Characterization of Novel Isocyanate-Derived Metabolites of the Formamide *N*-Formylamphetamine with the Combined Use of Electrospray Mass Spectrometry and Stable Isotope Methodology

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Bioactivation of the formamide *N*-formylamphetamine (NFA) to 1-methyl-2-phenylethyl isocyanate (MPIC) was investigated in rats by screening bile and urine for conjugates subsequent to the phase I event. NFA was administered to rats as a mixture of protio- and pentadeuteriophenyl analogues to gain insight into the carbamoylating activity of MPIC when traced by electrospray liquid chromatography/mass spectrometry (LC/MS). An LC/MS contour generated by recording the summed mass spectrum as a function of chromatographic retention time allowed four biliary metabolites to be identified from four sets of doublets, with the peak of each doublet offset by 5 amu and *ca.* 0.07 min. Tandem mass spectrometry experiments allowed these metabolites to be attributed structurally to the glutathione, cysteinylglycine, cysteine, and *N*-acetylcysteine conjugates of the isocyanate MPIC. These assignments were subsequently validated by comparison of the LC/MS properties of the metabolites to synthetic reference compounds. Only the carbamoylated *N*-acetylcysteine conjugate was detected in urine. The observed excretion in bile of all metabolites of the mercapturate pathway is novel for formamide metabolism. NFA can thus be added to the short list of compounds that are eliminated in this fashion. Factors envisioned as contributory to this metabolic profile in bile include hepatorenal, enterohepatic, and biliary–hepatic cycling, in addition to possible equilibrium exchange of the isocyanate from thiocarbamate conjugates to endogenous free thiols during the course of biliary transit.

Introduction

Human exposure to formamides usually occurs within laboratory or industrial settings where these compounds, primarily *N*-methylformamide (NMF)[1] and *N,N*-dimethylformamide, are routinely employed as solvents. NMF was also briefly implemented as a chemotherapeutic agent; however, a high incidence of severe hepatotoxicity rendered the drug unacceptable for therapeutic application (*1*). Strategies employed to uncover the mechanism of NMF hepatotoxicity strongly suggested that bioactivation of the parent compound was involved (*2*, *3*); and the discovery of *N*-acetyl-*S*-(methylcarbamoyl)cysteine (*4*) and *S*-(methylcarbamoyl)glutathione (*5*) as metabolites strongly implicated the reactive electrophile methyl isocyanate (MIC) as the offending agent. NMF toxicity is now generally viewed as a consequence of metabolic activation of the secondary formamide function to MIC (*6*).

Despite the interest shown in the metabolism of NMF and low molecular weight formamides, few studies in the literature have focused on high molecular weight formamides (*7*, *8*). Notwithstanding, large formamides are toxicologically relevant as evidenced by the occurrence of *N*-formyl derivatives as contaminants in illicit preparations of amphetamine analogues (*9–11*). Studies in our laboratory with *N*-(1-methyl-3,3-diphenylpropyl)formamide as a prototype high molecular weight formamide revealed that this compound, like NMF, is bioactivated in rats to *N*-(1-methyl-3,3-diphenylpropyl) isocyanate which, in turn, gives rise to carbamoylated glutathione (GSH) and *N*-acetylcysteine (NAC) metabolites (*8*). Interestingly, in vitro studies revealed that *N*-(1-methyl-3,3-diphenylpropyl) isocyanate, unlike MIC, was unusually stable and could be detected as such in equilibrium with its GSH conjugate.

The stability of *N*-(1-methyl-3,3-diphenylpropyl) isocyanate, when considered along with the fact that thiocarbamates act as transport forms of isocyanates (*12*), introduced the likelihood that large formamides could elicit widespread carbamoylation of biological molecules in vivo. In order to test this hypothesis, we directed our attention to *N*-formylamphetamine (NFA), which is metabolized in rats to biliary *S*-((1-methyl-2-phenylethyl)carbamoyl)glutathione (SMPG) and urinary *N*-acetyl-*S*-((1-methyl-2-phenylethyl)carbamoyl)cysteine (NMPC), quite likely through the intermediacy of 1-methyl-2-phenylethyl isocyanate (MPIC) (*13*). In our continuing effort to elucidate the involvement of MPIC in the metabolism of NFA, we elected to employ LC/MS, LC/MS/MS, and stable isotope methodology. The utility of

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[1] Abbreviations: CAD, collisionally activated dissociation; GSH, glutathione; LC/MS, liquid chromatography/mass spectrometry; LC/MS/MS, liquid chromatography/tandem mass spectrometry; MIC, methyl isocyanate; MPIC, 1-methyl-2-phenylethyl isocyanate; NAC, *N*-acetyl-L-cysteine; NFA, *N*-formylamphetamine; NMF, *N*-methylformamide; NMPC, *N*-acetyl-*S*-((1-methyl-2-phenylethyl)carbamoyl)cysteine; Q_1, quadrupole 1 (in the triple quadrupole MS); SMPC, *S*-((1-methyl-2-phenylethyl)carbamoyl)cysteine; SMPCG, *S*-((1-methyl-2-phenylethyl)carbamoyl)cysteinylglycine; SMPG, *S*-((1-methyl-2-phenylethyl)carbamoyl)glutathione; SRM, selected reaction monitoring; *t*-BOC, *tert*-butyloxycarbonyl; TFA, trifluoroacetic acid; TIC, total ion current; γ-GT, γ-glutamyltranspeptidase.

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these techniques in metabolite profiling has been aptly demonstrated by Weidolf and Covey (*14*), who recorded the LC/MS data of an entire chromatographic run as a summed background−subtracted mass spectrum. In order to improve on this approach, the study reported here implements LC/MS contour-mapping, which allows the presentation of chromatographic and mass spectral data together as a two-dimensional array. This method was used to decipher isotopic clusters attributable to metabolites in rats which were administered NFA as a mixture of *protio* and *deuterio* analogues.

Experimental Section

Materials. Samples of (±)-*N*-formylamphetamine and (±)-amphetamine sulfate were supplied by Dr. R. D. Hossie of the Bureau of Dangerous Drugs, Health Protection Branch (Ottawa). Other chemicals were obtained from the following sources: $AlCl_3$, C_6D_6, D_2O, dicyclohexylcarbodiimide, *N*-hydroxysuccinimide, and 4-nitrophenyl chloroformate: Aldrich Chemical Co. (Milwaukee, WI); *N*-*t*-BOC-*S*-(3,4-dimethylbenzyl)-L-cysteine: Bachem Inc. (Torrance, CA); glycine, analytical reagent grade solvents, glass distilled acetonitrile, and silica gel 60 (230−400 mesh): British Drug Houses, Inc. (Vancouver, BC); GSH, NAC, and urethane: Sigma Chemical Co. (St. Louis, MO); polyethylene tubing PE-10: Clay Adams (Parsippany, NJ); Spe octadecyl extraction columns (6 mL) and silica gel (40 μm particle diameter): J. T. Baker (Phillipsburg, NJ); and silica gel plates (AL SIL G/UV, 250 μm): Whatman Ltd. (Maidstone, Kent).

Instrumentation. LC/MS and LC/MS/MS experiments were performed on either a Sciex API *III* MS/MS system (Thornhill, ON) or a VG QUATTRO instrument (FISONS Instruments, Altrincham, Cheshire). All experiments, other than the analysis of some synthetic compounds, were performed on the SCIEX system. Settings for this instrument were ionspray voltage 4000 V, interface plate voltage 650 V, and orifice lens voltage 50 V. For Q_1 scan and LC/MS/MS experiments, dwell times were adjusted to provide a scan rate of 1.85−2.15 s/scan. The collision gas was a mixture of argon/nitrogen, 90:10 (v/v). The collision energy was set at 31 eV, and collision gas thickness was (400−450) × 10^{12} molecules/cm^2. Chromatography was performed on a Michrom BioResources UMA 600 HPLC fitted with a Reliasil C-18 (150 × 1 mm, 3 μm) column. The mobile phase consisted of solvent A: 2% acetonitrile in 0.05% trifluoroacetic acid (TFA) and solvent B: 80% acetonitrile in 0.05% TFA, and was delivered at a flow rate of 50 μL/min. Two chromatographic methods were used. Method 1: The mobile phase program was a gradient increase from 0% to 70% B at 15 min, followed by a gradient increase to 100% B at 17 min, a hold at 100% B to 29 min, a gradient decrease to 0% B at 31 min, and then a hold at 0% B to 43 min (Figures 1−5 and 7). This method was used throughout, except for the experiment in which metabolite and synthetic compounds were compared. In the latter experiment, method 2 was used. Method 2: The mobile phase consisted of 0% B for 2 min, followed by a gradient increase to 50% B at 14 min, a gradient increase to 100% B at 17 min, a hold at 100% B to 29 min, a gradient decrease to 0% B at 32 min, and then a hold at 0% B to 44 min (Figure 6). The column eluate was subject to a 1:5 proportioning through a flow-split "Tee" which introduced the reduced flow to the mass spectrometer.

