosyncratic reactions in vivo and progress only those compounds with low toxicity liability. The detection of reactive intermediates poses challenges however, in that they are generally hydrophilic, unstable and formed in small quantities.

Several reports have described using the ubiquitous tri-peptide glutathione (γ-L-glutamyl-L-cysteinylgly-cine) to conjugate reactive intermediates of drug compounds [11–15]. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) was used to detect glutathione-drug conjugates based on the inherent selectivity of this analytical combination. The presence of a labile glutamic acid moiety and its facile removal (−129 Da) when subjected to collision-induced dissociation (CID), allows the selective detection of glutathione-drug conjugates using the tandem MS scanning techniques selected reaction monitoring (SRM) and/or constant neutral loss (CNL) scanning [16–19]. A typical approach used in these reports to circumvent conjugate detection limitations was the use of high substrate concentrations to produce reactive intermediate levels which, following conjugation by glutathione, could be detected using standard LC–MS/MS techniques. However, when considering the implementation of a reactive intermediate screen in early drug discovery, this approach can potentially lead to solubility problems when attempting to screen large numbers of structurally diverse compounds.

Alternative LC–MS/MS techniques such as micro-bore (μ) and nano-bore (n) LC coupled with micro-electrospray ionization (μESI) tandem MS have been shown to achieve substantial decreases in limits of detection for broad classes of analytes when compared to standard LC–MS/MS [20–22]. Reports have also described the use of alternative reactive intermediate conjugating agents whose difference in physiochemical properties to glutathione resulted in substantial increases in mass spectrometric detection capabilities [23]. Combining the use of these two approaches when developing an assay to detect reactive intermediate formation could result in substantial reduction in limits of detection for reactive intermediate conjugates thus enabling the use of low assay substrate concentration and minimizing the potential for screening problems.

The objectives of the present study were to develop a reactive intermediate trapping assay and LC–MS/MS method that (i) had high sample capacity and throughput potential and (ii) could be used to screen compounds using a substrate concentration of $\leq$10 μM. In pursuit of these objectives, the close analog of glutathione, glutathione ethyl ester, was chosen as a surrogate reactive intermediate trapping agent due to its increased hydrophobicity and MS signal intensity as compared to glutathione. A micro-bore liquid chromatography–tandem mass spectrometry method was developed to further enhance reactive intermediate conjugate detection capabilities. Detection sensitivity was assessed via standard curve analysis using glutathione and glutathione ethyl ester conjugate standards. In vitro experiments were conducted to (i) confirm improvements in glutathione ethyl ester conjugate detection versus glutathione conjugates and (ii) test the detection capabilities of the assay using a set of literature drug compounds with known toxicity profiles.

2. Experimental

2.1. Materials

All solvents were HPLC grade and reagent purity >98% unless otherwise specified. *s*-(*p*-Nitrobenzyl)-glutathione, glutathione, glutathione ethyl ester, potassium phosphate, magnesium chloride, β-nicotinamide adenine dinucleotide phosphate, DL-isocitric acid, DL-dithiothreitol and isocitric dehydrogenase were purchased from Sigma (St. Louis, MO). Substrates acetaminophen, clozapine, amodiaquin, diclofenac, indomethacin, sulfamethoxazole, carbamazepine, felbamate, pioglitazone, imipramine, valproic acid, *N*-acetylbenzoquinoneimine were obtained from Sigma and *N*-acetylbenzoquinoneimine was purchased from Dalton Chemical Labs (Toronto, Canada). Rosiglitazone was obtained from Pfizer chemical library. Formic acid was obtained from J.T. Baker (Mallinckrodt Baker Inc., Phillipsburg, NJ). Methanol, acetonitrile (ACN) and water were obtained from J.T. Baker (Mallinckrodt Baker Inc., Phillipsburg, NJ).

2.2. Synthesis

Standards were purified by reversed-phase HPLC using a water (0.1% trifluoroacteic acid)–acetonitrile linear gradient, a 20 mm × 250 mm C_{18} column (Vydac™ Peptide and Protein column), a flow rate 10 mL/min, UV detection (280 nm or 310 nm), and automated fraction collection.

2.2.1. *s-p-Nitrobenzyl-glutathione ethyl ester (s-p-NBGSH-EE) mono TFA salt*

To a solution of 19 mg (88 μmol) *p*-nitrobenzyl bromide dissolved in 2.0 mL methanol was added 0.5 mL water and

31 mg (93 μmol) glutathione ethyl ester. Ammonium bicarbonate (60 mg) was added, the mixture was stirred for 1 h, filtered, and the filtrate purified directly by preparative HPLC (0% ACN to 20%ACN (1 min) to 60% ACN (20 min)) Appropriate fractions were pooled, concentration estimated by UV absorbance at 280 nm ($\varepsilon = 9200$ L/(mol cm)) and evaporated by rotary evaporator and high vacuum. UV estimated yield = 23 mg, actual dry yield of TFA salt = 24 mg (48%). Electrospray MS monoisotopic *m/z* 471.4 (expect 471.5). ^{1}H NMR (CD3OD) δ 1.22 t (3H), 2.05 m (1H), 2.15 m (1H), 2.50 m (H), 2.68 dd (1H), 2.93 dd (1H), 3.60 t (1H), 3.82 s (2H), 2.90 dd (2H), 4.15 q (2H), 4.55 dd (1H), 7.60, d (2H), 8.09 d (2H).

2.2.2. Acetaminophen-glutathione TFA salt

In a 1.5 mL Eppendorf tube, 5 mg (33 μmol) *N*-acetylbenzoquinoneimine and 9 mg (29 μmol) glutathione were dissolved in 0.40 mL methanol.

After 1 h, the reaction was purified directly by preparative HPLC (0% ACN to 40% ACN (20 min)). 5.0 mL fractions were collected and assayed by LC/MS. A single fraction contained a species with *m/z* 457.4 (expect 457.5), which was lyophilized. The ^{1}H NMR spectrum of this product showed the expected resonances but also a major contaminant related to the starting material.

