Disposition of Glutathione Conjugates in Rats by a Novel Glutamic Acid Pathway: Characterization of Unique Peptide Conjugates by Liquid Chromatography/Mass Spectrometry and Liquid Chromatography/NMR

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ABSTRACT

With the advent of liquid chromatography/mass spectrometry and liquid chromatography/NMR, it has become easier to characterize metabolites that were once difficult to isolate and identify. These techniques have enabled us to uncover the existence of an alternate pathway for the disposition of glutathione adducts of several structurally diverse compounds. Studies were carried out using acetaminophen as a model compound to investigate the role of the glutamic acid pathway in disposition of the glutathione adducts. Although the mercapturic acid pathway was the major route of degradation of the glutathione adducts, it was found that the conjugation of the glutathione, cysteinylglycine, and cysteine adducts of acetaminophen with the \( \gamma \)-carboxylic acid of the glutamic acid was both interesting and novel. The coupling of the glutathione adduct and the products from the mercapturic acid pathway with the glutamic acid led to unusual peptide conjugates. The natures of these adducts were confirmed unequivocally by comparisons with synthetic standards. This pathway (addition of glutamic acids) led to larger peptides, in contrast to the mercapturic acid pathway, in which the glutathione adducts are broken down to smaller molecules. The enzyme responsible for the addition of glutamic acid to the different elements of the mercapturic acid pathway is currently unknown. It is postulated that the \( \gamma \)-carboxylic acid is activated (perhaps by ATP) before enzymatic addition to the \( \alpha \)-amino group of cysteine or glutamate takes place. The discovery of these peptide conjugates of acetaminophen represents a novel disposition of glutathione adducts of compounds. The formation of such conjugates may represent yet another pathway by which drugs could produce covalent binding via their reactive intermediates.

Glutathione (GSH) is one of the most important molecules in the cellular defense against chemically reactive toxic compounds or oxidative stress. This protective function is due in part to its involvement in conjugation reactions. GSH is a tripeptide composed of \( L \)-glutamic acid, \( L \)-cysteine, and \( L \)-glycine (\( \gamma \)-Glu-Cys-Gly). The presence of cysteine in the tripeptide provides a sulfhydryl group that is nucleophilic, and hence GSH reacts with electrophiles as the thiolate ion, GS\(^{-}\). These electrophiles may be chemically reactive and are produced as metabolic products of a phase 1 reaction, or they may be more stable foreign compounds. Hence, GSH protects cells by removing reactive metabolites. The GSH adducts may then either be excreted, usually into the bile, or the conjugate may undergo further metabolism via the mercapturic acid pathway. This involves sequential removal of the glutamyl and glycine groups and acetylation of the cysteine amino group to produce the N-acetylcysteine conjugate or mercapturic acid. Alternate metabolic pathways or catabolism of these GSH conjugates has not been described before. In this report, we describe a unique and novel metabolic pathway for GSH adducts of acetaminophen. Acetaminophen is a widely used analgesic known to cause hepatotoxicity in animals and in humans exposed to high doses of this compound. The formation of the GSH conjugate from acetaminophen has been well documented (Hinson et al., 1982). The degradation of the GSH adduct via the mercapturic acid pathway has been the subject of several publications and reviews (Commandeur et al., 1995, and the references cited therein).

A previously unreported metabolic pathway for these GSH adducts, leading to the formation of peptide conjugates, is described in this report. These polar metabolites were iso-
lated from bile by solid phase extraction and by reversed phase HPLC. Often, the structures of such polar metabolites are not elucidated due to the difficulty in isolating and separating them from endogenous components in the biological matrices. With the advent of liquid chromatography/mass spectrometry (LC/MS) and LC/NMR, it has become possible to elucidate the structures of minor metabolites. Characterization of these polar conjugates can provide very useful information regarding the metabolic pathways and evidence for any potentially reactive metabolites of a compound. This report describes the unequivocal identification of novel peptide conjugates resulting from conjugation of GSH adducts and its breakdown products with glutamic acid using LC/MS/MS, LC/NMR, and NMR approaches. The γ-carboxylic acid coupling of glutamic acid to other amino acids of GSH-derived adducts was demonstrated by synthesizing authentic standards and comparing spectral data with isolated metabolites. These metabolites are biologically novel, and the formation of these types of metabolites may represent a previously unappreciated combination of drug metabolism reactions.

**Materials and Methods**

**Chemicals and Supplies.** Trifluoroacetic acid (TFA), phenol, ethanediithiol, thioanisole, acetaminophen, p-acetamidophenol-β-γ-gluconuride, N-acetylbenzoquinone imine (NAPQI), cysteinylglycine, and γ-glutamyleysteine were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Resin Fmoc-Gly and Fmoc-Cys (Trt) were obtained from Advanced ChemTech (Louisville, KY). Fmoc-γ-glu-taminic acid-ε-butyler ester was obtained from NovaBiochem (San Diego, CA). All other amino acids and synthesizer reagents were obtained from PE Biosystems (Foster City, CA). Bond-Elut C18 cartridges (10 g/60 ml) were obtained from Varian Sample Preparation Products (Habor City, CA). All general solvents and reagents were of the highest grade commercially available.