The HPLC flow for experiments on the VG QUATTRO instrument was delivered at 50 μL/min by a Hewlett-Packard 1090II HPLC (Avondale, PA) with a mobile phase of methanol/water (50:50). Samples were injected in volumes of 10 μL. The ionizing voltage was 3500 V, and fragmentation conditions involved argon as the target gas at an energy of 50 eV and collision cell pressure 1.2 × 10^{-3} Torr.

GC/MS was performed on a Varian MAT-111 MS interfaced to a HP 5700A GC fitted with a 1.8 m × 2 mm i.d. glass column packed with 3% Dexsil 300 on 100/120 mesh Supelcoport. Mass spectra were recorded at 12 scans/min for 20 min. The electron impact mode of ionization was used with an energy of 70 eV and emission current of 300 μA. Injection port, line, and separator temperatures were 250 °C.

^{1}H-NMR spectra were determined on a Bruker AC-200 (200 MHz) instrument in the Department of Chemistry, University of British Columbia, and chemical shifts were measured relative to an external tetramethylsilane standard. The following terminology is used: Ar, aromatic; s, singlet; bs, broad singlet; d, doublet; dd, doublet of doublets; q, quartet; m, multiplet; and *J*, coupling constant in Hz.

Syntheses. The compounds SMPG and NMPC were previously synthesized in our laboratory (*13*).

***N*-(1-Methyl-2-(pentadeuteriophenyl)ethyl)formamide (Pentadeuteriophenyl-*N*-formylamphetamine, [$^2H_5$]NFA).** Aromatic deuteration of the formamide NFA was accomplished by $AlCl_3$-catalyzed exchange with C_6D_6 (*15*). NFA (228 mg, 1.40 mmol) was dissolved in C_6D_6, and to the stirred solution was added $AlCl_3$ (608 mg, 4.6 mmol) followed by D_2O (50 μL, 2.7 mmol). The reaction was refluxed for 24 h and then terminated with the addition of 5 mL of D_2O. The product was extracted with ether, dried (Na_2SO_4), and concentrated *in vacuo* to a yellow oil. The oil was purified by flash chromatography on a silica gel column. The silica column was successively eluted with $CHCl_3$ and ether to remove contaminating compounds and product, respectively. The product was concentrated to a pale yellow wax in quantitative yield. TLC, ether (100%): R_f 0.32 (UV detection). GC/MS: *m*/*z* 168 (1) $(M)^+$, 123 (80), 96 (30), 72 (100), 44 (55). ^{1}H-NMR ($CDCl_3$): δ 1.10 (d, *J* = 8 Hz, 2H, CH_3), 1.30 (d, *J* = 8 Hz, 1H, CH_3), 2.65−2.90 (m, 2H, CH_2), 3.70 (m, 0.2H, CH_2CH), 4.30 (m, 0.8H, CH_2CH), 5.60 (bs, 1H, NH), 7.10−7.30 (m, 0.02H, Ar-H), 7.60 (d, *J* = 10 Hz, 0.2H, CHO), 8.10 (d, *J* = 2 Hz, 0.8H, CHO). Isotopic purity was 99 atom % D based on ^{1}H-NMR integration of the aromatic protons and ≥96 atom % D based on MS analysis. NMR indicated the presence of rotamers of the formamide.

Synthesis of Thiol Conjugates. (A) 4′-Nitrophenyl (1-Methyl-2-phenylethyl)carbamate. Amphetamine free base, obtained by extraction of a basified aqueous solution of the sulfate salt with $CHCl_3$, was dried ($MgSO_4$) and concentrated under mild vacuum (4 mmHg, 3 h) to a yellow oil. 4-Nitrophenyl chloroformate (707 mg, 3.51 mmol) was added to a solution of amphetamine (294 mg, 2.18 mmol) in dry CH_2Cl_2. The flask was purged with N_2, capped with a septum, and stirred for 24 h. The reaction was concentrated to an oil and purified on a silica column (15 × 1.5 cm) with $CHCl_3$/ethyl acetate (20:1 v/v) to afford 260 mg (40%) of product as pale yellow flakes. TLC, $CHCl_3$/ethyl acetate (20:1 v/v): R_f 0.44 (UV detection). ^{1}H-NMR ($CDCl_3$): δ 1.30 (d, *J* = 8 Hz, 3H, CH_3), 2.90 (t, 2H, CH_2), 4.05 (m, 1H, CH), 5.00 (d, *J* = 8 Hz, 1H, NH), 7.10−7.40 (m, 7H, amphetamine-ArH, $ArNO_2$ H-2/H-6), 8.25 (d, 2H, *J* = 10 Hz, $ArNO_2$ H-3/H-5).

(B) *S*-((1-Methyl-2-phenylethyl)carbamoyl)cysteine (SMPC). Cysteine (12.3 mg, 0.10 mmol) and 4′-nitrophenyl (1-methyl-2-phenylethyl)carbamate (0.11 mmol) were dissolved in acetonitrile/water (1:1 v/v), and the solution was adjusted to pH 8.6 and stirred for 90 min. The reaction was terminated by acidifying to pH 4.0. The acetonitrile was removed under vacuum and the aqueous fraction extracted with ether. The product in the aqueous fraction was applied to a C-18 solid phase extraction cartridge washed with water (3 × 6 mL) and eluted with methanol (5 × 6 mL). Concentration of the methanolic extract to dryness afforded 6 mg (19%) of product as white crystals. Direct injection LC/MS: *m*/*z* 321 (15) $(M + K)^+$, 283 (100) MH^+, 122 (25). LC/MS/MS, parent ion = *m*/*z* 283: *m*/*z* 283 (100) MH^+, 122 (85), 119 (45), 105 (8), 91 (65), 76 (20). In its acid form, SMPC could not be obtained in sufficiently concentrated solution in either methanol, water, acetonitrile, or DMSO for NMR analysis. The sodium salt was obtained by titrating to pH 8.5 with NaOH solution, concentrating the solution to dryness, and redissolving in D_2O. HPLC and NMR analysis revealed that decomposition had occurred. The following resonance signals were considered characteristic of the

product SMPC. ^{1}H-NMR (D_2O): δ 1.09 (d, J = 7 Hz, CH_3), 2.65 (m, CH_2CH), 2.75 (dd, J = 10 and 6 Hz, CH_2CH), 3.00 (m, Cysβ), 3.08 (dd, J = 18 and 4 Hz, Cysβ'), 3.92 (m, $CHCH_3$), 4.25 (m, Cysα), 7.25 (m, C_6H_5).

