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Enhanced Screening of Glutathione-Trapped Reactive Metabolites by In-Source Collision-Induced Dissociation and Extraction of Product Ion Using UHPLC-High Resolution Mass Spectrometry

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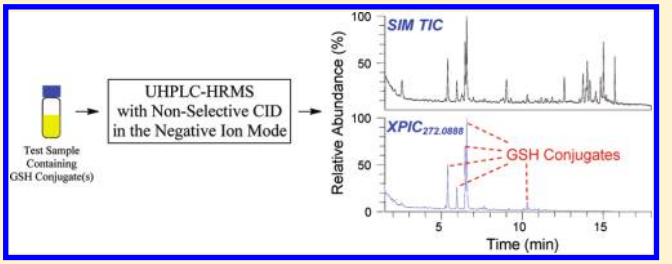
Supporting Information

ABSTRACT: A selective and sensitive approach, called extraction of product ion (XoPI) method, was developed for the detection of L-glutathione (GSH)-trapped reactive metabolites employing an Orbitrap high resolution mass spectrometer. Fragmentation of GSH conjugates in the negative ion mode leads to a product ion, deprotonated γ-glutamyl-dehydroalanyl-glycine (m/z 272.0888). As a means of utilizing this property, negative ion high resolution MS data were collected from *in vitro* incubations by monitoring ions from m/z 269.5 to 274.5 under in-source collision-induced dissociation. Extraction of product ions at m/z 272.0888 ± 5 ppm from this data resulted in a chromatogram exhibiting deprotonated γ-glutamyl-dehydroalanyl-glycine as the major peaks with no or very few interferences. Therefore, peaks in this extracted product ion chromatogram potentially came from GSH-trapped reactive metabolites. The GSH conjugate parent ions were then confirmed in the corresponding full scan MS data, and their structures were identified from their MS^2 fragmentation patterns. The effectiveness of the approach was assessed with four model compounds, amodiaquine, clozapine, diclofenac, and fipexide, all well-known to form GSH-trapped reactive metabolites, following incubation in human liver microsomes supplemented with β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH) and GSH. The results from XoPI method were compared to two other commonly employed liquid chromatography−mass spectrometry (LC-MS) methods: precursor ion scan method and mass defect filter method. Overall, the XoPI method was more selective and sensitive in detecting the GSH conjugates. Many GSH conjugates previously not reported were detected and characterized in this study.



Chemically reactive metabolites produced during metabolism of xenobiotics have been reported to react with cellular biomolecules, including DNA and proteins,[1,2] and are believed to contribute to drug induced toxicity.[3,4] To alleviate the potentially adverse effects caused by reactive drug intermediates, detection of reactive metabolites of lead compounds has routinely been employed in the drug discovery process. These electrophilic species have a short half-life and are not readily detected by analytical tools directly. Therefore, they are usually trapped with nucleophiles such as L-glutathione (GSH),[5−16] semicarbazide,[13] or cyanide[7,13,17,18] to form products that are readily amenable to liquid chromatography−mass spectrometry (LC-MS) analysis. GSH is the most commonly used trapping agent for reactive metabolites.

Many LC-MS methods have been developed to detect GSH-trapped reactive metabolites. The classic one is the constant neutral loss method which is based on the observation that the pyroglutamic acid moiety (m/z 129 Da) cleaves from GSH conjugates upon collision-induced dissociation (CID). A constant neutral loss scan (CNLS) of 129 Da using a triple quadrupole mass spectrometer in the positive ion mode is often used to screen for GSH conjugates.[7] Both the sensitivity and selectivity of this assay are poor due to a low duty cycle in full scan mode and a unit resolution of the Q1 and Q3 analyzers.[13] The poor selectivity but not the sensitivity of CNLS was improved using an equi-molar ratio of GSH and stable-isotope labeled GSH as the trapping agent in in vitro experiments. GSH conjugates could then be unambiguously identified by the presence of a characteristic doublet with a mass difference of 3 Da.[19−21] Better selectivity was also achieved via an exact mass neutral loss (129.0426 Da) acquisition using a quadrupole-time-of-flight (Q-TOF) mass spectrometer.[22] However, not all GSH conjugates produce a neutral loss of 129 Da by CID, which suggests that the CNLS method may not detect some classes of conjugates.[6]

This CNLS method limitation was later overcome by a precursor ion scan (PreIS) method. The PreIS method exploits the observation that a product ion at m/z 272, corresponding to deprotonated γ-glutamyl-dehydroalanyl-glycine, is formed upon CID from different classes of GSH conjugates in the negative ion mode. The implementation employed a triple quadrupole mass spectrometer in the negative ion mode and monitored Q1 for the precursor ion that formed m/z 272 ions in Q3.[6,9] Selectivity was improved by utilizing GSH ethyl ester as the trapping agent and

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performing a precursor ion scan at m/z 300 in the negative ion mode.[23] Selectivity was also enhanced by dual precursor ion scan at m/z 272 and 254 (another commonly observed product ion of GSH conjugates) in the negative ion mode.[12] However, the sensitivity of precursor ion detection is still limited by a low duty cycle of Q1 in the full scan mode on a triple quadrupole or a Q-TRAP mass spectrometer.

