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Detection of Reactive Metabolites Using Isotope-Labeled Glutathione Trapping and Simultaneous Neutral Loss and Precursor Ion Scanning with Ultra-High-Pressure Liquid Chromatography Triple Quadrupole Mass Spectrometry

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Abstract

Metabolic activation of drugs to electrophilic species is responsible for over 60% of black box warnings and drug withdrawals from the market place in United States. Reactive metabolite trapping using glutathione (GSH) and analysis using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) or HPLC with high resolution mass spectrometry (mass defect filtering) have enabled screening for metabolic activation to become routine during drug development. However, current MS-based approaches cannot detect all GSH conjugates present in complex mixtures, especially those present in extracts of botanical dietary supplements. To overcome these limitations, a fast triple quadrupole mass spectrometer-based approach was developed that can detect positively and negatively charged GSH conjugates in a single analysis without the need for advance knowledge of the elemental compositions of potential conjugates and while avoiding false positives. This approach utilized UHPLC instead of HPLC to shorten separation time and enhance sensitivity, incorporated stable-isotope labeled GSH to avoid false positives, and used fast polarity switching electrospray MS/MS to detect GSH conjugates that form positive and/or negative ions. The general new method was then used to test the licorice dietary supplement *Glycyrrhiza glabra* which was found to form multiple GSH conjugates upon metabolic activation. Among the GSH conjugates found in the licorice assay were conjugates with isoliquiritigenin and glabridin, which is an irreversible inhibitor of cytochrome P450 enzymes.

Keywords

Electrophilic metabolites; Tandem mass spectrometry; Glutathione; Precursor ion scanning; Neutral loss scanning

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INTRODUCTION

Although xenobiotic metabolism is usually a detoxification process rendering drugs and similar molecules more polar and more rapidly excreted, metabolic transformation may result in electrophilic products that are potentially more toxic than their precursors. There are many examples of metabolic activation of drugs to electrophiles that covalently bind to macromolecules causing cell damage, and this process is responsible for 60% of instances where drugs had to be withdrawn from the US market or had black box warnings added to their packaging due to hepatotoxicity.¹ To address this safety concern, lead compounds are typically evaluated early during drug discovery and development for the formation of reactive metabolites and then chemically modified or abandoned to avoid this potentially toxic pathway.²

Since reactive metabolites have short half-lives and are therefore challenging to isolate or even to detect, in vitro chemical trapping has been used to form stable products that may be analyzed using mass spectrometry and/or nuclear magnetic resonance.^{3–5} These experiments typically include the incubation of liver microsomes with NADPH and thiol trapping agents such as glutathione (GSH) or *N*-acetylcysteine. GSH is present in all mammalian tissues and serves as a natural scavenger for reactive metabolites by reacting with a broad range of electrophiles including epoxides, arene oxides, alkyl halides, quinones, and Michael addition acceptors.⁶

Tandem mass spectrometric fragmentation of GSH conjugates during collision-induced dissociation shows characteristic peptide product ions.^{7–9} For example, GSH conjugates generally undergo a neutral loss (NL) of pyroglutamic acid (weighing 129 Da) during positive ion electrospray tandem mass spectrometry (MS/MS), which enables GSH conjugates to be detected with considerable selectivity using constant NL scanning of 129 Da (Figure 1).^{9,10} Unfortunately, not all GSH conjugates form positive ions, and some that do ionize in positive mode do not produce a NL of 129 Da during collision-induced dissociation.¹¹ Another major drawback of this approach is false positive results due to interference from endogenous compounds. To overcome such false positives, Yan and Caldwell¹² used an equimolar mixture of GSH and isotope-labeled GSH(¹³C₂¹⁵N]glycine) (Figure 1) to trap reactive drug metabolites from liver microsomal incubations for detection using HPLC-MS/MS with constant NL scanning. This method produced unambiguous doublet isotopic peaks with a mass difference of 3 Da for GSH conjugates that formed positive ions during electrospray, but those conjugates that did not form positive ions or eliminate 129 Da remained undetected.

When deprotonated GSH conjugates are formed, negative ion MS/MS product ion scanning usually shows fragment ions of *m/z* 272 corresponding to deprotonated γ -glutamyl-dehydroalanyl-glycine (Figure 1). Therefore, precursor ion (PI) scanning of *m/z* 272 in negative ion mode is an approach to detect GSH conjugates that do not form positive ions while being less prone to false positive results.^{2,11} To reduce false negative results and to characterize the detected GSH conjugates, Wen, *et al.*,¹³ used polarity switching during HPLC-MS/MS to combine PI scanning of *m/z* 272 of GSH conjugates in negative ion mode with positive ion MS/MS product ion scanning. This approach to the detection and

characterization of GSH conjugates worked well for the subset of GSH conjugates forming both product ions of m/z 272 during negative ion electrospray as well as protonated molecules in positive ion mode.