(C) S-((1-Methyl-2-phenylethyl)carbamoyl)cysteinylglycine (SMPCG). *N-t*-BOC-S-(3,4-dimethylbenzyl)-L-cysteinylglycine. *N-t*-BOC-*S*-(3,4-dimethylbenzyl)-L-cysteine (338.3 mg, 1.0 mmol) and *N*-hydroxysuccimide (127.8 mg, 1.08 mmol) were dissolved in 3 mL of dry dioxane with stirring at 10 °C. Dicyclohexylcarbodiimide (243 mg, 1.17 mmol) was added to the solution at 10 °C, the reaction allowed to reach room temperature, and stirring continued for another 5 h to afford a white suspension. The suspension was filtered, the filtrate added to an aqueous solution (3 mL) of glycine (90.4 mg, 1.20 mmol) and $NaHCO_3$ (129 mg, 1.54 mmol), and the reaction mixture stirred for 24 h. The reaction was concentrated under vacuum to remove dioxane, extracted (basic) with ethyl acetate (2 × 10 mL), and adjusted to acidic pH with 4.0 M HCl, and the desired product extracted (acidic) with ethyl acetate (3 × 10 mL). The crude product was concentrated and purified by flash chromatography (ether/acetic acid, 50:0.1 v/v) to afford a pale yellow oil. TLC, ether/acetic acid (10:0.1): R_f 0.67 (UV). LC/MS: m/z 395 (40) $(M - H)^-$, 321 (50) $(M - \text{glycine})^-$.

Cysteinylglycine. *N-t*-BOC-*S*-(3,4-dimethylbenzyl)-L-cysteinylglycine was taken up in anisole and transferred to a Teflon tube for HF(l) deprotection. The tube was evacuated, and HF, introduced as a gas, was allowed to condense at −10 °C (ice/NaCl) in the vessel for the reaction to proceed. The reaction was stirred for 45 min at −10 °C and a further 2 h at room temperature. HF was removed under vacuum to afford the product as an insoluble waxy solid in anisole. The product was taken up into H_2O (1 mL), extracted with ether (4 × 3 mL), and concentrated *in vacuo* to afford 118 mg (67%) of product as a white powder. LC/MS/MS, parent ion = m/z 179: m/z 179 (100) MH^+, 162 (4), 87 (4), 59 (20), 43 (8).

S-Carbamoylation of cysteinylglycine was accomplished as described above to afford the product as a white powder in 25% yield. Direct injection LC/MS: m/z 378 (55) $(M + K)^+$, 340 (100) MH^+, 179 (10), 119 (10). LC/MS/MS, parent ion = m/z 340: m/z 340 (100) MH^+, 179 (45), 162 (28), 119 (25), 91 (20), 76 (75). ^{1}H-NMR (D_2O): δ 1.20 (d, J = 10 Hz, 3H, CH_3), 1.30 (m, 0.2H)[a], 2.65 (m, 1H, CH_2CH), 2.90 (m, 1H, CH_2CH), 3.2−3.5 (m, 2H, Cys$\beta\beta'$), 4.05 (d, 1H)[b], 4.10 (s, 2H, Gly$\alpha\alpha'$), 4.20 (m, 1H, $CHCH_3$), 4.45 (m, 1 H, Cysα). [a]Resonance characteristic of possible minor MPIC decomposition product. [b]Uncharacterized contaminant.

Animal Experiments. Three male Sprague-Dawley rats (Animal Care Facility, University of British Columbia, Vancouver) weighing 275−350 g were dosed ip with $[^2H_0]$NFA/$[^2H_5]$NFA (50:50 w/w) at 20 mg/kg (injection solution 20 mg/mL in DMSO) or with DMSO only (control). Rats were housed in metabolic cages with access to food and water, and urine was collected for 24 h postdose. Subsequent to urine collection, rats were prepared for the collection of bile. Rats were anesthetized with an intraperitoneal injection of urethane (1.2 g/kg) which was administered as an aqueous solution (0.4 g/mL). After anesthesia was induced (*ca.* 30 min), an incision was made on the ventral surface of the animal, the region of the bile duct at the duodenal junction cannulated with PE-10 tubing, and the incision closed. Immediately after cannulation, animals were dosed for a second time with $[^2H_0]$NFA/$[^2H_5]$NFA as described above and bile was collected for 4 h over sodium acetate buffer (1.0 M, pH 5.0). Throughout the collection of bile, animals remained under anesthesia.

Aliquots (2 mL) of pooled bile and pooled urine were extracted with ethyl acetate (4 × 3 mL), and the aqueous fraction was applied to a column of XAD-2 resin (7 × 1.5 cm). The column was washed with water (30 mL) and dried with air. The metabolite-containing fraction was eluted with methanol (50 mL), concentrated to dryness, and reconstituted in 0.5 mL of methanol for analysis.

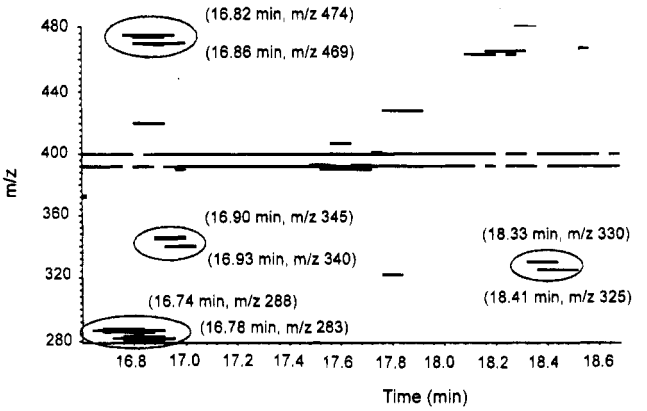


Figure 1. Partial LC/MS Q_1 scan of the bile of rats dosed with $[^2H_0]$NFA/$[^2H_5]$NFA plotted as a contour. The summed mass spectrum (y-axis) is plotted as a function of retention time (x-axis) with ion abundance depicted in the z-direction. This sector shows the appearance of the isotopically related doublets m/z 469/474, m/z 283/288, m/z 340/345, and m/z 325/330.

Results

The metabolism of NFA was examined with a specific emphasis on characterizing the biliary and urinary metabolites which could arise as carbamoylation products of the isocyanate MPIC. The procedure involved the combined use of LC/MS, LC/MS/MS, and stable isotope methodology. NFA, prepared as a mixture of *protio* and *deuterio* analogues ($[^2H_0]$NFA/$[^2H_5]$NFA, 50:50 w/w), was administered ip to rats, and the bile and urine were analyzed. A Q_1 scan of bile was performed in the mass range m/z 250−750, and the resulting TIC mass chromatogram (x-axis) and summed mass spectrum (y-axis) were constructed as a contour using SCIEX MacSpec3.2 software. The chromatographic run (acquisition duration 30 min) and mass range (500 amu) were divided into sectors as typified in Figure 1 in order to better identify isotopically related doublets as NFA metabolites. In the partial contour shown in Figure 1, four doublets at m/z 469, 474; m/z 283, 288; m/z 340, 345; and m/z 325, 330, each pair offset by 0.03−0.08 min, suggested the presence of potential biotransformation products of NFA. In order to identify the structures of the compounds related to these ion doublets, collisionally activated dissociation (CAD) experiments were performed, with each of the eight ions serving as the precursor species.

In order to systematize the fragmentation nomenclature of the conjugates of GSH and its derivatives, it was necessary to incorporate descriptions of fragment formation along the peptide chain and across the thiol linkage. Description of the fragmentation of GSH and NAC conjugates along the peptide backbone is based on the nomenclature of Roepstorff and Fohlman (*16*). In order to address the issue of thiol-associated cleavages, the nomenclature of Deterding *et al.* (*17*) is employed. According to this convention, fragmentation is partitioned on either side of the *S*-linkage. R_G denotes GSH-, cysteine-, or NAC-derived fragments, whereas R_X represents the xenobiotic-derived moiety. In this report, proton transfers are not detailed.