Recently, high resolution mass spectrometry (HRMS) acquired on a linear ion trap (LIT)-Orbitrap has been utilized to detect and characterize GSH-trapped reactive intermediates.[24] This hybrid mass spectrometer has good sensitivity and specificity due to large ion capacity traps and high resolution Fourier transform mass spectrometer (FTMS).[25,26] However, the mass spectrometer cannot perform a true CNLS or PreIS method described above, so a mass defect filter (MDF) based approach[24,27] has instead been developed to detect GSH conjugates. The success of the MDF approach is highly dependent on the width of the MDF window and similarity of the nominal mass between GSH conjugates and the predefined filter templates. Inappropriate MDF window and/or filter template may lead to either GSH conjugates not being detected or there being too many false positives. The analogous HRMS-MDF method has been implemented on data collected with a TOF mass spectrometer.[13]

In the present study, an efficient approach to screen for GSH-trapped reactive metabolites has been developed using LIT-Orbitrap HRMS. The method identifies GSH conjugates based on the extracted ion chromatogram of product ions at m/z 272.0888 from select ion monitored data acquired under nonselective in-source collision-induced dissociation (SCID; also called in-source fragmentation) conditions. Four model compounds, amodiaquine, clozapine, diclofenac, and fipexide (Figure S1 in Supporting Information), all known to produce reactive metabolites that are trapped with GSH, were used to evaluate the effectiveness of this method. The results were compared to those from PreIS method using a Q-TRAP mass spectrometer and MDF method using LIT-Orbitrap HRMS.

EXPERIMENTAL SECTION

General. Amodiaquine, fipexide, diclofenac, β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH), reduced GSH, and LC-MS grade acetonitrile and water were sourced from Sigma-Aldrich (St. Louis, MO). Clozapine and formic acid (99+% purity) were purchased from MP Biomedicals (Solon, OH) and Thermo Fisher Scientific (Rockfor, IL), respectively. Pooled human liver microsomes and blank human plasma were obtained from CellzDirect (Durham, NC) and Bioreclamation, LLC (Westbury, NY), respectively. Blank rat urine was collected at Amgen Inc. (Thousand Oaks, CA).

Microsomal Incubation. In a final volume of 500 μL, test compound (20 μM; amodiaquine, fipexide, clozapine or diclofenac), GSH (0 or 5 mM), and human liver microsomes (1 mg/mL protein) were incubated together in potassium phosphate buffer (100 mM, pH 7.4) supplemented with $MgCl_2$ (3 mM) at 37 °C. After a 3 min preincubation, the reaction was initiated by the addition of NADPH (1 mM). The reaction was quenched after 1 h with 500 μL of ice-cold acetonitrile and then centrifuged at 14000 rpm for 10 min at 4 °C. The supernatants were subjected to LC-MS analysis. Control samples were incubated and processed the same way but without addition of test compounds.

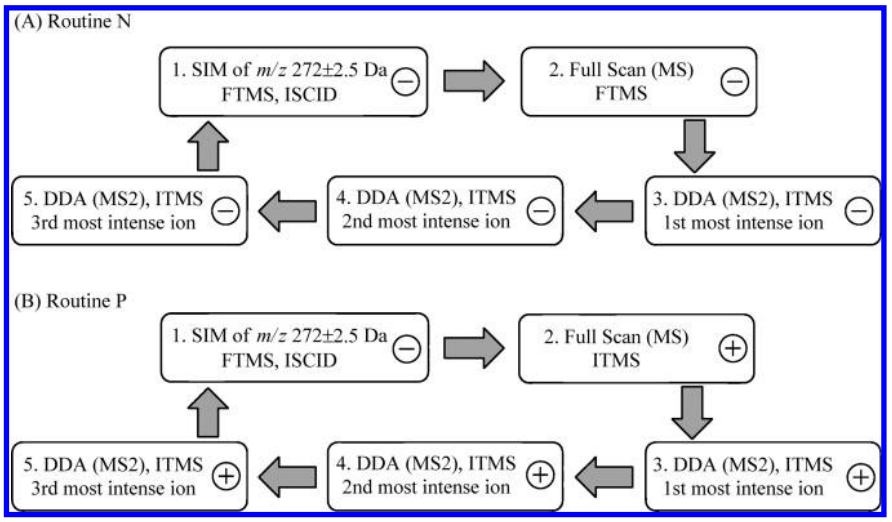
***Ex Vivo* Spiking Experiments.** Blank human plasma (1 mL) was precipitated with acetonitrile (3 mL) and centrifuged at 3000 rpm for 10 min at 4 °C. The extract was concentrated to approx 1 mL on a vacuum centrifuge (SpeedVac, Savant SC250EXP, Thermo Fisher Scientific Inc., San Jose, CA). An aliquot (150 μL) of supernatants from above quenched diclofenac or control liver microsomal incubations was mixed with 150 μL of blank human plasma extract or rat urine. The mixtures were subjected to LC-MS analysis.