Another MS-based approach for detecting GSH conjugates is mass defect filtering.^{14,15} Using high resolution mass spectrometry with accurate mass measurement, GSH conjugates can be detected by applying a mass defect filter, which takes into account the unique fractional mass (the mass to the right of the decimal point) of a GSH conjugate. In its original form, this approach requires that the approximate elemental composition of the GSH conjugate be known in advance. Then, a mass defect range could be calculated and applied to the high resolution mass spectra such that those ions with mass defects within the predicted range are determined to be GSH conjugates. Recognizing that mass defect filtering cannot be used to screen for GSH conjugates of unknown compounds (as in the case of botanical dietary supplements), Ruan and Zhu¹⁶ revised the approach to test for the negative ion electrospray GSH fragment ion of exact mass m/z 272.0888. Of course, mass defect filtering that is limited to negatively charged GSH conjugates will miss those that form only positive ions. Furthermore, mass defect filtering requires access to high resolution accurate mass mass spectrometry.

In the present study, selected features from several previous methods for GSH conjugate detection were combined with new approaches that included constant NL MS/MS scanning,⁸ stable isotope labeled GSH,¹¹ polarity switching,¹² PI MS/MS scanning,¹¹ and the new features of ultra-high pressure liquid chromatography (UHPLC) and fast-scanning triple quadrupole mass spectrometry. False positive results were avoided by using a 1:1 ratio of GSH and stable isotope labeled GSH to trap reactive metabolites. Fast positive ion NL MS/MS scanning of 129 Da was alternated on-line with negative ion PI MS/MS scanning of m/z 272 (unlabeled GSH) and m/z 275 (labeled GSH) for the unambiguous detection of GSH conjugates that formed either positive or negative ions (Figure 1). Compared with our previous GSH screening assay based on HPLC-MS/MS,¹³ UHPLC-MS/MS reduced analysis time from 30 min to less than 8 min.

The utility of this new approach was verified using 7 compounds known to form reactive metabolites (acetaminophen, ticlopidine, diclofenac, *p*-cresol, 4-ethylphenol, amodiaquine, and 17 α -ethinylestradiol) and 4 compounds do not form reactive metabolites as negative controls (dextromethorphan, testosterone, midazolam, and tolbutamide) (Figure 2). Finally, the method was used to study the bioactivation of a licorice extract from *Glycyrrhiza glabra*, which was found to form multiple GSH conjugates, including previously reported GSH conjugates with the chalcone isoliquiritigenin¹⁷ as well as new conjugates with the isoflavan glabridin (Figure 2). Glabridin is known to inactivate cytochrome (CYP) P450 3A4 and CYP2B6 in a time-dependent manner,¹⁸ although no reactive metabolites have yet been reported.

EXPERIMENTAL SECTION

Materials

Acetaminophen, diclofenac, *p*-cresol, ticlopidine, amodiaquine, 17 α -ethinyl estradiol, dextromethorphan, testosterone, midazolam, tolbutamide, reduced GSH, ([¹³C₂¹⁵N]-glycine) GSH (Figures 1 and 2), β -nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt (NADPH), glabridin, isoliquiritigenin, and trichloroacetic acid were purchased from Sigma-Aldrich (St. Louis, MO). A methanolic extract of botanically authenticated *Glycyrrhiza glabra* roots was prepared as described previously.¹⁹ Pooled human liver microsomes (20 mg/mL, 150 donors) were purchased from BD Biosciences (Woburn, MA). Bond Elute C₁₈ solid phase extraction cartridges (3 mL, 200 mg sorbent) were purchased from Agilent Technologies (Santa Clara, CA), and HPLC-grade solvents were purchased from Thermo Fisher (Pittsburgh, PA).

Microsomal Incubations

Human liver microsomes (0.5 mg/mL) and 1 mM GSH (equimolar unlabeled GSH and labeled GSH) were incubated separately with each test compound (10 to 50 μ M) or extract (500 μ g/mL) in 100 mM phosphate buffer (pH 7.4) at 37 °C in a final volume of 1 mL. NADPH (1 mM) was added to initiate oxidative metabolism after a 5 min pre-incubation. Control experiments were carried out without microsomes, NADPH, test compound or GSH. After 60 min, the reactions were terminated by the addition of 100 μ L of trichloroacetic acid (10%) followed by 15 min centrifugation at 12,000 g and 4 °C. Supernatants (1 mL) of each were removed and loaded onto solid phase extraction cartridges.

Solid Phase Extraction

Sample preparation was carried out using C₁₈ solid phase extraction cartridges that were pre-washed with 1 mL of methanol and then conditioned with 1 mL of water. After loading a supernatant from a microsomal incubation, the cartridge was washed with 2 mL water and then eluted with 2 mL of methanol. The methanol elute was evaporated to dryness under a stream of nitrogen and reconstituted in 100 μ L of acetonitrile/water (10:90, v/v) before analysis using UHPLC-MS/MS.