LC/MS/MS of MH^+ at m/z 469 and MH^+ at m/z 474 (Figure 2) suggested the presence of $[^2H_0]$SMPG and $[^2H_5]$-SMPG, respectively, consistent with the report of SMPG as a biliary metabolite of NFA (*13*). The ions m/z 179 and 162 which were common to both fragment ion spectra were attributed to R_GSY_2 and R_GSZ_2, derived from the peptide backbone of GSH. Two fragments of the precur-

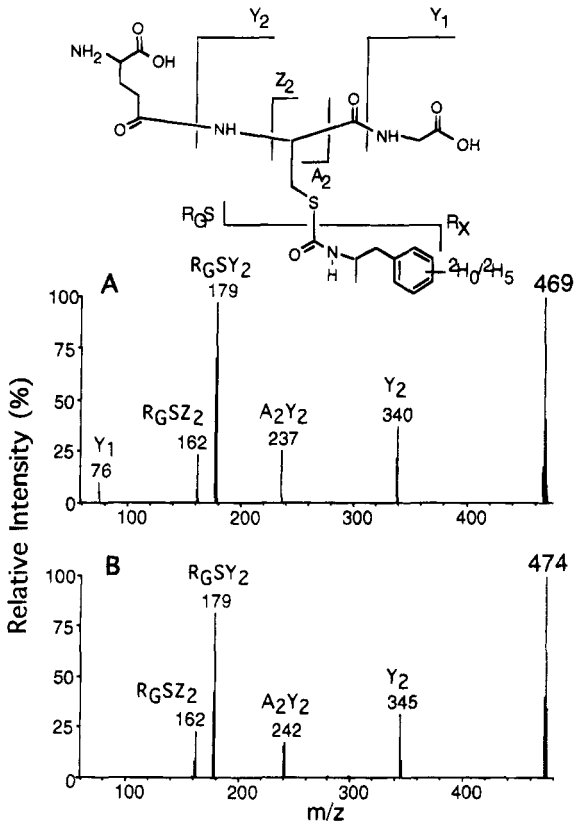


Figure 2. Fragment ion spectra obtained by CAD of the precursor ion MH^+ of the biliary metabolites (A) [$^2H_0$]SMPG, MH^+ at m/z 469; and (B) [$^2H_5$]SMPG, MH^+ at m/z 474.

sor ion m/z 469, namely, ions m/z 237 and 340, carried information which was suggestive of their retention of the carbamoyl moiety, in that each of these was offset by 5 amu when derived from m/z 474. Accordingly, these isotopically-related fragments were consistent with [$^2H_0$]-A_2Y_2, [$^2H_5$]A_2Y_2 (m/z 237, 242), and [$^2H_0$]Y_2, [$^2H_5$]Y_2 (m/z 340, 345).

CAD of the precursor ions with MH^+ at m/z 283 and 288 revealed common fragments at m/z 76, 105, and 122 which were thought to account for R_G-CH_2, R_GS-NH_3, and R_GS, respectively, derived from the cysteinyl backbone of the metabolite SMPC (Figure 3). Supporting evidence for the assignment of this structure to the metabolite was derived from the fragments which contained the aromatic nucleus. The phenyl ring-associated fragment ions m/z 91 and 119, arising from CAD of MH^+ at m/z 283, parallelled the formation of ions m/z 96 and 124 proceeding from CAD of m/z 288.

The compounds in the contour with paired precursor ions MH^+ at m/z 340, 345 and MH^+ at m/z 325, 330 were not present at sufficient concentrations for a complete fragment ion spectrum to be obtained over the mass ranges selected, *viz.*, m/z 30–355 for CAD of MH^+ at m/z 340, 345, and m/z 30–340 for MH^+ at m/z 325, 330. Subdivision of these ranges to improve ion statistics allowed diagnostic fragments to be ascertained in a piecemeal fashion. Fragment ions m/z 179 and 162 registered prominently for both precursor ions with MH^+ at m/z 340, 345 and were viewed as originating from the peptide backbone of a putative cysteinylglycine conjugate of MPIC. SRM of the transitions m/z 340/179, m/z 340/162 coincident at 17.12 min and m/z 345/179, m/z 345/162 coincident at 17.07 min substantiated the identities

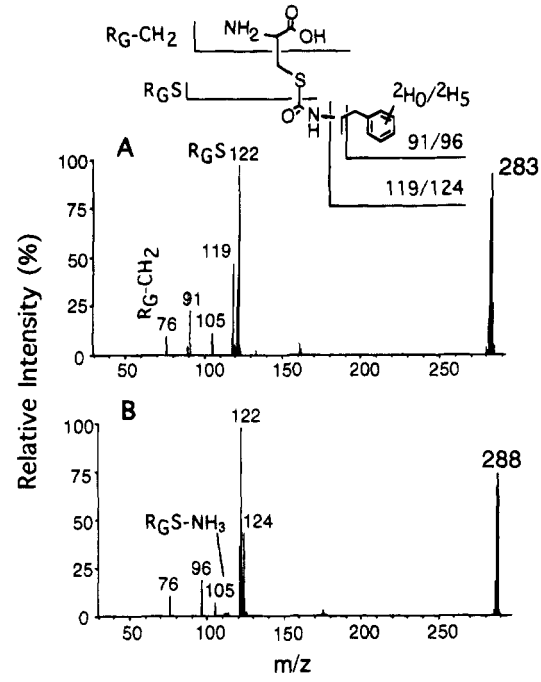


Figure 3. Fragment ion spectra obtained by CAD of the precursor ion MH^+ of the biliary metabolites (A) [$^2H_0$]SMPC, MH^+ at m/z 283; and (B) [$^2H_5$]SMPC, MH^+ at m/z 288.

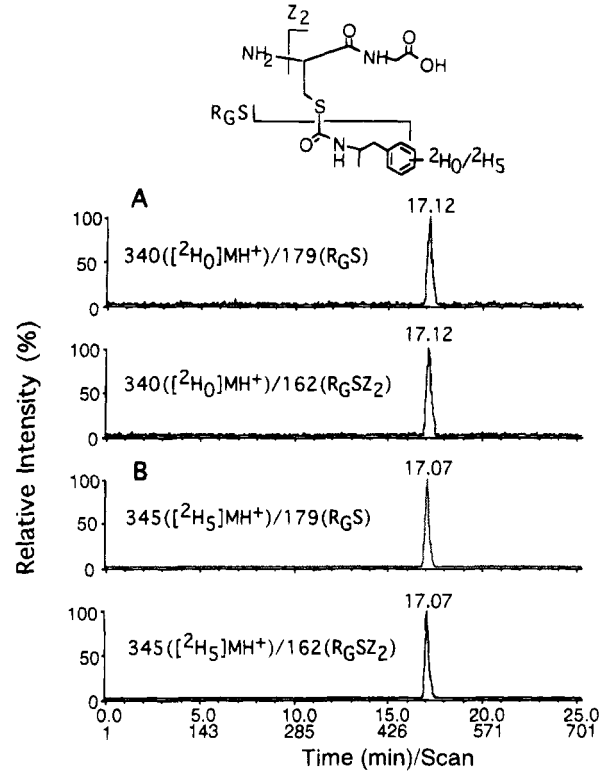


Figure 4. LC/MS/MS analysis of bile for the metabolite SMPCG performed by SRM for transitions proceeding from the precursor ion species (A) [$^2H_0$]SMPCG, MH^+ at m/z 340; and (B) [$^2H_5$]SMPCG, MH^+ at m/z 345.

of [$^2H_0$]- and [$^2H_5$]-*S*-((1-methyl-2-phenylethyl)carbamoyl)cysteinylglycine ([$^2H_0$]-, [$^2H_5$]SMPCG), respectively, in accordance with the observed precursor/fragment association, and the characteristic earlier retention time of the *deuterio* analogue before its *protio* partner (Figure 4). In an experiment of similar design, the putative metabolite *N*-acetyl-*S*-((1-methyl-2-phenylethyl)carbamoyl)cysteine (NMPC) was identified by monitoring of the

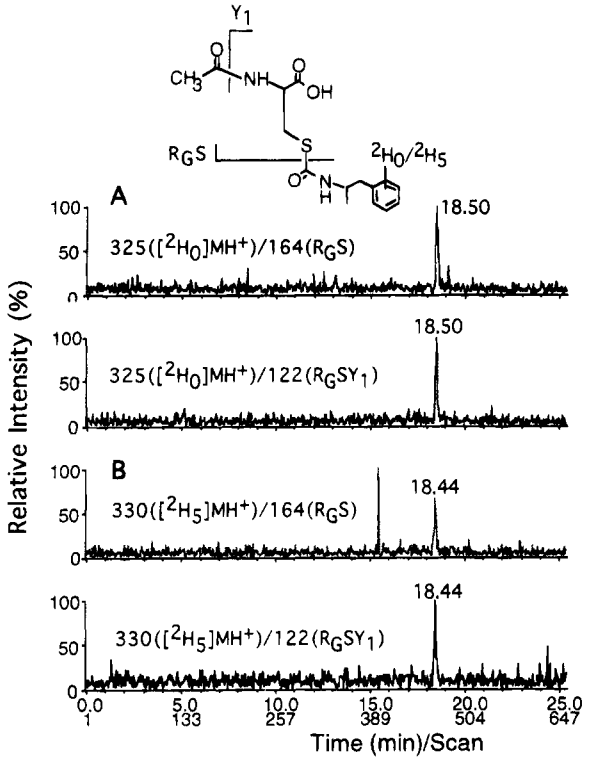


Figure 5. LC/MS/MS analysis of bile for the metabolite NMPC performed by SRM for transitions proceeding from the precursor ion species (A) $[^2H_0]$NMPC, MH^+ at m/z 325; and (B) $[^2H_5]$-NMPC, MH^+ at m/z 330.

transitions m/z 325/164 and m/z 325/122, ascribed to MH^+/R_GS and MH^+/R_GSY_1, respectively (Figure 5).