LC-Linear Ion Trap/Orbitrap HRMS. All samples were subjected to reverse phase chromatography on an UHPLC system (1290 Infinity; Agilent Technologies Inc., Wilmington, DE) that included a binary pump, an autosampler, a thermostatted column compartment, and a diode-array detector. Mobile phases A and B were 0.1% formic acid in water and acetonitrile, respectively. A C18 column (Kinetex, 2.6 μm particle size, 100 × 2.10 mm, Phenomenex Inc., Torrance, CA) was used, and two gradient programs were employed. Samples containing amodiaquine were separated by gradient 1: 0−2 min, 5% B, 400 μL/min; 2−14 min, 5−25% B, 400 μL/min; 14−15 min, 25−95% B, 400−600 μL/min; 15−17.5 min, 95% B, 600 μL/min; 17.5−18 min, 95−5% B, 600−400 μL/min; 18−20 min, 5% B, 400 μL/min. Samples containing fipexide, clozapine, or diclofenac were separated by gradient 2, which was the same as gradient 1 except that 55% B was used at 14 min. LC eluant from 1.5 to 18 min was directed to an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific Inc., San Jose, CA) equipped with a heated electrospray probe (HESI-II; Thermo Fisher Scientific Inc.). The ESI parameters were as follows: capillary temperature, 350 °C; source heater temperature, 300 °C; N_2 sheath gas flow rate, 55 (arbitrary units); N_2 auxiliary gas flow rate (20 arbitrary units); spray voltages, 3.00 kV in the positive ion mode, 2.60 kV in the negative ion mode. Selected ion monitoring (SIM) at m/z 272 ± 2.5 Da was performed in the negative ion mode under SCID at 30 V using FTMS. Full scan data from m/z 200 to 1000 in both negative and positive ion modes were acquired using FTMS or ion trap mass spectrometer (ITMS). FTMS or ITMS data dependent acquisition (DDA) of MS^2 spectra were triggered for either the top three most intense ions applying dynamic exclusion or the most intense ion from a predefined parent ion list with CID at normalized collision energy of 35%. All FTMS data were acquired within 1 week after an external mass calibration. FTMS resolution was 30 000 for the SIM and full scan events and 7500 for the MS^2 scan events. All spectra were processed with the Qual browser module of Xcalibur 2.1 (Thermo Fisher Scientific Inc.).

LC-Q-TRAP MS. The same HPLC method described above was executed on a system consisting of two HPLC pumps (LC20AD; Shimadzu Scientific Instruments Inc., Columbia, MD), an autosampler (CTC PAL; Leap Technologies, Cary, NC), and a column oven (CTO20A; Shimadzu Scientific Instruments Inc.). ESI-MS was performed with an API 4000 Q-TRAP mass spectrometer (AB Sciex LLC, Foster City, CA) using nitrogen as the nebulizer and auxiliary gas. PreIS of m/z 272 was acquired at unit resolution with a scan range of m/z 300−1000 for Q1 in the negative ion mode. The mass range pause was 5 ms; step size was 0.25 Da, and scan time was 1 s. Other parameters were set as follows: curtain gas, 10 (arbitrary units); collision gas, medium; ionspray voltage, −4.5 kV; temperature, 450 °C; ion source gas 1, 50 (arbitrary units); ion source gas 2, 50 (arbitrary units).

Mass Defect Filter Method. The MDF method[24,27] was implemented in an Amgen in-house program written using the R-language. The positive ion mode FTMS data acquired from LTQ Orbitrap Velos was converted to a NetCDF file using Xcalibur 2.1 and then processed with the MDF program. The processed

