Interaction of γ-Glutamyltranspeptidase with Clofibryl-*S*-acyl-glutathione in Vitro and in Vivo in Rat

Mark P. Grillo† and Leslie Z. Benet*

Department of Biopharmaceutical Sciences, University of California, San Francisco, California 94143-0446

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Clofibric acid (CA) is metabolized to chemically reactive acylating products that can transacylate glutathione to form clofibryl-*S*-acyl-glutathione (CA-SG) in vitro and in vivo. We investigated the first step in the degradation of CA-SG to the mercapturic acid conjugate, clofibryl-*S*-acyl-*N*-acetylcysteine (CA-SNAC), which is catalyzed by γ-glutamyltranspeptidase (γ-GT). After γ-GT mediated cleavage of glutamate from CA-SG, the product clofibryl-*S*-acyl-cysteinylglycine (CA-*S*-CG) should undergo an intramolecular rearrangement reaction [Tate, S. S. (1975) *FEBS Lett.* **54**, 319−322] to form clofibryl-*N*-acyl-cysteinylglycine (CA-*N*-CG). We performed in vitro studies incubating CA-SG with γ-GT to determine the products formed, and in vivo studies examining the products excreted in urine after dosing rats with CA-SG or CA. Thus, CA-SG (0.1 mM) was incubated with γ-GT (0.1 unit/mL) in buffer (pH 7.4, 25 °C) and analyzed for products formed by reversed-phase HPLC and electrospray mass spectrometry (ESI/MS). Results showed that CA-SG is degraded completely after 6 h of incubation leading to the formation of two products, CA-*N*-CG and its disulfide, with no detection of CA-*S*-CG thioester. After 36 h of incubation, only the disulfide remained in the incubation. Treatment of the disulfide with dithiothreitol led to the reappearance of CA-*N*-CG. ESI/LC/MS analysis of urine (16 h) extracts of CA-SG-dosed rats (200 mg/kg, iv) showed that CA-SG is degraded to CA-*N*-CG, CA-*N*-acyl-cysteine (CA-*N*-C) and their respective *S*-methylated products. The mercapturic acid conjugate (CA-SNAC) was found as a minor product. Analysis of urine extracts from CA-dosed rats (200 mg/kg, ip) resulted in the detection of clofibryl-*N*-acyl-cysteine (CA-*N*-C), but no evidence for the formation of CA-SNAC was obtained. These in vitro and in vivo experiments indicate that γ-GT mediated degradation of clofibryl-*S*-acyl-glutathione leads primarily to the formation and excretion of clofibryl-*N*-acyl-cysteine products rather than the *S*-acyl-NAC conjugate.

Introduction

Clofibric acid [2-(4-chlorophenoxyl)-2-methylpropionic acid, CA],[1] a lipid lowering drug, is metabolized to reactive acylating derivatives (Scheme 1) that have been shown to transacylate GSH forming clofibryl-*S*-acyl-glutathione (CA-SG) in vivo and in vitro (*1*, *2*). Metabolism of CA by acyl glucuronidation, leading to clofibryl-1-*O*-acyl glucuronide (1-*O*-CA-G), has been proposed to be a route whereby CA becomes metabolically activated to chemically reactive species that are able to bind irreversibly to protein (*3*, *4*) and endogenous nucleophiles such as GSH (*1*). Another metabolic route by which CA is converted to reactive acylating species is conversion to clofibryl-*S*-acyl-CoA [CA-SCoA (*5*−*7*)], an intermediary metabolite of CA which has also been shown to transacylate GSH in vitro (*6*). Covalent binding of electrophilic acyl-linked metabolites to protein has been proposed to be a mechanism for the hypersensitivity reactions associated with the use of CA (*3*, *8*−*11*). Transacylation of the cysteinyl thiol of GSH by reactive clofibric acid metabolites is likely to provide a detoxification mechanism that leads to the formation of CA-SG. Evidence for the formation of this glutathione conjugate in vivo comes from studies where it has been detected in the bile of CA-dosed rats (*1*), and, after conversion to the mercapturic acid derivative clofibryl-*S*-acyl-*N*-acetylcysteine (CA-SNAC), detected in the urine of CA-dosed patients (*2*).

For those glutathione conjugates that are not excreted into the bile, they must first be degraded in the kidneys before excretion into the urine as the mercapturate. The first step in the mercapturic acid pathway is catalyzed by the enzyme γ-glutamyltranspeptidase (γ-GT), whereby the enzyme cleaves off the γ-glutamyl portion of the conjugate (*12*) resulting in cysteinylglycine *S*-derivatives. Abundant levels of γ-GT are located in the proximal tubular cells of the kidney. The conjugates then are further processed to the corresponding cysteine *S*-derivatives by cysteinylglycine dipeptidase (*13*) or aminopeptidase-M (*14*) by cleavage of the glycine moiety. Finally, the cysteine *S*-derivatives are acetylated by cysteine *S*-conjugate *N*-acetyltransferase using acetyl-CoA as a cosubstrate to form the *N*-acetylcysteine conjugate (*15*).

* To whom correspondence should be addressed. Phone: (415) 476-3853. Fax: (415) 476-8887. E-mail: benet@itsa.ucsf.edu.

† Present address: Pharmacia Corporation, Global Metabolism and Investigative Sciences, Kalamazoo, MI 49007.

[1] Abbreviations: CA, clofibric acid; CA-SG, clofibryl-*S*-acyl-glutathione; γ-GT, γ-glutamyltranspeptidase; CA-SNAC, clofibryl-*S*-acyl-*N*-acetylcysteine; CA-*N*-CG, clofibryl-*N*-acyl-cysteinylglycine; CA-*S*-CG, clofibryl-*S*-acyl-cysteinylglycine; CA-*N*-C, clofibryl-*N*-acyl-cysteine; CA-*S*-C, clofibryl-*S*-acyl-cysteine; CA-SCoA, clofibryl-*S*-acyl-CoA; 1-*O*-CA-G, 1-*O*-acyl-clofibryl glucuronide; DTT, dithiothreitol; ECF, ethyl chloroformate; THF, tetrahydrofuran; ESI, electrospray ionization; SIM, selected-ion monitoring; NAC, *N*-acetylcysteine.