Mass Spectrometry

A Shimadzu (Kyoto, Japan) LCMS-8050 triple quadrupole mass spectrometer equipped with a Shimadzu Nexera UHPLC system was used with a Waters (Milford, MA) Acquity UPLC BEH Shield RP18, 2.1 $\times$ 50 mm, 1.7 μ m column for UHPLC-MS/MS analyses. The UHPLC mobile phase A was 5 mM ammonium acetate in water containing 0.1% formic acid, and mobile phase B was acetonitrile containing 0.1% formic acid. The gradient elution profile consisted of a 3.5 min linear gradient from 5% B to 100% B. The column was re-equilibrated with 5% B for at least 1 min between analyses. The UHPLC flow rate was 0.4 mL/min, the column temperature was 40 °C, and the injection volume was 10 μ L.

Electrospray was used for ionization during UHPLC-MS/MS while the following cycle of MS/MS scans was carried out: negative ion PI scanning for *m/z* 272 (unlabeled GSH

conjugates); negative ion PI scanning for m/z 275 (labeled GSH conjugates); and positive ion constant NL scanning for loss of 129 Da (labeled and unlabeled GSH conjugates). The scan range was m/z 400–700 at unit resolving power, the polarity switching speed was 5 ms, and each scan was recorded over 0.12 s. Additional mass spectrometer parameters included an electrospray voltage of 4.0 kV (positive ion) and –4.5 kV (negative ion), Q3 voltage of –17 V (positive ion) and 13 V (negative ion), collision energy –13 V (positive ion) and 12 V (negative ion), nebulizing gas flow 2 L/min, heating gas flow 10 L/min, drying gas flow 10 L/min, interface temperature 300 °C, desolvation line temperature 250 °C, and a heat block temperature of 400 °C.

As part of the method validation (although not required for routine GSH conjugate screening), each sample was also analyzed using high resolution accurate mass measurement with data-dependent product ion MS/MS on a Shimadzu LCMS-IT-TOF hybrid mass spectrometer equipped with a Shimadzu Prominence UFLC-XR HPLC system. Separations were obtained using a Waters XTerra C₁₈, 2.1×100 mm, 3.5 µm HPLC column. The mobile phase consisted of a 20 min linear gradient from 2 mM ammonium acetate in water containing 0.1% formic acid to acetonitrile containing 0.1% formic acid. The flow rate was 0.3 mL/min, and the column temperature was 30 °C. In the electrospray source, the nitrogen nebulizing gas flow was 1.5 L/min, the heating gas flow 10 L/min, the interface temperature 300 °C, the desolvation line 200 °C, and the heat block 300 °C. Positive ion electrospray mass spectra and product ion tandem mass spectra were recorded from m/z 100–700. Product ion mass spectra were recorded in 0.411 sec using unit resolution selection in the ion trap and a resolving power of 14,000 in the time-of-flight (TOF) sector. Ion accumulation times in the ion trap were 0.02 s for MS and 0.015 s for MS/MS. During collision-induced dissociation in the ion trap, the collision energy was set to 80%, the collision gas was 80%, and the frequency was 0.251 (45 kHz). The detector voltage was 1.65 kV.

RESULTS AND DISCUSSION

The MS/MS conditions for GSH conjugate detection were optimized using 1 µM GSH prior to the analysis of the GSH conjugates. By varying the concentration of formic acid from 0 to 0.2%, it was determined that 0.1% formic acid was optimum for the formation of abundant protonated GSH during positive ion electrospray as well as abundant deprotonated GSH during negative ion electrospray. The optimum CE values for MS/MS (–13 V for positive ion and 12 V for negative ion) that were determined using GSH were confirmed to be valid for GSH conjugates such as those of acetaminophen. Even though the triple quadrupole mass spectrometer that was used was capable of 0.01 s per scan over the selected mass range, scans of 0.10 s to 0.15 s produced tandem mass spectra with superior signal-to-noise in both NL and PI modes (data not shown). Subsequently, 0.12 s per scan was used for all GSH conjugate screening.

Acetaminophen

Cytochrome P450 enzymes in human liver catalyze the oxidation of acetaminophen to several metabolites including the electrophile *N*-acetyl-*p*-benzoquinone imine.³ Figure 3

shows NL and PI MS/MS chromatograms of acetaminophen metabolites after incubation with human liver microsomes, cofactor NADPH, unlabeled GSH, and [$^{13}\text{C}_2, ^{15}\text{N}$]-GSH. Two peaks were detected eluting at 1.75 min and 1.86 min in all 3 chromatograms using our new approach, indicating that these were GSH conjugates of acetaminophen.

