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Interaction of γ -Glutamyltranspeptidase with Ibuprofen-S-Acyl-Glutathione In Vitro and In Vivo in Human

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Running Title: Interaction of γ -GT with Ibuprofen-*S*-Acyl-Glutathione

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Abbreviations: I-SG, ibuprofen-*S*-acyl-glutathione; I-SCoA, ibuprofen-*S*-acyl-CoA; I-1- β -*O*-G, ibuprofen-1- β -*O*-acyl glucuronide; I-*S*-CG, ibuprofen-*S*-acyl-cysteinylglycine; I-*N*-CG, ibuprofen-*N*-acyl-cysteinylglycine; I-*N*-C, ibuprofen-*N*-acyl-cysteine; I-*S*-C, ibuprofen-*S*-acyl-cysteine; NAC, *N*-acetylcysteine; I-*S*-NAC, ibuprofen-*S*-acyl-*N*-acetylcysteine; γ -GT, γ -glutamyltranspeptidase; MRM, multiple reaction monitoring; CID, collision-induced dissociation; IAA, iodoacetamide; GSH, glutathione; ESI, electrospray ionization; LC-MS/MS, liquid chromatography-tandem mass spectrometry.

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ABSTRACT:

Ibuprofen is metabolized to chemically-reactive acyl glucuronide and *S*-acyl-CoA metabolites that are proposed to transacylate glutathione (GSH) forming ibuprofen-*S*-acyl-GSH (I-SG) in vivo. Herein, we report the detection of novel metabolites of ibuprofen, namely ibuprofen-*N*-acyl-cysteinylglycine (I-*N*-CG), ibuprofen-*N*-acyl-cysteine (I-*N*-C), and the mercapturic acid conjugate, ibuprofen-*S*-acyl-*N*-acetylcysteine (I-*S*-NAC), in urine from an ibuprofen-dosed volunteer. Thus, analysis of ibuprofen-dosed (Advil[®], 800 mg) human urine extracts by sensitive liquid chromatography tandem mass spectrometric detection resulted in the identification of I-*N*-CG, I-*N*-C, and I-*S*-NAC derivatives as minor metabolites (6.0, 1.7, and 0.2 μ g excreted 10-h post administration, respectively). I-*N*-CG is proposed to be formed from the degradation of I-SG by γ glutamyltranspeptidase (γ GT)-mediated cleavage of the γ glutamyl group leading to an unstable ibuprofen-*S*-acyl-cysteinylglycine (I-*S*-CG) intermediate which undergoes spontaneous *S* to *N* intramolecular rearrangement. Then, dipeptidase-mediated cleavage of glycine from I-*N*-CG leads to the formation of I-*N*-C. Treatment of racemic I-SG (100 μ M) in vitro with commercially available bovine kidney γ GT (0.1 units/ml) in buffer at pH 7.4 and 37 degrees C resulted in its complete degradation yielding (*R*)- and (*S*)-I-*N*-CG after 15-min of incubation. In vitro enzyme kinetic studies with bovine kidney γ GT incubated separately with (*R*)- and (*S*)-I-SG isomers revealed no enantioselective degradation. Results from these studies provided evidence that ibuprofen is metabolized in human to reactive transacylating-type intermediates that react with GSH forming I-SG thioester which, following degradation by γ GT and dipeptidase enzymes and following *S* to *N* intramolecular rearrangement, leads to the urinary excretion of the I-*N*-CG and I-*N*-C amide-linked conjugates, respectively.

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Introduction

Carboxylic acid-containing drugs can be metabolized to chemically-reactive 1-*O*-acyl glucuronide and/or *S*-acyl-CoA thioester derivatives capable of transacylating cellular nucleophiles including GSH *in vitro* and *in vivo* (Boelsterli, 2002; Faed, 1984; Grillo, 2011; Skonberg et al., 2008). Ibuprofen, a nonsteroidal antiinflammatory drug, is metabolized by both acyl glucuronidation and acyl-CoA formation leading to ibuprofen-1- β -*O*-acyl glucuronide (I-1- β -*O*-G, Kepp et al., 1997) and ibuprofen-*S*-acyl-CoA (I-SCoA; Knadler and Hall, 1990), respectively (Fig. 1). Since ibuprofen has been shown to become covalently bound to protein in patients treated with the drug (Castillo et al., 1995), we propose that it may also form adducts with GSH through chemically-reactive acyl glucuronide and/or *S*-acyl-CoA metabolites formed in human. Recently, we demonstrated that I-SCoA transacylates GSH in buffer forming ibuprofen-*S*-acyl-GSH (I-SG) thioester and that I-SCoA, but not I- β -1-*O*-G, plays the central role in the transacylation of GSH forming I-SG in incubations with rat hepatocytes (Grillo and Hua, 2008).

GSH-adducts undergo degradation *in vivo* by the mercapturic acid pathway which occurs initially via an extracellular process catalyzed by γ -glutamyltranspeptidase (γ -GT) primarily in the liver and kidney (Hinchman and Ballatori, 1994). Thus, once formed in the liver, GSH-adducts can be transported out of the hepatocyte and into the plasma where they undergo interorgan transport to the kidney and are degraded first by γ -GT which functions by hydrolyzing the γ -glutamyl bond leading to the corresponding cysteinyl-glycine-*S*-linked-adducts (Tate and Meister, 1985; Hinchman and Ballatori, 1994). Following the γ -GT hydrolysis step, cysteinyl-glycine-*S*-linked-adducts undergo further hydrolysis by a Cys-Gly dipeptidase, or aminopeptidase N, leading to the formation of the corresponding L-cysteine-*S*-linked-adducts (Kozak and Tate, 1982). L-Cysteine-*S*-linked-adducts then serve as substrates for *S*-cysteine conjugate *N*-acetyltransferase which catalyzes the *N*-acetylation of the cysteinyl-amine residue (*via* the cofactor acetyl-CoA) affording *N*-acetylcysteine (NAC)-adducts prior to excretion. Similar to thioether-linked GSH-adducts, thioester-linked *S*-acyl-GSH adducts formed *in vivo* would be predicted to undergo sequential enzyme-catalyzed degradation steps of the mercapturic acid pathway yielding the

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corresponding *S*-acyl-linked mercapturic acid (*S*-acyl-NAC) conjugates prior to excretion. However, only one report on the urinary excretion of an *S*-acyl-NAC conjugate of a carboxylic acid-containing drug has been shown in which clofibryl-*S*-acyl-NAC was detected as a minor metabolite in the urine of clofibric acid-dosed patients (Stogniew et al., 1982). By contrast, results from *in vivo* studies in clofibric acid-dosed rats failed to detect clofibryl-*S*-acyl-NAC thioester in urine extracts by sensitive liquid chromatography-mass spectrometric (LC-MS) detection (Grillo and Benet, 2001).

A report published by Tate (1975) provided an explanation for a potential lack of *S*-acyl-NAC-adduct detection in urine when it was discovered that γ -GT interacts with an *S*-acyl-GSH thioester leading to cleavage of the γ -glutamyl group and formation of an unstable *S*-acyl-cysteinylglycine (*S*-acyl-Cys-Gly) thioester-linked intermediate. Importantly, due to the proximity of the α -amino group to the thioester functionality, the *S*-acyl-Cys-Gly-thioester undergoes spontaneous intramolecular *S* to *N* acyl transfer leading to the corresponding *N*-acyl-Cys-Gly amide-linked conjugate (Fig. 1). Through this mechanism, further metabolism to the corresponding mercapturic acid conjugate would not occur due to the blocked α -amino group which might explain a lack, or low level, of detection of drug-*S*-acyl-NAC-adducts in urine for carboxylic acid-containing drugs. Thus, the sensitive LC-MS analysis of urine extracts from clofibric acid-dosed rats resulted only in the detection of clofibryl-*N*-acyl-Cys and *N*-acyl-Cys-Gly conjugates as their corresponding *S*-methylated products and/or *N*-acyl-cystinyl- and *N*-acyl-cystinylbisglycine-disulfide dimers (Grillo and Benet, 2001), but not as the *S*-acyl-linked mercapturate (Grillo and Benet, 2001). Other reports have demonstrated the excretion in bile of *S*-methyl-*N*-acyl-Cys and *N*-acyl-Cys-Gly disulfide (*N*-acyl-cystinylbisglycine-disulfide) conjugates of aromatic carboxylic acid-containing prostaglandin I_2 -preferring receptor antagonist analogs (Fitch et al., 2004), however with no report of corresponding *S*-acyl-NAC detection. In addition, *in vivo* studies with diclofenac in bile-duct cannulated rats showed that diclofenac-*N*-acyl-Cys-Gly and diclofenac-*N*-acyl-cys were excreted in bile (Grillo et al., 2008), but where no diclofenac-*S*-acyl-NAC was detected (Grillo et al., 2003). From observations such as these, we proposed that *N*-acyl-Cys-Gly and *N*-acyl-Cys amide derivatives serve as markers of drug-*S*-acyl-GSH conjugate formation occurring *in vivo*, especially when examining urine

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extracts following administration of carboxylic acid-containing drugs for evidence of drug-*S*-acyl-GSH conjugate formation in dosed patients (Grillo and Benet, 2001).

In the present work, we tested this proposal by examining urine extracts from an ibuprofen-dosed volunteer for the presence of the I-SG γ -GT-mediated degradation product, ibuprofen-*N*-acyl-cysteinylglycine (I-*N*-CG), and the subsequent dipeptidase generated metabolite, ibuprofen-*N*-acyl-cysteine (I-*N*-C), as well as for the mercapturic acid adduct, I-*S*-NAC. In addition, we examined the interaction of commercially available bovine kidney γ -GT with I-SG in vitro to determine the products formed and also to determine the affinity and enantioselectivity of (*R*)- and (*S*)-I-SG for the enzyme.