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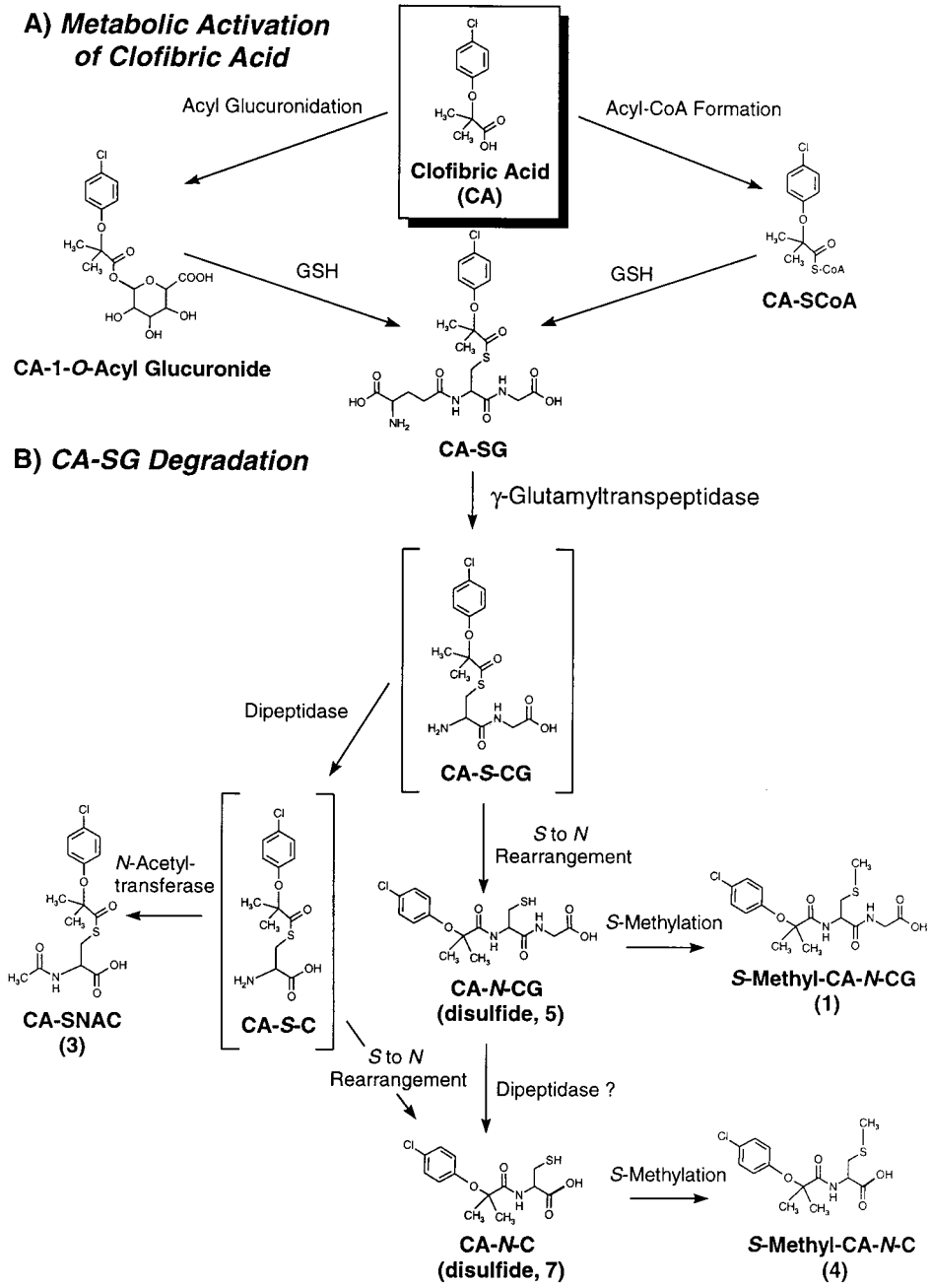
Scheme 1. Proposed Scheme for the (A) Metabolic Activation of Clofibric Acid Followed by Reaction with GSH and (B) the Pathways in the Degradation of CA-SG in Vivo, Which Includes the Proposed *S* to *N* Rearrangement Reaction that Occurs after γ-Glutamyltranspeptidase-Mediated Degradation of *S*-Acyl-Linked Glutathione Conjugates (*16*). Intermediates with Structures Shown in Brackets Have Not Been Identified[a]



[a] Structures of disulfide products, formed ex vivo, are not shown.

The interaction of γ-GT with *S*-acyl-linked glutathione conjugates of lactic acid, acetic acid, and benzoic acid leads to *S*-acylated cysteinylglycine products that have been shown to undergo an intramolecular rearrangement reaction from the *S*-acyl-derivative to the *N*-acyl-derivative in in vitro experiments with purified γ-GT enzyme (*16*). The high reactivity of the thioester bond (*17*) of the *S*-acylated cysteinylglycine product allows for the cysteinylamine to react in an intramolecular fashion with the carbonyl carbon of the thioester linkage resulting in the formation of a more stable *N*-acyl-cysteinylglycine amide product. If such a rearrangement occurs in vivo for γ-GT-mediated products of *S*-acyl-linked glutathione conjugates, then the respective mercapturic acid derivatives would not be expected to be the only degradation products of the *S*-acyl-glutathione derivatives excreted into the urine. Depending on the extent of rearrangement of the *S*-acyl-cysteinylglycine products formed by degradation of the *S*-acyl-glutathione conjugates by γ-GT in the kidney, the relative amount of excreted mercapturates versus rearrangement products would vary. To our knowledge, *N*-acylated cysteinylglycine conjugates have not yet been detected as urinary products of *S*-acylated glutathione conjugates. The formation of CA-SNAC in humans represents the only example of an *S*-acyl-linked mercapturate of an acidic drug found in man (*2*). Similar

experiments performed in our laboratory could not find in human urine (unpublished observations)[2] *S*-acyl-linked mercapturates of ibuprofen and tolmetin, two carboxylic-acid-containing drugs known to form reactive acyl glucuronides (*18*, *19*). Therefore, we hypothesized that the lack of detection of the respective *S*-acylated mercapturates may have resulted from an extensive rearrangement reaction of the γ-GT mediated *S*-acyl-cysteinylglycine products to the *N*-acylated cysteinylglycine derivatives of these drugs.

In the present work, we examined the degradation of CA-SG by γ-GT in vitro with purified enzyme to determine the extent of intramolecular rearrangement of the *S*-acylated cysteinylglycine intermediate. Second, the degradation of synthetic CA-SG in vivo in CA-SG-dosed rats was studied by looking particularly for the urinary *S*-linked mercapturate and *N*-acylated cysteinylglycine rearrangement products. Finally, we examined the urine extracts from CA-dosed rats for derivatives related to the γ-GT mediated degradation of biologically formed CA-SG.

Experimental Section

Chemicals. CA, GSH, dithiothreitol (DTT), *N*-acetylcysteine (NAC), cysteinylglycine, and bovine kidney γ-glutamyltranspeptidase (γ-GT) were purchased from Sigma Chemical Co. (St. Louis, MO). Triethylamine, ethyl chloroformate, monobasic potassium phosphate, potassium bicarbonate, methyl iodide, and THF (anhydrous) were purchased from Aldrich Chemical Co. (Milwaukee, WI). CA-SG and CA-SNAC were synthesized as described below. All solvents used for HPLC were of chromatography grade.