Scheme 1. XoPI Method Data Acquisition Flow Chart: (A) Routine N; (B) Routine P



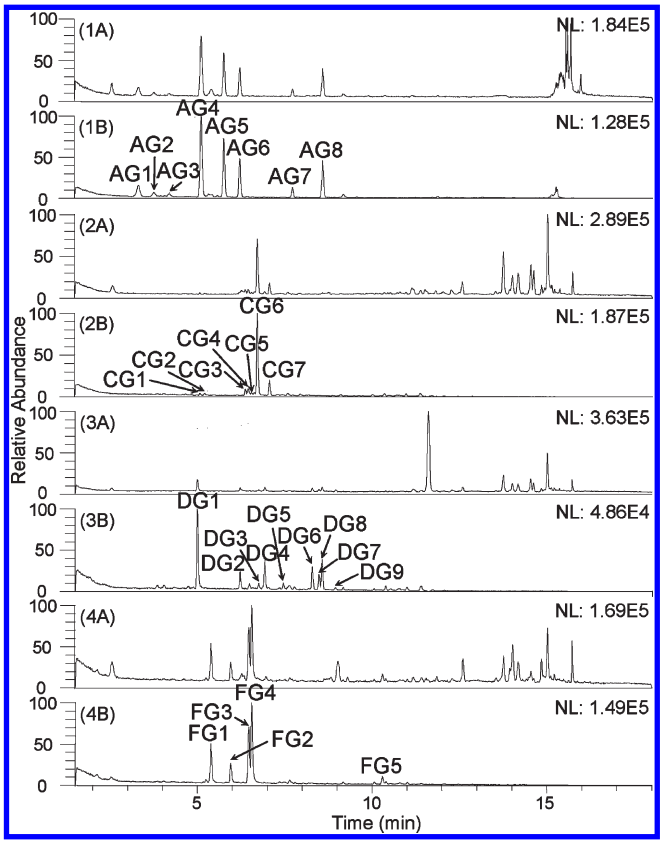


Figure 1. LC-MS profiles of *in vitro* incubations with test compounds acquired with selected ion monitoring at m/z 272 ± 2.5 Da under SCID in the negative ion mode. Labels 1−4 refer to amodiaquine, clozapine, diclofenac, and fipexide, respectively. The total ion chromatograms from m/z 272 ± 2.5 Da are displayed in panel A. The corresponding $XPIC_{272.0888}$, i.e., XPIC of 272.0888 ± 5 ppm, are displayed in panel B. The peak assignments are listed in Table 1.

NetCDF file was then converted back to the original format to be viewed and analyzed in Xcalibur. Each test compound had a GSH conjugate template (MH^+ of parent + GSH − 2H), i.e., m/z 661.2206, m/z 632.2053, m/z 601.0921, and m/z 694.1944 for amodiaquine, clozapine, diclofenac, and fipexide, respectively. An additional filter template was applied for the three compounds that had the potential to form GSH conjugates upon N- or O-dealkylation of the parent drug: amodiaquine, m/z 605.1580 (MH^+ of parent − C_4H_8 + GSH − 2H); clozapine, m/z 549.1318 (MH^+ of parent − C_5H_9N + GSH − 2H); and fipexide, m/z 434.0783 (MH^+ of parent − $C_{14}H_{16}N_2O_3$ + GSH − 2H). The MDF window and mass range were set to ±40 mDa and ±50 Da around each filter template, respectively.

RESULTS AND DISCUSSION

Analytical Strategy. The principal idea of the current approach, called extraction of product ion (XoPI) method, is detection of putative GSH conjugates by the appearance of peaks in the extracted product ion chromatogram (XPIC) at m/z 272.0888 from HRMS data acquired in the negative ion mode under nonselective SCID. The putative GSH conjugates are then confirmed and their structures are elucidated from the corresponding full scan and MS^2 data acquired in either negative ion or positive ion mode. The principles are demonstrated with data acquired on an LTQ Orbitrap Velos mass spectrometer; however, the strategy can be applied with other HRMS instruments.

The XoPI method is implemented via either routine N or routine P shown in Scheme 1. MS data collection for both routines consists of five events and is performed in separate chromatographic runs. In the first event, common for both routines, high resolution FTMS data are obtained by selected ion monitoring for ions at m/z 272 ± 2.5 Da under SCID in the negative ion mode. The remaining four events are all acquired without SCID. In the second event, full scan data are acquired from m/z 200 to 1000. In the third to fifth events, MS^2 spectra are acquired for the top three intense ions detected in the second event. In the P routine (Scheme 1B), polarity is switched going from the first to second event and, again, at the end of the cycle (after the fifth event).

In the first event, all components passing the ion source are fragmented exclusively under SCID[28] with GSH conjugates expected to generate a product ion at m/z 272.0888.[6] Extraction of ions

Table 1. Summary of MS Properties for the Identified GSH Conjugates Employing the XoPI Method. [a]