Materials and Methods

Materials. Advil[®] (200 mg caplets) was used in the present study. Iodoacetamide (IAA), L-cysteine, L-cysteinylglycine, *N*-acetylcysteine, glycyl-glycine, carbamazepine (CBZ), 5,5'-dithiobis(2-nitrobenzoate), tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), and bovine kidney γ -GT were purchased from Sigma-Aldrich (St. Louis, MO). *S*-Acyl-GSH adducts (*R,S*)-I-SG, (*R*)-I-SG, and (*S*)-I-SG were available from previous studies (Grillo and Hua, 2008). Racemic ibuprofen- β -1-*O*-acyl glucuronide was purchased from LC Scientific Inc. (ID005, lot ERG20651, 99% pure; Concord, Ontario, Canada). The derivatives I-*N*-C, I-*N*-CG, and I-*S*-NAC were synthesized as described below. Stock solutions of I-SG (1 and 10 mM), I-*N*-C (1 mM), I-*N*-CG (1 mM), and I-*S*-NAC (1 mM) were prepared as fresh solutions in acetonitrile containing 3% formic acid in water (1/1, v/v). All solvents used for liquid chromatography-tandem mass spectrometric (LC-MS/MS) analyses were of chromatographic grade.

Instrumentation and Analytical Methods. Ibuprofen derivatives I-SG, I-*N*-C, I-*N*-CG, and I-*S*-NAC were characterized by LC-MS/MS on a Thermo Fisher TSQ Quantum Discovery Max mass spectrometer (Thermo Fisher Scientific, Waltham, MA), linked to an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara CA) with in-line UV detection (226 nm), and using a CTC HTS PAL Autosampler (LEAP Technologies, Carrboro, NC). Electrospray ionization (ESI) was employed with the needle potential held at 4.5 kV. Positive ion mode full scan (m/z 50 to m/z 1000) LC-MS analysis was

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conducted with a scan time of 0.73-sec and source collision energy of 10 V. The tandem MS/MS conditions used were 2 mTorr argon collision gas and a collision potential of 35 eV. Xcalibur software (version 2.0, Thermo Electron Corporation, Waltham, MA) was used to acquire all data. Urine extracts were chromatographed on a reverse-phase column (4.6 mm I.D. x 250 mm, Shiseido Capcell Pak C-18, Shiseido Co., Ltd., Japan Fine Chemicals) using an Agilent 1100 series HPLC with a flow rate of 1 ml/min (0.3 ml/min flow into the ESI source) over 60-min. The gradient aqueous mobile phase (solvent-A) consisted of water with 0.1% formic acid (v/v) and the organic mobile phase (solvent-B) contained acetonitrile with 0.1% formic acid (v/v). Elution was achieved by holding the aqueous solvent-A mobile phase constant at 100% for 5-min, and then, in a linear fashion, decreasing to 30% solvent-A over 52-min of elution. Quenched samples from in vitro studies on the degradation of I-SG to I-N-CG by γ GT (Fig. 5) were chromatographed on a Phenomenex Luna, 5 μ , C18(2), 100Å, 2.0 mm I.D. x 150 mm, reverse-phase column (Torrance, CA), and eluted with a mobile phase flow rate of 0.3 ml/min. The mobile phases used for these LC-MS/MS analyses were the same as described above, and where gradient elution was achieved by increasing solvent-B from 0 to 95% over 13-min. NMR spectra were acquired in ²H₆-DMSO on a 500-MHz spectrometer equipped with a 5-mm cryoprobe (Bruker, Newark, DE).

Synthesis of I-N-CG and I-N-C Amide Derivatives. I-N-CG and I-N-C amides were synthesized by reacting (*R,S*)-, (*R*)- or (*S*)-I-SG (50 mg, 0.1 mmol) with either L-cysteine (500 mg, 4.1 mmol) or L-cysteinylglycine (500 mg, 2.8 mmol), respectively, dissolved in potassium phosphate buffer (pH 8, 50 ml) in a 100 ml glass beaker and stirred at 37°C for 1-h. The reaction mixtures then were acidified to pH 3 by the drop-wise addition of 1N HCl and then extracted three times with ethyl acetate (50 ml). Organic extracts were combined, washed with acidic water (pH 3, three times with 50 ml), dried with anhydrous magnesium sulfate, filtered through filter paper, and evaporated to dryness under reduced pressure at room temperature. I-N-CG and I-N-C derivatives were obtained as clear colorless oils in 50-60% yield. LC-MS positive ion scanning analysis with UV detection at 226 nm showed the synthetic (*R,S*)-, (*R*)- and (*S*)-I-N-CG derivatives (MH⁺ *m/z* 367) eluting at retention times

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28.0-min for (*S*)-I-*N*-CG and 28.8-min for (*R*)-I-*N*-CG and both to be >99% pure. LC-MS/MS analyses were performed for both (*R*)- and (*S*)-I-*N*-CG derivatives by collision-induced dissociation (CID) of the MH^+ at m/z 367 and showed essentially identical product ion spectra: m/z (%): 292 ($[M+H - Gly]^+$, 28%), 264 ($[M+H - Gly-C=O]^+$, 20%), m/z 161 ([4-isobutylethylbenzene] $^+$, 100%), and m/z 76 ([glycine] $^+$, 3%). HPLC analyses with UV detection at 226 nm showed the synthetic (*R,S*)-, (*R*)- or (*S*)-I-*N*-C derivatives eluting at retention times 30.3-min for (*S*)-I-*N*-C and 31.4-min for (*R*)-I-*N*-C and both to be >99% pure. Tandem LC-MS/MS analyses were performed for both (*R*)- and (*S*)-I-*N*-C derivatives by CID of the MH^+ at m/z 310 and showed nearly identical product ion spectra: m/z (%): m/z 161 ([4-isobutylethylbenzene] $^+$, 100%), m/z 119 (*p*-ethyl-methylbenzene] $^+$, 80%), m/z 105 ([ethylbenzene] $^+$, 42%), and m/z 57 ([isobutyl] $^+$, 7%). 1H NMR analysis of (*R*)-I-*N*-C (2H_6 -DMSO): δ 0.82-0.85 (d, 6H, isopropyl -CH₃ groups), δ 1.31-1.33 (d, 3H, R-CH₃), δ 1.75-1.82 (m, 1H, isopropyl-CH), 2.40-2.42 (d, 2H, isopropyl -CH₂), 2.68-2.81 (m, 2H, Cys- β,β'), 3.70-3.75 (q, 1H, ibuprofen α -CH-), 4.29-4.35 (m, 1H, Cys- α), 7.10-7.30 (m, 4H, phenyl ring), 8.18-8.24 (m, 1H, Cys -NH). 1H NMR analysis of (*S*)-I-*N*-C (2H_6 -DMSO): δ 0.83-0.88 (d, 6H, isopropyl -CH₃ groups), δ 1.32-1.35 (d, 3H, R-CH₃), δ 1.76-1.85 (m, 1H, isopropyl-CH), 2.39-2.42 (d, 2H, isopropyl -CH₂), 2.73-2.89 (m, 2H, Cys- β,β'), 3.70-3.75 (q, 1H, ibuprofen α -CH-), 4.35-4.40 (m, 1H, Cys- α), 7.06-7.25 (m, 4H, phenyl ring), 8.28-8.30 (m, 1H, Cys -NH). 1H NMR analysis of (*R*)-I-*N*-CG (2H_6 -DMSO): δ 0.82-0.85 (d, 6H, isopropyl -CH₃ groups), δ 1.31-1.33 (d, 3H, R-CH₃), δ 1.75-1.82 (m, 1H, isopropyl-CH), 2.40-2.42 (d, 2H, isopropyl -CH₂), 2.68-2.81 (m, 2H, Cys- β,β'), 3.70-3.81 (m, 2H, Gly- α,α'), 3.70-3.75 (q, 1H, ibuprofen α -CH-), 4.29-4.35 (m, 1H, Cys- α), 7.10-7.30 (m, 4H, phenyl ring), 8.18-8.20 (d, 1H, Gly -NH), 8.30-8.35 (m, 1H, Cys -NH). 1H NMR analysis of (*S*)-I-*N*-CG (2H_6 -DMSO): δ 0.84-0.87 (d, 6H, isopropyl -CH₃ groups), δ 1.32-1.35 (d, 3H, R-CH₃), δ 1.75-1.83 (m, 1H, isopropyl-CH), 2.38-2.41 (d, 2H, isopropyl -CH₂), 2.65-2.83 (m, 2H, Cys- β,β'), 3.61-3.67 (m, 2H, Gly- α,α'), 3.70-3.77 (q, 1H, ibuprofen α -CH-), 4.40-4.46 (m, 1H, Cys- α), 7.06-7.25 (m, 4H, phenyl ring), 8.18-8.20 (d, 1H, Gly -NH), 8.30-8.35 (m, 1H, Cys -NH).

