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# STUDIES ON THE CHEMICAL REACTIVITY OF DICLOFENAC ACYL GLUCURONIDE WITH GLUTATHIONE: IDENTIFICATION OF DICLOFENAC-*S*-ACYL-GLUTATHIONE IN RAT BILE

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**ABSTRACT:**

**Diclofenac, a nonsteroidal anti-inflammatory drug, is metabolized to a reactive acyl glucuronide that has been proposed to mediate toxic adverse drug reactions associated with its use. In the present study, we examined the ability of diclofenac acyl glucuronide (D-1-*O*-G) to transacylate glutathione (GSH) in vitro in buffer and in vivo in rats. Thus, in vitro reactions of D-1-*O*-G (100 $\mu$M) with GSH (10 mM) at pH 7.4 and 37°C showed a linear time-dependent formation of diclofenac-*S*-acyl-glutathione (D-SG, 3 $\mu$M/h) through 60 min of incubation, reaching a maximum of 3.7 $\mu$M after 2 h of incubation. The major reaction that occurred was acyl migration of D-1-*O*-G ($t_{1/2}$, 54 min) to less reactive isomers. The D-SG thioester product was shown to be unstable by degrading primarily to 1-(2,6-dichlorophenyl)indolin-2-one and by hydrolysis to diclofenac. After administration of diclofenac to rats (200 mg/kg), bile was collected and analyzed for D-SG by liquid chromatography-tandem mass spectrometry. Results indicated the presence of D-SG, which was confirmed by coelution with synthetic standard and by its tandem mass spectrum. When the reactivity of D-SG (100 $\mu$M) was compared with D-1-*O*-G (100 $\mu$M) in vitro in reactions with *N*-acetylcysteine (NAC, 10 mM), results showed the quantitative reaction of D-SG with NAC after 30 min of incubation, whereas only ~1% of D-1-*O*-G reacted to form diclofenac-*S*-acyl-NAC at the same time point. Results from these studies indicate that GSH reacts with D-1-*O*-G in vitro, and presumably in vivo, to form D-SG, and that the product D-SG thioester is chemically more reactive in transacylation-type reactions than the D-1-*O*-G metabolite.**

Diclofenac (*o*-[(2,6-dichlorophenyl)amino]phenylacetic acid; Fig. 1) is a carboxylic acid-containing nonsteroidal anti-inflammatory drug used for the treatment of osteoarthritis, rheumatoid arthritis, or ankylosing spondylitis (Small, 1989; Banks et al., 1995). Use of diclofenac has been associated with a rare, but serious, hepatic injury that is proposed to be mediated by chemically reactive metabolites of the drug (Hargus et al., 1994; Banks et al., 1995). Diclofenac is metabolized to a reactive acyl glucuronide metabolite, namely diclofenac-1-*O*-acyl glucuronide (D-1-*O*-G[1]; Fig. 1; Kretz-Rommel and Boelsterli, 1993; Ebner et al., 1999), which has been implicated as playing an important role in the mechanism of diclofenac-mediated toxicity. Acyl glucuronide metabolites of acidic drugs, in general, have been shown to bind covalently to protein by two different mechanisms, including transacylation reactions of protein nucleophiles with the 1-*O*-acyl glucuronide isomer and through a glycation mechanism involving the reaction of protein amino groups with open-chain aldehyde forms of the acyl migration isomers of the glucuronide (Fig. 1; Faed, 1984; Spahn-Langguth and Benet, 1992). Acidic drug-protein adducts then may be recognized by the immune system as foreign, resulting in an immune response and thereby leading potentially to the associated idiosyncratic hepatotoxicity (Banks et al., 1995; Zia-Amirhosseini et al., 1995).