compound (exact mass)	GSH conjugate	observed accurate m/z of GSH conjugate in −/+ mode	error in −/+mode (ppm)[e]	retention time (min)	proposed GSH conjugate	was MS^2 acquired? routine N	was MS^2 acquired? routine P	was parent ion detected? PreIS[f]	was parent ion detected? MDF[g]
Amodiaquine (355.1451)	AG1[b]	936.2416, 467.6171[c]/469.6328[c], 313.4251[d]	1.4/0.9	3.32	P + 2GSH − 2CH2 − 4H	yes	yes	yes	no
	AG2[b]	527.1015[c]/529.1153[c]	1.3/−1.7	3.78	P + 3GSH − C5H11N − C5H7NO3 − 6H	yes	yes	no	no
	AG3[b]	964.2722, 481.6326[c]/483.6486[c], 322.7692[d]	−2.1/1.2	4.22	P + 2GSH − 4H	yes	yes	no	no
	AG4[b]	591.6223[c]/593.6377[c], 396.0945[d]	−1.2/0.3	5.12	P + 3GSH − C5H11N − 6H	yes	yes	yes	yes[h]
	AG5	631.1738/633.1896, 317.0996[c], 211.7350[d]	−1.4/0.5	5.77	P + GSH − 2CH2 − 2H	yes	yes	yes	yes
	AG6	659.2048/661.2211, 331.1154[c], 221.0793[d]	−1.8/0.8	6.22	P + GSH − 2H	yes	yes	yes	yes
	AG7[b]	879.1840, 439.0882[c]/881.1991, 441.1039[c], 294.4059[d]	−1.1/−0.6	7.73	P + 2GSH − C5H11N − 4H	yes	yes	yes	no
	AG8[b]	574.1160/576.1322, 288.5706[c]	−1.6/1.4	8.59	P + GSH − C5H11N − 2H	yes	yes	yes	yes
Clozapine (326.1298)	CG1	648.2001/650.2177, 325.6133[c]	−1.9/2.9	5.08	P + GSH + O	yes	no	yes	no
	CG2	648.2007/650.2164, 325.6131[c]	−0.9/0.9	5.22	P + GSH + O	yes	yes	yes	yes
	CG3	630.1896/632.2056, 316.6077[c]	−1.7/0.5	6.39	P + GSH − 2H	yes	yes	yes	yes
	CG4	616.1742/618.1901, 309.5998[c]	−1.5/0.8	6.46	P + GSH − CH2 − 2H	yes	yes	no	yes
	CG5	646.1844/648.2005, 324.6053[c], 216.7392[d]	−1.9/0.5	6.56	P + GSH + O − 2H	yes	yes	no	yes
	CG6	630.1904/632.2064, 316.6078[c]	−0.5/1.7	6.72	P + GSH − 2H	yes	yes	yes	yes
	CG7	646.1845/648.2007, 324.6052[c], 216.7390[d]	−1.7/0.8	7.07	P + GSH + O − 2H	yes	yes	yes	yes
Diclofenac (295.0167)	DG1	578.1391[c]/580.1544[c], 387.1074[d]	−1.0/0.2	5.01	P + 3GSH + O − 2HCl − 2H	yes	yes	yes	no
	DG2	852.2174, 425.6052[c]/854.2335, 427.6212[c]	−1.4/0.5	6.23	P + 2GSH + O − 2HCl	yes	yes	no	no
	DG3[b]	936.1388, 467.5652[c]/469.5800[c]	3.4/2.8	6.76	P + 2GSH + 2O − 4H	yes	no	no	no
	DG4	886.1786, 442.5853[c]/888.1951, 444.6014[c]	−1.1/1.0	6.94	P + 2GSH + O − HCl − 2H	yes	yes	no	no
	DG5[b]	631.0667/633.0818	−1.1/−0.3	7.46	P + GSH + 2O − 2H	no	no	yes	no
	DG6	581.1110/583.1266	−0.9/1.0	8.29	P + GSH + O − HCl	yes	yes	yes	yes
	DG7	615.0721/617.0876	−0.7/1.0	8.48	P + GSH + O − 2H	yes	yes	yes	no
	DG8	615.0720/617.0876	−0.8/1.0	8.58	P + GSH + O − 2H	yes	yes	yes	yes
	DG9	615.0740, 307.9898[c]/617.0891	2.4/3.4	8.96	P + GSH + O − 2H	yes	no	no	no
Fipexide (388.1190)	FG1[b]	448.0580/450.0741	−1.6/2.0	5.39	P + GSH + O − 2H after O-dealkylation	yes	yes	yes	yes
	FG2[b]	985.2466, 492.1193[c]/987.2642, 494.1356[c], 329.7605[d]	−1.4/1.6	5.96	P + 2GSH − CH2 − 2H	yes	yes	yes	no
	FG3	680.1787/682.1951, 341.6025[c]	−1.8/1.0	6.47	P + GSH − CH2	yes	yes	yes	yes
	FG4[b]	680.1788/682.1952, 341.6025[c]	−1.6/1.2	6.56	P + GSH − CH2	yes	yes	yes	yes
	FG5[b]	706.1581/708.1739	−1.4/0.3	10.30	P + GSH + O − 4H	yes	no	no	no

[a] The four test compounds were incubated in human liver microsomal incubations supplemented with NADPH and GSH. [b] GSH conjugates not reported previously. [c] Doubly charged ion. [d] Triply charged ion. [e] Calculated from the observed lowest charged parent ion. [f] From data acquired on an API 4000 Q-TRAP MS. [g] MDF algorithm applied to positive ion full scan FTMS data acquired on an LTQ Orbitrap Velos MS. [h] m/z 593.6377 is a doubly charged parent ion. It was inferred from its isotopic peak at m/z 594.1395 detected from the MDF template, m/z 605.1580 (MH^+ of parent − C_4H_8+ GSH − 2H).

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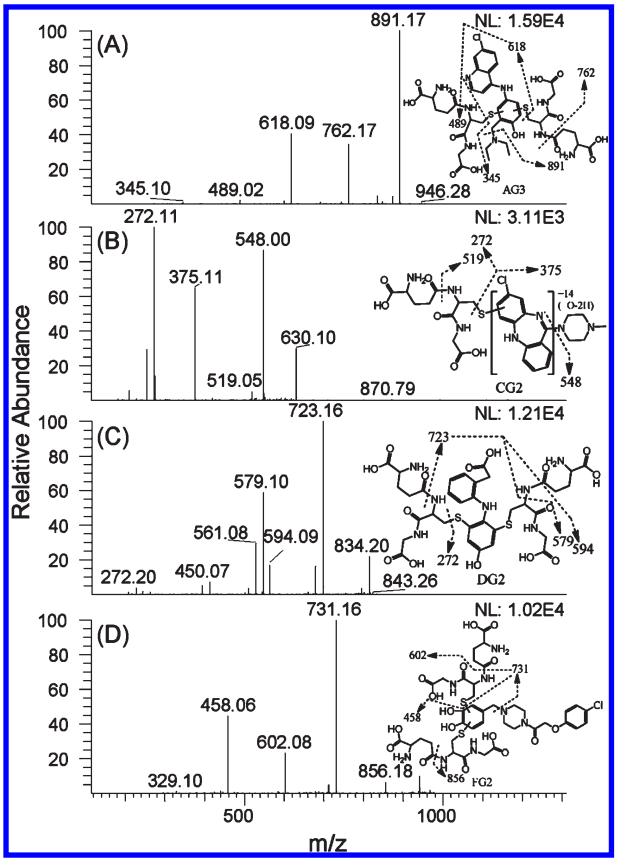