Synthesis I-*S*-NAC Thioester. I-*S*-NAC was obtained by reacting racemic I-SG (50 mg, 0.1 mmol) with NAC (200 mg, 1.2 mmol) in buffer (pH 7.4, 37°C) in a total volume of 10 ml and in

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shaking water bath incubator for 4-hr. The incubation then was acidified to pH 2.5 by the drop-wise addition of 1N HCl, followed by extraction of the I-S-NAC conjugate with ethyl acetate (2 x 10 ml). Then the ethyl acetate layers were combined, dried (anhydrous MgSO₄), and evaporated at room temperature under a stream of N₂ gas to afford 26 mg (75% yield) of product as a clear, colorless oil. Positive ion scanning LC-MS analysis of the I-S-NAC product (MH⁺ *m/z* 352, retention time 32.0-min) showed it to have less than 1% impurity of ibuprofen. LC-MS/MS mass spectrometric analysis was performed for the I-S-NAC derivative by CID of the MH⁺ at *m/z* 352: *m/z* (%): 164 ([N-acetylcysteine + H]⁺, 8%), *m/z* 161 ([4-isobutylethylbenzene]⁺, 100%), *m/z* 122 ([cysteine]⁺, 12%), *m/z* 118 ([N-acetylcysteine - CO₂H]⁺, 21%), *m/z* 119 ([*p*-ethyl-methylbenzene]⁺, 65%), *m/z* 105 ([ethylbenzene]⁺, 42%), *m/z* 91 ([CH₃COSH]⁺, 50%), and *m/z* 57 ([isobutyl]⁺, 7%).

In Vitro Experiments With γ -GT and I-SG. (*R,S*)-I-SG (100 μ M), (*R*)- and (*S*)-I-SG derivatives (50 μ M) were incubated separately with γ -GT (EC 2.3.2.2; type I from bovine kidney, 0.1 units/ml) in potassium phosphate buffer (0.05 M, pH 7.4, 4 ml total volume, 37°C, triplicate incubations) in screw-capped glass vials in a shaking incubator. One unit of the transpeptidase will liberate 1 μ mol of *p*-nitroaniline from L- γ -glutamyl-*p*-nitroaniline/min at pH 8.5 and 25°C. The γ -GT preparation was used without further purification and was reported to have less than 0.5% creatine phosphokinase, glutamic-oxalacetic transaminase, and glutamic pyruvic transaminase activity from by the supplier. Aliquots (100 μ l) of the incubations were taken at times 0-, 1-, 2-, 4-, 6-, 8-, 10-, and 15-min and added directly to 100 μ l of quench solution consisting of acetonitrile containing 3% formic acid (v/v) and CBZ internal standard (1 μ M). Quenched samples then were analyzed directly by LC-MS with UV detection at 226 nm. Aliquots (25 μ l) of quenched samples were diluted 100-fold with a solution of acetonitrile in water (1/1, v/v) prior to LC-MS/MS analysis. I-SG, I-*N*-CG, and CBZ were detected by LC-MS/MS with gradient elution as described above and by multiple reaction monitoring (MRM) at MH⁺ *m/z* 496 to *m/z* 161 for I-SG, MH⁺ 367 to *m/z* 161 for I-*N*-CG, and MH⁺ *m/z* 237 to *m/z* 194 for CBZ detection. Quantitative measurements were determined from linear standard curves generated from I-SG/CBZ and I-*N*-CG/CBZ peak area ratios. In order to provide more confirmation of the identity of the

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S to *N* rearranged products from the γ -GT-mediated degradation of racemic (*R,S*)-I-SG, at the 15-min time-point 50 μ l of a DMSO solution containing 1 mg of the sulfhydryl-reactive alkylating reagent iodoacetamide (IAA) was added to a 1 ml aliquot of the incubation mixture. This mixture then was incubated at 37°C in a shaking water bath for 30-min and processed for LC-MS (as described above) with UV detection at 226 nm. LC-MS/MS analysis was performed on the *S*-carbamidomethylated products by CID of the MH^+ ion at m/z 424.

Determination of K_m and v_{max} . Measurements of the apparent K_m and v_{max} values for (*R*)- and (*S*)-I-SG with γ -GT were performed as follows with commercially available bovine kidney γ -GT (0.1 units/ml) using the method of Tate (1975). Briefly, experiments were performed in 96-well plates where the absorbance at 412 nm (due to the absorbance of thionitrobenzoate) over time (3-min with one data point every 10-sec) was determined on a Molecular Devices SpectaMax Plus absorbance microplate reader (Technical Instruments, Burlingame, CA) at 37°C. In the reaction, the interaction of I-SG with γ -GT led to the formation of the reduced sulfhydryl (*I-N*-CG), which then reacted with 5,5'-dithiobis(2-nitrobenzoate) (DTNB) and led to the formation of thionitrobenzoate. Reaction mixtures (300 μ l, triplicate) contained 0.1M Tris-HCl buffer (pH 7.5), 20 mM glycyl-glycine, 1 mM DTNB, and increasing concentrations of (*R*)-I-SG or (*S*)-I-SG derivatives (3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, 500, and 1000 μ M).

In Vivo Studies. On three separate occasions, each separated by 1-week, a 800 mg dose of ibuprofen (Advil[®], four 200 mg caplets) was given orally to one male volunteer followed by urine collection every 2-h over a 10-h time period into a 250 ml glass bottle. Then, at each time-point, an equivalent volume (~100 ml) of acetonitrile containing 3% formic acid (v/v) and 1 μ M CBZ was added to the collected urine sample and mixed vigorously for 1-min. In triplicate, 2 ml of the quenched urine mixture were added to microcentrifuge tubes and centrifuged (14,000 rpm, 10-min). Supernatants then were analyzed for *I-N*-C, *I-N*-CG (as described above), and for *I-S*-NAC (positive ion MRM analysis for MH^+ m/z 352 to m/z 161) by LC-MS/MS. The concentrations of *I-N*-C, *I-N*-CG, and *I-S*-NAC derivatives

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in urine were determined from linear standard curves generated in pre-dose urine and from corresponding analyte/CBZ peak area ratios.

Reactions of I-SCoA and I-1- β -O-G with GSH in Buffer. Incubations (4 ml, $n=3$) containing both racemic I-SCoA (1 μ M) and racemic I-1- β -O-G (1 μ M) were performed in phosphate buffer (0.1 M, pH 7.4) at 37°C with 10 mM GSH in 20 ml glass vials. Aliquots (200 μ l) were removed from the incubations at 0-, 15-, 30, 45-, and 60-min and added to a quench solution (acetonitrile containing 3% formic acid [v/v] and 1 μ M CBZ, 200 μ l) in a 96-well plate. The quenched mixtures then were analyzed for I-SG concentration (as described above) by LC-MS/MS detection (Grillo and Hua, 2008). The concentration of I-SG was determined from a linear standard curve generated from I-SG/CBZ peak area ratios.

Results

Interactions of I-SG With γ GT In Vitro. Analysis of extracts from the incubation of racemic I-SG (100 μ M) with bovine kidney γ GT (type I, 0.1 units/ml) in buffer (pH 7.4) at 37°C showed the formation of two γ GT-mediated degradation products that were detected by UV analysis (Fig. 2A), by LC-MS positive ion scanning, and quantified (Fig. 5) by LC-MS/MS with MRM analysis of the MH^+ m/z 367 to m/z 161 transition. The chromatographic peaks (UV detection at 226 nm) eluting at retention times 28.0- and 28.8-min were derived from the γ GT-mediated degradation of (*S*)-I-SG and (*R*)-I-SG, respectively (Fig. 2B). LC-MS positive ion scanning showed the products to be isobaric with protonated molecular ions at m/z 367 (data not shown) which was consistent with the loss of the glutamic acid portion (-129 amu) from the racemic I-SG derivatives. LC-MS/MS analysis of the reactions mixture from the 15-min time-point confirmed both products to be I-*N*-CG derivatives (Fig. 3). Thus, both isomeric products provided identical tandem mass spectra when analyzed by CID of the MH^+ ion at m/z 367 and which were consistent with the product ion spectra of synthetic (*R*)- and (*S*)-I-*N*-CG standards (Fig. 3). Treatment of an aliquot of the incubation at the 15-min time-point with IAA led to a complete disappearance of free sulfhydryl containing I-*N*-CG products from the incubation mixture and

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showed the corresponding formation of a two *S*-carbamidomethylated derivatives eluting at 25.2- and 25.9-min (Fig. 2A). Tandem LC-MS/MS analysis of the *S*-carbamidomethylated products by positive ion CID of the MH^+ ion at m/z 424 provided identical tandem mass spectra consistent with their chemical structures, i.e., *S*-carbamidomethylated-*I-N*-CG (Fig. 4). Thus, the major fragment ion at m/z 206 provided strong evidence that ibuprofen was connected by an amide linkage to L-cysteinylglycine on the cysteine-moiety amino-group, since the m/z 206 fragment ion retained the nitrogen atom. The time-course of the degradation of (*R,S*)-*I-SG* by γ -GT showed a nearly linear formation of the corresponding *I-N*-CG products up to 6-min of incubation and complete degradation of (*R,S*)-*I-SG* and a corresponding quantitative formation of the *I-N*-CG products after 15-min of incubation (Fig. 5).

Determination of K_m and V_{max} . Studies performed to determine the activity of bovine kidney γ -GT (0.1 units/ml) towards the degradation of (*R*)- and (*S*)-*I-SG* showed that both isomers are equally good substrates for the enzyme. The apparent K_m and v_{max} values were determined to be nearly identical and were $K_m=130\pm 8 \mu M$ and $v_{max}=24\pm 0.3$ units/l for (*R*)-*I-SG*, and $K_m=130\pm 9 \mu M$ and $v_{max}=23\pm 0.4$ units/l for (*S*)-*I-SG* (Fig. 6). The apparent K_m and v_{max} for both (*R*)- and (*S*)-*I-SG* are of the same order of magnitude as determined for other *S*-linked glutathione conjugates studied with rat kidney γ -GT and cross-species for liver γ -GT (Tate, 1975; Tate and Meister, 1985; Petitchlerc et al., 1980; Verhoeff et al., 1988).