Acyl glucuronidation of diclofenac, which has been shown to be catalyzed by rat UGT2B1 and human UGT2B7 UGT isoforms (King et al., 2001), leads to a time- and concentration-dependent covalent binding to hepatocyte protein in vitro (Kretz-Rommel and Boelsterli, 1993). Immunochemical detection of hepatic protein adducts formed from reactive metabolites of diclofenac revealed selective adduct formation to 50-, 70-, 110-, and 140-kDa proteins in the livers of diclofenac-treated mice (Pumford et al., 1993). The authors of that study proposed the acyl glucuronide of diclofenac as a likely reactive metabolite, at least in part for the formation of the hepatic protein adducts. In another study, Hargus et al. (1995) showed the diclofenac/110-kDa protein adduct to be a canalicular plasma membrane protein identified as dipeptidyl peptidase IV. Other reactive metabolites of diclofenac that may have contributed to the covalent binding to hepatic protein come from cytochrome P450-mediated aromatic hydroxylation of diclofenac followed by further oxidation to reactive benzoquinone imine intermediates (Shen et al., 1999; Tang et al., 1999). In the latter study, glutathione (GSH) conjugates of these benzoquinone imine intermediates that were discovered were identi-

A preliminary account of this work was presented at the XIVth World Congress of Pharmacology (IUPHAR) in San Francisco, CA in July 2002.

[1] Abbreviations used are: D-1-*O*-G, diclofenac-1-*O*-acyl glucuronide; UGT, uridine diphosphate glucuronosyltransferase; GSH, glutathione; D-SG, diclofenac-*S*-acyl-glutathione thioester; D-SCoA, diclofenac-*S*-acyl-CoA thioester; CBZ, carbamazepine; $\beta$-glucuronidase, $\beta$-D-glucuronide glucuronosohydrolase; D-SNAC, diclofenac-*S*-acyl-*N*-acetylcysteine thioester; HPLC, high-performance liquid chromatography; SVC, superior vena cava; ACN, acetonitrile; LC-MS/MS, liquid chromatography-tandem mass spectrometry; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran; NAC, *N*-acetylcysteine; CID, collision-induced dissociation; MRM, multiple reaction monitoring; SRM, selected reaction monitoring.

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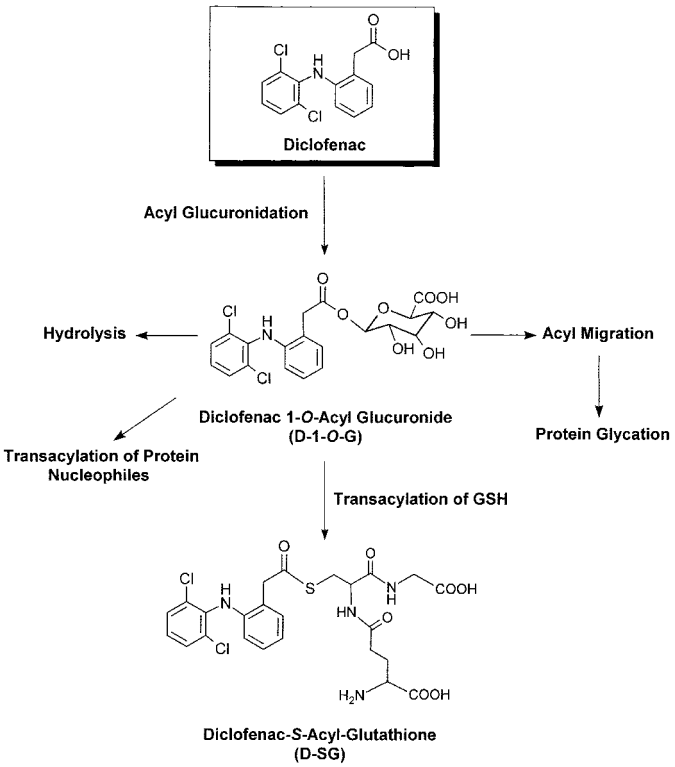


FIG. 1. *Proposed scheme for the metabolic activation of diclofenac by acyl glucuronidation leading to covalent binding to protein and the transacylation of glutathione.*

fied in the bile of diclofenac-dosed rats and in incubations of diclofenac with human hepatocytes.