**Synthesis of γ-Glu-γ-Glu-Cys-Gly (Tetrapeptide) and γ-Glu-γ-Glu-Cys (Tripeptide).** The tetrapeptide γ-Glu-γ-Glu-Cys-Gly and the tripeptide γ-Glu-γ-Glu-Cys were made using i-isoemers of each amino acid. The α-carboxylic acid was protected on the glutamic acid, allowing the γ-carboxylic acid to be coupled with the amino functional group of the coupling amino acid. The peptides were synthesized on solid phase using Fmoc protection and 2-(1-tetrazole) N-coupling amino acid. The peptides were synthesized as described for the isolation of the tetrapeptide conjugate, M6 (see earlier). A second HPLC purification was done using an isocratic mobile phase (10:90 v/v acetonitrile/0.05% TFA) delivered at a rate of 3.5 ml/min. Approximately 4.5 mg of the tetrapeptide conjugate was recovered after purification.

**Synthesis of Dipeptide Conjugate (M8) of Acetaminophen.** The synthesis of 3-(γ-glutamylcysteinyl-S-yl)acetaminophen (M8, see Scheme 1) was done by reacting freshly prepared solution of NAPQI with γ-glutamylcysteine in the same manner as described for metabolite M6. NAPQI (15 mg) was dissolved in 2 ml of acetonitrile in a scintillation vial, and the entire sample was transferred to a vessel containing 5 ml of ice-cold water and 1 ml of 0.2 M ammonium acetate solution (pH 6.0). Cysteinylglycine (18 mg) was dissolved in 2 ml of water and added to the NAPQI solution. The reaction was carried out as described, after which the solvent was removed under nitrogen at room temperature. The residue was reconstituted in water and chromatographed on a semipreparative HPLC column. The HPLC system used to separate the dipeptide conjugate was same as that used for isolation of the tetrapeptide conjugate, M6 (see earlier). A second HPLC purification was done using an isocratic mobile phase (10:90 v/v acetonitrile/0.05% TFA) delivered at a rate of 3.5 ml/min. Approximately 18 mg of the cysteinylglycine conjugate was recovered after purification.

**Synthesis of Tripeptide Conjugate (M9) of Acetaminophen.** The synthesis of 3-(γ-glutamylcysteinyl-S-yl)acetaminophen (M9, see Scheme 1) was done by reacting freshly prepared solution of NAPQI with γ-glutamylγ-glutamylcysteine in the same manner as described for metabolites M6 and M8. Due to the low yield of the product, the tripeptide conjugate was not isolated for NMR analyses. Instead, the product was analyzed directly by LC/MS, and comparisons were made with the in vivo metabolite.

**LCMS.** LC/MS was carried out by coupling a Hewlett-Packard HPLC system (HP 1100) to a Finnigan LCQ ion trap mass spectrometer. The HPLC eluent was introduced to the mass spectrometer using a pneumatically assisted electrospray source. The mass spectrometer was operated in both the negative and positive ion modes. MS (MS/MS experiments with n = 0–4) were done with relative collision energy set to 20 to 25%. Metabolites that could not be isolated in pure form for NMR analyses were subjected to several LC/MS experiments for the purpose of structure elucidation.
For analyses of the acetaminophen metabolites, HPLC was carried out using a Hewlett-Packard HPLC system (HP 1100) coupled in sequence to a Waters symmetry C18 column (150 × 3.9 mm, 5 μm). The metabolites were separated by a gradient solvent system consisting of a mixture of acetonitrile and 0.05% TFA with the flow rate set at 0.7 ml/min. The initial conditions consisted of a mixture of acetonitrile and 0.05% TFA (5:95 v/v) and was maintained for 5 min after the sample was injected. The percentage of acetonitrile was increased from 5 to 30% in the next 15 min. After 20 min, the column was washed with 90% acetonitrile for 5 min before re-equilibrating with the initial mobile phase. Aliquots of bile and urine samples were injected directly onto the HPLC, and the eluent was introduced into the source of the mass spectrometer.

To detect the metabolites in the fractions from C18 cartridges and from semipreparative HPLC column, aliquots (20–50 μl) were introduced into the mass spectrometer using the flow injection analysis method. The mobile phase consisted of a mixture of acetonitrile and 10 mM ammonium formate (1:1 v/v) delivered at a rate of 0.25 ml/min.

**LC/NMR.** The tetrapeptide conjugate of acetaminophen was characterized by LC/NMR. All of the spectra were obtained using a Bruker Avance 600 MHz NMR spectrometer equipped with an 1H/13C LC/NMR flow probe with a cell volume of 120 μl. Suppression of the residual water and acetonitrile signals was carried out using the WET solvent suppression method in all of the LC/NMR experiments. Chemical shifts were referenced to DMSO at δ of 2.49 ppm and to acetonitrile at δ of 2.0 ppm. An HP 1100 LC system was used with a Bruker Diode-Array detector set at 254 nm. A 3.9 × 150 mm HPLC Waters Symmetry C18 5-mm column was used. The initial HPLC conditions consisted of isocratic elution using 95% D₂O and 5% acetonitrile-d₃ for the first 5 min, followed by a gradient from 95% D₂O and 5% acetonitrile-d₃ to 70% D₂O and 30% acetonitrile-d₃ over 15 min at a flow rate of 0.7 ml/min. Both solvents contained 0.05% TFA.