Identification of *I-N-C*, *I-N-CG*, and *I-S-NAC* in Vivo in Human. Sensitive and selective LC-MS/MS MRM techniques facilitated the identification of *I-N-C*, *I-N-CG*, and *I-S-NAC* derivatives present in urine extracts from a healthy ibuprofen-dosed (Advil[®], 800 mg) volunteer (Figs. 7-9). The LC-MS/MS transitions used for MRM analysis were MH^+ m/z 310 to m/z 161 for *I-N-C*, MH^+ m/z 367 to m/z 161 for *I-N-CG*, and MH^+ m/z 352 to m/z 161 for *I-S-NAC* derivatives, where in each case the m/z 161 fragment ion was the, or a, major product ion (Figs. 7C, 8C, and 9C). LC-MS/MS analysis showed the presence of both (*R*)- and (*S*)-*I-N-C* conjugates in urine extracts which co-eluted with authentic standards at retention times of 31.3- and 30.3-min, respectively (Fig. 7A). Tandem LC-MS/MS analysis of (*R*)-*I-N-C* and (*S*)-*I-N-C* in the same urine extracts by CID of the MH^+ ion at m/z 310 provided

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product ions that were identical for both (*R*)- and (*S*)-*I-N-C* isomers (Fig. 7B) and with the mass spectra of authentic standards (Fig. 7C), which were consistent with their chemical structures (Fig. 7D). LC-MS/MS MRM analysis showed the presence of both (*R*)- and (*S*)-*I-N-CG* in urine extracts and which co-eluted with authentic standards at retention times of 29.0- and 28.2-min, respectively (Fig. 8A). Tandem LC-MS/MS analysis of (*R*)-*I-N-CG* and (*S*)-*I-N-CG* conjugates in the urine extract by CID of the MH^+ ion at m/z 367 provided product ions that were nearly identical for both (*R*)- and (*S*)-*I-N-CG* isomers (Fig. 8B) and with the mass spectra of authentic standard (Fig. 8C) and were consistent with their chemical structures (Fig. 8D). LC-MS/MS MRM analysis also showed the presence of *I-S-NAC* in urine extracts and which co-eluted with authentic standard at a retention time of 32.0-min (Fig. 9A). Tandem LC-MS/MS analysis of *I-S-NAC* in the urine extract by CID of the MH^+ ion at m/z 352 provided a product ion spectrum (Fig. 9B) that was nearly identical to the tandem mass spectra of authentic standard (Fig. 9C) and which was consistent with its chemical structure (Fig. 9D). The quantitative analysis of *I-N-CG*, *I-N-C*, and *I-S-NAC* present in human urine extracts (triplicate studies) showed linear accumulation in urine up to the 6-h time-point post-administration for each analyte (Fig. 10). A total of 6.0 ± 0.8 , 1.7 ± 0.4 and 0.2 ± 0.1 μg of *I-N-CG*, *I-N-C*, and *I-S-NAC*, respectively, were excreted into urine over a 10-h collection period post-administration.

Reaction of GSH with I-SCoA and I-1- β -O-G 1 μM in Buffer. Separate incubations of racemic *I-SCoA* (1 μM) and racemic *I-1- β -O-G* (1 μM) with GSH (10 mM) in buffer (0.1 M potassium phosphate, pH 7.4, 37°C) resulted in the transacylation of GSH forming 0.56 μM and 0.035 μM *I-SG*, respectively, after 60-min of incubation (Fig. 11). The reaction of *I-SCoA* with GSH forming *I-SG* occurred at a rate of 11 nM *I-SG*/min over the first 30-min of incubation, which was 17-fold more rapid than the corresponding reaction of *I-1- β -O-G* with GSH forming *I-SG* over the same incubation time-period.

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Discussion

Carboxylic acid-containing drugs such as ibuprofen can be metabolized to reactive acyl glucuronide and *S*-acyl-CoA metabolites that can mediate the transacylation of nucleophilic residues on protein and GSH in vitro and in vivo (Castillo and Smith, 1995; Castillo et al., 1995; Grillo and Hua, 2008; Grillo, 2011). For ibuprofen, most attention has focused on the chemical reactivity of its acyl glucuronide metabolite, where reports demonstrated it to mediate the covalent binding of ibuprofen to plasma protein and human serum albumin in vitro (Castillo and Smith, 1995; Castillo et al., 1995). However, in addition to I-1- β -O-G formation, metabolism by *S*-acyl-CoA formation leads to a reactive transacylating *S*-acyl-CoA thioester, namely I-SCoA, where it has also been proposed to be reactive with protein nucleophiles in vitro and in vivo leading to covalent binding to protein (Xiaotao and Hall, 1993; Grillo and Hua, 2008). Thus, in human, ibuprofen acyl glucuronide and/or acyl-CoA thioester metabolites are proposed to react with GSH in a transacylation-type fashion leading to the formation of I-SG thioester (Fig. 1). Recent in vitro studies in buffer and in incubations with rat hepatocytes showed that I-SCoA thioester to be reactive with GSH forming I-SG (Grillo and Hua, 2008). Results from rat hepatocyte studies with (*R*)- and (*S*)-ibuprofen enantiomers showed I-SG thioester formation to be 26-fold enantioselective in favor of the (*R*)-isomer, which was consistent with an 11-fold enantioselectivity of I-SCoA formation in favor of the (*R*)-isomer, but not with bioactivation by acyl glucuronidation. In addition, it was shown that inhibition of acyl glucuronidation by coincubation with the glucuronidation inhibitor (-)-borneol did not lead to decreased I-SG thioester formation in incubations with rat hepatocytes. These results led to the proposal that metabolism to reactive I-SCoA thioester was more important than I-1- β -O-acyl glucuronide formation in mediating the production of I-SG thioester in vitro and therefore potentially in vivo. The I-SG metabolite formed in vivo then may either be excreted unchanged in bile or, *via* the classical mercapturic acid pathway, undergo sequential enzyme catalyzed degradation steps to the mercapturic acid conjugate (I-S-NAC) prior to excretion in bile and/or urine. However, other than one report on the detection of clofibril-*S*-acyl-NAC in human urine (Shore et al., 1995), we have searched unsuccessfully for *S*-acyl-linked mercapturic acid conjugates of carboxylic

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acid drugs as a marker for *S*-acyl-GSH conjugate formation in vivo (Grillo and Benet, 2001; Grillo et al., 2008). The first report on the γ -GT-mediated degradation of *S*-acyl-GSH thioesters in vitro showed γ -GT to hydrolyze the glutamic acid moiety from *S*-acyl-GSH conjugates of acetic, lactic and benzoic acid yielding specifically *N*-acyl-cysteinylglycine products (Tate, 1975). The γ -GT-mediated cleavage of the γ -glutamyl group led to chemically unstable *S*-acyl-cysteinylglycine products that rapidly rearranged to the corresponding *N*-acyl-cysteinylglycine conjugates. Therefore, the rearrangement of an *S*-acyl-cysteinylglycine to the α -amino group blocked *N*-acyl-cysteinylglycine derivative would preclude its further degradation to the corresponding mercapturate and provide an explanation for a lack of mercapturic acid adduct detection in urine. In the present studies, we determined indirectly if ibuprofen is metabolized to I-SG in vivo in human by examining urine extracts from an ibuprofen-dosed volunteer for the presence of I-*S*-NAC and the rearranged I-*N*-CG and I-*N*-C amide conjugates. Prior to the in vivo studies, we first examined the γ -GT-mediated degradation of I-SG thioester in vitro with commercially available bovine kidney γ -GT.

Results from the present in vitro studies showed that racemic I-SG was efficiently and quantitatively degraded by γ -GT resulting solely in the formation of the rearranged I-*N*-CG amide-linked isomeric products (Figs. 2-5). We did not obtain evidence for I-*S*-CG thioester formation during incubations of (*R,S*)-I-SG with γ -GT (Fig. 2A). Thus, from the LC-UV and LC-MS analysis of incubation extracts, we were only able to detect and chromatographically resolve two isomeric products, (*R*)- and (*S*)-I-*N*-CG (Figs. 2 and 3), that when treated with the *S*-alkylating reagent IAA led to formation of two corresponding *S*-carbamidomethylated derivatives (Figs. 2A and 4). Therefore, the γ -GT-mediated degradation of (*R,S*)-I-SG in vitro at pH 7.4 led to hydrolysis of the γ -glutamic acid group followed by the rapid intramolecular *S* to *N* rearrangement resulting in a free cysteinyl-sulfhydryl that was able to react quantitatively with IAA. All together, these results are in agreement with the observations reported by Tate (1975) from studies with varied *S*-acyl-GSH derivatives, where in the present experiments the rearranged ibuprofen-*N*-acyl-cysteinylglycine conjugate, and not the ibuprofen-*S*-acyl-cysteinylglycine derivative, was the product detected in vitro from γ -GT-mediated degradation of the I-SG thioester. The

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γ -GT-mediated degradation of racemic I-SG was shown not to be enantioselective, where chromatographic analysis with UV detection (226 nm) showed equal I-*N*-CG product formation (Fig. 2). In addition, in vitro kinetic studies to determine the activity of γ -GT for the (*R*)- and (*S*)-I-SG isomers showed no significant difference in K_m and v_{max} values (Fig. 6).