In order to validate the proposed structural assignments for each of the putative metabolites, authentic reference compounds were made available by synthesis. Comparison of the LC/MS properties of the biliary metabolites to the synthetic standards under SRM confirmed structural assignments (Figure 6). Screening of urine for the thiol conjugates by monitoring their corresponding transitions, SMPC (m/z 283/122), NMPC (m/z 325/122), SMPCG (m/z 340/179), and SMPG (m/z 469/179), revealed the presence of only the NAC conjugate (Figure 7).

Discussion

Mass Spectrometry. The metabolism of NFA in rats was examined with a view to characterizing the chemical species that arise as a consequence of in vivo carbamoylation. Phenyl-deuterated NFA was synthesized for administration to rats in order to screen for metabolites downstream of the primary product MPIC by LC/MS and LC/MS/MS. $[^2H_5]$NFA was considered to be an ideal candidate as a stable isotope-labeled analogue because aromatic protons are chemically inert under physiological conditions, and biotransformations involving aromatic proton removal are subject to modest isotope effects (*18*) and consequently minimal metabolic switching.

The first step in the profiling strategy as applied to bile involved the plotting of Q_1 scan data as a contour using SCIEX MacSpec3.2 software (Figure 1). By this approach the summed mass spectrum (y-axis) was resolved along a chromatographic time axis. The advantage of presenting these data together in a two-dimensional array is that it allows the facile discernment of the m/z value(s) associated with each chromatographic peak in a single display (*19*). In a previously reported isotope-cluster study, the entire duration of a chromatographic run was recorded as a single, summed, background-subtracted mass spectrum to decipher metabolite-related doublets (*14*). Because the isotope clusters varied dramatically in ion intensity, those which were present at low abundance were not readily apparent. LC/MS contour-mapping can specifically address this type of situation since the chromatographic resolution of a two-dimensional plot can unmask low intensity doublets.

Distinctly apparent in the display of LC/MS data as a contour was the fact that doublets were not completely symmetrical but skewed to the lower molecular weight with increasing retention time (Figure 1). *Deuterio* analogues preceded their *protio* partners by 0.04–0.07 min, consistent with the higher polarity displayed by the former under reverse phase HPLC conditions (*20*). One of the inherent limitations of this approach is the uncorrected registration of background interference as was apparent at m/z 390–394 and m/z 398–402 (Figure 1). Nonetheless, once doublets were identified in the contour, subtraction of background elements at time intervals before and after the doublet allowed a useful summed mass spectrum to be reconstructed if necessary.

This report, as well as previous studies (*14*, *21*, *22*), serves to underscore the synergistic utility of LC/MS, LC/MS/MS, and stable isotope methodology in the characterization of metabolite entities. In the work here, SMPG was confirmed as a biliary metabolite of NFA (*13*), and interestingly, MPIC derivatives of cysteinylglycine, cysteine, and NAC were identified as novel metabolites in bile. Only the latter metabolite was detected in urine.

Excretion of Carbamoylated GSH and NAC Conjugates. The formation of SMPG as a biliary metabolite was thought to be a consequence of NFA bioactivation to MPIC which reacted with hepatic GSH to afford the conjugate (Scheme 1). However, the additional finding of carbamoylated cysteinylglycine, cysteine, and NAC conjugates as metabolites in bile was very interesting. In the usual course of the mercapturic acid cascade, GSH and NAC conjugates are preferentially excreted in bile and urine, respectively, because of their physicochemical properties (*23*). This principle holds true for carbamoylated GSH and NAC conjugates as excretory products of nitrosoureas (*24*, *25*) and formamides (*4*, *5*, *8*, *13*). The biosynthesis and disposition of mercapturic acids are, for the most part, explained by the hepatorenal coordinated model proposed by Inoue *et al.* (*26*, *27*). According to this model, GSH conjugates formed in the liver, where the activities of cytochrome P450-mediated xenobiotic bioactivation and GSH conjugation are relatively high (*28*), are transported by the circulatory system to the kidney where the sequential activity of γ-GT and nonspecific peptidases continues the cascade to the cysteinyl conjugate (*29*). The cysteinyl conjugate, in turn, is either *N*-acetylated in the kidney or returned *via* the circulatory system to the liver for this biotransformation to occur before final renal elimination (*26*, *27*).

The construct of this model does not account for the presence of *all* the mercapturic acid pathway conjugates of MPIC found as biliary metabolites of NFA. A review of the literature revealed that the biliary excretion of GSH, cysteinylglycine, cysteine, and NAC conjugates observed here for NFA was an uncommon phenomenon and has been reported for the in vivo metabolism of very few compounds including pentachlorothioanisole (*30*),

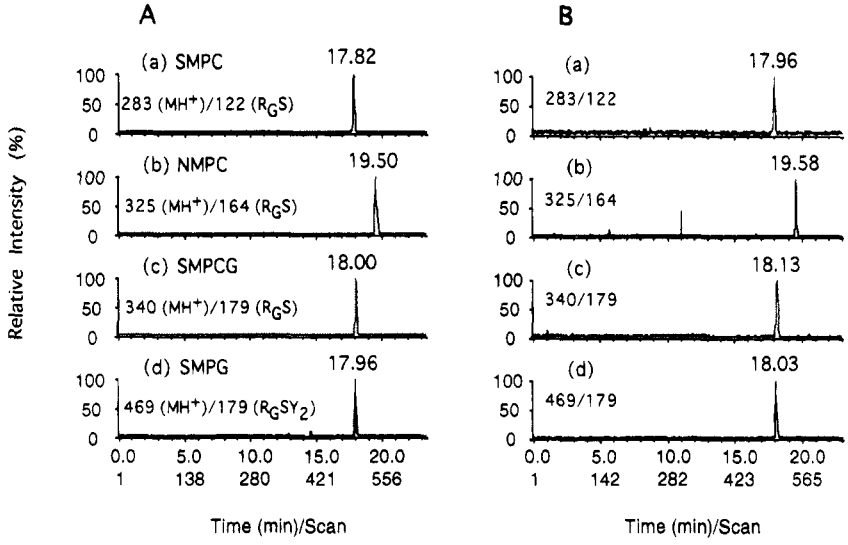


Figure 6. Ion current chromatograms for (A) the synthesized reference compounds (a) SMPC, m/z 283/122; (b) NMPC, m/z 325/164; (c) SMPCG, m/z 340/179; and (d) SMPG, m/z 469/179, obtained by SRM of the appropriate precursor (MH^+)/fragment ion transitions. These were compared to the corresponding transitions (B) from bile in order to confirm the identities of the putative metabolites.