Figure 2. Negative ion MS^2 spectra of the parent ions acquired upon collision induced dissociation using routine N and their proposed structure assignments. (A) AG3, m/z 964.27; (B) CG2, m/z 648.20; (C) DG2, m/z 852.22; and (D) FG2, m/z 985.25.

at m/z 272.0888 ± 5 ppm from the high resolution data acquired in the first event is then used to construct a XPIC, referred to as $XPIC_{272.0888}$, where peaks potentially represent GSH conjugates. The corresponding precursor ion for each of the peaks in the $XPIC_{272.0888}$ is then identified in the full scan mass spectrum obtained from the second event. Thus, the $XPIC_{272.0888}$ serves as a map to facilitate identification of the parent m/z ion of GSH conjugates in the negative or positive ion full scan mass spectrum. The structure characterization is then performed by analyzing the corresponding MS^2 spectra acquired from the third to fifth events. Identification of precursor ion of the putative GSH conjugates not detected by either routine can be implemented by applying background subtraction function (available in the Xcalibur software) at the retention times corresponding to the peaks discerned in the $XPIC_{272.0888}$.

Method Validation. The analytical strategy was validated with amodiaquine, fipexide, clozapine, and diclofenac, all incubated in human liver microsomes supplemented with NADPH and GSH. The SIM total ion chromatogram (TIC) at m/z 272 ± 2.5 Da acquired in the negative ion mode under SCID from the test compounds incubations are displayed in Figure 1, panels 1A–4A. The corresponding $XPIC_{272.0888}$ are shown in Figure 1, panels 1B–4B. Each peak in the $XPIC_{272.0888}$ represented a potential GSH conjugate. The precursor ions for the peaks in the $XPIC_{272.0888}$ were then identified in the corresponding full scan MS, and the MS^2 data (acquired with routine N or routine P) were used to elucidate the GSH conjugate structures.

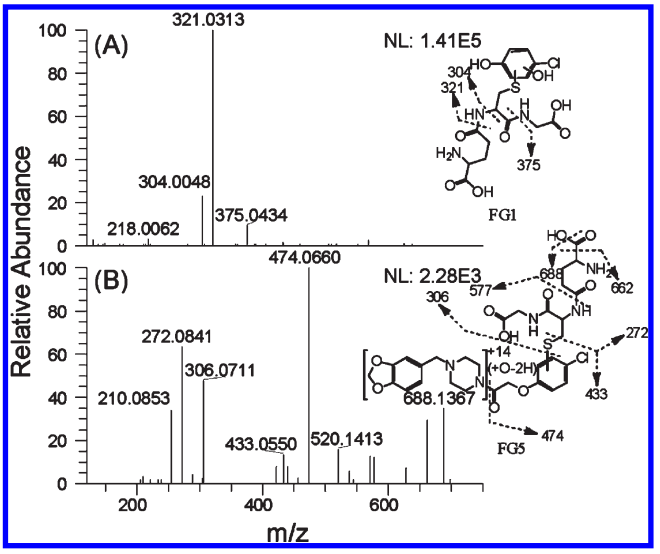

Figure 3. MS^2 FTMS spectra for fipexide GSH conjugates and their proposed structure assignments. (A) FG1 at m/z 450.07 in the positive ion mode; (B) FG5 at m/z 706.16 in the negative ion mode.

GSH conjugates identified for each of the test compounds are annotated in Figure 1, panels 1B–4B; their MS and MS^2 properties are catalogued in Tables 1 and S1 (in Supporting Information), respectively. Using routine N, all GSH conjugates except DG5 were detected and confirmed by MS^2 spectra. Negative ion MS^2 mass spectra for selected GSH conjugates (AG3, CG2, DG2, and FG2) are shown in Figure 2. Each of these four conjugates had only a minor product ion peak in the $XPIC_{272.0888}$ (Figure 1, panels 1B–4B), but their parent ions were still captured for MS^2 fragmentation by the data dependent acquisition algorithm. In routine P, a majority of the GSH conjugates were captured; however, some minor peaks were missed for MS^2 fragmentation. Positive ion MS^2 mass spectra for GSH conjugates AG3, CG2, DG2, and FG2 are shown in Figure S2 (in Supporting Information).