In the present investigation, which employed sensitive LC-MS/MS techniques, we report the detection of mercapturic acid pathway-mediated degradation products of ibuprofen-*S*-acyl-GSH in ibuprofen-dosed (Advil[®], 800 mg) healthy volunteer urine extracts. These degradation products included ibuprofen-*N*-acyl-amide-linked cysteine (I-*N*-C) and cysteinylglycine (I-*N*-CG) conjugates, as well as the mercapturic acid conjugate, ibuprofen-*S*-acyl-NAC. To our knowledge, *N*-acyl-amide-linked cysteine and cysteinylglycine conjugates have not been observed previously as metabolites for 2-arylpropionic acid NSAIDs nor for other carboxylic acid-containing drugs in human.

In these studies, the LC-MS/MS analysis of ibuprofen-dosed human urine extracts showed that both the *R*- and *S*-isomers of the I-*N*-C and I-*N*-CG metabolites were detected (Figs. 7 and 8); however, the relative importance of I-SCoA versus I-1- β -*O*-G in mediating the formation of I-SG in vivo is not yet known. Ibuprofen is known to be metabolized to I-SCoA in human liver homogenate (Tracy et al., 1993) and to undergo enantioselective acyl-CoA mediated chiral inversion in human in favor of the *R*-isomer as well as enantioselective acyl glucuronidation in favor of the *S*-isomer (Tan et al., 2002; Lee et al., 1985; el Mouelhi et al., 1987). In these studies, we showed that the I-SCoA derivative to be 17-fold more reactive than the I-1- β -*O*-G in incubations with GSH in buffer (Fig. 11). Based on these results, and on results from published studies in rat hepatocytes incubated with enantiomerically pure (*R*)- and (*S*)-ibuprofen isomers showing that chemically-reactive I-SCoA thioester, and not the I-1- β -*O*-G metabolite, led to the transacylation of hepatocyte GSH (Grillo and Hua, 2008), we propose that the I-*N*-C and I-*N*-CG products detected in human urine come primarily from the degradation of I-SG formed in vivo from the reaction of I-SCoA with GSH. Although it may be possible that I-SCoA could react with free hepatocellular cysteine in vivo leading to I-*S*-C, and after *S* to *N* rearrangement to I-*N*-C, the low

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concentration of cysteine in liver tissue (20 to 100 μM ; Stipanuk et al., 2006) makes the transacylation reaction with GSH (5-10 mM in liver tissue) more likely.

Although the analysis of ibuprofen-dosed human urine extracts in the present studies showed that the I-*N*-C, I-*N*-CG, and I-*S*-NAC conjugates were quantitatively very minor metabolites of the drug that contribute less than a fraction of a percent to its in vivo clearance, the detection of these conjugates does provide strong evidence that ibuprofen is metabolized in human to reactive metabolites that transacylate GSH. The observation that the I-*S*-NAC mercapturate was detected in ibuprofen-dosed human urine (Fig. 9) implied that the interaction of γ -GT with I-SG led to the thioester product I-*S*-CG that was able to escape the active-site of the enzyme and serve as a substrate for dipeptidase-mediated hydrolysis leading to the *S*-acylated cysteine derivative, I-*S*-C, which underwent *N*-acetylation (Fig. 1). Likewise, the formation of I-*N*-C detected in urine could potentially be due to the *S* to *N* intramolecular rearrangement of I-*S*-C (Fig. 1). We did not investigate the products of the γ -GT reaction under slightly acidic conditions, which might occur in vivo. It is known that *S*-acylated cysteine derivatives are stable at slightly acidic pH, where the cysteine α -amino-group is protonated and unreactive (Katritzky et al., 2009), and which might provide an explanation for the subsequent *N*-acetylation of I-*S*-C and the detection of I-*S*-NAC in human urine in the present studies.

In summary, results from the present studies have shown that I-SG undergoes γ -GT-mediated degradation in vitro and in vivo to an unstable and undetected *S*-acylated-cysteinylglycine intermediate that rearranges to the *N*-acyl-cysteinylglycine amide derivative detected in human urine extracts. Evidence for the formation and excretion in urine of the mercapturic acid conjugate of ibuprofen, I-*S*-NAC, in human was also found, however at 30- and 9-fold lower amounts relative to I-*N*-CG and I-*N*-C, respectively. Therefore, based on these data and on related reports, we propose that the mercapturate conjugate should not be the primary metabolite searched for in urine extracts as a marker for *S*-acyl-GSH-adduct formation occurring in vivo. Finally, from the results of the present studies on the degradation of I-SG thioester by γ -GT, we propose that drug-*N*-acyl-cysteinylglycine and/or drug-*N*-acyl-

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cysteine derivatives be used as markers of *S*-acyl-GSH thioester formation occurring in vivo when examining urines extracts from patients dosed with carboxylic acid-containing drugs.

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Authorship Contributions

Participated in research design: Grillo.

Conducted experiments: Grillo, Lohr, and Khera.

Contributed new reagents or analytic tools: Grillo.

Performed data analysis: Grillo, Lohr, and Khera.

Wrote or contributed to the writing of the manuscript: Grillo.

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Figure Legends

FIG. 1. Proposed scheme for the metabolic activation of ibuprofen in vivo followed by reaction with GSH forming I-SG and its subsequent degradation by the enzymes of the mercapturic acid pathway leading to the elimination of I-N-CG, I-N-C, and I-S-NAC in urine.

FIG. 2. Representative reverse-phase gradient LC-UV (226 nm) chromatograms of incubation extracts containing γ GT (0.1 units/ml in 0.1 mM potassium phosphate buffer, pH 7.4, 37°C) and: A) (*R,S*)-I-SG (100 μ M) incubated for (a) 0.2-min, 1(b) 5-min, (c) for 15-min followed by reaction with iodoacetamide and (d) compared to authentic standard (*R,S*)-I-N-CG (d); and B) (*R*)- and (*S*)-I-SG (50 μ M) incubated separately as above for 0.2-min (a and c) and for 15-min (b and d), respectively.

FIG. 3. LC-MS/MS tandem mass spectra of A) (*R*)-I-N-CG and C) (*S*)-I-N-CG formed in incubations of (*R,S*)-I-SG (100 μ M) with γ GT (0.1 units/ml in 0.1 mM potassium phosphate buffer, pH 7.4, 37°C, 15-min); and B) authentic (*R*)-I-N-CG, and D) (*S*)-I-N-CG standards obtained by CID of the protonated molecular ion at m/z 367. Origins of the diagnostic fragment ions are as noted.

FIG. 4. LC-MS/MS tandem mass spectra of *S*-carbamidomethylated-I-N-CG obtained by positive ion CID of the MH^+ ion at m/z 424 of chromatographic peaks eluting at A) 25.2- and B) 25.9-min which provided identical tandem mass spectra consistent with their chemical structures. Origins of the diagnostic fragment ions are as noted. I-N-CG derivatives were obtained from the treatment of (*R,S*)-I-SG (100 μ M) with γ GT (0.1 units/ml in 0.1 mM potassium phosphate buffer, pH 7.4, 37°C) followed by reaction of incubation aliquots with iodoacetamide for 30-min leading to the formation of the *S*-carbamidomethylated products.

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FIG. 5. Time-course of the degradation of (*R,S*)-I-SG (100 μ M) by γ GT (0.1 units/ml) in buffer (0.1 M potassium phosphate, pH 7.4, 37°C). Values are expressed as the mean \pm SD from triplicate incubations.

FIG. 6. Michaelis-Menten plot for the determination of the K_m and v_{max} for (*R*)- and (*S*)-I-SG in interactions with bovine kidney γ -GT (0.1 units/ml). Values are expressed as the mean \pm SD from triplicate incubations.

FIG. 7. A) Representative reverse-phase gradient LC-MS/MS MRM chromatograms of I-*N*-C from a) the analysis of pre-dose urine spiked with (*R*)- and (*S*)-I-*N*-C authentic standard (1/1 mixture), b) the analysis of combined human urine (0-10-h) extracts from an ibuprofen-dosed (800 mg) volunteer, and c) the analysis of pre-dose urine extract. The LC-MS/MS MRM transition used for the detection of (*R*)- and (*S*)-I-*N*-C was MH^+ m/z 310 to m/z 161. B) LC-MS/MS tandem mass spectrum of (*R*)-I-*N*-C present in ibuprofen-dosed human urine extract obtained by CID of the protonated molecular ion at m/z 310 (both (*R*)- and (*S*)-I-*N*-C provided essentially identical product ion mass spectra). C) Corresponding LC-MS/MS tandem mass spectrum of authentic (*R*)-I-*N*-C standard. D) The chemical structure and origins of the characteristic fragment ions for (*R*)- and (*S*)-I-*N*-C are as shown.

FIG. 8. A) Representative reverse-phase gradient LC-MS/MS MRM chromatograms of I-*N*-CG from a) the analysis of pre-dose urine spiked with (*R*)- and (*S*)-I-*N*-CG authentic standard (1/1 mixture), b) the analysis of combined human urine (0-10-h) extracts from an ibuprofen-dosed (800 mg) volunteer, and c) the analysis of pre-dose urine extract. The LC-MS/MS MRM transition used for the detection of (*R*)- and (*S*)-I-*N*-CG was MH^+ m/z 367 to m/z 161 310. B) LC-MS/MS tandem mass spectrum of (*R*)-I-*N*-CG present in ibuprofen-dosed human urine extract obtained by CID of the protonated molecular ion at m/z 367 (both (*R*)- and (*S*)-I-*N*-CG provided essentially identical product ion mass spectra). C) Corresponding LC-MS/MS tandem mass spectrum of authentic (*R*)-I-*N*-CG standards. D) The chemical structure and origins of the characteristic fragment ions for (*R*)- and (*S*)-I-*N*-CG are as shown.