2.2.3. Acetaminophen-glutathione ethyl ester TFA salt

In a 1.5 mL Eppendorf tube, 2 mg (13.4 μmol) *N*-acetylbenzoquinoneimine in 0.5 mL of chloroform and a solution of 25 mg (75 μmol) glutathione ethyl ester in 1 mL of 50 mM ammonium bicarbonate buffer were shaken together at 37 °C. After 0.5 h, the reaction aqueous phase was separated, treated with 10 mg DL-dithiothreitol at 37 °C for 10 min, and purified directly by preparative HPLC (0% ACN to 50% ACN (25 min)). 5.0 mL fractions were collected and assayed by LC/MS. A fraction containing *m/z* 485.2 (expect 485.5) was evaporated to afford 11 mg desired product. The ^{1}H NMR spectrum of this product showed the expected resonances.

2.3. In vitro reactive intermediate microsomal assay

Test compound (10 μM), glutathione or glutathione ethyl ester (1 mM) was added to human liver microsomes (1 mg/mL protein) in phosphate buffer (100 mM, pH 7.4). Following pre-incubation at 37 °C for 3 min, the reaction was initiated by the addition of an NADPH-generating system (0.54 mM $NADP^+$, 11.5 mM $MgCl_2$, 6.2 mM DL-isocitric acid and 0.5 units/mL isocitric dehydrogenase). The final incubation volume was 250 μL. Samples without substrate were used as negative controls. After 30 min incubation at 37 °C, 375 μL of acetonitrile was added into the incubation mixture and it was centrifuged at 3500 rpm for 10 min. Supernatant (200 μL) was transferred to a 96-well plate and placed in a nitrogen evaporator (Evaporex EVX-192, Apricot designs, Monrovia, CA). N_2 gas evaporation was conducted for approximately 1 h to reduce the percent organic prior to solid phase extraction (SPE).

2.4. 96-Well solid phase extraction

Glutathione and glutathione ethyl ester-drug conjugates were extracted from each sample using a Waters Oasis™ HLB 96-well μelution™ solid phase extraction plate. A 384-channel personal-150 pipettor fitted with a 96 channel head (Apricot Designs Inc., Monrovia, CA) was used during SPE extraction to facilitate solvent transfer. The SPE plates were conditioned by passing 200 μL methanol followed by 200 μL water through the SPE plate. To initiate solvent flow through the sorbent, vacuum was applied to the receiving side of the SPE plate using a 96-well extraction manifold (Tomtec Inc., Hampden, CT). Sample (150 μL) was added to the 96-well plate and washed with 200 μL water. Analyte was desorbed using 50 μL acetonitrile:isopropanol (40:60, v/v). The desorption solvent was collected in a 96-well plate (Analytical sales and service, NJ). Solvent was evaporated using a 96-channel Evaporex EVX-192 evaporator (Apricot Designs, Monrovia, CA) that utilized nitrogen as a drying gas. Each sample was reconstituted with 40 μL mobile phase, acetonitrile:ammonium formate (5 mM):formic acid (10:90:0.05, v/v/v) (pH* 3.5).

2.5. Instrumentation

2.5.1. Liquid chromatography

An LC packings capillary liquid chromatography system (Dionex Corp., Sunnyvale CA) was used during this study. The autosampler module was configured to inject sample from 96-well plates. Chromatography was performed in a Vydac™ 300 μm i.d. × 5 cm C_{18} column that contained 5 μm particles with a pore size of 300 Å (Grace Vydac, Hesperia, CA). The tubing connecting the injection port to the analytical column and analytical column to ESI source housing was made of polyetheretherketone (PEEK) and had a i.d. of 75 μm and o.d. of 365 μm. The lengths of tubing used to connect injection port to column and column to ESI source housing were 10 and 30 cm, respectively. GSH and GSH-EE conjugates were isolated from endogenous sample components using a binary mobile phase (MP) system consisting of solvent system-A; acetonitrile:ammonium formate (5 mM):formic acid (10:90:0.05, v/v/v) and solvent system-B; acetonitrile:ammonium formate (5 mM):formic acid (80:20:0.05, v/v/v), at a flow rate of 5 μL/min. Following injection of 0.6 μL sample, a starting MP composition of 90% solvent system A–10% solvent system B was increased linearly to 40% solvent system A–60% solvent system B from 0 to 5 min. From 5 to 10 min, the % solvent system B was held isocratic. At 10 min, the solvent system was returned to 90% solvent system A–10% solvent system B and the column allowed to re-equlibrate

for 5 min prior to the next injection (total cycle time/analysis = 15 min).

2.5.2. Micro-electrospray ionization-tandem mass spectrometry

A Thermo-Finnigan Quantum triple quadrupole mass spectrometer with an orthogonal electrospray ionization interface (Thermo-Finnigan Corp., San Jose, CA) was used during this study. PEEK tubing (75 μm i.d. × 365 μm o.d. × 15 cm) was used to transfer chromatographic effluent from the MS source housing to the orthogonal ESI probe assembly. A 60 μm i.d. stainless steel needle was used in place of the standard bare fused silica ESI capillary to achieve efficient and stable micro-electrospray ionization. μESI was initiated by applying voltage of 3.8 kV (positive polarity). μESI spray stability was enhanced using a sheath gas (nitrogen) setting of 5 psi. The auxiliary gas pressure and source transfer capillary temperature were maintained at 0 and 250 °C, respectively, throughout the study. The ESI probe was held at a position that placed it as close to 90° from the entrance of the sweep cone as possible. Optimal tandem MS parameters for GSH and GSH-EE conjugates were established using 15 μM *s-p*-nitrobenzyl-glutathione and glutathione ethyl ester (*s-p*-NB-GSH/GSH-EE) standard solutions. Operating the MS in product ion mode (Q1 transmit MH^+ *m/z* 443 and 471 for GSH and GSH-EE, respectively, while Q3 scanned from 100–500 Da), the efficient loss of the 129 Da neutral fragment from the GSH and GSH-EE moiety of was achieved using a Q2 offset voltage of −18 V while maintaining the Q2 cell pressure at 1.0 mtorr.