Instrumentation and Analytical Methods. HPLC was carried out on a Shimadzu LC-600 isocratic system coupled to a Shimadzu SPD-6AV UV−Vis detector. All isocratic HPLC analyses were performed on a reversed-phase column (Beckman C8, 15 cm, 5 μm, 1 mL/min). Electrospray mass spectrometry of synthetic standards and in vitro biological extracts was performed on a Hewlett-Packard HP 1100 LC/MSD benchtop electrospray mass spectrometer. Analysis was conducted in the positive ion mode at a fragmentor voltage of 80 and by direct infusion of the sample [dissolved in methanol/1% acetic acid in distilled water (50/50)] into the ion source. ESI/LC/MS analysis of reconstituted urine extracts was conducted by gradient elution (from 40 to 70% methanol in 1% aqueous acetic acid over 30 min) on a Beckman C8 reverse-phase column (15 cm × 4.6 mm, 5 μm, 1 mL/min), which functioned to separate the compounds-of-interest. ^{1}H NMR spectra were recorded on a General Electric QE300 spectrometer operating at 300 MHz. Chemical shifts are reported in parts per million as referenced to the residual solvent peak (2.49 ppm for $^2H_6$-DMSO).

Synthesis of CA-SG. Synthesis of CA-SG was performed by conventional procedures employing ethyl chloroformate (ECF) (*20*). Briefly, to CA (342 mg, 1.6 mmol) dissolved in anhydrous THF (25 mL) was added, at room temperature and while stirring, triethylamine (220 μL, 1.6 mmol) followed by ECF (100 μL, 1.6 mmol). After 30 min of continued stirring, the precipitate which formed (triethylamine hydrochloride) was removed by passing through a glass funnel, fitted with a glass wool plug, and added directly into a solution containing GSH (500 mg, 1.6 mmol), $KHCO_3$ (100 mg, 1.6 mmol), distilled water (10 mL), and THF (15 mL). The solution was stirred continuously under nitrogen gas at room temperature for 2 h, after which the reaction was terminated by the addition of concentrated HCl (8 drops). The THF then was removed by evaporation under reduced pressure and the remaining aqueous phase extracted with diethyl ether (4 × 50 mL). After the first evaporation step and subsequent extractions with diethyl ether, a white precipitate, the *S*-acyl-glutathione conjugate, was formed. This precipitate was washed with distilled water (4 × 50 mL), to remove remaining GSH, followed by acetone (4 × 50 mL), to wash away remaining CA and water. Finally, the precipitate was dried under a stream of nitrogen gas (25 °C, 1 h) to afford 161 mg of a white solid (20% yield) of CA-SG. The poor yield of CA-SG probably resulted from the washing procedure at the acetone-wash step. Unlike most glutathione conjugates (*20*), CA-SG has a slight solubility in acetone. HPLC analysis with UV detection at 226 nm showed the synthetic CA-SG to be 100% pure. ESI mass spectrometric analysis was performed for CA-SG, *m*/*z* (%): 504 (100%, MH^+), 429 ($[M + H - Gly]^+$, 4%), 375 ($[M + H -$ pyroglutamic acid$]^+$, 9%), 307 ($[GSH]^+$, 2%), 272 ($[p\text{-Cl-}C_6H_4\text{-}OC(CH_3)_2COSCH_2\text{-}CH{=}NH_2]^+$, 2%), 169 ($[p\text{-Cl-}C_6H_4OC(CH_3)_2]^+$, 3%). These fragments are characteristic of those found during the ESI/MS analysis of glutathione conjugates (*21*). ^{1}H NMR ($^2H_6$-DMSO): δ 1.42 (s, 6H, $-C(CH_3)_2$-), 2.28 (m, 2H, Glu-β,β′), 2.5 (m, 2H, Glu-γ,γ′), 2.95 (m, 2H, Cys-β,β′), 3.67 (t, 2H, $J = 6.4$ Hz, Glu-α), 3.72 (s, 2H, Gly-α,α′), 4.46 (m, 1H, Cys-α), 6.9−7.4 (m, 4H, Ar).

Synthesis of CA-SNAC Thioester. CA-SNAC was obtained by reacting CA-SG (1 mM) with NAC (20 mM) in buffer (pH 7.4, 37 °C) in a volume of 10 mL until the reaction was complete, as indicated by HPLC analysis of the reaction mixture. The incubation then was acidified (pH 2.5) by the addition of HCl (1 N), followed by extraction of the CA-SNAC conjugate with ethyl acetate (1 × 10 mL). The ethyl acetate layer was dried (anhydrous $MgSO_4$) and evaporated (N_2 gas, 25 °C) to afford 2.7 mg (75% yield) of product as a clear colorless oil. HPLC analysis of the CA-SNAC product showed it to have less than 1% impurity of CA. ESI/MS for CA-SNAC, *m*/*z* (%): 360 (MH^+, 11%), 382 (MNa^+, 318 ($MH^+ - CH_3CO$, 3%), 169 ($[p\text{-Cl}-C_6H_4\text{-}OC(CH_3)_2]^+$, 100%).

In Vitro Experiments With γ-GT and CA-SG. Synthetic CA-SG (0.1 mM) was incubated with γ-glutamyltranspeptidase (type II from bovine kidney, 0.1 unit/mL) in potassium phosphate buffer (0.05 M, pH 7.4, total volume of 4 mL, 25 °C, duplicate incubations) in screw-capped glass vials in a shaking incubator. One unit of the transpeptidase will liberate 1 mmol of *p*-nitroaniline from L-γ-glutamyl-*p*-nitroaniline/min at pH 8.5 and 25 °C, as reported by the supplier. The γ-GT preparation, from the supplier, was reported to have less than 0.5% creatine phosphokinase, glutamic-oxalacetic transaminase, glutamic pyruvic transaminase activity, but with no mention of any dipeptidase activity. The γ-GT preparation was used without further purification. Aliquots (50 μL) of the incubation were taken at times 0, 1, 2, 3, 4, 5, 6, 7, 8, and 36 h and were analyzed directly by isocratic reversed-phase HPLC (50% methanol in 0.05 M potassium phosphate, pH 4.2) for the loss of CA-SG and the respective formation of CA-*S*-CG, CA-*N*-CG or its disulfide. CA-SG and degradation products were detected by UV absorbance at 226 nm. Quantitative measurements were made using a standard curve generated from absolute peak areas of CA-SG. In the present experiments, it was assumed that the UV characteristics of CA-SG and its degradation products are the same. At the 5 and 36 h time points of incubation, aliquots (1 mL) of the incubations were acidified to pH 2.5 with HCl (1 N) and then extracted with ethyl acetate (1 × 5 mL). The extracts were then dried ($MgSO_4$) and evaporated to dryness under a stream of N_2 gas. Identification of the products formed was obtained by ESI/MS analysis of the reconstituted (50% methanol in distilled water with 1% acetic acid, 0.5 mL) by direct infusion of the sample into the ion source (25 μL/min) using a syringe pump (Applied Biosystems, Foster City, CA).