Although GSH conjugate formation from the test compounds has been extensively studied,[9,10,13,14,20,23,24,29−33] many novel conjugates were identified and characterized in the current study (Table 1). Two previously unreported GSH conjugates, FG1 and FG5, were detected from fipexide incubations (Table 1 and Figure 3). FG1, formed upon an *O*-dealkylation of fipexide, was identified as the GSH conjugate of chloro-catechol based on its accurate mass data (Table 1), monochlorine isotopic pattern of the parent ion (data not shown), and the MS^2 spectrum (Figure 3A). For FG5, GSH was inferred to be on the chlorophenoxy moiety due to the abundant product ion at m/z 474.0660 in its MS^2 FTMS spectrum shown in Figure 3B. Fipexide was withdrawn from the market due to adverse drug reactions,[34,35] and catechol reactive intermediates formed upon oxidation of the methylene dioxyphenyl moiety were implicated.[14,32] Detection of FG1 and FG5 in our study suggested that catechol intermediate was not the only reactive metabolite generated from fipexide.

Interestingly, the parent ion of FG5 (m/z 708.1739) was barely discernible in the positive ion full scan spectrum (Figure S3B in Supporting Information), and no MS^2 spectrum could be acquired. In contrast, the parent ion of FG5 (m/z 706.1581) was readily observed in the negative ion full scan spectrum (Figure S3A in Supporting Information). GSH moiety contains two carboxylic acid functional groups that are easily ionized and

readily detected in the negative ion mode at the employed mobile phase pH. Vice versa, if the drug candidate contains strongly basic groups, its GSH conjugate may be ionized better in the positive ion mode. Previously, most of the methods utilized positive[10,11,13−15,19,20,22,24] or negative[6] but not both ionization modes. The absence of data from the opposite polarity in those studies implicated that some GSH conjugates might have been missed. However, a GSH conjugate, regardless of its parent drug structure, always appears to produce γ-glutamyl-dehydroalanyl-glycine which can be detected in the negative ion mode. Therefore, in the XoPI method, no matter what the precursor ion charge state is, the product ion m/z 272.0888 arising from a GSH conjugate is always present in the $XPIC_{272.0888}$. The precursor ion not observed in one ion mode can then be intentionally searched for in the full scan data from the opposite polarity.

A dihydroxylated diclofenac GSH conjugate, DG5, was readily identified in the $XPIC_{272.0888}$ (Figure 1, panel 3B). However, the precursor ion was not triggered for data dependent MS^2 acquisition in routine N or routine P. Upon application of the background subtraction to the negative and positive ion full scan MS data, the precursor ions were identified at m/z 631.0667 and m/z 633.0818, respectively. The DG5 tandem mass spectrum from a predefined parent ion list directed acquisition in a separate chromatographic run helped identify it as a GSH conjugate of dihydroxy−diclofenac (Table 1).

The proposed approach was very selective in detecting the GSH conjugates. Almost all of the peaks in the $XPIC_{272.0888}$ (Figure 1, panels 1B−4B) were found to arise from GSH conjugates for each of the test compounds. There were a few minor GSH conjugate product ion peaks in the $XPIC_{272.0888}$ (Figure 1), such as those from 10 to 14 min in panels 1B−4B (Figure 1), each of which was also observed in control samples supplemented with GSH but without test compound and not observed in control samples without addition of GSH (data not shown). Therefore, those peaks were inferred to have formed upon reaction of GSH and an endogenous component in the reaction mixture. Also, many GSH conjugates which existed in multiply charged states (Table 1) with no singly charged precursor ion were also observed.

The implementation of the routines N and P in an LIT-Orbitrap mass spectrometer have their own advantages and drawbacks. In routine N, negative ion full scan FTMS data provide the accurate m/z of detected ions, which can be used for confirming the elemental composition. However, the negative MS^2 spectra contained fewer parent drug related product ions and, therefore, afforded less structure information on the parent molecule.[6] In routine P, more structure information can be obtained due to increased product ions from the parent drug in the positive ion MS^2 spectra. Nonetheless, the positive ion accurate m/z cannot be obtained since the polarity switching on FTMS causes the mass accuracy to be lost for several minutes on an LIT-Orbitrap.

In routine N, the cycle time for five events is approximately 1.3 s. In routine P, the polarity switching increases the cycle time to approximately 2.5 s. Including negative ion full FTMS scan into routine P will provide high resolution m/z for the parent ion but will also increase cycle time (to approximately 3 s) which in turn increases the possibility of missing the MS^2 data for peaks of interest from a single UHPLC run. The increased cycle time in routine P may also explain why a fewer number of parent m/z ions was captured for MS^2 in the data dependent acquisition events.

The combined results indicate that $XPIC_{272.0888}$ could be readily used for the agnostic detection of all the GSH-trapped reactive metabolites in the four test compounds. The sites of bioactivation could then be characterized from MS^2 data acquired by routine N or routine P mostly within a single chromatographic run. Conversely, we infer that if no peaks are observed in the $XPIC_{272.0888}$, the compound may not be producing any reactive metabolites that can be trapped by GSH. The proposed methodology is selective, sensitive, and amenable to automation and can be routinely used for the GSH conjugate screening.