The positive ion electrospray constant NL (129 Da) MS/MS chromatogram of the acetaminophen GSH conjugate eluting at 1.75 min showed two protonated molecules of equal abundance at m/z 473 and m/z 476 (Table 1), corresponding to conjugation with unlabeled and labeled GSH, respectively, and confirming that this peak was not a false positive result. The corresponding deprotonated molecules of m/z 471 and m/z 474 at equal abundance observed during negative ion PI scanning (Table 1) also indicated that this peak was a GSH conjugate. This compound is consistent with a previously reported monooxygenated acetaminophen quinone imine that forms a GSH conjugate (acetaminophen +O -2H +GSH).¹³

The most intense peak (retention time 1.86 min; Figure 3) showed protonated molecules of m/z 457 and m/z 460 at equal abundance during positive ion NL scanning and also formed the corresponding deprotonated molecules of m/z 455 and m/z 458 at approximately equal abundance during negative PI scanning of m/z 272 (Table 1). These masses are consistent with the reaction of GSH with an *N*-acetyl-*p*-benzoquinone imine metabolite of acetaminophen (acetaminophen -2H +GSH) as reported previously.^{12,20}

For method validation, positive ion electrospray HPLC-MS/MS analyses with product ion scanning of the ions of m/z 473, m/z 476, m/z 457 and m/z 460 were carried out using a high resolution hybrid mass spectrometer. For example, accurate mass measurements of the ions of m/z 457 and m/z 460 were within 5 ppm of expected formula $\text{C}_{18}\text{H}_{24}\text{N}_4\text{O}_8\text{S}$, and both conjugates shared a common fragment ion of m/z 382 (Figure 4), which represented the loss of labeled/unlabeled glycine from the GSH moiety. Another common fragment ion of m/z 182 corresponded to cleavage of the S-peptide bond with loss of the GSH residue and retention of the positive charge on the unlabeled acetaminophen metabolite. Other abundant fragment ions of m/z 328 (unlabeled) and m/z 331 (labeled) originated from the loss of a GSH-characteristic pyroglutamate (129 Da) group, and ions of m/z 311 and m/z 314 corresponded to loss of unlabeled amino-pyroglutamate (146 Da) from the protonated molecules (Figure 4). Therefore, this new screening assay correctly detected and characterized the known quinoid GSH conjugates of acetaminophen oxidative metabolites.

A minor chromatographic peak was observed at a retention time of 2.95 min only in the negative ion PI scanning chromatogram (Figure 3). The corresponding precursor ion scans indicated ions of m/z 443.0 and m/z 445.5 ($M = 2$) (Table 1) which are inconsistent with GSH conjugates differing by 3 u. Also, the relative abundances of these two signals were 40% and 100%, respectively (Table 1), which are also inconsistent with the expected equal abundances of conjugates formed by reaction of an acetaminophen metabolite with equimolar GSH and labeled GSH. Therefore, the signal detected at 2.95 min may be dismissed as a false positive without the need for any additional experimentation.

4-Ethylphenol

UHPLC-MS/MS analysis of metabolites of 4-ethylphenol after incubation with human liver microsomes, NADPH, and labeled and unlabeled GSH showed five peaks (Figure S-1), and each of these five peaks showed signals separated by 3 u corresponding to the isotope signatures for labeled and unlabeled GSH (Table 1). The peak eluting at 3.31 min corresponded to a previously reported quinone methide metabolite of 4-ethylphenol that eliminated 2H atoms before reacting with GSH (4-ethylphenol -2H +GSH).²⁰ The most abundant GSH conjugate (retention time 3.85 min) has also been reported previously²⁰ and consisted of a catechol metabolite of 4-ethylphenol that was oxidized to an *ortho*-quinone before reacting with GSH (4-ethylphenol +GSH -2H +O). The GSH conjugate eluting at 4.14 min (Table 1) was a previously unreported minor isomer of the *ortho*-quinone conjugate.

The peaks eluting at 2.18 min and 2.38 min (Figure S-1) corresponded to previously unreported isomeric GSH conjugates of 4-ethylphenol metabolites which had gained 2 oxygen atoms and lost 2 hydrogen atoms (4-ethylphenol +2O -2H +GSH). These two peaks were only observed in negative PI scan mode, which emphasizes the need for measuring negative ions and not just positively charged GSH conjugates. A peak was detected at 5.24 min during positive ion NL screening but not during negative ion PI screening, and without any additional experimentation, it could be determined that this peak was not a GSH conjugate. Although the positive ion constant neutral loss ion scanning indicated ions of both m/z 458.3 and m/z 461.2 ($M = 3$), the relative abundances of these two signals were 100% and 48%, respectively (Table 1), which is inconsistent with the equal abundances of conjugates that would be formed by reaction of an electrophilic metabolite with equimolar GSH and labeled GSH.