The spectra of isolated metabolites of acetaminophen were obtained using a 2.5-mm ¹H/¹³C inverse probe. The samples were dissolved in DMSO-d₆ and filtered to remove particulate matter. The structures of these metabolites (approximately 1–2 mg of each) were determined from proton and carbon one-dimensional NMR as well as two-dimensional proton-proton correlated spectroscopy (COSY), proton-carbon heteronuclear single quantum coherence (HSQC), and long-range proton-carbon heteronuclear multiple bond coherence (HMBC) NMR experiments.

**Animal Studies.** Six male Sprague-Dawley rats with cannulated bile ducts (weighing between 250–400 g) were dosed orally with acetaminophen (100 mg/ml, made in propylene glycol/water 8:2 v/v) once daily at 500 mg/kg for 2 days. Serial bile and urine samples were collected over ice and pooled from all of the animals on a daily basis and stored frozen at −20°C until analyzed. The dosing volume was 5 ml/kg. In another study, a single male Sprague-Dawley rat was dosed i.v. (60 mg/kg) with the cysteinylglycine conjugate of acetaminophen (M4), and bile and urine were collected over 0- to 6- and 6- to 24-h time intervals.

**Isolation of GSH-Derived Conjugates from Rat Bile.** Bile obtained from the rats was pooled, diluted 1:1 with distilled water, and loaded onto a C18 cartridge (10 g/60 ml). The sample was allowed to elute under gravity at a rate of less than 1 ml/min. After
the sample had been loaded, the column bed was washed with 30 ml of deionized water followed by elution with 5, 10, and 15% methanol in water (20 ml volume of each). Aliquots from all the fractions were analyzed by LC/MS. The fractions containing the GSH related adducts were subsequently loaded onto C18 cartridges (10 g/60 ml) for further repurification. The fractions containing the metabolites were pooled and dried under vacuum. The dried residues were reconstituted in water and rechromatographed on a semipreparative HPLC column as described later.

Semipreparative HPLC separation of the polar metabolites of acetaminophen isolated from bile was carried out on a Waters Symmetry C18 (300 × 7.5 mm, 7 μm) using acetonitrile and 0.05% TFA as the eluent. The initial HPLC conditions consisted of isocratic elution using 95% H2O and 5% acetonitrile for first 5 min, followed by a gradient from 95% H2O and 5% acetonitrile to 85% H2O and 15% acetonitrile over 8 min. The percentage of acetonitrile was increased to 60% over the next minute and maintained at this for an additional 5 min before re-equilibrating the column with the initial mobile phase. The flow rate was set at 3.5 ml/min. The eluent was monitored at 254 nm using a variable wavelength detector. Five peaks were collected corresponding to metabolites M1 (m/z 328), M2 (m/z 232), M3 (m/z 271), M4 (m/z 328), and M5 (m/z 457). Samples corresponding to each metabolite were pooled and dried under vacuum. Final purification was done by rechromatographing each isolated metabolite on C18 cartridges. Repurification of M5 on an analytical column (3.9 × 150 mm, Waters Symmetry C18, 5 μm) resulted in separation of the tetrapeptide conjugate, M6 with MH+ at m/z 586 (see later). After pooling the fractions containing the metabolites, the samples were dried under vacuum and submitted for NMR analyses.

Isolation of Tetrapeptide Conjugate of Acetaminophen from Rat Bile. The tetrapeptide conjugate, M6 (MH+ at m/z 586), coeluted with the GSH adduct of acetaminophen during the initial purification steps. This metabolite was isolated from the GSH adduct on an analytical column (Waters Symmetry C19, 3.9 × 150 mm) using an isocratic mobile phase consisting of a mixture of acetonitrile and 0.05% TFA (5:95 v/v) delivered at 0.7 ml/min. The peak corresponding to this metabolite (11.2 min) was collected, pooled from several injections, dried, and submitted for LC/NMR analyses. Approximately 1 to 2 mg of each metabolite M1 to M5 were isolated for NMR analyses.

Incubations of 3-(γ-Glutathion-S-yl)acetaminophen with Rat Liver/Kidney Subcellular Fractions and Bile. The purified GSH conjugate of acetaminophen, M5, was incubated in the presence of rat bile and rat liver/kidney subcellular fractions. The absence of any other metabolites in the purified metabolite, M5, was confirmed by LC/MS experiments before incubations. Approximately 250 μg of M5 was incubated with 0.5 ml of freshly collected rat bile or with freshly prepared liver/kidney S9 fractions (5 mg/ml). The samples were incubated at 37°C for 1 h, after which the samples were analyzed by LC/MS. Bile samples (25 μl) were analyzed by LC/MS without any sample treatment. The liver/kidney incubations were precipitated with 3 ml of acetonitrile, and the supernatant was dried, reconstituted in the HPLC mobile phase, and injected onto LC/MS.