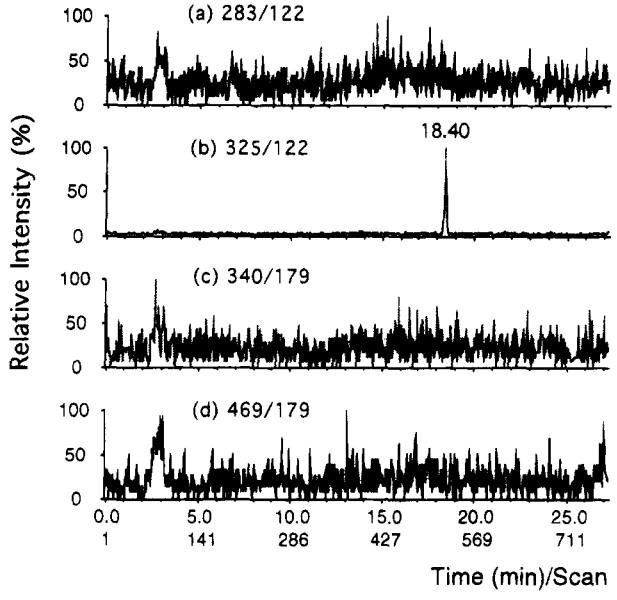


Figure 7. LC/MS/MS analysis of urine for the metabolites (a) SMPC, m/z 283/122; (b) NMPC, m/z 325/122; (c) SMPCG, m/z 340/179; and (d) SMPG, m/z 469/179 performed by SRM of the appropriate precursor (MH^+)/fragment ion transitions. Only NMPC was detected in urine.

1,2,4-trichlorobenzene (*31*), and benzyl chloride (*32*). Thus, the underlying mechanism for this pattern of metabolism and excretion needs to be addressed.

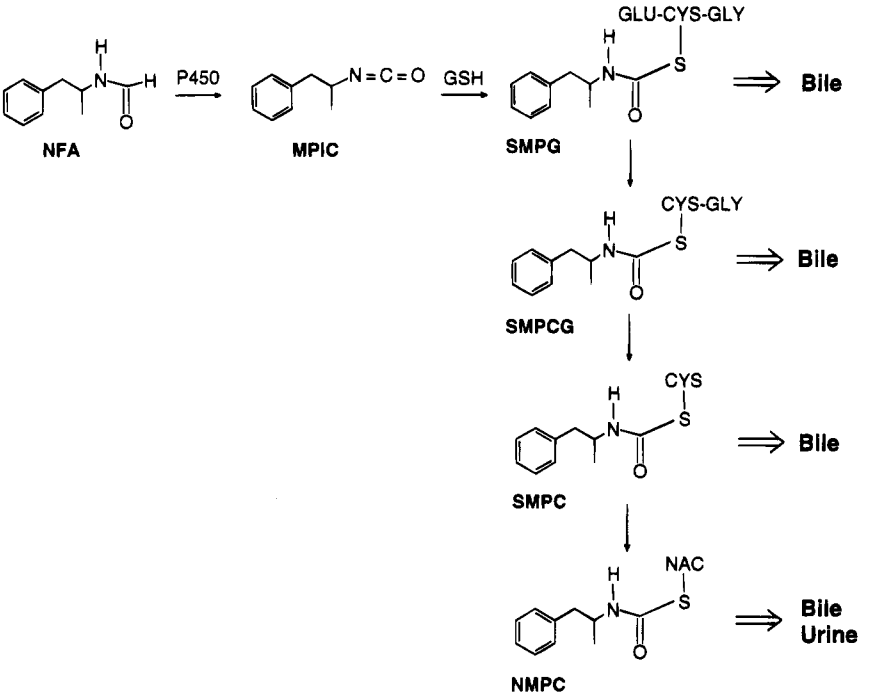
The design of this study with respect to the times of dosing and the collection of bile undoubtedly invited participation from both renal and enteric systems in the ultimate biliary elimination of mercapturate pathway metabolites. The collection of bile after two doses of NFA—one at 24 h before bile duct cannulation and another immediately thereafter—allows for extensive metabolism of MPIC conjugates by hepatorenal cycling (*26, 27*) (*vide supra*), as well as enterohepatic cycling (*28*). With respect to enterohepatic cycling, it is conceivable that the γ-GT, peptidase, and N-acetyltransferase activities present in the intestinal mucosa account for substantial conversion of SMPG to its secondary metabolites which could be transported back to the liver for further mercapturate pathway metabolism upon their second hepatic transit (*28, 33*). It is interesting that the liver possesses significant N-deacetylase activity (*34*) which, by opposing N-acetyltransferase, conceivably modulates the cysteine/NAC conjugate ratio which finally emerges upon biliary elimination.

In a recent review Hinchman and Ballatori (*35*) address the concept of the liver assuming a dominant role in the mercapturate pathway without recruitment of the kidney. When these workers infused either 0.3 or 3.0 μmol of 1-chloro-2,4-dinitrobenzene (CDNB) into isolated rat and guinea pig liver, it was found that GSH, cysteinylglycine, cysteine, and NAC conjugates were all excreted in the bile of both species (*36*). NAC conjugate formation was more efficient in the guinea pig and became saturated at the higher concentration of CDNB. It was concluded that the GSH conjugate was excreted into bile and metabolized to the cysteine conjugate, and the latter was transported back into the hepatocyte across the canalicular membrane for N-acetylation and ultimate reexport as the mercapturic acid. This suggestion is certainly tenable in the light of reported γ-GT activity in hepatocyte canaliculi (*37*), biliary tract (*38*), and bile (*39*). Thus, consistent with the findings of Hinchman and Ballatori (*35*), it is conceivable that the metabolite profile of NFA observed here could partly be the result of biliary–hepatic cycling and possibly be affected by dose.

The study by Hinchman *et al.* (*36*) brings other aspects of the metabolism of GSH conjugates into focus. Among animal species, the ratio of kidney/hepatic γ-GT activity ranks high in the rat (*40*). In this light, there is speculation that the uncommon occurrence of NAC conjugates as metabolites in the bile of rats at relatively high doses of a xenobiotic could be reflective of the saturation of hepatic γ-GT by elevated levels of GSH conjugates which are diverted from hepatic to renal mercapturate pathway metabolism and excretion.

Another consideration related to the disposition of metabolites of the mercapturic acid pathway is the route of xenobiotic administration. In the study reported here, NFA was injected ip and consequently destined for the portal circulation (*41*) and extensive first-pass metabolism (*42*). Because the bioactivation of formamides to

Scheme 1. Proposed Pathway for the Metabolism of *N*-Formylamphetamine in Rats to Carbamoylated GSH, Cysteinylglycine, Cysteine, and NAC Conjugates



isocyanates takes place to a large extent in the liver (*2*, *3*, *43*), the rapid sequence of events involving NFA bioactivation to MPIC, GSH conjugation to form SMPG, and the elimination of the GSH conjugate and its secondary mercapturate pathway metabolites is quite likely to be favored by the hepatic route.

Formamide-derived isocyanates, as a consequence of being generated in the liver, are likely to be predisposed to hepatic excretion. On this note, it is worthwhile to consider how isocyanates could themselves be metabolized and eliminated when exposure arises by extrahepatic routes. For example, inhalational exposure to the isocyanate (*44*) could conceivably result in rapid conjugation of the isocyanate to pulmonary reserves of GSH (*45*). In this situation, GSH conjugates proceeding from the lung could forseeably undergo substantial renal metabolism to NAC conjugates and be excreted in the urine because of the high levels of mercapturate pathway enzymes in the kidney (*29*, *40*).

The fact that thiol conjugation of isocyanates is an equilibrium process (*12*) could also play a role in the biliary metabolite profile of NFA. This consideration becomes apparent when it is borne in mind that GSH, cysteinylglycine, and cysteine are excreted in the bile of rats at rates of 100, 50, and 20 nmol/(min·kg), respectively (*46*). It is conceivable that these free thiols and mercapturate pathway conjugates, at the alkaline pH of bile (*23*), could be involved in an equilibrium exchange of the isocyanate species, thereby nonspecifically altering the thiol metabolite profile upon biliary transit.