2.6. Selected reaction monitoring battery calculation

A microsoft excel macro generated a table of prospective SRM transitions for each compound in the test set (Fig. 1). The SRM table was imported into template MS methods and the final SRM scanning method used during MS/MS analysis. The calculated MH^+ masses for each compound were based on potential metabolic changes to the parent structure as a result of characteristic bioactivation pathways that lead to reactive intermediate formation and subsequent conjugation with either GSH or GSH-EE. Since the intended use of the macro would be to generate SRM tables for large numbers of structurally diverse compounds, the macro calculations covered a broad class of molecular structures so as to minimize the possibility of not detecting reactive intermediate formation due to incorrect *m/z* monitoring.

3. Results

3.1. Determination of MS detection sensitivity for glutathione versus glutathione ethyl ester conjugated metabolites

The product ion spectra of the $[M + H]^+$ ion of *s-p*-NB-GSH and *s-p*-NB–GSH-EE acquired during infusion analysis of 10 μM solutions of each compound are shown in Fig. 2. The product ion spectrum of *s-p*-NB-GSH (Fig. 2a) contains three prominent ions that are characteristic of glutathione-conjugated metabolites [17]. The ions at *m/z* 368 ($[MH - 75]^+$) and *m/z* 314 ($[MH - 129]^+$) represent the loss of glycine and pyroglutamate, respectively, while the prominent ion at *m/z* 296 ($[MH - 146]^+$) represents the loss of pyroglutamate and a water molecule. The product ion spectrum of *s-p*-NB–GSH-EE (Fig. 2b), in addition to containing ions representing loss of 129 and 146 Da from the parent ion at *m/z* 471, shows an ion at *m/z* 368 ($[MH - 103]^+$) that results from the loss of the glycylethylester portion of the moiety. Tandem MS collision-induced dissociation (CID) parameters were optimized to maximize the efficiency of parent $[M + H]^+$ to product ion $[MH - 129]^+$ transition for both *s-p*-NB-GSH and *s-p*-NB–GSH-EE.

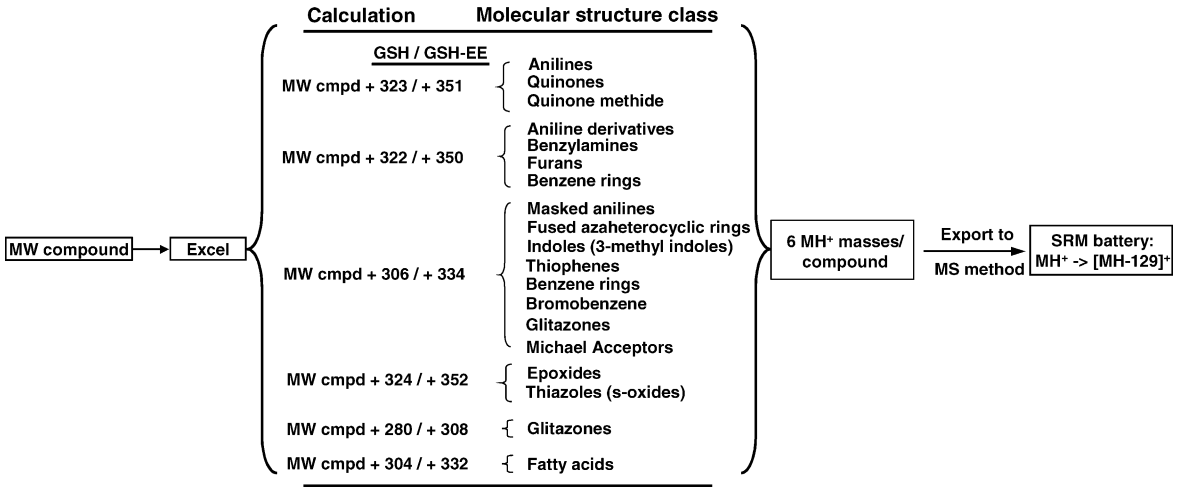


Fig. 1. Multiple selected reaction monitoring (SRM) battery calculation diagram.