17 α -Ethinyl estradiol

Five GSH conjugates were detected during positive ion NL and/or negative ion PI MS/MS scanning of the liver microsomal metabolites of 17 α -ethinyl estradiol (Figure S-2; Table 1). Each of these peaks was confirmed as a GSH conjugate by the presence of ions differing by 3 u that corresponded to the isotope signatures for labeled and unlabeled GSH (Table 1). The precursor ions for the peaks eluting at 3.82, 4.00 and 4.13 min were m/z 618/621 in positive ion mode (NL) and m/z 616/619 in negative mode (PI), which corresponded to an unlabeled molecular mass of 617 (17 α -ethinyl estradiol +O -2H +GSH). This mass is consistent with the known *ortho*-quinone metabolite of 17- α -ethinyl estradiol.²¹

The precursor ions of the peaks eluting at 3.47 min and 3.68 min (Figure S-2) showed doublet ions of equal intensity at m/z 634 and m/z 637 during positive ion NL scanning and at m/z 632 and m/z 635 during negative ion PI scanning (Table 1), which indicated that these peaks corresponded to GSH conjugates. Previously unreported GSH conjugates of 17 α -ethinyl estradiol, these isomers corresponded to the addition of two oxygen atoms and loss of two hydrogen atoms before conjugation with GSH (17 α -ethinyl estradiol +2O -2H +GSH). Although a peak was observed at 3.29 min during negative ion PI, it was inconsistent with a GSH conjugate since the precursor ions of m/z 632.1 and m/z 636.0

differed by 4 u instead of 3 u as would be expected for conjugates containing unlabeled and labeled GSH, respectively (Table 1).

Diclofenac

After incubation of diclofenac with human liver microsomes, NADPH and GSH, seven peaks were detected during UHPLC-MS/MS with positive ion NL and negative ion PI scanning (Figure S-3; Table 1). The peak eluting at 4.00 min was confirmed as a GSH conjugate by the precursor ions of equal abundance at m/z 583 and m/z 586 during positive ion NL scanning and the corresponding doublet of m/z 581 and m/z 584 observed during negative ion PI scanning (Table 1). This GSH conjugate corresponds to a previously reported metabolite of molecular mass 582 formed by monooxygenation of diclofenac, loss of HCl and addition of GSH (diclofenac +O -HCl +GSH).^{13,20,22,23} Peaks eluting at 4.14 min, 4.27 min and 4.60 min during UHPLC-MS/MS produced protonated molecules of equal abundance of m/z 617/620 during positive ion NL scanning and of m/z 615/618 during negative ion PI scanning (Table 1), which confirmed that they were isomeric diclofenac GSH conjugates (diclofenac +O -2H +GSH) as had been reported previously.^{13,20,22,23}

p-Cresol

Three GSH conjugates of *p*-cresol metabolites were detected during UHPLC-MS/MS at retention times 2.93 min, 3.21 and 3.39 min, respectively (Figure S-4; Table 1). The peak eluting at 2.93 min formed a pair of protonated molecules of m/z 414/417 of equal abundance during positive ion NL MS/MS and a pair of deprotonated molecules of m/z 412/415 during negative ion PI MS/MS, which confirmed that this was a GSH conjugate. The molecular mass of this compound (413 u) corresponded to a known quinone methide of *p*-cresol (*p*-cresol -2H +GSH).^{20,24} The peaks eluting at 3.21 min and 3.39 min were isomeric GSH conjugates as indicated by the pairs of protonated and deprotonated molecules of equal relative abundance at m/z 430/433 and m/z 428/431, respectively (Table 1). With a molecular mass of 429 u, these two isomers corresponded to GSH conjugates of known catechol metabolites of *p*-cresol (*p*-cresol +O -2H +GSH).^{20,24}

Ticlopidine

During analysis of the ticlopidine incubation mixture, two peaks were detected at retention times 1.83 min and 2.02 min in the UHPLC-MS/MS chromatograms (Figure S-5; Table 1). Pairs of precursor ions ($M = 3$) of equal abundance were detected in both positive mode (m/z 587/590) and negative mode (m/z 585/588) for each peak corresponding to conjugates with light and heavy GSH, respectively. These isomeric compounds were formed by monooxygenation of ticlopidine followed by conjugation with GSH (ticlopidine +O +GSH). GSH conjugates of monooxygenated ticlopidine have been reported previously.¹³

Amodiaquine

As reported previously,²² two GSH conjugates of electrophilic metabolites of amodiaquine (retention times 3.63 and 3.84 min) were detected during negative ion electrospray UHPLC-MS/MS PI scanning, and one peak eluting at 3.84 min was detected during positive ion UHPLC-MS/MS NL scanning (Figure S-6; Table 1). The peak eluting at 3.63 min

corresponded to a GSH conjugate of amodiaquine that had undergone oxidative *N*-deethylation (amodiaquine -C₂H₆+GSH). The presence of a pair of ions of equal abundance that differed by 3 u (*m/z* 632/635) confirmed that this peak was a GSH conjugate (Table 1). The abundant peak eluting at 3.84 min corresponded to an amodiaquine quinonimine that had reacted with GSH (amodiaquine -2H +GSH). Pairs of ions of equal abundance differing by 3 u in positive mode (*m/z* 662/665) and in negative mode (*m/z* 660/663) confirmed that this compound was a GSH conjugate (Table 1).