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FIG. 9. A) Representative reverse-phase gradient LC-MS/MS MRM chromatograms of I-S-NAC from a) the analysis of pre-dose urine spiked with I-S-NAC authentic standard, b) the analysis of combined human urine (0-10-h) extracts from an ibuprofen-dosed (800 mg) volunteer, and c) the analysis of pre-dose urine extract. The LC-MS/MS MRM transition used for the detection of I-S-NAC was MH^+ m/z 352 to m/z 161. B) LC-MS/MS tandem mass spectrum of I-S-NAC present in ibuprofen-dosed human urine extract obtained by CID of the protonated molecular ion at m/z 352. C) Corresponding LC-MS/MS tandem mass spectrum of authentic I-S-NAC standard. D) The chemical structure and origins of the characteristic fragment ions for I-S-NAC are as shown.

FIG. 10. Cumulative excretion of I-N-CG, I-N-C, and I-S-NAC into urine following a single oral dose of ibuprofen (800 mg) to a volunteer (data represent the mean \pm SD from three studies and triplicate measurements at each time-point).

FIG. 11. Time-dependent formation of the I-SG thioester obtained from the incubation of racemic I-SCoA (1 μ M) and racemic I-1- β -O-G (1 μ M) with GSH (10 mM) in potassium phosphate buffer (0.1 M, pH 7.4, 37°C). Values are expressed as the mean \pm SD from three incubations.