We propose that diclofenac is metabolized to diclofenac-*S*-acyl-glutathione (D-SG) thioester from transacylation reactions of GSH with D-1-*O*-G (Fig. 1) or from similar reactions with other potential acylating metabolites of the drug, such as diclofenac-*S*-acyl-CoA thioester (D-SCoA). Evidence supporting this proposal comes from studies in which it was shown that clofibric acid, a lipid lowering drug, is metabolized to reactive acylating glucuronide and acyl-CoA thioester conjugates that can transacylate GSH-forming clofibryl-*S*-acyl-glutathione in vivo (as detected in rat bile) and in vitro (Stogniew and Fenselau, 1982; Shore et al., 1995; Grillo and Benet, 2002). The formation of D-SCoA in vitro in rat hepatocytes and in vivo in rat liver, as well as the chemical reactivity of the thioester with GSH, will be the focus of future studies in our laboratory on the metabolic activation of diclofenac. Therefore, we contend that diclofenac may also form a glutathione thioester derivative that would confirm the in vivo formation of reactive acylating metabolites of the drug. The D-SG thioester conjugate could be used for further mechanistic studies, in vivo and in vitro, to examine the importance of D-1-*O*-G or other reactive acylating metabolites, with respect to the acylation of cellular nucleophiles.

In the present experiments: 1) we incubated biosynthetic D-1-*O*-G with GSH, in vitro in buffer, to characterize the time-dependent transacylation reaction of D-1-*O*-G with GSH-forming D-SG; and 2) we examined the bile of diclofenac-dosed rats for the in vivo formation of D-SG. Results from these studies show that D-1-*O*-G reacts with GSH forming D-SG in vitro under physiological conditions, but to a minor extent relative to competing intramolecular acyl migration reactions, which result in less reactive acylating isomers. The data also show the excretion of D-SG in bile of diclofenac-dosed rats as a minor, but mechanistically important, metabolite in terms of understanding the toxicology of the drug.

## Materials and Methods

**Materials.** Diclofenac sodium, CBZ, reserpine, and β-D-glucuronide glucuronosohydrolase (β-glucuronidase, EC 3.2.1.31) were purchased from Sigma-Aldrich (St. Louis, MO). D-SG, D-SNAC, 1-(2,6-dichlorophenyl)indolin-2-one, and D-1-*O*-G were synthesized as described below. All solvents used for HPLC and LC/MS were of chromatographic grade. Stock solutions of diclofenac (10 mM), D-SG (10 mM), D-1-*O*-G (1.3 mM), and D-SNAC (10 mM) were prepared as solutions in 50:50 acetonitrile/water (3% formic acid).

**Animal Treatment.** Male Sprague-Dawley rats ($N = 2$; 250–275 g) were purchased from Charles River Breeding Laboratories (Portage, MI). Upon arrival, animals were acclimated following a 12-h light/dark cycle in a humidity- and temperature-controlled environment for 2 weeks in accordance with the National Institutes of Health publication *Guide for the Care and Use of Laboratory Animals*. After the first week of acclimation, surgically implanted venous and biliary catheters were placed into the superior vena cava (SVC) or bile duct. After a 1-week recovery, rats were dosed with diclofenac sodium (50 or 200 mg/kg) via SVC. All procedures in this study have been approved and conducted in compliance with the Animal Welfare Act Regulations (9 CFR, Parts 1, 2, and 3) and the *Guide for the Care and Use of Laboratory Animal*s (Institute for Laboratory Animal Research, 1996), as well as with all internal company policies and guidelines.