In Vivo Experiments in Rats. Male Sprague−Dawley rats (200−220 g) were given doses of CA-SG (200 mg/kg, iv, tail vein)

[2] Preliminary in vivo experiments were performed with ibuprofen and tolmetin, where, after an oral dose of 600 or 400 mg to volunteers, respectively, 8 h urines were collected, processed, and analyzed for *S*-acyl-mercapturic acid conjugates by reported procedures (*2*). ESI/LC/MS and analysis of the urine extracts did not show the presence of *S*-acyl-mercapturates relative to authentic standards.

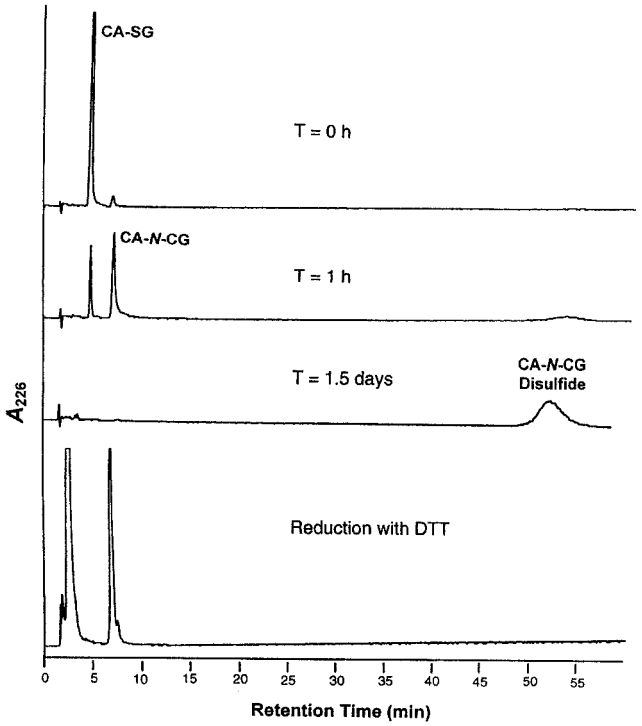


Figure 1. Representative reversed-phase isocratic HPLC chromatograms of 50 μL of the in vitro reaction mixture containing CA-SG (0.1 mM, pH 7.4, 25 °C, retention time of 5 min) and γ-GT (0.1 unit/mL) after 0, 1, and 36 h of incubation. The bottom HPLC chromatogram was obtained 30 min after the addition of DTT (1 mM final concentration) to the incubation at the 36 h time-point. DTT functioned to reduce the CA-*N*-CG disulfide (retention time of 53 min) to the free cysteinyl sulfhydryl form (retention time of 7 min).

or CA (200 mg/kg, ip), both dissolved in phosphate-buffered saline (0.5 mL, pH 7.0). Sixteen hours postadministration, during which time the animals (one rat used for each treatment) were kept unrestrained in metabolic cages, total urines (~15 mL) were collected, acidified (pH 2.5, 1 N HCl), and extracted with ethyl acetate (3 × 15 mL). The combined extracts were dried ($MgSO_4$) and evaporated to dryness with N_2 gas at room temperature. Residues were dissolved in 50% methanol in potassium phosphate buffer (0.05 M, pH 4.2, 4 mL) and vortex mixed. Portions (100 μL) of dissolved extracts were analyzed by reversed-phase gradient ESI/LC/MS with in-line UV detection (226 nm).

Results

HPLC Analysis of the Products Formed in Incubations of CA-SG with γ-GT. Incubation of CA-SG (0.1 mM) with γ-GT (0.1 unit/mL) led to the formation of two products, the rearrangement product clofibryl-*N*-acyl-cysteinylglycine (CA-*N*-CG) and its disulfide. Reversed-phase isocratic HPLC analysis of 50 μL of the incubation mixture (Figure 1) shows that CA-SG elutes before CA-*N*-CG, having retention times of 5 and 7 min, respectively. The disulfide of CA-*N*-CG elutes much later as a broad peak at approximately 53 min. Treatment of the incubation mixture, at the 36 h time point, with dithiothreitol (DTT, final concentration 1 mM, 30 min at 25 °C) led to the complete disappearance of the disulfide peak and the reappearance of the CA-*N*-CG free cysteinyl sulfhydryl product. Electrospray mass spectrometric analysis of an extract of the reaction mixture at the 1 h time point gave a mass spectrum as shown in Figure 2. Mass spectra for all degradation products and fragments

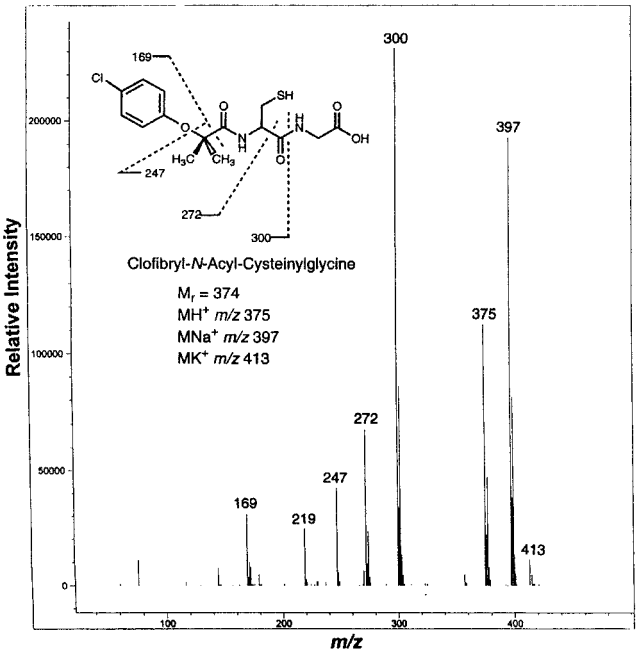


Figure 2. ESI/MS positive ion mass spectrum of in vitro formed CA-*N*-CG obtained from extracts of the incubation of CA-SG (0.1 mM, pH 7.4, 25 °C) with γ-GT (0.1 unit/mL) after 1 h of incubation. The origins of the characteristic ions are as shown.

containing clofibric acid show the characteristic ^{35}Cl and ^{37}Cl isotope cluster pattern. The mass spectrum of the peak that eluted at 5 min was consistent with the degradation product having lost glutamate and giving a protonated molecular (MH^+) ion at *m/z* 375 (less 129 AMU) and the corresponding sodium and potassium adduct ions at *m/z* 397 and 413, respectively. The mass spectrum also shows major ion fragments at *m/z* 300 (loss of glycine), 272, 247 and 169, which are consistent with its structure (Figure 2). The spectrum cannot differentiate between the *N*-acyl- or *S*-acyl-cysteinylglycine products, although the data showing that the product readily oxidizes to the disulfide (Figure 1) provides strong evidence for the formation of the rearranged *N*-acyl product.