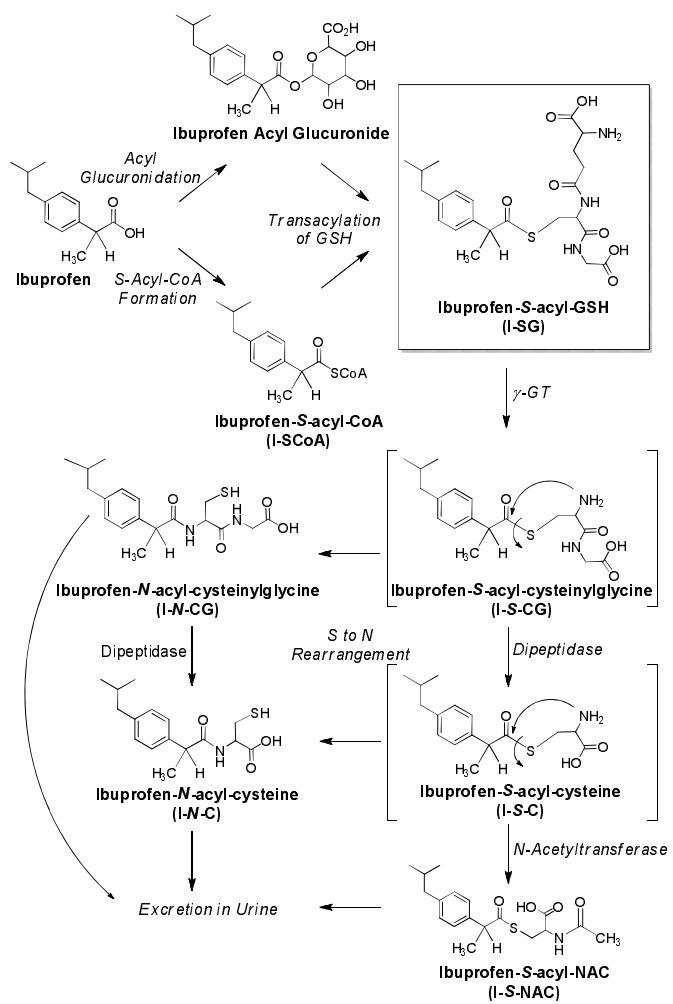


Fig. 1

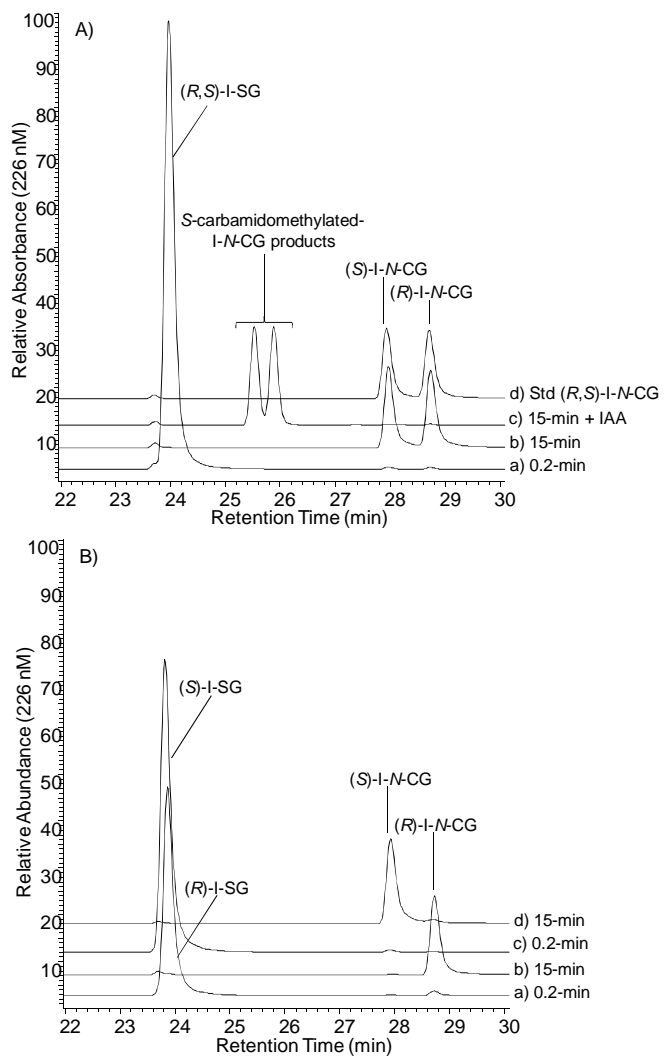


Fig. 2

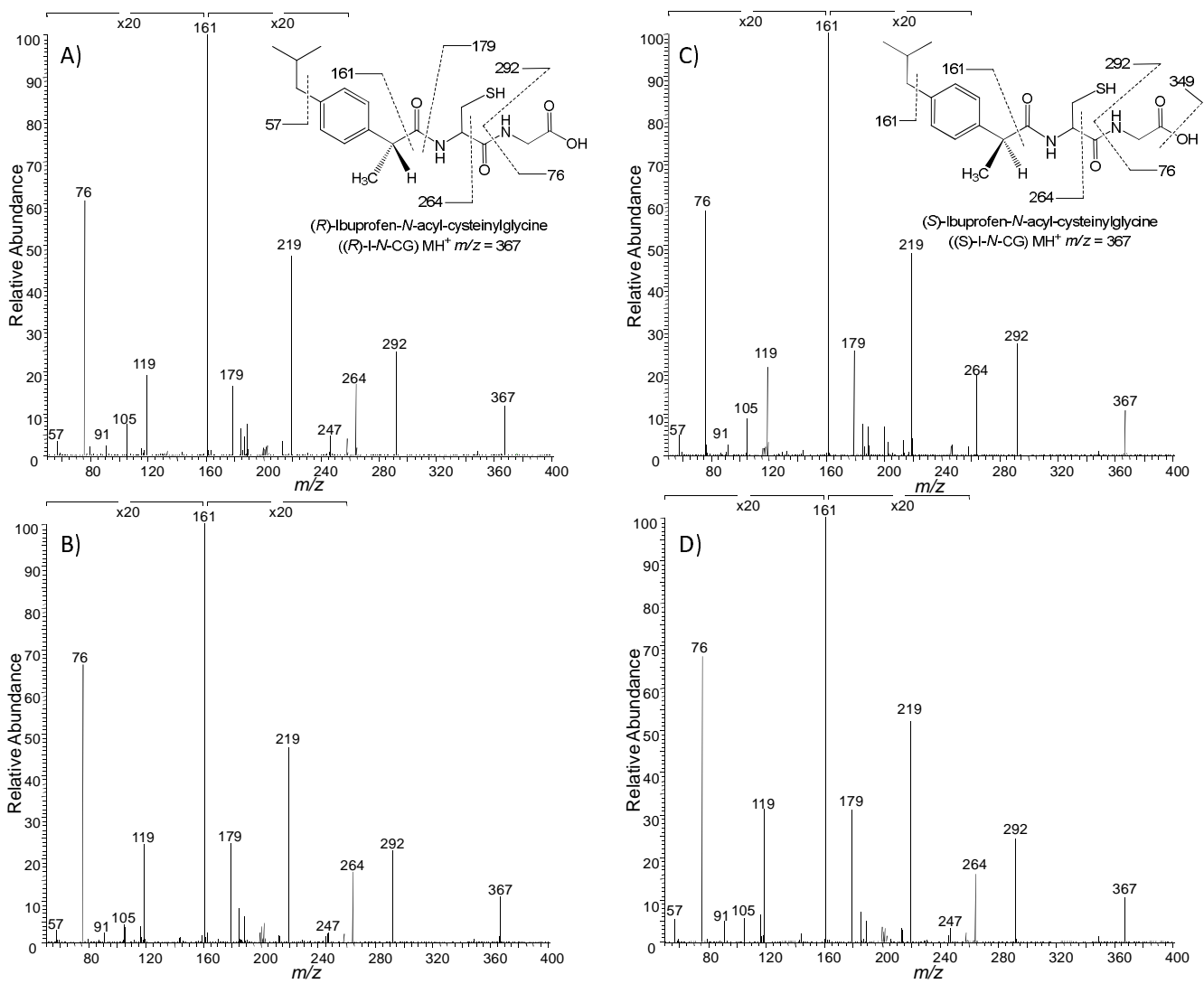


Fig. 3

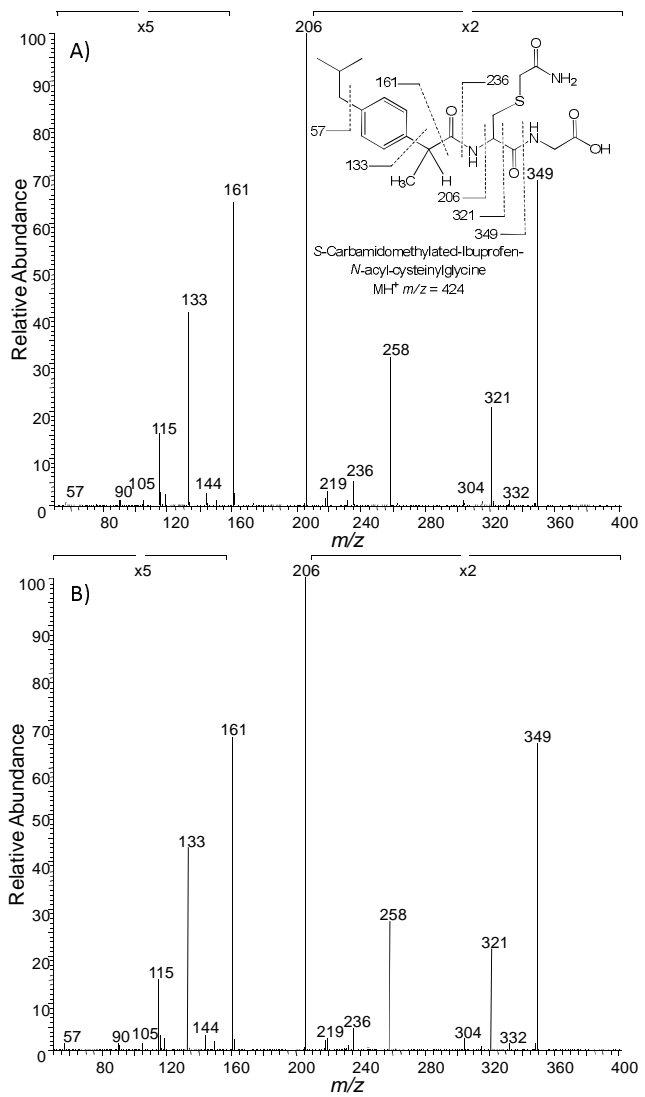


Fig. 4

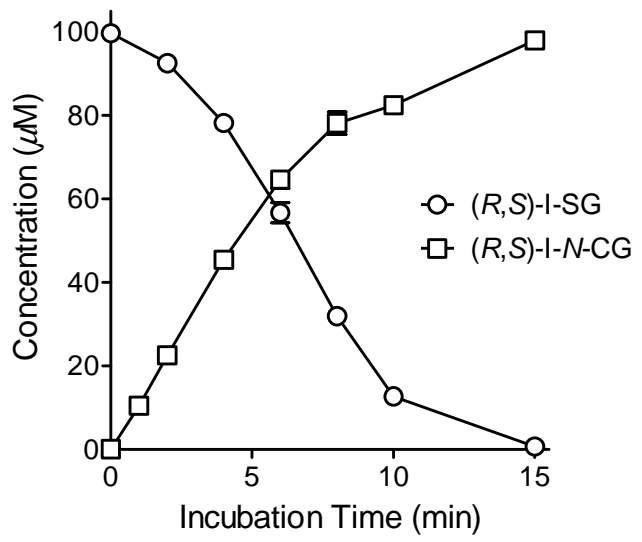


Fig. 5

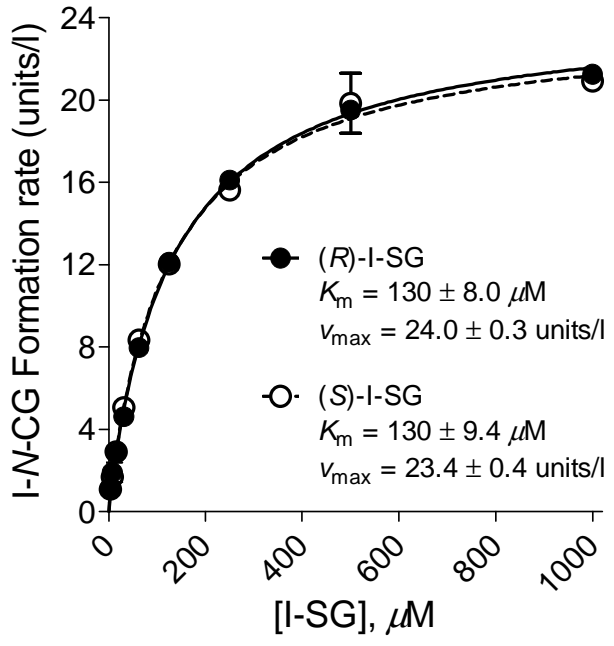