As a corollary to the issue of reversible thiocarbamate formation, it is apparent from the present study that the GSH conjugate could act as a vector for the carbamoylating activity of the isocyanate (*8*, *12*) in parallel with secondary mercapturate pathway metabolites. Consequent upon biliary excretion, these metabolites could immediately target the biliary epithelium for injury by depletion of cellular GSH. Moreover, it should be noted that the vulnerability of biliary epithelial cells to GSH depletion is made more pronounced by the fact that this hepatic cell population contains relatively low levels of both GSH and glutathione reductase (*47*). Indeed, it has been proposed that the biliary pathogenesis of 1-naphthyl isothiocyanate, a structurally analogous carbamoylating agent to MPIC, is linked to its GSH conjugate which acts as a latent form of the isothiocyanate (*48*).

Screening of the urine of NFA dosed rats revealed detectable levels of only NMPC in accordance with what is known about the excretion of thiol conjugates by this route. Because of their high molecular weight, SMPG and SMPCG are selectively favored by the biliary route (*23*). NMPC, on the other hand, is a likely candidate for urinary excretion for two reasons: First, this metabolite lies at the lower molecular weight threshold for biliary excretion (*23*), and second, mercapturic acids are good substrates for the organic anion transporter of renal proximal epithelial cells (*49*). As our identification of biliary NMPC indicates, however, it is possible that a portion of this metabolite could escape renal extraction and be transported back to the liver for excretion. The fact that SMPC was not detected in urine could be illustrative of the fact that cysteinyl conjugates, unlike NAC conjugates, are unsuitable candidates for transport mechanisms present in the kidney (*33*).

Summary and Conclusions

LC/MS contour-mapping of isotope clusters in conjunction with LC/MS/MS provided a valuable approach to the detection and ultimate characterization of NFA-derived thiol conjugates. Apart from the expected identification of carbamoylated GSH and NAC conjugates as biliary and urinary metabolites, respectively, cysteinylglycine, cysteine, and NAC conjugates were characterized as novel formamide metabolites in bile. The issue of whether this pattern is unique to NFA, or is characteristic of formamides as a class of xenobiotics, is under investigation.

The fact that the metabolite NMPC is able to partition between excretion in both bile and urine is germane to the noninvasive analysis of urinary NAC conjugates as an index of the primary event of GSH conjugation with a reactive chemical species (*50*, *51*). It may be necessary to interpret with caution urinary NAC conjugate profiles used for this purpose, because mercapturate pathway metabolism could be modulated by the dose of the xenobiotic, animal species, route of xenobiotic exposure (or administration), the type of thiol conjugate formed, and circulating levels of endogenous thiols.

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References

(1) Eisenhauer, E. A., Weinerman, B. H., Kerr, I., and Quirt, I. (1986) Toxicity of oral *N*-methylformamide in three phase II trials: A report from the National Cancer Institute of Canada clinical trials group. *Cancer Treat. Rep.* **70**, 881–883.

(2) Pearson, P. G., Gescher, A., and Harpur, E. S. (1987) Hepatotoxicity of *N*-methylformamide in mice—I. Relationship to glutathione status. *Biochem. Pharmacol.* **36**, 381–384.

(3) Pearson, P. G., Gescher, A., Harpur, E. S., and Threadgill, M. D. (1987) Hepatotoxicity of *N*-methylformamide in mice—II. Covalent binding of metabolites of [^{14}C]-labelled *N*-methylformamide to hepatic proteins. *Biochem. Pharmacol.* **36**, 385–390.

(4) Kestell, P., Gledhill, A. P., Threadgill, M. D., and Gescher, A. (1986) *S*-(*N*-methylcarbamoyl)-*N*-acetylcysteine: A urinary metabolite of the hepatotoxic experimental antitumour agent *N*-methylformamide (NSC 3051) in mouse, rat and man. *Biochem. Pharmacol.* **35**, 2283–2286.

(5) Threadgill, M. D., Axworthy, D. B., Baillie, T. A., Farmer, P. B., Farrow, K. C., Gescher, A., Kestell, P., Pearson, P. G., and Shaw, A. J. (1987) Metabolism of *N*-methylformamide in mice: Primary kinetic deuterium isotope effect and identification of *S*-(*N*-methylcarbamoyl)glutathione as a metabolite. *J. Pharmacol. Exp. Ther.* **242**, 312–319.

(6) Gescher, A. (1993) Metabolism of *N*,*N*-dimethylformamide: Key to the understanding of its toxicity. *Chem. Res. Toxicol.* **6**, 245–251.

(7) Slatter, J. G., Mutlib, A. E., and Abbott, F. S. (1989) Biotransformation of aliphatic formamides: Metabolites of (±)-*N*-methyl-*N*-(1-methyl-3,3-diphenylpropyl)formamide in rats. *Biomed. Environ. Mass Spectrom.* **18**, 690–701.

(8) Mutlib, A. E., Talaat, R. E., Slatter, J. G., and Abbott, F. S. (1990) Formation and reversibility of *S*-linked conjugates of *N*-(1-methyl-3,3-diphenylpropyl)isocyanate, an *in vivo* metabolite of *N*-(1-methyl-3,3-diphenylpropyl)formamide, in rats. *Drug Metab. Dispos.* **18**, 1038–1045.

(9) LeBelle, M., Sileika, M., and Romach, M. (1973) Identification of a major impurity in methamphetamine. *J. Pharm. Sci.* **62**, 862.

(10) Frank, R. S. (1983) The clandestine laboratory situation in the United States. *J. Forensic Sci.* **28**, 18–31.

(11) Renton, J. R., Cowied, J. S., and Oon, M. C. H. (1993) A study of the precursors, intermediates and reaction by-products in the synthesis of 3,4-methylenedioxymethylamphetamine and its application to forensic drug analysis. *Forensic Sci. Int.* **60**, 189–202.

(12) Baillie, T. A., and Slatter, J. G. (1991) Glutathione: A vehicle for the transport of chemically reactive metabolites *in vivo*. *Acc. Chem. Res.* **24**, 264–270.

(13) Mutlib, A. E., Iagallo, M., and Abbott, F. (1991) GC/MS and LC/MS characterization of metabolites of *N*-formylamphetamine, a potent hepatotoxic contaminant of illegally synthesized amphetamine. *Proceedings of the 39th ASMS Conference on Mass Spectrometry and Allied Topics, Nashville, TN*, pp 585–586.

(14) Weidolf, L., and Covey, T. R. (1992) Studies on the metabolism of omeprazole in the rat using liquid chromatography/ionspray mass spectrometry and the isotope cluster technique with [^{34}S]-omeprazole. *Rapid Commun. Mass Spectrom.* **6**, 192–196.

(15) Garnett, J. L., Long, M. A., Vining, R. F. W., and Mole, T. (1972) A new simple method for rapid, selective aromatic deuteration using organoaluminum dihalide catalysts. *J. Am. Chem. Soc.* **94**, 5913–5914.

(16) Roepstorff, P., and Fohlman, J. (1984) Proposal for a common nomenclature for sequence ions in mass spectra of peptides. *Biomed. Mass Spectrom.* **11**, 601.

(17) Deterding, L. J., Srinivas, P., Mahmood, N. A., Burka, L. T., and Tomer, K. B. (1989) Fast atom bombardment and tandem mass spectrometry for structure determination of cysteine, *N*-acetylcysteine, and glutathione adducts of xenobiotics. *Anal. Biochem.* **183**, 94–107.

(18) Ortiz de Montellano, P. R. (1986) Oxygen activation and transfer. In *Cytochrome P450* (Ortiz de Montellano, P. R., Ed.) pp 217–271, Plenum Press, New York.

(19) Rudewicz, P. J., Stack, R. F., Kennedy, C. D., Covey, T. R., and Sakuma, T. (1993) Comparison of thermospray and ionspray for the analysis of drug conjugates. *Proceedings of the 41st ASMS Conference on Mass Spectrometry and Allied Topics, San Francisco, CA*, p 292.

(20) Honma, S., Iwamura, S., Kobayashi, R., Kawabe, Y., and Shibata, K. (1987) The metabolism of roxatidine acetate hydrochloride. Liberation of deuterium from the piperidine ring during hydroxylation. *Drug Metab. Dispos.* **15**, 551–559.

(21) Baillie, T. A., Pearson, P. G., Rashed, M. S., and Howald, W. N. (1989) The use of mass spectrometry in the study of chemically-reactive drug metabolites. Application of MS/MS and LC/MS to the analysis of glutathione- and related *S*-linked conjugates of *N*-methylformamide. *J. Pharmacol. Biomed. Anal.* **7**, 1351–1360.