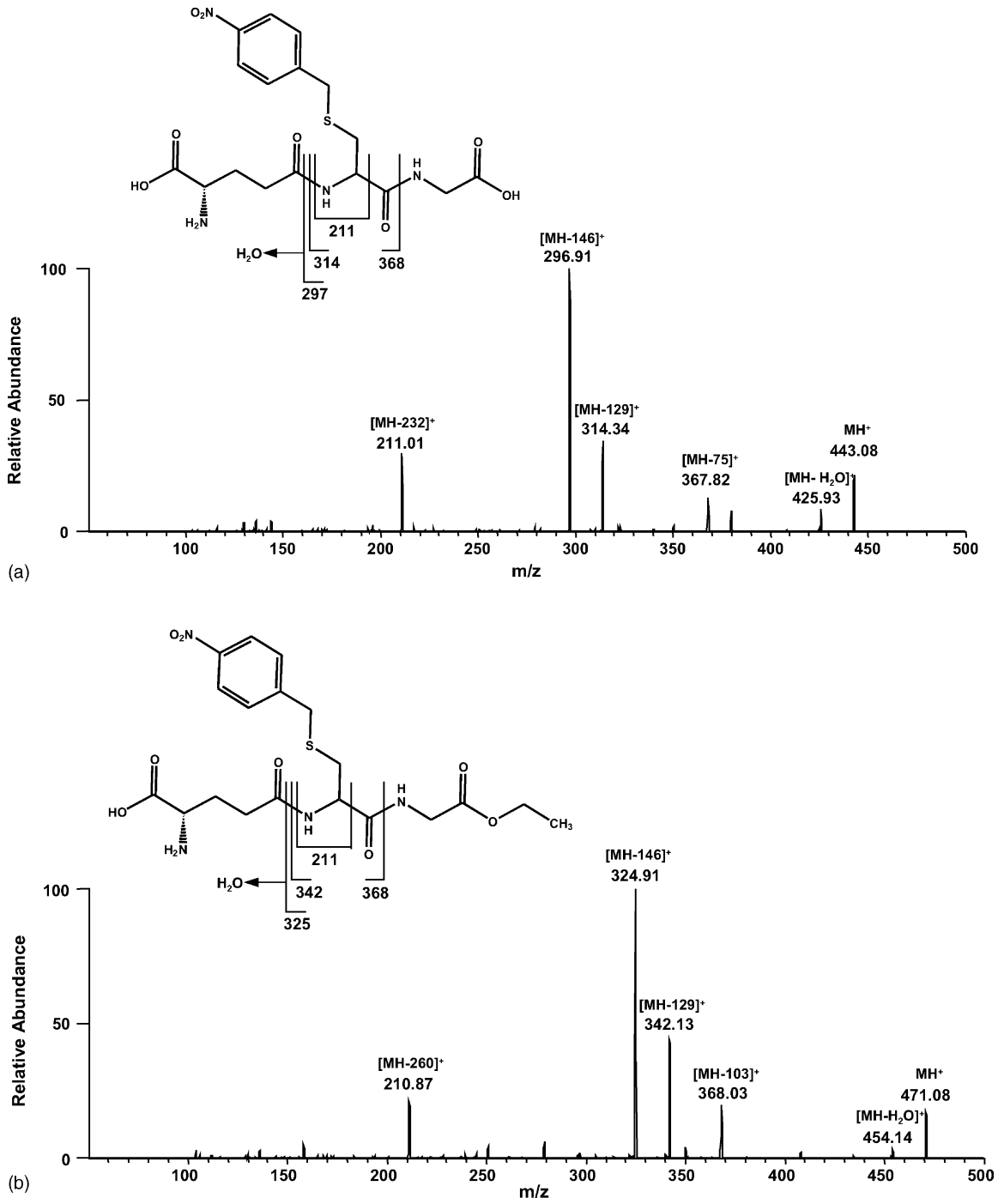


Fig. 2. Product ion spectrum obtained by CID of the MH^+ ion: (a) (*m*/*z* 443) of *s*-*p*-nitrobenzylglutathione and (b) (*m*/*z* 471) of *s*-*p*-nitrobenzylglutathione ethyl ester. Spectrum obtained by direct infusion of a 10 μM standard solution.

Standard curve solutions were prepared for each compound via serial dilution. The concentration range of both standard curves was identical and covered approximately two orders of magnitude from 5 to 300 nM. The diluent used to prepare the standard curves was the same as the solution used to reconstitute samples following sample preparation (SPE). Both standard curves were run sequentially and in duplicate. The resulting peak areas for both *s*-*p*-NB-GSH and *s*-*p*-NB–GSH-EE were plotted versus concentration and the results shown in Fig. 3. Least squares regression was used to generate the best-fit line and mathematical equation ($y = mx + b$) adjacent to each curve. Evident in the equations is an approximate 10-fold increase in the slope of the line for *s*-*p*-NB–GSH-EE versus *s*-*p*-NB-GSH. By definition, this

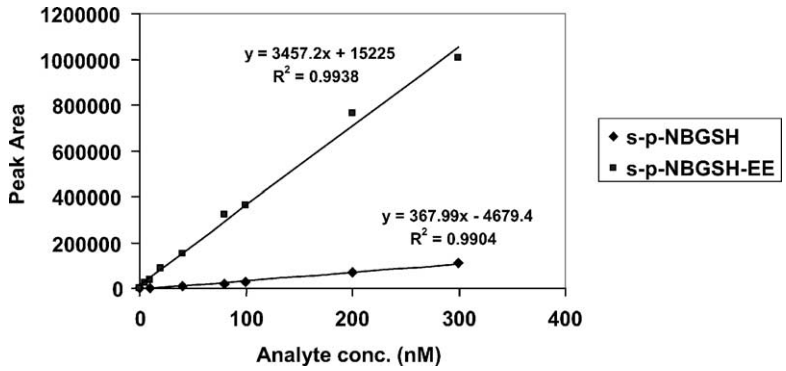


Fig. 3. *s-p*-Nitrobenzylglutathione and *s-p*-nitrobenzylglutathione ethyl ester standard curve slope comparison. Standard curves were prepared via serial dilution of identical concentration working solutions. SRM scanning ($[MH - 146]^+$, collision offset potential −18 V, collision cell pressure 1.0 mtorr) was used during sample analysis. Data points represent mean peak area ($n = 2$).

10-fold increase in slope equates to a 10-fold increase in MS sensitivity for *s-p*-NB–GSH-EE versus *s-p*-NB-GSH. The 10-fold increase in MS sensitivity was confirmed by infusing equi-molar/same solvent solutions of *s-p*-NB–GSH-EE and *s-p*-NB-GSH and comparing MH^+ ion signal intensities (data not shown). Analytical method figures of merit including limits of detection, intra- and inter-day reproducibility were as follows. The limits of detection for *s-p*-NB-GSH and *s-p*-NB–GSH-EE standards based on peak signal to noise level of greater than three (S/N > 3) were 3 and 0.34 nM, respectively. *s-p*-NB–GSH-EE standard intra-day peak retention time and area reproducibility was 1 and 5% relative standard deviation (R.S.D.), respectively. *s-p*-NB–GSH-EE standard inter-day peak retention time and area inter-day reproducibility was 3 and 10% R.S.D., respectively.

In addition to increased MS sensitivity, a substantial increase in chromatographic retention time was observed for the *s-p*-NB–GSH-EE conjugate (Fig. 4). This change in conjugate hydrophobicity was anticipated due to the reduction in acidic sites from two (GSH) to one (GSH-EE) as a result of esterification. The observed increase in GSH-EE conjugate hydrophobicity, when compared to GSH conjugates, was verified by the observa-