Analysis of extracts of the incubation mixture after 36 h of incubation by ESI/MS gave a protonated molecular ion (MH^+) at *m/z* 747, as well as the corresponding sodium (*m/z* 769) and potassium (*m/z* 785) adduct ions, which are consistent with the molecular weight of the CA-*N*-CG disulfide (Figure 3). The disulfide is more stable than the reduced product under the ESI/MS conditions used as indicated by the lack of intense fragment ions produced during the ESI/MS analysis.

The time course of the degradation of CA-SG, with aliquots of the incubation mixture taken every hour for 24 h, showed the glutathione derivative to be completely degraded after 6 h of incubation (Figure 4). The rearranged free sulfhydryl *N*-acyl-cysteinylglycine product reached its maximum concentration (63 μM) at 6 h, but then slowly oxidized to the disulfide after about 24 h of incubation.

In Vivo Experiments with CA-SG. Sixteen hours after dosing rats with CA-SG (200 mg/kg, iv, tail vein), total urine (0−16 h, ~15 mL) was collected and the extract analyzed by reversed-phase ESI/LC/MS with in-line UV detection. Analysis of the urine extract at a UV

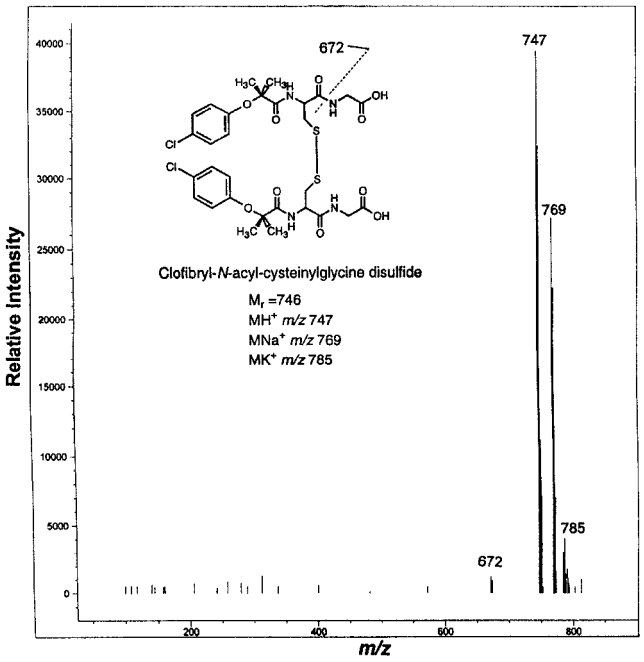


Figure 3. ESI/MS positive ion mass spectrum of in vitro formed CA-*N*-CG disulfide obtained from extracts of the incubation of CA-SG (0.1 mM, pH 7.4, 25 °C) with γ-GT (0.1 unit/mL) after 36 h of incubation. The origins of the characteristic ions are as shown.

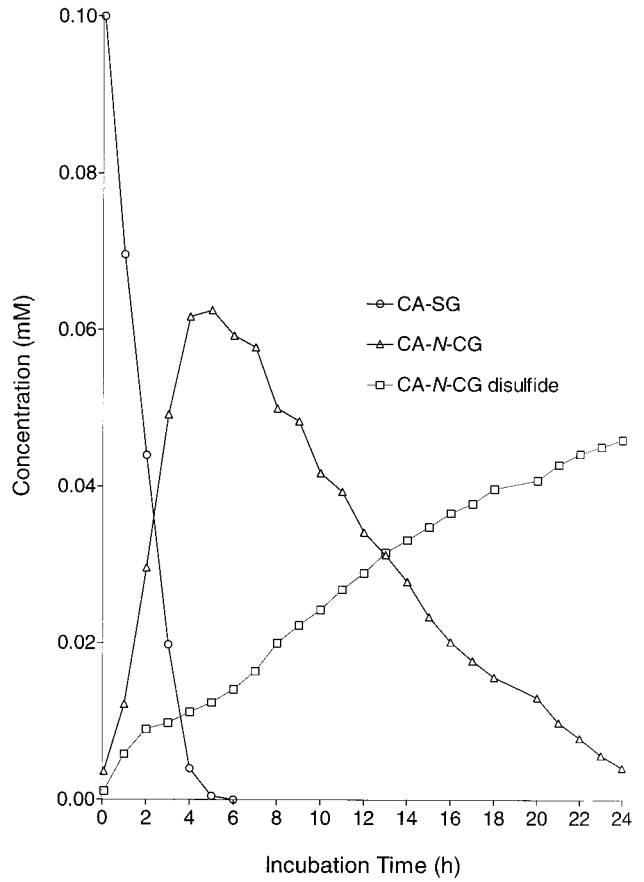


Figure 4. Time course of the degradation of CA-SG (0.1 mM) by γ-GT (0.1 unit/mL) in buffer (0.05 M potassium phosphate, pH 7.4, 25 °C). Values are expressed as the average of duplicate experiments.

absorbance of 226 nm showed the formation of substances eluting after a retention time of 10 min, which were the focus of the present study (Figure 5). The mass spectrum

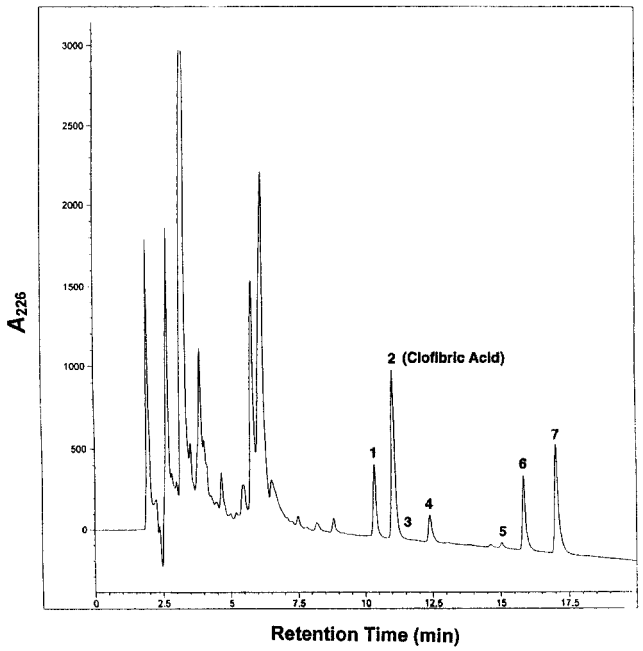


Figure 5. Representative reversed-phase gradient HPLC chromatogram with UV detection of urine extracts from CA-SG dosed rats. An aliquot of the reconstituted urine extract (100 μL) was injected onto a Beckman C8 column and analyzed by in-line UV (A_{226}) and ESI/MS detection. The peaks-of-interest are labeled **1** (*S*-methyl-CA-*N*-CG), **2** (CA), **4** (*S*-methyl-CA-*N*-C), **5** (CA-*N*-CG disulfide), **6** (CA-*N*-CG/CA-*N*-C mixed disulfide), and **7** (CA-*N*-C disulfide). The label **3** (CA-SNAC) indicates the approximate retention time of the authentic mercapturate (11.2 min). The biologically formed CA-SNAC, as shown, was not detected in the extract by UV analysis.