**Instrumentation and Analytical Methods.** HPLC was carried out on a Hewlett-Packard 1090 Series II HPLC equipped with diode array detection (Palo Alto, CA). All analytical HPLC analysis was performed on a reverse-phase column (Zorbax, 3 μm, C18, 150 × 2.1 mm; Agilent Technologies, Palo Alto, CA). HPLC (with UV detection at 275 nm) and LC/MS analyses were performed using a gradient system of 0.1% formic acid with elution from 5% acetonitrile (ACN) to 100% over 20 min at a flow rate of 0.3 ml/min. LC/MS and LC-MS/MS were performed on a Finnigan TSQ-7000 equipped with API2 and running Excalibur version 1.2 (Thermo Finnigan, San Jose, CA). Electrospray ionization was used with the needle potential held at ~4.5 kV. MS/MS conditions used were ~2 mtorr of argon collision gas and 25 eV of collision potential. High-resolution LC/MS was performed on a Micromass Q-TOF instrument (Manchester, UK) running MassLynx software (version 3.5; Micromass). The instrument resolution was approximately 10,000 resolving power (50% valley definition). Exact mass measurement was accomplished through the use of postcolumn addition of a standard solution of reserpine (1 nM in methanol with 3% acetic acid, mol. wt. 609.2812 atomic mass units). The appropriate HPLC peak was averaged, and the data mass was converted using reserpine as the lock mass. The resulting measured mass for the unknown analyte was exported to the exact mass/molecular formula software within MassLynx. Parameters for this program were set to include possible molecular formulae lying well above the apparent molecular weight of the unknown but limited to the suspected heteroatoms of the unknown. Based on this construct, the molecular weight of the unknown was found to be within 0.3 millidaltons of the suspected molecular weight. $^1$H NMR spectra were recorded on a Bruker Avance 400 spectrometer (Bruker Optics, Inc., Billerica, MA) operating at 400 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 Diclofenac Thioesters.** D-SG and D-SNAC thioesters were synthesized as described previously (Grillo and Benet, 2002) and provided D-SG as a white solid (13% yield) and D-SNAC as a clear,

colorless oil (70% yield). Synthesis of D-SG: briefly, diclofenac-free acid (1.6 mmol, 473.6 mg) was dissolved in anhydrous THF (20 ml) followed by the addition of triethylamine (1.6 mmol, 220 $\mu$l), then ethyl chloroformate (1.6 mmol, 160 $\mu$l), and stirred at room temperature for 3 min. The white precipitate that formed (triethylamine hydrochloride) was removed by filtration through a glass funnel fitted with a glass wool plug. The filtered clear mixture was added to a solution containing GSH (1.6 mmol, 500 mg) and $KHCO_3$ (1.6 mmol, 160 mg) dissolved in distilled water (10 ml, pH 8.1) and THF (15 ml). The solution was stirred continuously at room temperature for 30 min, 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 was extracted with ethyl acetate (4 $\times$ 20 ml) to wash away any unreacted organics. The white D-SG precipitate then was washed with distilled water (pH ~5.0, 4 $\times$ 20 ml) to remove any remaining GSH and $KHCO_3$. The D-SG precipitate was washed with acetone (4 $\times$ 20 ml) to remove water. Finally, the product precipitate was dried under a stream of nitrogen gas at room temperature (1 h). The *S*-acyl-*N*-acetylcysteine derivative of diclofenac, D-SNAC, was synthesized by reacting a solution of D-SG (1 mM) in aqueous buffer ($KHCO_3$, 100 mM, 10 ml) with *N*-acetylcysteine (NAC, 32.6 mg, 20 mM final concentration) at pH 7.4 and 37°C. The solution was stirred for 0.5 h and then quenched by the drop-wise addition of 1 mM HCl (final pH ~3). The D-SNAC conjugate then was isolated by extraction with ethyl acetate (2 $\times$ 10 ml). The ethyl acetate extracts were combined, dried (anhydrous $MgSO_4$), and evaporated to afford product D-SNAC. D-SG and D-SNAC thioesters were characterized by tandem mass spectrometry on a Finnigan-MAT TSQ 7000 tandem mass spectrometer, and LC-MS/MS analysis was performed by gradient elution as described above. Synthetic D-SG and D-SNAC standards eluted at retention times of 12.6 and 12.0 min, respectively, and showed no significant impurities (less than 0.5% relative peak area) when analyzed by both positive and negative ion LC/MS scan modes (data not shown) via reverse-phase gradient elution (described above).