**Results**

LC/MS and NMR Characterization of Acetaminophen Metabolites in Rat Bile. LC/MS analyses of bile samples from rats dosed with acetaminophen showed that the polar metabolites, including the glucuronide (M1), sulfate (M2), cysteine (M3), cysteinylglycine (M4), and GSH (M5) conjugates, were well resolved from each other and from the endogenous materials using a gradient HPLC system. The metabolites were monitored by using the LCQ mass spectrometer and a variable-wavelength UV detector. A representative total ion current (TIC) and an HPLC/UV trace obtained simultaneously during the analysis of a bile sample are shown in Fig. 2. A number of unusual metabolites (M6–M9) were also found during the analysis. These metabolites either coeluted with other known metabolites of acetaminophen and/or were present in very low quantities. The extracted ion chromatograms showing the presence of these metabolites in bile are shown in Fig. 2.

Metabolites M1 to M6 found in the bile of rats dosed with acetaminophen produced pseudomolecular ions ([M + H]+) at m/z 328, 232, 271, 328, 457, and 586, respectively, were isolated in sufficient quantities for unambiguous confirmation of the structures by NMR experiments. Metabolites M7 to M9 were isolated chromatographically during LC/MS analyses for several MS/MS experiments.

Metabolite M1 was found to be the glucuronide conjugate of acetaminophen (MH+ at m/z 328). The identity of this metabolite was confirmed by comparing the retention time and mass spectral fragmentation pattern with commercially available synthetic standard.

Metabolite M2 was determined to be the sulfate conjugate of acetaminophen with MH+ at m/z 232. The pseudomolecular ion at m/z 230 in the negative ion mode showed a characteristic loss of sulfate moiety (Weidolf et al., 1988). The 1H NMR data showed proton signals of an intact aromatic ring [δ 7.05 (d) and 7.43 (d)]. The aromatic signals were characteristic of a para-substituted ring system. The signal for the amide proton appeared at δ 9.82, whereas the acetyl group appeared as a singlet at δ 2.0 ppm.

Metabolite M3 was found to be the cysteine conjugate with MH+ at m/z 271. The 1H NMR data and the mass spectral fragmentation pattern confirmed the structure of this metabolite. The 1H NMR spectrum showed signals at δ 7.60 (1H, Ar-H, d), 7.24 (1H, Ar-H, dd), 6.80 (1H, Ar-H, d), 4.00 (1H, cys α, m), 3.30 (1H, cys β, m), 3.20 (1H, cys β’, m), 2.00 (3H, COCH3, s). The aromatic proton signals had changed as a result of substitution on the ring.

Metabolite M4 was found to be the cysteinylglycine adduct of acetaminophen. The 1H NMR showed signals for the cysteine and glycine protons as reported for cysteinylglycine conjugates (Mutlib et al., 1999). The 1H NMR spectrum showed signals at δ 7.63 (1H, Ar-H, d), 7.24 (1H, Ar-H, dd), 6.82 (1H, Ar-H, d), 4.00 (1H, cys α, m), 3.78 (2H, gly α, m), 3.30 (1H, cys β, m), 3.18 (1H, cys β’, m), 2.00 (3H, COCH3, s). The aromatic proton signals were found to be similar to those obtained for the cysteine conjugate. The MH+ for the cystei-
nylglycine adduct was found at \( m/z \) 328. The LC/MS retention times and mass spectral fragmentation patterns for the metabolite and the synthetic standard were found to be identical.

Metabolite M5 was the GSH adduct of acetaminophen with the mass spectral data showing the pseudomolecular ion \( (M^+\) at \( m/z \) 457. The \(^1\)H NMR of the isolated metabolite clearly showed the presence of the GSH and the loss of one of the aromatic protons. The assignment of the proton signals were done by TOCSY experiments; \( \delta \) 7.45 (1H, Ar-H, d), 7.22 (1H, Ar-H, d), 6.75 (1H, Ar-H, d), 4.48 (1H, cys \( \alpha \), m), 3.98 (1H, glu \( g \), m), 3.76 (2H, gly \( a \), d), 3.23 (1H, cys \( \beta \), m), 2.98 (1H, cys \( \beta' \), m), 2.41 (1H, glu \( \gamma \), m), 2.35 (1H, glu \( \gamma' \), m) 2.05 (2H, glut \( b \), b, m), 2.00 (3H, COCH\(_3\), s). The \(^1\)H NMR results obtained for M5 was found to be consistent with data reported previously (Hinson et al., 1982). The MS/MS fragmentation ions are shown in Fig. 3. A number of MS\(^n\) experiments with the fragment ions derived from MH\(^+\) (\( m/z \) 457) and [M − H]\(^−\) (\( m/z \) 455) were done, and the results are shown in Tables 1 and 2. Hence, M5 was confirmed as 3-{\( \gamma \)-glutathion-S-yl}acetaminophen.