Fig. 6

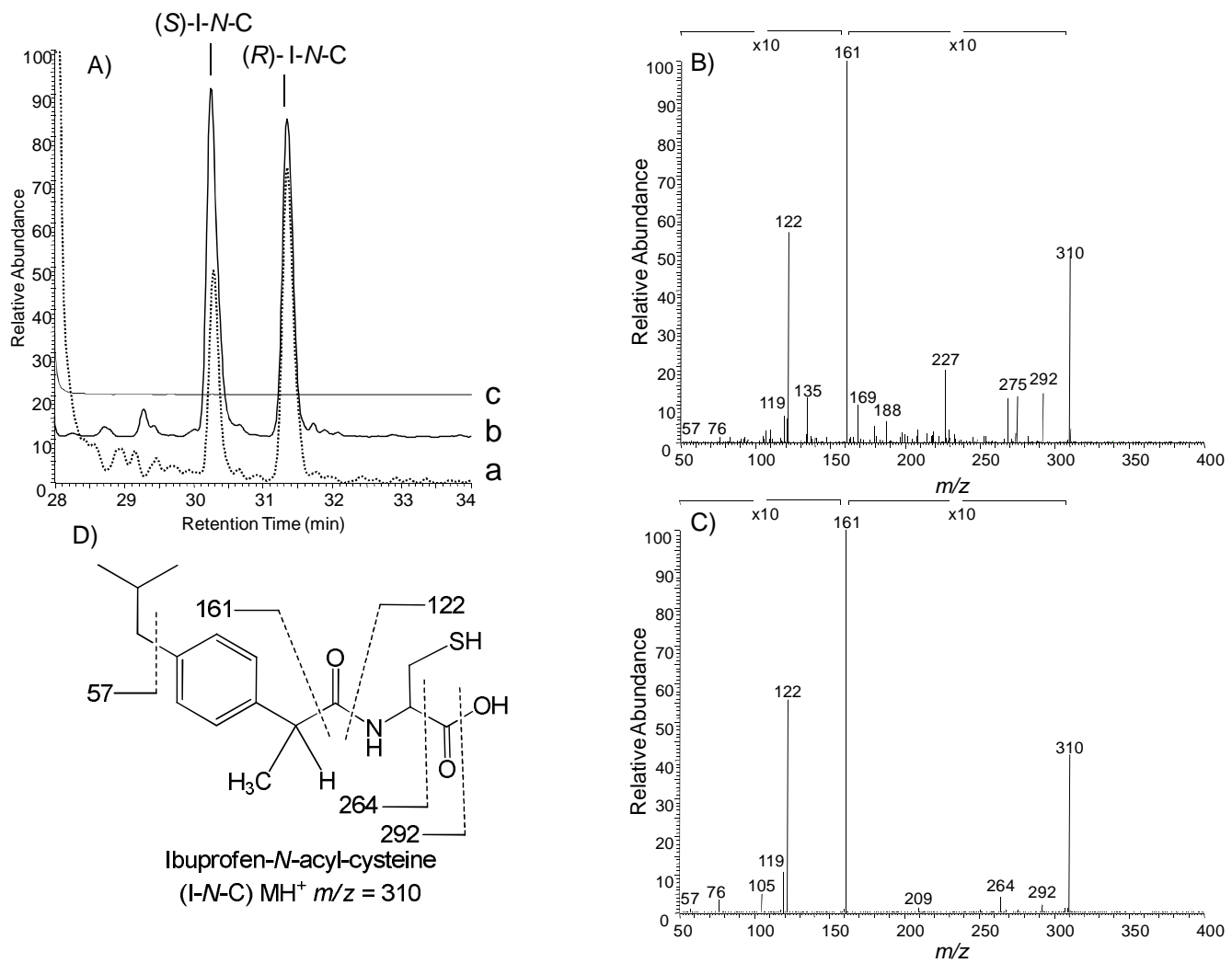


Fig. 7

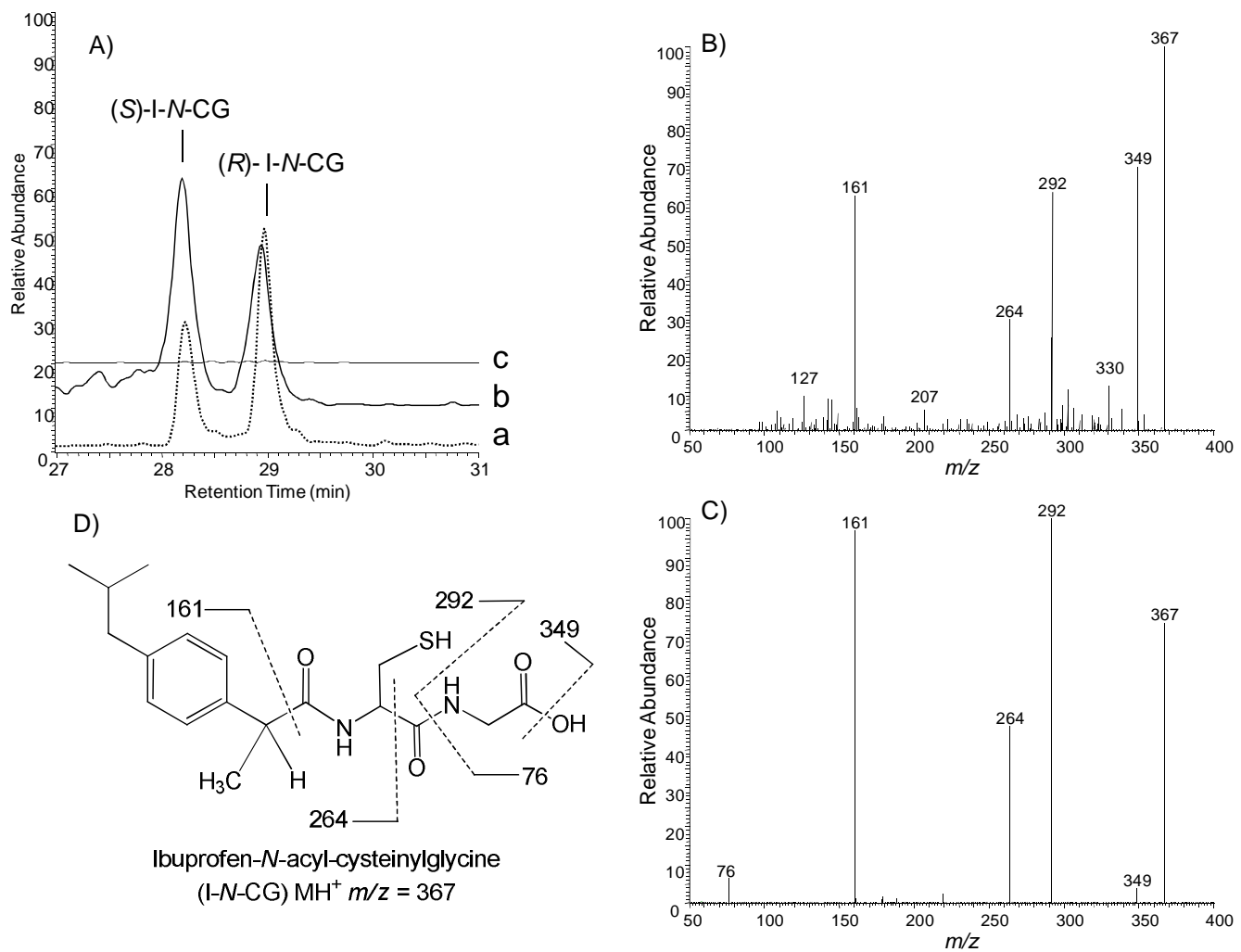


Fig. 8

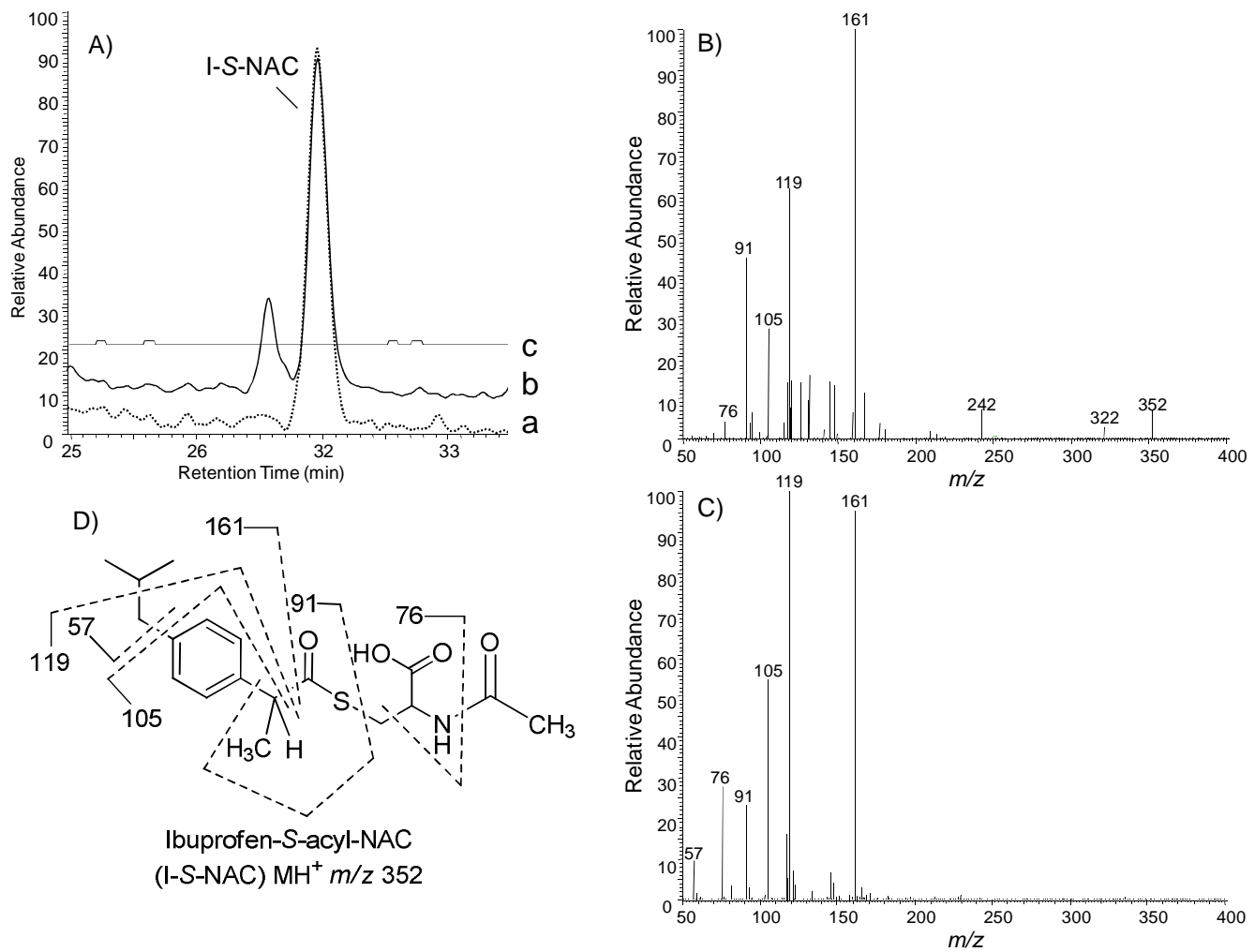


Fig. 9

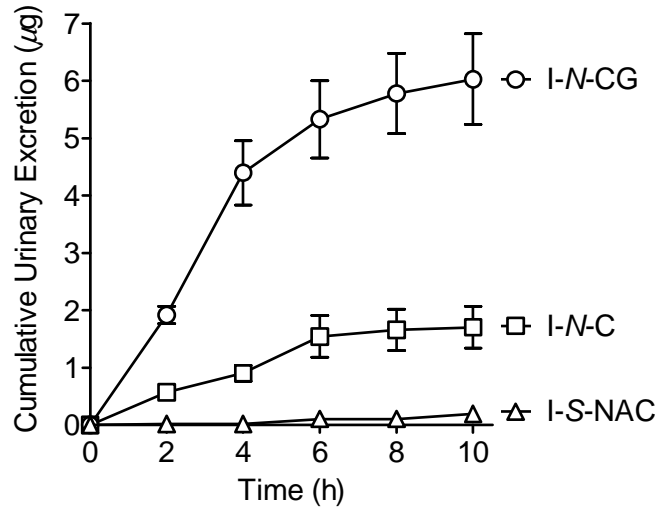


Fig. 10

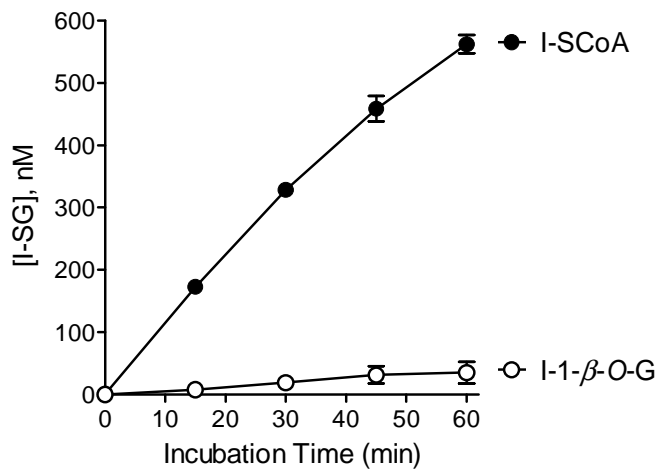


Fig. 11