Tandem LC-MS/MS analysis of D-SG: [collision-induced dissociation (CID) of $MH^+$ ion [$^{35}$Cl] at *m/z* 585], *m/z* (%): *m/z* 510 ([M + H − Gly]$^+$, 10%), *m/z* 438 ([M + H − pyroglutamic acid − water]$^+$, 100%), *m/z* 308 ([glutathione + H]$^+$, 8%), *m/z* 278 ([2-([2,6-dichlorophenyl]amino)benzene-CHCO]$^+$, 23%), *m/z* 250 ([2-([2,6-dichlorophenyl]amino)benzene-$CH_2$]$^+$, 25%). $^1$H NMR ($^2H_6$-DMSO): diclofenac portion of D-SG: $\delta$ 3.7 (s, 2H, $CH_2$C-S), $\delta$ 6.84–7.54 (m, 6H, 4-CH to 6-CH and 3′-CH to 5′-CH), $\delta$ 6.28 (d, 1H, 3-CH, $J$ = 7.9 Hz); GSH portion of D-SG: $\delta$ 2.30 (m, 2H, Glu-$\beta$,$\beta'$), $\delta$ 2.5 (m, 2H, Glu-$\gamma$,$\gamma'$), $\delta$ 2.98 (m, 2H, Cys-$\beta$,$\beta'$), $\delta$ 3.70 (t, 2H, $J$ = 6.4 Hz, Glu-$\alpha$), $\delta$ 3.72 (s, 2H, Gly-$\alpha$,$\alpha'$), $\delta$ 4.42 (m, 1H, Cys-$\alpha$), $\delta$ 8.5 (d, 1H, $J$ = 8.5 Hz, Cys-amide-NH), $\delta$ 8.8 (t, 1H, Gly-amide-NH). Tandem LC-MS/MS analysis of D-SNAC: (CID of $MH^+$ ion [$^{35}$Cl] at *m/z* 441), *m/z* 278 ([2-([2,6-dichlorophenyl]amino)benzene-CHCO]$^+$, 100%), *m/z* 250 ([2-([2,6-dichlorophenyl]-amino)benzene-$CH_2$]$^+$, 50%), *m/z* 215 ([2-([2,6-dichlorophenyl]amino)benzene-$CH_2$- Cl]$^+$, 23%), *m/z* 164 ([*N*-acetylcysteine + H]$^+$, 5%), *m/z* 122 ([cysteine + H]$^+$, 10%). $^1$H NMR ($^2H_6$-DMSO): diclofenac portion of D-SNAC: $\delta$ 3.7 (s, 2H, $CH_2$C-S), $\delta$ 6.84–7.54 (m, 6H, 4-CH to 6-CH and 3′-CH to 5′-CH), $\delta$ 6.28 (d, 1H, 3-CH, $J$ = 7.9 Hz); NAC portion of D-SNAC; $\delta$ 1.82 (s, 3H, $CH_3$CONH), $\delta$ 3.0 (m, 2H, Cys-$\beta$,$\beta'$), $\delta$ 4.42 (m, 1H, Cys-$\alpha$).