Metabolite M6 was the tetrapeptide conjugate formed by condensation of \( \gamma \)-carboxyl group of the glutamic acid with the \( \alpha \)-amino group of the glutamate present in the GSH adduct of acetaminophen. The proposed structure of this metabolite is shown in Scheme 1. The mass spectral data showed MH\(^+\) at \( m/z \) 586, which was consistent with an addition of a glutamic acid onto the GSH adduct of acetaminophen. LC/MS analysis of bile samples obtained from rats dosed with acetaminophen showed that this metabolite eluted after the GSH conjugate on the HPLC column. The structure of this adduct was supported by MS\(^n\) experiments that showed that the glutamic acid was linked to the GSH

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**Fig. 2.** LC/MS and HPLC/UV profiles (A and B, respectively) of acetaminophen metabolites present in rat bile showing the presence of glucuronide conjugate (M1), sulfate conjugate (M2), cysteine (M3), cysteinylglycine (M4), GSH adduct (M5), and tetrapeptide and pentapeptide conjugates (M6 and M7, respectively). The extracted ion chromatograms of metabolites M6 (\( m/z \) 586), M7 (\( m/z \) 715), M8 (\( m/z \) 400), and M9 (\( m/z \) 529) showed that these metabolites coeluted with each other or with other metabolites of acetaminophen.

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**Fig. 3.** MS/MS fragmentation patterns of the GSH adduct (M5) and the tetrapeptide conjugate (M6). The fragment ions \( a \) (\( m/z \) 382) and \( b \) (\( m/z \) 152) are formed by an elimination of glutamic acid and by cleavage at the C—S bond of cysteine moiety, respectively. The mass spectral analysis was done in the positive ion mode.
The fragment ions obtained from these experiments are listed in Table 1 and 2. The fragmentation pathways confirming the structure of this conjugate is shown in Fig. 3. Based on the fragment ion spectra of a series of model GSH adducts, Haroldsen et al. (1988) identified routes of fragmentation of these conjugates. Subsequent studies with other GSH adducts have confirmed the generality of these pathways, regardless of ionization technique adopted to generate the pseudomolecular ion. The MS/MS favors the cleavage of specific peptide bonds in the GSH residue, notably the γ-glutamyl linkage to yield ion a (Fig. 3). In the positive ion mode, MS/MS of the pseudomolecular ion (m/z 457) of the GSH conjugate produced the major fragment ion at m/z 328 formed by the loss of the glutamoyl moiety. Loss of glycine residue (−75 amu) from the parent ion led to a fragment ion at m/z 382. This characteristic loss of glycine residue from the GSH adduct has been described previously (Haroldsen et al., 1988) and was found to be very diagnostic in confirming the structures of M6 and M7 (see later). The ion at m/z 182 is derived from the cleavage of carbon-sulfur bond leading to retention of sulfur on the aromatic ring of acetaminophen. Such fragmentation pathway has been described for GSH adducts of aromatic compounds (Haroldsen et al., 1988). The aromatic thioether conjugates usually display a fragment resulting from the cysteinyl C—S bond with proton transfer to the aromatic moiety to yield a stabilized aryl-SH₂⁺ species (ion b, Fig. 3). The MS/MS fragmentation of the pseudomolecular ion (m/z 586) of the tetrapeptide produced ions at m/z 457 and 511 formed by losses of glutamate and glycine residues, respectively. Loss of glutamate from ion m/z 511 yielded a significant ion at m/z 382. The fragmentation pattern produced from different ions after several MS² experiments was consistent with the structure proposed in Fig. 3 and Scheme 1. The negative ion MS showed a fragmentation pattern dominated by fragments resulting from the cleavage of amino acid residues from the Cys-Gly-Glu entity. For comparison, the MS/MS data for the GSH adduct is shown in Table 2. The MS/MS of the pseudomolecular ion ([M – H]⁻ at m/z 584) of the tetrapeptide conjugate M6 produced a major fragment ion at m/z 401 (Table 2). This ion represents the Gly-Cys-Glu-Glu fragment with the sulfur of cysteine being retained by the aromatic ring of the acetaminophen (fragment ion at m/z 182). The MS/MS data obtained from MS² experiments with different fragment ions are shown in Table 2. The MS/MS data for intact reduced GSH is included in Table 2 for comparison purposes. The fragment ions arising from MS/MS of ions at m/z 401 and 272 confirmed that the GSH moiety linked to acetaminophen was intact (compare data with those of M5 and GSH). Results from further MS/MS experiments confirming the structure of the tetrapeptide are shown in Table 2.

The ¹H NMR and TOCSY data for the tetrapeptide conjugate M6 isolated from rat bile are shown in Figs. 4 and 5, respectively. The ¹H NMR spectrum clearly showed the presence of additional resonances for the extra glutamic acid at δ 4.28 (1H, glu a, m), 2.3 (2H, glu γ, m), and 1.90–2.00 (2H, glu β, m), in addition to the usual GSH protons. The correlation between the α, β, and γ protons of the two glutamic acids in the molecule is shown by TOCSY data (see Fig. 5). The LC/MS and NMR data obtained for the synthetic standard were found to be identical with those obtained from M6. Based on the spectroscopic data and comparison with synthetic standard, M6 was confirmed as 3-(γ-glutamyl-glutamyl-S-yl)acetaminophen.

Metabolite M7 was found to be the pentapeptide conjugate of acetaminophen with [M – H]⁻ at m/z 713. The structure of this conjugate was determined to be 3-(γ-glutamyl-γ-glutamyl-glutathion-S-yl)acetaminophen as shown in Scheme 1. MS/MS of ion at m/z 713 produced sequential losses of two glutamates resulting in ions at m/z 584 and 455 (see Table 2). Further MS/MS studies with ions at m/z 584 and 455 supported the proposed structure shown in Scheme 1. M7 was not produced in sufficient quantities to be isolated for characterization by NMR.