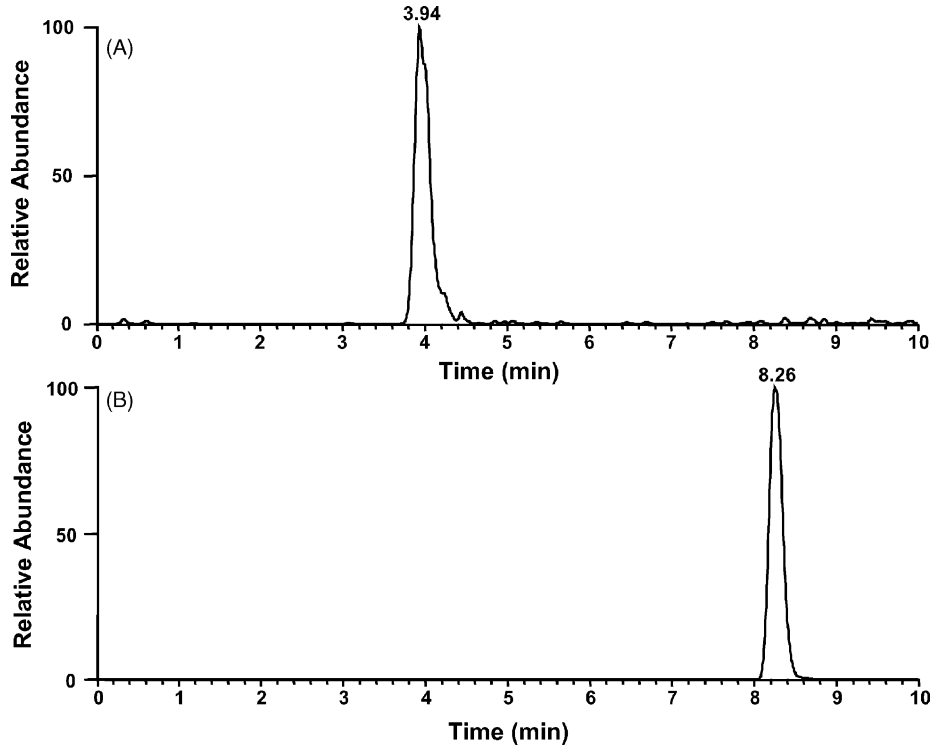
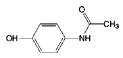
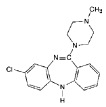
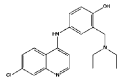
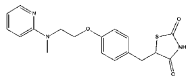
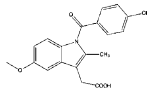
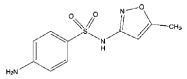
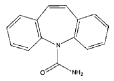
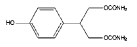
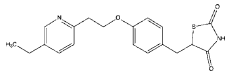
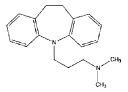
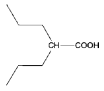


Fig. 4. Total ion current chromatograms obtained from μLC–MS/MS analysis of a 200 nM standard solution of *s-p*-nitrobenzylglutathione (A) and 40 nM standard solution of *s-p*-nitrobenzylglutathione ethyl ester (B). Trace (A) SRM, 443 → 314 at −18 V, 1.0 mtorr. Trace (B) SRM, 471 → 342 at −18 V,1.0 mtorr.

Table 1
Literature compound test set

Compound	Bioactivation structure class	Structure	Reactive intermediate
Acetaminophen[a]	Masked aniline		Quinoneimine
Clozapine[a]	Fused azaheterocycle		Nitrenium ion
Amodiaquine[a]	Masked aniline		Quinoneimine
Diclofenac[a]	Aniline derivative		Quinoneimine
Rosiglitazone[b]	Glitazone		α-keto-isocyanate
Indomethacin[a]	Indole		Iminoquinone
Sulfamethoxazole[a]	Aniline		Nitrosoamine
Carbamazepine[a]	Benzene rings		Arene oxide
Felbamate[a]	Michael acceptor		Atropaldehyde
Pioglitazone[b]	Glitazone		α-Keto-isocyanate
Imipramine[a]	Benzene rings		Arene oxide
Valproicacid[a]	Fatty acids		α-β-Unsaturated-carbonyl

[a] Positive control compounds.
[b] Negative control compounds.

tion of an increased chromatographic retention time for acetaminophen–GSH-EE versus acetaminophen-GSH standards (data not shown).

3.2. Improvement in reactive intermediate assay detection capabilities using GSH-EE as the conjugating agent

The literature compound test set used to assess the detection capabilities of the assay described herein is shown in Table 1. This literature compound set was chosen based on structural diversity, hepatotoxicity profiles and diversity in bioactivation mechanism. Of the 12 compounds, 10 have been reported to cause idiosyncratic toxicity and form reactive intermediates while two do not cause idiosyncratic reactions at their therapeutic dose but do form reactive intermediates. Since all of the literature compounds form reactive intermediates and could be trapped using conjugating species, the assay detection capability was based on the number of positive responses observed out of the entire set of 12 compounds. A response in the assay was deemed positive if there were no co-eluting species observed in control (blank) samples and analyte peak signal to noise ratio was >10.

The detection capabilities of the assay were first assessed using GSH as the trapping agent and substrate concentration of 10 μM. Of the 12 literature compounds, four were detected as being conjugated by GSH following microsomal incubation (Fig. 5). A separate study using the same compounds was conducted with the samples incubated in the presence of either GSH or GSH-EE. Scheme 1 shows the results of the study in terms of increase in MS response using GSH-EE as the trapping