(22) Lanting, A. B., Bruins, A. P., Drenth, B. F., de Jonge, K., Ensing, K., de Zeeuw, R. A., and Meijer, D. K. (1993) Identification with liquid chromatography–ionspray mass spectrometry of the metabolites of the enantiomers *N*-methyl dextrorphan and *N*-methyl levorphanol after rat liver perfusion. *Biol. Mass Spectrom.* **22**, 226–234.

(23) Levine, W. G. (1978) Biliary excretion of drugs and other xenobiotics. *Annu. Rev. Pharmacol. Toxicol.* **18**, 81–96.

(24) Borel, A. G., and Abbott, F. S. (1993) Identification of carbamoylated thiol conjugates as metabolites of the antineoplastic 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, in rats and humans. *Drug Metab. Dispos.* **21**, 889–901.

(25) Davis, M. R., Kassahun, K., Jochheim, C. M., Brandt, K. M., and Baillie, T. A. (1993) Glutathione and *N*-acetylcysteine conjugates of 2-chloroethyl isocyanate. Identification as metabolites of *N*,*N*′-bis(2-chloroethyl)-*N*-nitrosourea in the rat and inhibitory properties toward glutathione reductase *in vitro*. *Chem. Res. Toxicol.* **6**, 376–383.

(26) Inoue, M., Okajima, K., and Morino, Y. (1982) Metabolic coordination of liver and kidney in mercapturic acid biosynthesis *in vivo*. *Hepatology* **2**, 311–316.

(27) Inoue, M., Okajima, K., and Morino, Y. (1984) Hepato-renal cooperation in biotransformation, membrane transport, and elimination of cysteine *S*-conjugates of xenobiotics. *J. Biochem.* **95**, 247–254.

(28) Stevens, J. L., and Wallin, A. (1990) Is the toxicity of cysteine conjugates formed during mercapturic acid biosynthesis relevant to the toxicity of covalently bound drug residues. *Drug Metab. Rev.* **22**, 617–635.

(29) Tate, S. (1980) Enzymes of mercapturic acid formation. In *Enzymatic basis of detoxication* (Jakoby, W. B., Ed.) Vol. 2, pp 95–120, Academic Press, New York.

(30) Bakke, J. E., Feil, V. J., and Mulford, D. J. (1990) Biliary excretion and intestinal metabolism in the intermediary metabolism of pentachlorothioanisole. *Xenobiotica* **20**, 601–605.

(31) Bakke, J. E., Huwe, J. K., Mulford, D. J., and Bergman, A. (1992) Metabolism of 1,2,4-trichlorobenzene in rats: examination of thiol formation. *Xenobiotica* **22**, 199–210.

(32) Caldwell, J., Weil, A., and Tanaka, Y. (1989) Species differences in xenobiotic conjugation. In *Xenobiotic metabolism and disposition* (Kato, R., Estabrook, R. W., and Cayen, M. N., Eds.) pp 217–224, Taylor & Francis, London.

(33) Monks, T. J., Anders, M. W., Dekant, W., Stevens, J. L., Lau, S. S., and van Bladeren, P. J. (1990) Glutathione conjugate mediated toxicities. *Toxicol. Appl. Pharmacol.* **106**, 1–19.

(34) Commandeur, J. N. M., Stijntjes, G. J., Winjngaard, J., and Vermeulen, N. P. E. (1991) Metabolism of L-cysteine *S*-conjugates and *N*-(trideuteroacetyl)-L-cysteine *S*-conjugates of four fluoroethylenes in the rat. Role of balance of deacetylation and acetylation in relation to the nephrotoxicity of mercapturic acids. *Biochem. Pharmacol.* **42**, 31–38.

(35) Hinchman, C. A., and Ballatori, N. (1994) Glutathione conjugation and conversion to mercapturic acids can occur as an intrahepatic process. *J. Toxicol. Environ. Health* **41**, 387–409.

(36) Hinchman, C. A., Matsumoto, H., Simmons, T. W., and Ballatori, N. (1991) Intrahepatic conversion of a glutathione conjugate to its mercapturic acid. Metabolism of 1-chloro-2,4-dinitrobenzene in isolated perfused rat and guinea pig livers. *J. Biol. Chem.* **266**, 22179–22185.

(37) Meister, A., Tate, S. S., and Ross, L. L. (1976) Membrane-bound γ-glutamyl transpeptidase. In *The enzymes of biological membranes* (Martonosi, A., Ed.) Vol. 3, pp 315–347, Plenum Press, New York.

(38) Ballatori, N., Jacob, R., Barrett, C., and Boyer, J. L. (1988) Biliary catabolism of glutathione and differential reabsorption of its amino acid constituents. *Am. J. Physiol.* **254**, G1–G7.

(39) Rosalki, S. B. (1975) Gamma-glutamyl transpeptidase. *Adv. Clin. Chem.* **17**, 53–107.

(40) Hinchman, C. A., and Ballatori, N. (1990) Glutathione-degrading capacities of liver and kidney in different species. *Biochem. Pharmacol.* **40**, 1131–1135.

(41) Lukas, G., Brindle, S. D., and Greengard, P. (1971) The route of absorption of intraperitoneally administered compounds. *J. Pharmacol. Exp. Ther.* **178**, 562–566.

(42) Pang, K. S. (1983) Fate of xenobiotics: Physiologic and kinetic considerations. In *Biological Basis of Detoxication* (Caldwell, J., and Jakoby, W. B., Eds.) pp 213–249, Academic Press, New York.

(43) Tulip, K., and Timbrell, J. A. (1988) Comparative hepatotoxicity and metabolism of *N*-methylformamide in rats and mice. *Arch. Toxicol.* **62**, 167–176.

(44) Vandenplas, O., Cartier, A., Ghezzo, H., Cloutier, Y., and Malo, J.-L. (1993) Response to isocyanates: Effect of concentration, duration of exposure, and dose. *Am. Rev. Respir. Dis.* **147**, 1287–1290.

(45) Cantin, A. M., North, S. L., Hubbard, R. C., and Crystal, R. G. (1987) Normal alveolar epithelial lining fluid contains high levels of glutathione. *J. Appl. Physiol.* **63**, 152–157.

(46) Madhu, C., Mitchell, D. Y., and Klaassen, C. D. (1993) Effect of P-450 inducers on biliary excretion of glutathione and its hydrolysis products: Correlation between hepatic gamma-glutamyltranspeptidase activity and the proportion of glutathione hydrolysis products in bile. *Drug Metab. Dispos.* **21**, 342–349.

(47) Parola, M., Cheeseman, K. H., Biocca, M. E., Dianzani, M. U., and Slater, T. F. (1990) Menadione and cumene hydroperoxide induced cytotoxicity in biliary epithelial cells isolated from rat liver. *Biochem. Pharmacol.* **39**, 1727–1734.

(48) Carpenter-Deyo, L., Marchand, D. H., Jean, P. A., Roth, R. A., and Reed, D. J. (1991) Involvement of glutathione in 1-naphthylisothiocyanate (ANIT) metabolism and toxicity to isolated hepatocytes. *Biochem. Pharmacol.* **42**, 2171–2180.

(49) Zhang, G. H., and Stevens, J. L. (1989) Transport and activation of *S*-(1,2-dichlorovinyl)-L-cysteine and *N*-acetyl-*S*-(1,2-dichlorovinyl)-L-cysteine in rat kidney proximal tubules. *Toxicol. Appl. Pharmacol.* **100**, 51–61.

(50) Vermeulen, N. P. E. (1989) Analysis of mercapturic acids as a tool in biotransformation, biomonitoring and toxicological studies. *Trends Pharmacol. Sci.* **10**, 177–181.

(51) Mraz, J., Gescher, A., Cross, H., Shaw, A. J., and Flek, J. (1991) New findings in the metabolism of *N*,*N*-dimethylformamide—consequences for evaluation of occupational risk. *Sci. Total Environ.* **101**, 131–134.

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