Metabolite M8 was found to be the dipeptide conjugate of acetaminophen with MH⁺ at m/z 400. The structure of this conjugate was confirmed as 3-(γ-glutamylcystein-S-yl)acetaminophen (see Fig. 6). MS/MS of the pseudomolecular ion at

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**TABLE 1**

Mass spectral data (MS/MS in positive ion mode) for the glutathione (M5) and the peptide conjugate M6 of acetaminophen

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>M/z</th>
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<tr>
<td>M5 Metabolite</td>
<td>457→</td>
<td>457→</td>
<td>457→</td>
</tr>
<tr>
<td>(MH⁺ 457)</td>
<td>382,328,311,269,225,182</td>
<td>311,269,225,208,182,140</td>
<td>269,225,208,182,140</td>
</tr>
<tr>
<td>M6 Metabolite</td>
<td>586→</td>
<td>586→</td>
<td>586→</td>
</tr>
<tr>
<td>(MH⁺ 586)</td>
<td>568,511,457,382,328,311,269</td>
<td>493,448,382,365,373,328,310,241</td>
<td>493,382,328,311,269,225,182</td>
</tr>
</tbody>
</table>

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**TABLE 2**

Mass spectral data (MS/MS in the negative ion mode) for the glutathione (M5) and peptide conjugates (M6 and M7) of acetaminophen

<table>
<thead>
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<th>Metabolite</th>
<th>M/z</th>
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<th>M/z</th>
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<td>M5 Metabolite</td>
<td>457→</td>
<td>457→</td>
<td>457→</td>
</tr>
<tr>
<td>[M – H]⁻ 455</td>
<td>272,254,210,182,179</td>
<td>254,210,179,143,128</td>
<td>210,179,167,126,87</td>
</tr>
<tr>
<td>M6 Metabolite</td>
<td>584→</td>
<td>584→</td>
<td>584→</td>
</tr>
<tr>
<td>M7 Metabolite</td>
<td>713→</td>
<td>713→</td>
<td>713→</td>
</tr>
<tr>
<td>[M – H]⁻ 713</td>
<td>695,619,584,566,530,512,455,401,272</td>
<td>516,455,401,383,272,254</td>
<td>272,254,210,182</td>
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<tr>
<td>Glutathione</td>
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</tr>
<tr>
<td>[M – H]⁻ 306</td>
<td>288,272,254,210,179,143,128</td>
<td>254,210,179,143</td>
<td>210,179,143</td>
</tr>
</tbody>
</table>

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Base peak in the mass spectrum.
m/z 400 produced an ion at m/z 271 formed by the characteristic loss of glutamate residue. The other fragments included ions at m/z 140, 182, 208, 225, and 254. An ion at m/z 337 was postulated to be formed by subsequent losses of NH₃ and formic acid from the α-position of the attached glutamic acid. Hence, the data suggested that the glutamic acid was linked to the amino group of the cysteine via the γ-carboxylic terminal group. The structure of this conjugate was confirmed by synthesizing the dipeptide conjugate of acetaminophen. The 1H NMR (see Fig. 6) and HMBC experiments confirmed the structure of the synthetic standard. The LC/MS retention time and mass spectral data of the metabolite matched those of the standard as shown in Fig. 7.

Metabolite M9 was found to be the tripeptide conjugate of acetaminophen with MH⁺ at m/z 529. The proposed structure of this conjugate is 3-(γ-glutamylcystein-S-yl)acetaminophen (see Scheme 1). MS/MS of the pseudomolecular ion at m/z 529 produced a characteristic ion at m/z 466 that probably was formed by the losses of ammonia and formic acid from the terminal glutamate moiety. Further MS/MS experiments with the fragment ions at m/z 400 and 271 confirmed the postulated sequence of amino acids as proposed in Scheme 1.

**LC/MS Characterization of Metabolites Produced by Cysteinylglycine Conjugate (M4) of Acetaminophen.**

Analyses of bile samples from the rat dosed with M4 showed that most of the metabolite was converted to N-acetylcysteine conjugate via the mercapturic acid pathway. Interestingly, when acetaminophen was given orally to the rats, cysteine and not N-acetylcysteine conjugate was detected as one of the major metabolites. The purpose of administering M4 was to show that GSH could be formed from this metabolite via the glutamic acid pathway. Indeed, the GSH adduct was detected as a minor metabolite in the bile. In addition to these metabolites, the 3-(γ-glutamylcystein-S-yl)acetaminophen conjugate M8 was found in significant quantities in bile. Overall, the conversion of M4 via the mercapturic acid pathway was the dominant route of metabolism.

**Incubation of 3-(γ-Glutathionyl-S-yl)acetaminophen with Rat Liver/Kidney Subcellular Fractions and Bile.**

It was found that the GSH adduct, M5, was converted to the cysteinylglycine and cysteine conjugates, M4 and M3, respectively, in the presence of rat bile and liver/kidney subcellular fractions. The formation of M6 or any of the peptide conjugates M7, M8, and M9 were not detected in any of these experiments. The LC/MS profiles suggested that mercapturic acid pathway predominated under the experimental conditions used during the incubations.