(not shown) of peak **1** (retention time 10.3 min) is consistent with the structure being *S*-methyl-clofibryl-*N*-acyl-cysteinylglycine (*S*-methyl-CA-*N*-CG, Figure 6A, MH^+ at *m*/*z* 389, with fragments at *m*/*z* of 169, 261, 186, and 314). A synthetic *S*-methyl-CA-*N*-CG conjugate standard was synthesized by reacting CA-*N*-CG free sulfhydryl, obtained by reducing CA-*N*-CG disulfide with DTT, with methyl iodide in buffer at pH 7.4. Confirmation of the structure of this product was obtained by comparing its mass spectrum with that of the synthetic derivative, which were identical, and by having the same HPLC retention times (data not shown).

The mass spectrum of peak **2** (retention time 11.0 min) showed it to be identical to that of standard CA (Figure 6B). CA proved to be poorly suited to analysis by ESI/MS by giving a weak mass spectrum (not shown) with no MH^+ ion detected. The mass spectra of CA did provide major fragment ions at *m*/*z* 169 (loss of CO_2), which is a characteristic fragment for clofibric acid derivatives, and *m*/*z* 129 (loss of dimethylacetic acid).

Authentic mercapturic acid conjugate, CA-SNAC, elutes at a retention time of ~11.2 min (just after CA and approximately where the label **3** is placed on the chromatogram in Figure 5), but the biologically formed product was not detected by UV analysis of the CA-SG-dosed rat urine extract (Figure 5). Equal amounts of CA-SNAC and CA give the same peak areas when analyzed by HPLC at 226 nm (data not shown), indicating the relatively low amount of CA-SNAC formed in vivo upon administration of CA-SG. The mercapturate was detected in the extract by ESI/MS detection, in that baseline subtraction of the ESI/LC/MS chromatogram around the 11.2 min retention time provided a mass spectrum of CA-

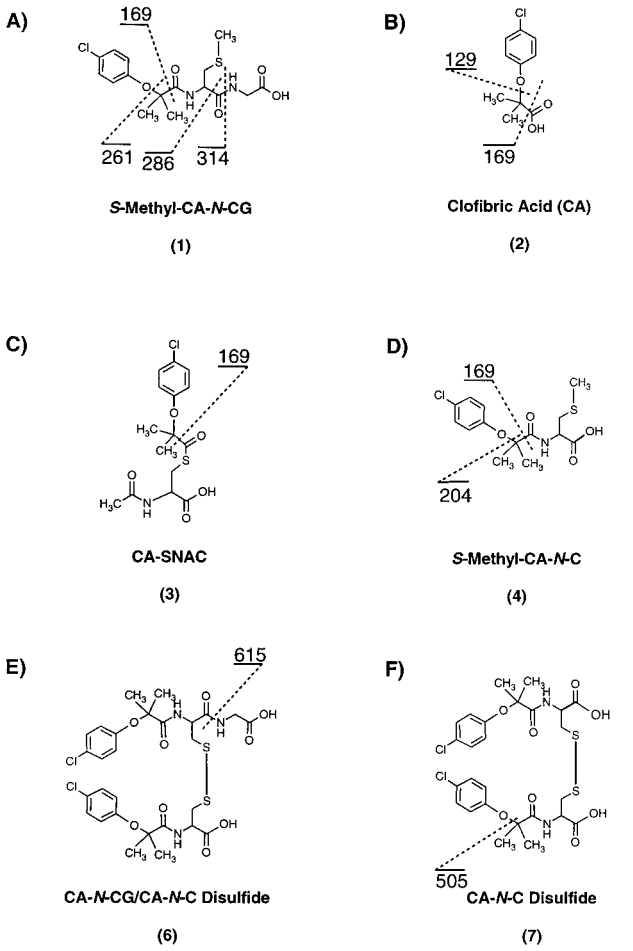


Figure 6. Structures and proposed ESI/MS fragmentation of the in vivo formed products **1**, **2**, **3**, **4**, **6**, and **7**, isolated from the urine of a CA-SG-dosed rat.

SNAC (not shown) that was identical to the synthetic standard. The mass spectrum of the biologically formed CA-SNAC (**3**) exhibited an MH^+ ion at *m*/*z* 360 and one major fragment ion at *m*/*z* 169, the characteristic CA fragment (Figure 6C).

The mass spectrum (not shown) of peak **4** eluting at 12.4 min (Figure 5) provided evidence for the formation of *S*-methyl-clofibryl-*N*-acyl-cysteine (*S*-methyl-CA-*N*-C) by showing the MH^+ ion at *m*/*z* 332 and fragment ions at *m*/*z* 204 (loss of *p*-chlorophenol), and *m*/*z* 169 (Figure 6D).

The disulfide of CA-*N*-CG (peak **5**) eluted at 15.0 min as a minor peak (Figure 5), which is the same HPLC retention time of the disulfide formed upon in vitro degradation of CA-SG by γ-GT (data not shown). The mass spectrum (not shown) for this disulfide, provided ions at *m*/*z* 747 (MH^+), 769 and 785 (sodium and potassium adduct ions), and 672 (MH^+-75, loss of glycine), which was identical to the spectrum shown for the in vitro formed product (Figure 3).

The substance labeled peak **6** eluting at 15.8 min (Figure 5) gave an ESI mass spectrum (not shown), containing the MH^+ ion at *m*/z 690, and a fragment ion at *m*/z 615 (MH^+-75, loss of glycine). The mass spectrum is consistent with the substance being the mixed disulfide of CA-*N*-CG and clofibryl-*N*-acyl-cysteine (CA-*N*-C) (Figure 6E).