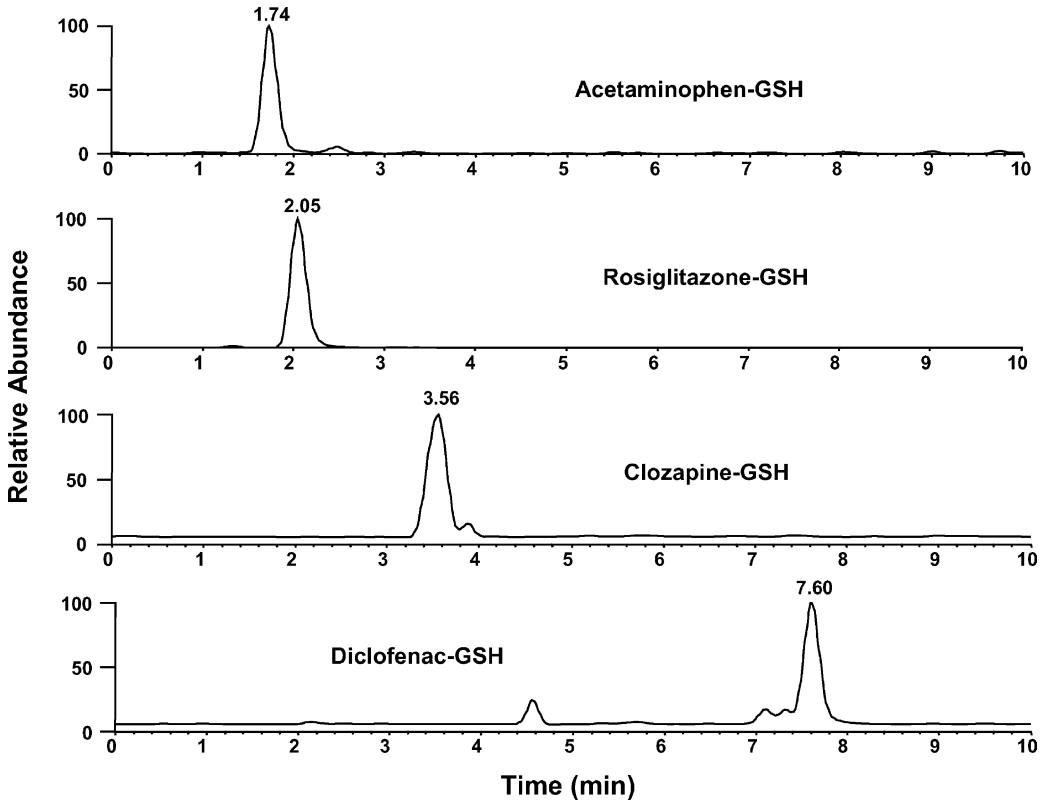
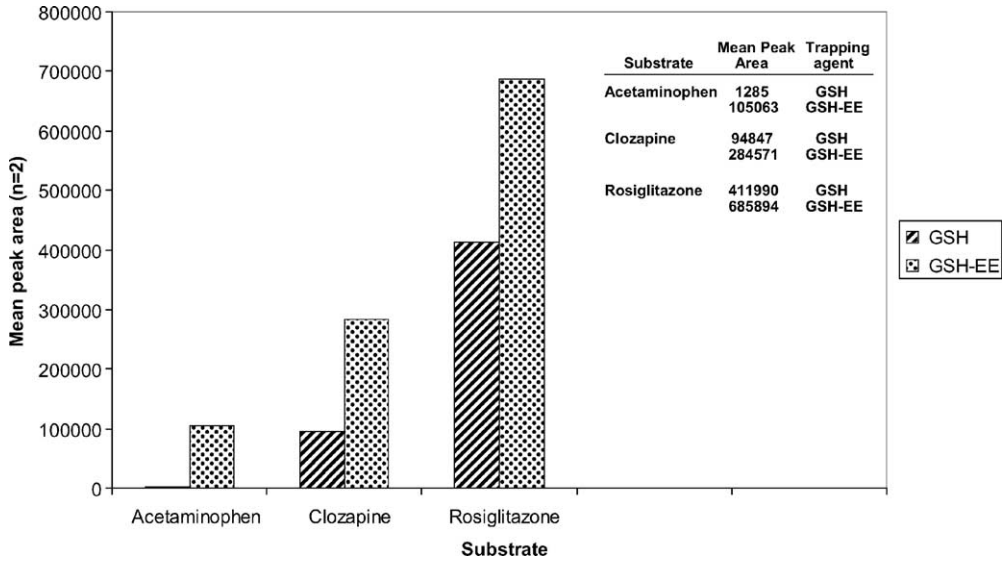


Fig. 5. Total ion current chromatograms obtained from μLC–MS/MS analysis of processed in vitro assay samples where glutathione was used in the assay to conjugate reactive intermediates. Multiple SRM scanning was used during sample analysis. SRM mass lists were generated for each test compound using an excel multiple SRM battery calculation macro.

agent versus GSH. As evident, a minimum of two-fold increase in peak area was observed using GSH-EE as the trapping agent. For compounds such as acetaminophen, the increase in conjugate peak area was greater than 80-fold.

3.3. Assay detection capabilities using GSH-EE as the conjugating agent

The detection capabilities of the assay were assessed using GSH-EE as the reactive intermediate trapping agent. Fig. 6



Scheme 1. Glutathione vs. glutathione ethyl ester conjugate MS/MS response comparison. Substrate concentration in the assay was 100 μM. Mean peak area ($n = 2$) obtained from μLC–MS/MS analysis of processed incubation samples. SRM scanning was used during analysis.