**Discussion**

Numerous reports have appeared in the literature describing the formation of GSH adducts and their subsequent metabolism to cysteinylglycine, cysteine, and N-acetylcysteine conjugates via the mercapturic acid pathway (Commandeur et al., 1995, and the literature cited therein). The GSH conjugates are formed by the interaction of an electrophilic compound, such as those produced by bioactivation of drugs by cytochrome P450, with the endogenous pool of GSH. These reactions are either mediated by specific GSH transferases or are nonenzymatic in nature (Habig et al., 1974; Ketterer, 1986). The resulting GSH adducts are excreted in bile or are
further catabolized via the mercapturic acid pathway to cysteinylglycine, cysteine, or N-acetylcysteine adducts (Commandeur et al., 1995). The cleaved cysteine conjugates are often reabsorbed through the gastrointestinal tract and are converted to the N-acetylcysteine derivatives by N-acetyltransferases present in kidneys. The formation of GSH conjugates of electrophilic compounds has generally been regarded as a detoxification process. However, conjugation with GSH has also been implicated in the activation of some xenobiotics to mutagenic and carcinogenic electrophiles (Lash and Anders, 1986; Vamvaka et al., 1987; Anders et al., 1988; Monks and Lau, 1989; Koob and Dekant, 1991). Furthermore, evidence has been presented showing that GSH adducts of a variety of compounds and/or their corresponding cysteine conjugates are nephrotoxic (Elfarra and Anders, 1984; Nash et al., 1984; Monks et al., 1985; Anders et al., 1987; Monks and Lau, 1989).

Acetaminophen, a commonly used analgesic, has been shown to produce a GSH adduct via an interaction between its metabolic intermediate, NAPQI, and endogenous GSH (Dahlin et al., 1984). The GSH adduct of acetaminophen, 3-(glutathion-S-yl)acetaminophen (M5) has been characterized before (Hinson et al., 1982). In this study, the GSH adduct of acetaminophen and the products resulting from further processing of this conjugate via the mercapturic acid pathway were characterized spectroscopically. In addition to these metabolites, a number of peptide conjugates were isolated from rat bile and characterized spectroscopically. The MS and NMR analyses of these conjugates confirmed the presence of additional glutamic acid moieties linked to the intact GSH, cysteinylglycine, and cysteine adducts of acetaminophen. The addition of one and two glutamic acids to the GSH adduct of acetaminophen produced metabolites M6 and M7, respectively. The addition of glutamic acid was also observed with the cysteinylglycine and cysteine conjugates of acetaminophen to produce M5 (GSH adduct) and M8, respectively. Metabolites M8 and M9 were produced as a result of conjugation of one and two glutamic acids to the cysteine conjugate (M3) of acetaminophen, respectively. Metabolites M8 and M9 were produced as a result of conjugation of one and two glutamic acids to the cysteine conjugate (M3) of acetaminophen, respectively. The enzyme responsible for mediating the addition of glutamic acid to cysteine, cysteinylglycine, and GSH adducts in the glutamic acid pathway is currently unknown. It is postulated that γ-glutamyltranspeptidase could be involved in the formation of these conjugates. The natural occurrence of γ-glutamyl-GSH, a polyanionic tetrapeptide, has been described previously (Abbott et al., 1986). The formation of γ-Glu-GSH from endogenous GSH was demonstrated to have been mediated by γ-glutamyltranspeptidase (Abbott et al., 1986). Studies are ongoing to determine the nature of the enzyme or enzymes responsible for the “glutamic acid pathway”.

The coupling of glutamic acid to other amino acids was demonstrated to occur via its γ-carboxylic acid group. This was confirmed by comparing the NMR and MS data of the isolated metabolites with those of synthetic standards. The

![Fig. 5. TOCSY of the tetrapeptide conjugate, 3-(γ-glutamyl-glutathion-S-yl)acetaminophen (M6), isolated from rat bile.](image-url)
identification of metabolites M8 and M9 was important because it confirmed that the enzymatic addition of glutamate was taking place on amino acids other than just the glutamate of the GSH adducts. Furthermore, it was demonstrated from in vivo studies that the cysteinylglycine conjugate itself could be converted to the GSH conjugate via the aforementioned glutamic acid pathway. However, it was shown that mercapturic acid pathway is more dominant than the glutamic acid pathway.