Finally, the major glutathione related product (peak **7**) eluting at 17.0 min (Figure 5) gave a mass spectrum

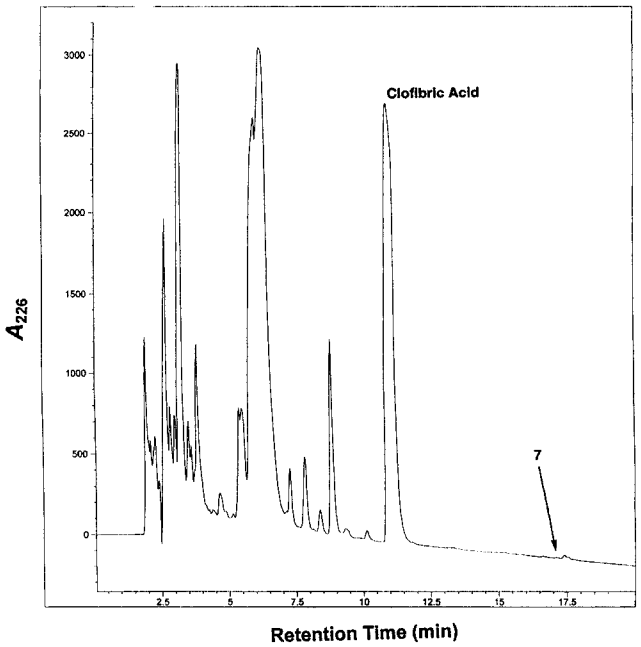


Figure 7. Representative reversed-phase gradient HPLC chromatogram with UV detection of urine extracts from CA-dosed rats. An aliquot of the reconstituted urine extract (100 μL) was injected onto a Beckman C8 column and analyzed by in-line UV (A_{226}) and ESI/MS detection. Peak **7** (labeled on the chromatogram) refers to the retention time of the CA-*N*-C disulfide product (shown as a very small peak).

(not shown) indicative for the disulfide of CA-*N*-C (Figure 6F) by providing masses at *m*/*z* 633 (MH^+) and *m*/*z* 505 (MH^+-*p*-chlorophenol).

In Vivo Experiments with CA. Sixteen hours post-administration of CA (200 mg/kg, ip), total urine (~15 mL) was collected, processed, and analyzed for glutathione related metabolites. Analysis of a portion (100 μL) of the reconstituted extract by ESI/LC/MS, with in-line UV detection, showed the presence (by UV detection) of a very small peak at 17.0 min (Figure 7). This substance has the same HPLC retention time as the CA-*N*-C disulfide derivative (peak **7**, Figure 5) found in the CA-SG-dosed rat urine extract. The ESI/MS mass spectrum (not shown) of this in vivo metabolite of CA was identical to the mass spectrum of the in vivo product detected in the CA-SG-dosed rat urine extract (peak **7**, Figures 5 and 6F).

Analysis of the urine extracts from CA-SG and CA treated rats by selected-ion monitoring (SIM) gave the ESI/LC/MS chromatogram shown in Figure 8. Selected-ions monitored were for the protonated molecular ions of peaks **1**, **3**, **4**, **5**, **6**, and **7**, and were as follows: *m*/*z* 389 (**1**, *S*-methyl-CA-*N*-CG, Figure 6A), 360 (**3**, CA-SNAC, Figure 6C), 332 (**4**, *S*-methyl-CA-*N*-C, Figure 6D), 747 (**5**, CA-*N*-CG disulfide, Figure 3), 690 (**6**, mixed disulfide of CA-*N*-CG and CA-*N*-C, Figure 6E), and 633 (**7**, CA-*N*-C disulfide, Figure 6F). SIM analysis of the CA-SG urine extract gave a chromatogram with a similar profile of eluting substances as detected by UV analysis at 226 nm (Figure 5). The chromatogram also showed the presence of CA-SNAC (**3**) in the extract eluting at 11.0 min, the same retention time of the CA-SNAC synthetic standard when analyzed during the same SIM experiment (data not shown). Analysis of the CA-dosed rat urine extract showed peak **7** (CA-*N*-C disulfide) as the most abundant ion. Other peaks in this SIM chro-

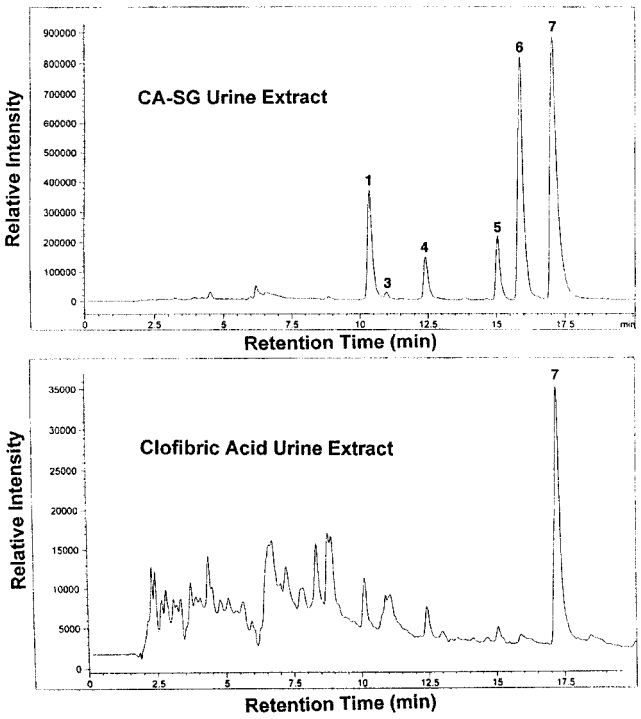


Figure 8. Representative reverse-phase gradient ESI/LC/MS SIM chromatogram of (top) CA-SG- and (bottom) CA-dosed rat urine extracts. The labeled peaks-of-interest are **1** (*S*-methyl-CA-*N*-CG), **3** (CA-SNAC), **4** (*S*-methyl-CA-*N*-C), **5** (CA-*N*-CG disulfide), **6** (CA-*N*-CG/CA-*N*-C mixed disulfide), and **7** (CA-*N*-C disulfide). Selected-ions used corresponded to the protonated molecular ions of substances **1**, **3**, **4**, **5**, **6**, and **7** (*m/z* 389, 360, 332, 747, 690, and 633, respectively). CA (**2**) was not measured during the SIM ESI/LC/MS analysis of the urine extracts.

matogram were not different than analysis performed of control rat urine extracts (data not shown).

Discussion

It has been proposed that CA is metabolized to reactive metabolites that can acylate proteins and lead to the associated hepatotoxic effects (*1*−*4*). Greatest attention has focused on the acyl glucuronide metabolite of the drug, 1-*O*-CA-G, in view of reports that this metabolite transacylates the nucleophilic cysteinyl-thiol of GSH, in vitro in buffer, to form CA-SG (Scheme 1) (*1*). In addition, studies have been performed showing that the corresponding mercapturic acid conjugate, CA-SNAC, is excreted from the urine of CA-dosed volunteers (*2*). The proposed metabolic activation of CA by acyl glucuronidation is illustrated in Scheme 1. Also shown in this scheme is another proposed, and understudied, bioactivation mechanism, namely acyl-CoA formation, resulting in CA-SCoA (*5*, *6*). We reported that this thioester metabolite is also reactive with GSH forming CA-SG (*6*). According to Scheme 1, CA, after conversion to the acyl glucuronide or acyl-CoA thioester, reacts with GSH to form the glutathione thioester CA-SG. This thioester glutathione derivative, a proposed detoxification product (*2*), then is either excreted unchanged in bile (*1*) or undergoes sequential enzyme catalyzed degradation steps to the mercapturic acid conjugate, prior to excretion in the urine [Scheme 1 (*2*)]. Although of mechanistic importance in understanding chemical reactivity of CA metabolites, the formation of CA-SG is only of minor quantitative importance, in that the amount of CA-SG excreted in bile was reported to be only 0.1% of the amount of 1-*O*-CA-G excreted in bile of CA-dosed rats (*1*). Unlike the observations reported for CA, we have searched unsuccessfully for mercapturic acid conjugates of carboxylic-acid-containing drugs as a marker for metabolism to reactive acyl glucuronides or acyl-CoA thioester derivatives in vivo.