Although the peak observed at 5.69 min formed ion of *m/z* 576.4/579.4 during positive ion NL MS/MS and negative ions of *m/z* 574.0/577.5 during PI MS/MS, the relative abundances of each pair of ions were unequal. Therefore, this compound was not a GSH conjugate. Finally, the peak detected at 6.12 min during positive ion NL MS/MS was not a GSH conjugate, since its precursor ions did not differ by 3 u or show equal abundances (Table 1).

Dextromethorphan, Midazolam, Testosterone, and Tolbutamide

Since dextromethorphan, midazolam, testosterone, and tolbutamide have not been reported to form electrophilic metabolites or GSH conjugates,¹² these compounds were used as negative controls to validate the UHPLC-MS/MS method. As expected, no GSH conjugates were detected using either positive ion NL scanning or negative ion PI scanning, indicating that the new GSH conjugate screening approach is highly accurate at preventing false positive results.

Licorice

As a general approach, GSH screening using UHPLC-MS/MS requires no advanced knowledge of the compounds that might form GSH conjugates. To demonstrate its suitability for testing complex botanical extracts, a methanolic extract of the botanical dietary supplement licorice root (*Glycyrrhiza glabra*) was tested. Licorice is used as a dietary supplements as well as a flavoring agent and has been reported to inhibit the drug metabolizing enzyme CYP3A4.¹⁸ A large number of peaks were detected during UHPLC-MS/MS analysis (Figure 5; Table 1), and all were GSH conjugates except for the peak eluting at 3.0 min, which showed ions of *m/z* 511/514 that were not of equal abundance (100% and 68%, respectively).

Based on dereplication of the measured masses using NAPRALERT, SciFinder, and Reaxys, we hypothesized that at least some of the conjugates were formed through reaction of GSH with metabolites of the isoflavan glabridin and with the chalcone isoliquiritigen and its metabolites. These peaks were observed at retention times 2.38, 2.65, 2.95, 3.05, 3.55, and 3.62 min (Figure 5, Table 1). The other GSH conjugates are still under investigation. An electrophilic α,β -unsaturated ketone, isoliquiritigenin (Figure 2) has been reported to form a GSH adduct without metabolic activation,¹⁷ and a peak of *m/z* 564/567 corresponding to unchanged isoliquiritigenin plus GSH was observed at 2.38 min in all incubations including control incubations of licorice extract without liver microsomes or without NADPH (data not shown). The peak eluting at 2.65 min was a GSH conjugate of isoliquiritigenin after monooxygenation and loss of two hydrogen atoms (isoliquiritigenin +O -2H +GSH) and was observed only in the presence of GSH, liver microsomes and NADPH. Separate incubation

of purified isoliquiritigenin confirmed the formation of the GSH conjugates eluting at both 2.38 min and 2.65 min (Figure S-7; Table 1).

The natural product glabridin from licorice can irreversibly inhibit CYP3A4, although no reactive metabolites of glabridin have yet been reported.¹⁸ UHPLC-MS/MS analysis of a glabridin incubation mixture after incubation with human liver microsomes showed peaks at retention times of 2.95 min and 3.05 min only in the positive ion NL MS/MS chromatogram (Figure S-8; Table 1). The precursor ions of each peak corresponded to a pair of ions of equal abundance at m/z 630 and m/z 633 ($M = 3$) (Table 1). Therefore, these peaks, which were also present in the licorice extract incubation (Figure 5), were confirmed as isomeric GSH conjugates. Accurate mass measurements of these two peaks were within 5 ppm of the elemental composition $C_{30}H_{35}N_3O_{10}S$, which is consistent with a quinone methide of glabridin that had reacted with GSH to form two isomers (glabridin -2H +GSH). Note that these glabridin GSH conjugates were observed only during positive ion PI MS/MS scanning, which emphasizes the need for measuring positive ions as well as negative ions.

GSH conjugates of mono-oxygenated glabridin were detected at 3.55 min and 3.62 min during both positive ion NL and negative ion PI MS/MS scanning of the glabridin incubation mixture as well as the licorice extract incubation mixture (Figure S-8; Figure 5). Pairs of precursor ions of approximately equal abundance were observed for both peaks, confirming that they were GSH conjugates (Table 1). With a molecular mass of 645 Da and an elemental composition of $C_{30}H_{35}N_3O_{11}S$ (M 4.05 ppm), the compound eluting at 3.55 min corresponded to a conjugate of GSH with either a mono-oxygenated glabridin quinone methide or a mono-oxygenated glabridin *ortho*-quinone (glabridin +O -2H +GSH). The compound eluting at 3.62 min (Figure 5) had a molecular mass of 643 Da and an elemental composition of $C_{30}H_{33}N_3O_{11}S$ (M 3.35 ppm), which corresponded to a GSH conjugate of mono-oxygenated glabridin that had eliminated 4H atoms (glabridin +O -4H +GSH). The observation of four GSH conjugates formed from at least three different glabridin metabolites supports the hypothesis that electrophilic metabolites of glabridin might be responsible for irreversible inactivation of CYP3A4.^{25,26} Confirmation of the structures of these glabridin GSH conjugates and other licorice GSH conjugates is in progress. Finally, the peak eluting at 3.00 min (Figure S-8; Figure 5) in the glabridin and licorice UHPLC-MS/MS chromatograms was not a GSH conjugate based on the absence of a pair of ions ($M = 3$) of equal abundance (Table 1).