The discovery of these tetrapeptide and pentapeptide conjugates of acetaminophen represents a novel disposition of GSH adducts of compounds. The degradation of GSH adducts via the mercapturic acid pathway is the only one that has been described in the literature (Commandeur et al., 1995, and the literature cited therein). GSH adducts undergoing catabolism via mercapturic pathway lead to smaller molecules such as cysteinylglycine and cysteine conjugates, which are normally excreted in bile. In this study, we have uncovered an alternate disposition pathway for GSH adducts (and for their breakdown products formed via the mercapturic acid pathway) in which the conjugates were taken as substrates by an unknown enzyme that added glutamic acids in an ordered manner (see Scheme 1). This pathway seems to act in the opposite manner compared with the mercapturic acid pathway. Although the mercapturic acid pathway represented essentially a degradation route for GSH adducts, the newly discovered glutamic acid pathway appears to build on the GSH adducts (and their breakdown products from mercapturic acid pathway) by adding elements of glutamic acid. As shown in Scheme 1, glutamic acid was added sequentially to the GSH adducts of acetaminophen to give metabolites M6 and M7. The addition of glutamic acid was not only confined to the glutamic acid moiety of the GSH adduct; instead, products were found in which the glutamic acid was added sequentially to the cysteine to give metabolites M8 and M9 or to the cysteinylglycine adduct to give M5. The structures of these dipeptides, tripeptides, tetrapeptides, and pentapeptides suggested that the γ-carboxyl group of glutamic acid was activated and subsequently added to the α-amino group of the conjugates. This type of reaction has gone unnoticed for a number of reasons. Analytical techniques, such as LC/MS and LC/NMR, were not available in the past to characterize these products that were once considered trivial and nonidentifiable. In addition, because of their low abundance in bile and their close proximity to the GSH adducts during chromatography, these peptides were easily overlooked (see Fig. 2). Numerous metabolism studies in the past with radiolabeled compounds have shown the existence of radioactive peaks in radiochromatograms that were not characterized. It is quite possible that some of the radioactivity could have been due to products resulting from glutamic acid pathway. With the advent of LC/MS, LC/NMR, and high field NMR, it has become much easier to characterize compounds that were once considered unidentifiable by previously existing techniques. LC/MS has been found to be useful tool in characterizing polar metabolites such as the GSH adducts (Pallante et al., 1986; Haroldsen et al., 1988, Draper et al., 1989; Muck and Henion, 1990; Burlingame et al., 1992, and the references cited therein; Baillie and Davis, 1993). However, there are limitations with MS in obtaining complete.
structural information on these metabolites. More frequently, NMR has been found to be a complementary tool in elucidating structures of complex metabolites such as those described in this report. Metabolites formed as very polar dicongjugates have recently been confirmed by using a combination of such techniques (Spraul et al., 1993; Mutlib et al., 1995, 1999; Shockcor et al., 1996; Tang and Abbott, 1996; Lindon et al., 1997).

The characterization of these peptides provides very convincing evidence of the existence of a competing pathway ("glutamic acid pathway") for GSH conjugates. Although the levels of these peptides appeared low in bile compared with the GSH adducts and the mercapturic acid products, the amount of these products formed intracellularly is not known. The formation of tetrapeptides and pentapeptides suggests the possibility that larger peptides were made by cells (probably elongation of the peptide chain by the sequential addition of glutamic acid via the γ-carboxylic acid linkage) that were not detected in this study. These probably were not identified because they were confined to cells (i.e., could not be transported out) and hence were not eliminated in bile. In vivo studies with M4 and in vitro studies with M5 incubated with rat bile or rat kidney/liver S9 fractions suggested that the mercapturic acid pathway was the predominant route for the disposition of GSH adducts. The contribution toward disposition of the GSH adducts by the glutamic acid pathway appears to be minor in comparison with the mercapturic acid pathway. A number of in-house discovery compounds that were structurally different from acetaminophen and were capable of producing GSH adducts were found to produce metabolites via the glutamic acid pathway.

The formation of such conjugates may represent another pathway by which drugs could produce covalent binding via their reactive metabolic intermediates. Previous reports have suggested the direct interaction of reactive metabolic intermediate of compounds such as acetaminophen with proteins and cellular components leading to covalent binding and possibly toxicity (Jollow et al., 1973; Streeter et al., 1984). The results from these studies suggest an alternate route for the interaction of reactive metabolites with cellular components may exist. Because the cellular concentration of endogenous GSH is fairly high (approximately 5 mM in hepatocytes), there is a good possibility that a reactive metabolic intermediate will interact with the thiolate of GSH to a greater extent than with other protein components. This in turn leads to the GSH adducts such as metabolites M5 of acetaminophen. Subsequent processing of these adducts via this novel metabolic pathway could lead to longer peptides (e.g., M6 and M7) in which the incorporated moiety includes the trapped reactive metabolic intermediate already linked to cysteine or GSH. The consequence of having peptides such as the ones described in this report is not known. It is possible that these peptides could disrupt cellular functions with toxicological consequences. Furthermore, the retention of exogenous compounds linked to peptides could potentially have immunological consequences such as the production of allergies or idiosyncratic reactions. The metabolic and physiological significance of existence of such drug-peptide conjugates
requires additional study. One must also consider the possibility that these conjugates have no function and that their formation represents a side reaction catalyzed by the \( \gamma \)-glutamyltranspeptidase.

The formation of peptide conjugates of acetaminophen was confirmed by MS and NMR analyses of isolated metabolites. It was shown that these peptides were formed by the addition of (activated) glutamic acid via the \( \gamma \)-carboxylic acid functional group to the \( \alpha \)-amino group of the adducts. The glutamic acid pathway appears to function in the opposite manner to mercapturic acid pathway. Longer peptide conjugates are produced via this metabolic pathway compared with the mercapturic acid pathway, which essentially degrades the GSH adducts to smaller molecules.

References


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