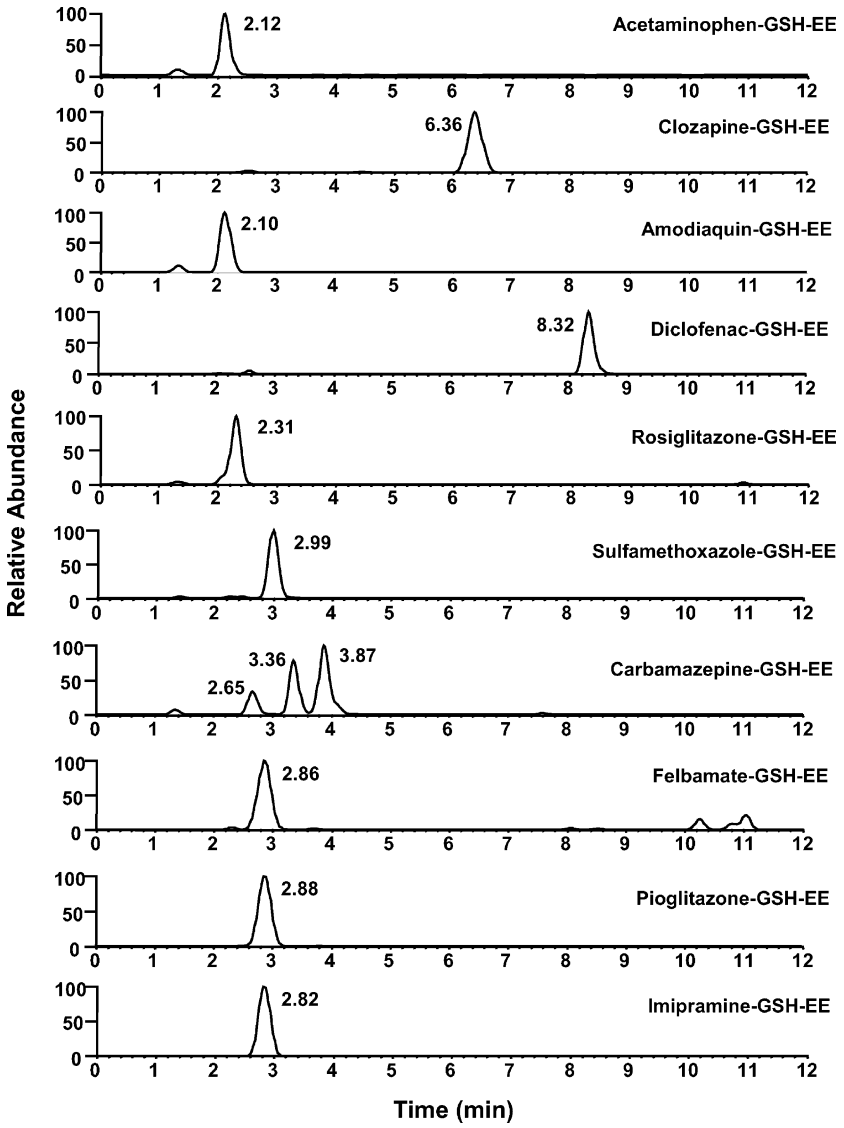


Fig. 6. Total ion current chromatograms obtained from μLC–MS/MS analysis of processed in vitro assay samples where glutathione ethyl ester was used in the assay to conjugate reactive intermediates. Multiple SRM scanning was used during sample analysis. SRM mass lists were generated for each test compound using an excel multiple SRM battery calculation macro.

shows the total ion current chromatograms of each literature compound showing a positive peak response. Of the 12 literature compounds, a positive response was observed for 10 (Table 2). Product ion scanning was used to obtain structural information on clozapine–GSH-EE due to its relatively high response in the assay (Fig. 7). Prominent MH^+ (*m/z* 660) and $[MH - 129]^+$ (*m/z* 531) ions were observed in the spectrum with the remaining ions representing characteristic fragmentation of the GSH-EE moiety.

4. Discussion

The widely accepted approach to studying reactive intermediate formation of drug-like compounds includes in vitro incubation to generate and subsequently trap electrophilic intermediates using the ubiquitous tripeptide glutathione and then isolate, detect and characterize glutathione conjugates using LC–MS/MS. This approach provided the framework upon which this in vitro assay was developed. However, considering the assay parameter constraint placed upon substrate concentration, efforts were focused on optimizing both the LC–MS/MS technique and in vitro biology portions of the assay to increase detection capability (i.e. decrease detection limits) for conjugates.

Micro-electrospray ionization/tandem mass spectrometry was employed to take advantage of the increased analyte ionization efficiency afforded by the reduced solvent flow rate and better ion transfer due to optimal probe positioning. A complementary micro-bore liquid chromatography method incorporating the use of ultra low volume tubing and valves was developed to minimize solvent change delays as a re-

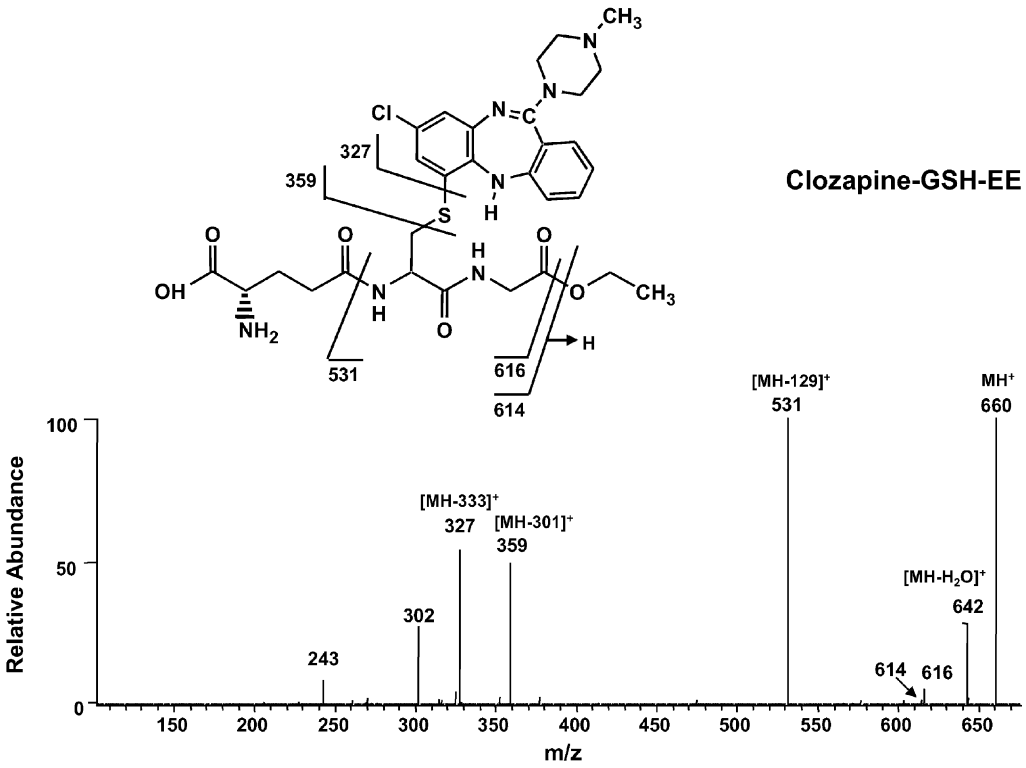


Fig. 7. Product ion spectrum obtained by CID of the MH^+ (*m*/*z* 660) ion of clozapine-glutathione ethyl ester formed in vitro. Substrate concentration of clozapine in the assay was 100 μM.

sult of using lower mobile phase flow rates. In addition, the choice of column i.d. (300 μm), flow rate (5 μL/min) and gradient profile were all made to keep chromatographic run times to a minimum. These choices were made to achieve the required throughput. A shortcoming of this approach was reduced column peak capacity, which had the potential to result in poor resolution of peaks (conjugates) during analysis. Since the purpose of the assay was to provide a clear yes or no answer as to the formation of reactive intermediate(s), chromatography was optimized to achieve adequate resolution between analyte(s) and endogenous sample components yet keep the runtime to less than 10 min. In consideration of peak capacity, conjugate peak shape was optimized during chromatography methods development. While an improvement in conjugate peak shape was observed using GSH-EE versus GSH in the assay, significant peak tailing was observed for several of the test compounds. Both the mobile phase additive and column type had significant effects on conjugate peak shape. The combination of the presence of the ammonium ion from ammonium formate and the resulting pH of the ammonium formate:formic acid mixture (pH* 3.5), significantly reduced peak tailing for conjugates. The use of a micro-bore column intended for protein and peptide separations (Vydac™) also helped to reduce peak tailing and increase column plate number (*N*). In looking at the total ion chromatograms shown in Fig. 5, the chromatographic method was adequate in its intended purpose of unambiguous detection of GSH-conjugate peaks with a signal to noise level of greater than 10.