CONCLUSIONS

To validate this new GSH screening assay, seven compounds known to form electrophilic intermediates and GSH conjugates and four compounds that do not form such metabolites were tested. All of these positive control and negative control compounds produced the expected results. In addition to the detection of known metabolites, three new GSH conjugates of 4-ethyl phenol and two new GSH conjugates of 17 α -ethinyl estradiol were detected. This indicated that the new GSH screening assay is extremely sensitive. Licorice which had been reported to inactivate CYP3A4 but had not been reported previously to form electrophilic metabolites, was tested to demonstrate how the new method may be applied to complex mixtures without advanced knowledge of elemental compositions of possible

conjugates. The licorice chalcone isoliquiritigenin and one of its metabolites were found to form GSH conjugates as did metabolites of glabridin, which confirmed that licorice contains compounds capable of forming electrophilic metabolites that react with biological nucleophiles.

Since some GSH conjugates might produce only positive ion signals while others might form only negation ions, our approach to measuring GSH conjugates using both positive ion and negative ion MS/MS in a single assay provides both higher throughput and consumes less sample than two separate analyses. This approach also detects more GSH conjugates than would UHPLC-MS/MS screening using only positive ion or only negative ion electrospray. For example, GSH conjugates of 17 α -ethinyl estradiol eluting at 3.47 min and amodiaquine eluting at 3.63 min (Table 1) were detected only when using negative ion electrospray PI MS/MS, while GSH conjugates of glabridin eluting at 2.95 min and 3.05 min (Table 1) were observed only during positive ion electrospray NL MS/MS.

In addition to the 2-fold enhancement of throughput by combining negative ion and positive ion MS/MS into a single analysis, the use of UHPLC instead of HPLC increases the throughput of the assay from ~30 min²⁷ to <8 min per injection. The incorporation of labeled and unlabeled GSH eliminates the need for subsequent measurements to determine if a peak is actually a GSH conjugate or a false positive result and thereby enhances throughput by at least another 2-fold. Therefore, by using fast polarity switching, fast MS/MS, and UHPLC instead of HPLC, this new approach increases the throughput of GSH screening by >10-fold.

Supplementary Material

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ABBREVIATIONS

UHPLC-MS/MS	ultra-high pressure liquid chromatography-tandem mass spectrometry
GSH	glutathione
CYP450	cytochrome P450
NL	neutral loss
PI	precursor ion

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Figure 1.
UHPLC-MS/MS approach to screening for GSH conjugates.

Figure 2.
Structures of compounds tested for metabolic activation to electrophilic metabolites.

Figure 3.

UHPLC-MS/MS chromatograms of GSH conjugates formed during incubation of acetaminophen with human liver microsomes, NADPH, GSH, and [$^{13}\text{C}_2, ^{15}\text{N}$]-GSH. A) Positive ion electrospray neutral loss MS/MS scanning of 129 Da; B) negative ion electrospray MS/MS scanning for precursors of m/z 272 (unlabeled GSH); and C) negative ion electrospray MS/MS precursor ion scanning for precursors of m/z 275 (labeled GSH).

*Denotes GSH conjugate.

Figure 4. High resolution product ion MS/MS spectra of protonated molecules of acetaminophen GSH conjugates eluting at a retention time of 1.86 min in Figure 3 containing A) unlabeled GSH (m/z 457); and B) isotope-labeled GSH (m/z 460).

Figure 5.

UHPLC-MS/MS chromatograms of GSH conjugates formed during incubation of a methanolic extract of licorice root (*Glycyrrhiza glabra*) with human liver microsomes, NADPH, GSH, and [$^{13}\text{C}_2, ^{15}\text{N}$]-GSH. A) Positive ion electrospray neutral loss MS/MS scanning of 129 Da; B) negative ion MS/MS scanning for precursors of m/z 272 (unlabeled GSH); and C) negative ion MS/MS scanning for precursors of m/z 275 (labeled GSH).

GSH conjugates and corresponding precursor ions detected during UHPLC-MS/MS with positive ion electrospray constant neutral loss (NL) scanning and with negative ion electrospray precursor ion (PI) scanning.