A report by Tate (*16*) offered an explanation for our finding by showing that γ-glutamyltranspeptidase (γ-GT), the enzyme that catalyzes the first step in the degradation of glutathione conjugates to mercapturic acid derivatives, interacts with *S*-acyl derivatives of glutathione to yield *N*-acyl-cysteinylglycine conjugates. The degradation of the *S*-acyl-derivative by γ-GT, via cleavage of the γ-glutamyl group, leads to *S*-acyl-cysteinylglycine products that quickly and quantitatively convert to the *N*-acyl-dipeptide conjugate (Scheme 1) (*16*). *S*-Acyl-glutathione conjugates used by Tate (*16*), where colorimetric techniques were employed to detect the free sulfhydryl formed in the *S* to *N* transfer, included *S*-acyl-glutathione conjugates of acetic, lactic, and benzoic acid. We propose that the rearrangement reaction that occurs in vitro for these *S*-acyl-thioester derivatives via the transpeptidase may also occur in vivo for glutathione thioester detoxification products of reactive acidic drug metabolites such as CA-1-*O*-G or CA-SCoA. We propose that rearrangement of dipeptide *S*-acyl-cysteinylglycine conjugates to *N*-acyl-cysteinylglycine derivatives would preclude further degradation to the corresponding mercapturates and explain their lack of detection.

In the present studies, we focused on the γ-GT mediated degradation of the GSH thioester conjugate of clofibric acid in vitro and in vivo. We found that CA-SG was degraded by γ-GT in vitro to give the rearranged CA-*N*-CG product rather than the *S*-acylated CA-*S*-CG dipeptide. This conclusion was based on ESI/MS data showing the protonated molecular ion (*m/z* 375) having lost 129 amu (loss of γ-glutamic acid) and that the rearrangement product readily oxidizes to its disulfide, which indicates the presence of a free cysteinyl sulfhydryl. The reaction that occurs is an intramolecular transacylation reaction between the cysteinylamine and the carbonyl-carbon of the thioester linkage forming the more stable amide. It has been proposed that γ-GT can facilitate the *S* to *N* rearrangement reaction (*16*), although we found that the intermediate *S*-acyl-thioester can rearrange rapidly in the absence of the γ-GT enzyme. We reached this conclusion since attempts to synthesize CA-*S*-CG thioester, by reacting clofibryl acyl-chloride with cysteinylglycine at pH 7.4, resulted only in the formation of the rearrangement product, CA-*N*-CG, (unpublished observations).[3] All together, these results are in agreement with the literature report by Tate (*16*) indicating that the rearranged *N*-acyl-cysteinylglycine conjugate is formed in vitro by γ-GT-mediated degradation of CA-SG.

To directly examine the degradation of CA-SG in vivo, and to bypass the bioactivation steps (Scheme 1), we administered CA-SG (iv) to rats and looked for the degradation products excreted in urine. ESI/LC/MS analysis with in-line UV detection (Figure 5) showed that CA-SG, in addition to being hydrolyzed to CA, is degraded

[3] An attempt to synthesize CA-*S*-CG thioester was made by reacting clofibryl acyl-chloride (1 mM) (obtained quantitatively by conventional procedures employing oxalyl chloride) with cysteinylglycine (1 mM) in phosphate buffer (0.1 M, pH 7.4 and 25 °C).

by γ-GT to products that are consistent with the rearrangement of the unstable *S*-acyl-cysteinylglycine intermediary product. As shown in Scheme 1, CA-SG is cleaved by γ-GT forming the unstable, and unidentified, *S*-acylated derivative. This derivative then undergoes an *S* to *N* transfer forming the *N*-acylated dipeptide, CA-*N*-CG. The subsequent formation of clofibryl-*N*-acyl-cysteine (CA-*N*-C) could happen by two routes, including dipeptidase mediated cleavage of glycine from CA-*N*-CG, or from CA-*S*-CG followed by an *S* to *N* rearrangement of CA-*S*-C thioester (Scheme 1). Since such rearrangement reactions occur rapidly (*16*), we believe that CA-*N*-CG serves as the actual substrate of the dipeptidase enzymes, although we have no knowledge of *N*-acetylated derivatives of cysteinylglycine being degraded by dipeptidases. The clofibryl-*N*-acylated products were also found to be metabolized to their corresponding *S*-methylated derivatives, *S*-methyl-CA-*N*-CG (**1**) and *S*-methyl-CA-*N*-C (**4**). *S*-Methylation occurs in general for degradation products of glutathione conjugates and is mediated by *S*-methyltransferase enzymes. The free sulfhydryl forms of CA-*N*-CG and CA-*N*-C were not found in the urine extracts as such, but were detected as disulfide products. These disulfides were most likely formed ex vivo in the urine collection tubes that were left open to air and untreated, but not formed in vivo. The classic urinary mercapturic acid conjugate, CA-SNAC, was found only as a minor metabolite of CA-SG. The major degradation product was clofibryl-*N*-acyl-cysteine disulfide and, therefore, should be the metabolite-of-interest to detect in the urine extracts of rats treated with CA.

Finally, during the ESI/LC/MS analysis of urine extracts from CA-dosed rats for the presence of CA-SG degradation products, no mercapturic acid conjugate was detected (Figure 8). As predicted by observations from studies showing the urinary products from CA-SG-dosed rats, the disulfide (**7**) of the rearranged γ-GT/dipeptidase product, CA-*N*-C, was the only observed glutathione-derived conjugate detected in urine. This is the first example in our laboratory of a carboxylic acid-containing drug being excreted as a glutathione-related derivative, and, we believe, the first example of an *N*-acyl-cysteine amide metabolite of an acidic drug.

In summary, clofibric acid *S*-acyl-glutathione has been shown to undergo a γ-GT mediated degradation in vitro and in vivo to *S*-acylated products that are unstable and rearrange from *S*- to *N*-acyl-cysteine amide derivatives. The formation of the mercapturic acid conjugate of CA occurs only to a minor extent, and therefore may not be the metabolite that should be searched for in urine extracts as a marker for reactive metabolite formation in vivo. From the results of the present studies on the degradation of CA-SG thioester by γ-GT, we propose that drug-*N*-acyl-cysteine derivatives be used as markers of *S*-acyl-glutathione conjugate formation occurring in vivo, especially when studying urine extracts from patients dosed with carboxylic acid-containing drugs.

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