Table 2
Reactive intermediate assay detection capabilities using either glutathione or glutathione ethyl ester as conjugating agents

Compound	Response observed using GSH as trapping agent	Response observed using GSH-EE as trapping agent
Acetaminophen	+	+
Clozapine	+	+
Amodiaquine	–	+
Diclofenac	+	+
Rosiglitazone	+	+
Indomethacin	–	–
Sulfamethoxazole	–	+
Carbamazepine	–	+
Felbamate	–	+
Pioglitazone	–	+
Imipramine	–	+
Valproic acid	–	–

(1) $N = 2$ assays using each conjugating agent. (2) $N = 3$ samples per compound; $N = 2$ substrate and co-factor, $N = 1$ co-factor only (control).

While successful in detecting conjugate formation for four out of the 12 compounds in the literature compound list using GSH as an in vitro conjugating agent and the μLC–μESI–MS/MS method, it was evident that further optimization was required to increase the detection capabilities of the assay. Changing the P450 and GSH concentrations did not significantly alter the levels of reactive intermediate formation (data not shown). The independence of the assay

from GSH and enzyme concentration was beneficial for the following reasons: (i) the use of P450 enzyme concentration equivalent to 1 mg/mL minimized protein binding, (ii) the assay was complementary to in-house microsomal lability HTS assays and (iii) resources were saved. Since changing keys components of the in vitro biology assay did not significantly alter reactive intermediate levels, attention was directed towards identifying alternative trapping agents that could potentially increase sample extraction recovery and conjugate detection sensitivity during MS. GSH is a tripeptide containing two acidic moieties making its isolation or extraction from sample matrix components difficult due to its inherent hydrophilicity. Sample extraction can be optimized using several techniques designed for molecules with acidic sites. However, addition of reagents (i.e. ion pairing agents) imparts an added level of complexity to the assay and, in the case of this assay, did not prove fruitful. The presence of the ethyl ester moiety on GSH-EE makes this molecule inherently less polar than GSH. This increase in hydrophobicity was confirmed by increased retention during reversed-phase chromatography for both *s*-*p*-NBGSH-EE (Fig. 4) and acetaminophen–GSH-EE standards (data not shown). While SPE recovery studies were not performed for the entire list of test compounds due to lack of conjugate standards, the inherent physiochemical property differences between GSH and GSH-EE would strongly indicate an increase in SPE recovery for GSH-EE versus GSH-conjugates. Furthermore, increased SPE recovery in addition to increased MS detection sensitivity for GSH-EE conjugates provides a plausible explanation for the observed 80-fold increase in peak area of acetaminophen–GSH-EE versus acetaminophen-GSH (Scheme 1). Since the only structural difference between GSH-EE and GSH was the presence of the ethyl ester moiety on the glycine portion of the molecule, the observed increase in MS detection sensitivity for GSH-EE versus GSH conjugates (Fig. 3) was believed to be due to the decrease in acidic sites from two to one making MS detection sensitivity inversely proportional to the number of acidic moieties present on the conjugating molecule (GSH = 2, GSH-EE = 1).

Of the 12 literature compounds, 10 were detected as forming reactive intermediates due to the detection of their GSH-EE conjugates. Based on these results, the use of GSH-EE as the in vitro assay-conjugating agent improved the detection capabilities of the assay almost three-fold to 83%. A plausible reason for not detecting GSH-EE conjugate(s) of indomethacin is that the reactive intermediate structure differs significantly from the parent compound. The major metabolic pathway of indomethacin involves demethylation and subsequent deacylation to form the metabolite desmethyldeschlorobenzylindomethacin (DMBI) [24]. Studies have shown that it is DMBI that undergoes further oxidation to form a reactive iminoquinone that can be trapped using GSH [11]. Because DMBI has a structure differing significantly from indomethacin, conjugation of the reactive intermediate would result in a molecular mass that would not coincide with masses calculated using the multiple SRM battery technique used in these studies. In regards to valproic acid, the initial bioactivation of the parent compound to the metabolite 2-*n*-propyl-4-pentenoic acid (4-ene-VPA) is a microsomal P450 catalyzed event [25]. Studies suggest that mitochondrial enzymes involved in the β-oxidation of fatty acids are involved in additional biotransformation steps of this metabolite into the ultimate hepatotoxic species [26]. The lack of mitochondrial enzymes in the in vitro assay described here could be the reason reactive intermediate(s) of valproic acid were not formed and GSH or GSH-EE conjugate(s) not detected in this study. An expanded literature compound test set is currently being formed and the occurrence of these “false negatives” using the MRM battery calculation technique will be assessed.

5. Conclusions

An in vitro reactive intermediate assay, incorporating micro-bore liquid chromatography–micro-electrospray ionization–tandem mass spectrometry and glutathione ethyl ester as a conjugating agent, was developed and presented in this report. The combination of optimized analytical methodology and use of the novel conjugating agent GSH-EE resulted in a significant improvement in detection capabilities for reactive intermediates. Low assay detection limits enabled the use of a substrate concentration of 10 μM. The use of low substrate concentration minimizes; (i) compound solubility issues, (ii) the chance of enzyme saturation and (iii) compound use. The assay was designed to accommodate screening large numbers of compounds in a higher sample throughput manner than previous assays. Based on its detection and throughput capabilities, the assay is suited for use in screening compounds for reactive intermediate formation earlier in the drug discovery process. Identifying reactive intermediate formation earlier in drug discovery could allow progression of only those compounds with low potential for reactive intermediate mediated toxicity liability.

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