Table 1

Compound	GSH conjugate	Retention time (min)	NL 129 Da (+)		PI m/z 275/278 (-)	
			GSH	[¹³ C ₂ , ¹⁵ N]-GSH	GSH	[¹³ C ₂ , ¹⁵ N]-GSH
Acetaminophen	Y	1.75	473.3 ^a (100) ^b	476.2 (92)	471.6 (91)	474.5 (100)
	Y	1.86	457.3 (100)	460.3 (94)	455.5 (100)	458.6 (90)
	N	2.95	-	-	443.0 (40)	445.5 (100)
Amodiaquine	Y	3.63	-	-	631.7 (100)	634.7 (95)
	Y	3.84	661.5 (98)	664.5 (100)	659.7 (100)	662.7 (97)
	N	5.69	576.4 (35)	579.4 (100)	574.0 (100)	577.5 (55)
	N	6.12	441.4 (100)	445.0 (32)	-	-
p-Cresol	Y	2.93	414.2 (100)	417.2 (91)	412.6 (100)	415.7 (86)
	Y	3.21	430.2 (100)	433.2 (95)	428.6 (100)	431.4 (95)
	Y	3.39	430.4 (100)	433.5 (98)	428.6 (100)	431.6 (84)
	N	4.11	444.5 (12)	447.2 (100)	-	-
Diclofenac	Y	4.00	583.5 (89)	586.4 (100)	581.6 (87)	584.5 (100)
	Y	4.14	617.5 (97)	620.4 (100)	615.6 (100)	618.6 (92)
	Y	4.27	617.4 (96)	620.4 (100)	615.2 (100)	618.3 (97)
	Y	4.60	617.3 (84)	620.4 (100)	615.3 (92)	618.4 (100)
	N	2.75	454.3 (100)	457.1 (11)	-	-
	N	5.71	465.7 (100)	468.4 (17)	-	-
17 α -Ethinyl estradiol	N	5.89	466.0 (100)	469.5 (35)	-	-
	Y	3.47	-	-	632.8 (100)	635.8 (88)
	Y	3.68	634.5 (100)	637.5 (83)	632.8 (100)	636.0 (88)
	Y	3.82	618.4 (100)	621.3 (88)	616.8 (95)	619.8 (100)
	Y	4.00	618.4 (100)	621.4 (75)	616.8 (100)	619.6 (73)
	Y	4.13	618.2 (100)	621.3 (85)	616.8 (100)	619.8 (91)
	N	3.29	-	-	632.1 (100)	636.0 (82)

Compound	GSH conjugate	Retention time (min)	NL 129 Da (+)		PI m/z 275/278 (-)	
			GSH	$^{13}\text{C}_2, ^{15}\text{N}_1\text{[L]}$	GSH	$^{13}\text{C}_2, ^{15}\text{N}_1\text{[L-GSH]}$
4-Ethylphenol	Y	2.18	460.3 (100)	463.4 (88)	458.6 (100)	461.6 (95)
	Y	2.38	460.4 (95)	463.1 (100)	458.6 (98)	461.7 (100)
	Y	3.31	428.3 (100)	431.2 (89)	426.6 (100)	429.6 (88)
	Y	3.85	444.3 (90)	447.3 (100)	442.6 (96)	445.6 (100)
	Y	4.14	444.3 (100)	447.2 (97)	442.5 (100)	445.6 (98)
	N	5.24	458.3 (100)	461.2 (48)	-	-
Tidipidine	Y	1.83	587.2 (82)	590.3 (100)	585.8 (100)	588.7 (77)
	Y	2.02	587.3 (88)	590.2 (100)	585.7 (100)	588.5 (85)
<i>G. glabra</i> extract	Y	2.24	528.4 (100)	531.4 (96)	526.4 (98)	529.4 (100)
	Y	2.38	564.3 (100)	567.3 (94)	562.3 (91)	565.3 (100)
	Y	2.65	578.3 (100)	581.2 (88)	576.4 (100)	579.5 (95)
	Y	2.95	630.3 (100)	633.3 (91)	-	-
	N	3.00	-	-	511.2 (100)	514.2 (68)
	Y	3.05	630.3 (88)	633.3 (100)	-	-
	Y	3.35	664.3 (97)	667.4 (100)	662.3 (100)	665.3 (95)
	Y	3.55	-	-	644.5 (100)	647.3 (85)
	Y	3.62	-	-	642.3 (100)	645.5 (90)
	Y	3.77	602.5 (98)	605.4 (100)	600.3 (100)	603.3 (82)
Isoliquiritigenin	Y	2.38	564.3 (100)	567.3 (98)	562.3 (100)	565.3 (96)
		2.65	578.3 (94)	581.2 (100)	576.4 (100)	579.5 (96)
Glabridin	Y	2.95	630.3 (94)	633.3 (100)	-	-
	N	3.00	-	-	647.2 (100)	649.2 (25)
	Y	3.05	630.3 (87)	633.3 (100)	-	-
	Y	3.55	646.5 (100)	649.5 (96)	644.5 (100)	647.5 (92)
	Y	3.62	644.3 (92)	647.3 (100)	642.3 (87)	645.3 (100)

^a apparent m/z value of precursor ion

^b (relative abundance)