Comparison with PreIS Method. Precursor ion scan at m/z 272 in the negative ion mode followed by a positive ion mode MS^2 scan on an API 4000 Q-TRAP mass spectrometer has been reported to be an effective method for the detection and characterization of GSH-trapped reactive metabolites.[9] Incubation supernatants from all four test compounds were subjected to PreIS method on the same model hybrid Q-TRAP mass spectrometer, and the result is displayed in Figure S4 (in Supporting Information). Many of the GSH conjugates identified in the $XPIC_{272.0888}$ (Figure 1) were detected in the m/z 272 precursor ion chromatogram (Table 1 and Figure S4 in Supporting Information). However, the PreIS method was less selective and less sensitive than the XoPI method. The real GSH conjugate peaks could not be easily distinguished because a large number of false positives were observed in the TIC of PreIS (Figure S4 in Supporting Information). Several GSH conjugates including AG2, AG3, CG4, CG5, DG2, DG3, DG4, DG9, and FG5 were not detected in the PreIS method (Table 1).

Comparison with MDF Method. The MDF method[24] has been applied to detect GSH-trapped reactive metabolites from HRMS data collected on an LTQ Orbitrap Velos mass spectrometer. Figure S5 (in Supporting Information) displays the pre- and post-MDF processed TIC from the positive ion full scan FTMS data for all four test compounds. MDF effectively removed the background interfering peaks outside the defined MDF windows, and the underlying GSH conjugate ions were easily discerned (Figure S5, panels 1B-4B, in Supporting Information).

Approximately half the number of GSH conjugates detected by the XoPI method was missed by the MDF method (Table 1). The MDF method requires some supposition of mass range and mass defect window and both are user input parameters in the filter template settings. Any peaks outside either the mass range or the mass defect window would be undetected. For example, the MDF template for diclofenac was set for a mono-GSH conjugate (m/z 601.0921) with a mass range from 551 to 651 Da and a mass defect window from 0.0521 to 0.1321 Da. Metabolite DG2, a di-GSH conjugate, appearing at m/z 854.2335 was not detected because both its nominal mass and its mass defect were outside the set range in the MDF template. Using multiple mass defect filters or more filter templates with a wider mass range and/or larger mass defect window may help to detect the missed conjugates. However, it may also result in more false positives. Also, many GSH conjugates in the positive ion mode take on multiple charges in ESI, appear as a doubly or triply charged ion, and are not easily detected if the corresponding singly charged ion is weak or absent. For example, GSH conjugates AG1, AG2, and DG1 occur as multiply charged ion and were not detected using the MDF method because their nominal mass and mass defect were outside the MDF template settings.

Analysis of Spiked *Ex Vivo* Samples. The spiked human plasma extract and rat urine samples were analyzed by XoPI,

PreIS, and MDF methods. The GSH conjugates identified from each method are listed in Table S2 (in Supporting Information). Only a subset of GSH conjugates detected in the direct analysis of the *in vitro* incubations were identified in the spiked human plasma and rat urine samples. Five GSH conjugates (DG1, DG2, DG4, DG6, and DG8) were detected in the human plasma extract, and three (DG4, DG6, and DG8) were observed in rat urine employing the XoPI method. GSH conjugates, DG6 and DG8, were detected in both matrixes by the MDF method. In contrast, no GSH conjugate was detected in either matrix by PreIS method. Overall, the results demonstrate that XoPI method is more selective and sensitive than PreIS or MDF methods for *ex vivo* spiked samples.

Although both XoPI and MDF methods worked in rat urine, more false positives in the $XPIC_{272.0888}$ and MDF filtered chromatogram were observed from this biomatrix than the *in vitro* sample (data not shown). The false positives in the $XPIC_{272.0888}$ were product ion peaks arising from endogenous GSH conjugates. However, the false positives in the MDF filtered chromatogram were endogenous matrix peaks captured by the MDF setting(s). The false positives in both methods can be identified by comparing against the corresponding control data.

Applicability of the XoPI Method in HRMS Spectrometers. The detection of GSH conjugates based on $XPIC_{272.0888}$ is agnostic, and their elution map can be obtained from any of the available HRMS platforms (Orbitrap/TOF/FTICR etc) as long as a nonselective CID is available for ions before they reach the HRMS detector. The acquisition of full scan MS and MS^2 data described in routines N or P would depend on HRMS instrument.

CONCLUSION

Fragmentation of all classes of GSH conjugates unbiasedly produces a deprotonated γ-glutamyl-dehydroalanyl-glycine (m/z 272.0888) in the negative ion mode. The proposed XoPI method detects the GSH conjugate product ion m/z 272.0888 formed upon in-source collision induced dissociation to produce an $XPIC_{272.0888}$ map of putative GSH conjugates. The precursor ions of peaks in the $XPIC_{272.0888}$ are identified and their structures are characterized from positive/negative ion full scan MS and MS^2 data sets. No stable-isotope labeled or a derivatized GSH is required. Routines N and P provide high throughput and simple ways to detect and characterize a majority of the GSH conjugates all within a single UHPLC run. The proposed methodology is both selective and sensitive in detecting GSH-trapped reactive intermediates and can be routinely used for the GSH conjugate screening.

ASSOCIATED CONTENT

Supporting Information. Tables S1 and S2 and Figures S1−S5 as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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