**Synthesis of (1-[2,6-Dichlorophenyl]indolin-2-one).** Diclofenac-free acid (1.6 mmol, 472 mg) was added to a round bottom flask (100 ml) followed by the addition of anhydrous tetrahydrofuran (25 ml), triethylamine (1.6 mmol, 220 $\mu$l), and last, ethyl chloroformate (1.6 mmol, 160 $\mu$l). The reaction was stirred at room temperature overnight and under a stream of nitrogen gas. After 16 h of reaction, the mixture was evaporated to dryness under reduced pressure. To the resulting residue potassium bicarbonate buffer (10 ml, 0.1 M, pH 9.2) was added, and the mixture was shaken for 1 min. This solution then was extracted with ethyl acetate (3 $\times$ 20 ml), the extracts combined, dried (anhydrous magnesium sulfate), filtered, and then evaporated to dryness to afford the indolinone product (white solid, 82% yield). Tandem LC-MS/MS analysis of the indolinone: (CID of $MH^+$ ion [$^{35}$Cl] at *m/z* 278), *m/z* (%): *m/z* 243 ([M + H − Cl]$^+$, 8%), *m/z* 215 ([M + H − Cl − CO]$^{+\cdot}$, 32%), *m/z* 214 ([M + H − HCl − CO]$^{+\cdot}$, 49%), *m/z* 208 ([M + H − 2Cl]$^+$, 18%), *m/z* 180 ([M + H − 2Cl − CO]$^+$, 5%), *m/z* 172 ([1-(2,6-dichlorophenyl)-nitrile]$^+$, 12%), *m/z* 132 ([indolin-2-one]$^+$, 4%). Exact mass 278.0143; calculated mass 278.0139; formula $[MH]^+$ $C_{14}H_{10}NOCl_2$.

**Biosynthesis of D-1-*O*-G.** D-1-*O*-G was obtained by preparative reverse-phase HPLC purification of rat bile (0–6 h collection) extracts from a rat dosed with diclofenac (50 mg/kg) through SVC. Bile was collected over glacial acetic acid (1 M) and on ice in microcentrifuge tubes (1.5 ml). Collected bile was pooled and extracted with 1 volume of ethyl acetate three times. The ethyl acetate extracts were combined and dried with anhydrous magnesium sulfate, filtered, and evaporated to dryness under reduced pressure. The residue obtained was dissolved in solution containing ACN and 3% formic acid in water (5 ml, 1:1, v/v), vortex mixed (1 min), and treated by sonication (1 min). The resulting mixture was filtered and then used for the purification of D-1-*O*-G by preparative HPLC on a Phenomenex, 5 $\mu$m, 250 $\times$ 21.2 mm, phenyl-hexyl reverse-phase column (Torrance, CA). The purification was performed on a Rainin Dynamax solvent delivery system (model SD1) linked to a Dynamax absorbance detector (model UV1, set at a wavelength of 275 nm) and a Dynamax fraction collector (model FC-1) (Rainin Instruments, Woburn, MA). Gradient elution of 2.5-ml injection volumes was performed by increasing ACN (with 0.1% acetic acid) from 30 to 50% over 30 min, followed by another increase from 50% ACN to 85% over 10 min, and finally isocratic elution at 85% ACN for 5 min. The aqueous phase used was 0.1% acetic acid in water, and elution was performed at a flow rate of 20 ml/min. Fractions were collected every 30 s into 40-ml amber glass vials. Fractions containing D-1-*O*-G (fractions, 25–28 min) were pooled, and the mobile phase evaporated to dryness under reduced pressure. The resulting residue was dissolved with sonication (1 min) in a solution of ACN and 3% formic acid in water (5 ml, 1/1, v/v), followed by vortex mixing (1 min). Product D-1-*O*-G was obtained, and its identity was confirmed by treatment of an aliquot of the extract with $\beta$-glucuronidase at pH 5.0 and 37°C (per the manufacturer's instructions) followed by HPLC analysis (as below, UV 275 nm) after 0 and 30 min of incubation. Reverse-phase HPLC analysis was performed on the extract using a Phenomenex phenyl-hexyl 250 mm $\times$ 2.0 mm, 5-$\mu$m column, with a flow rate of 0.3 ml/min and a gradient of 30% acetonitrile (containing 0.2% formic acid) to 50% over 30 min, 50 to 85% over 10 min, followed by isocratic elution at 85% ACN for another 5 min. The aqueous phase used was 0.2% formic acid in water. Results from this analysis showed the peak corresponding to D-1-*O*-G (retention time, 35 min) was completely absent in the 30-min $\beta$-glucuronidase-treated extract (data not shown). Tandem LC-MS/MS analysis of D-1-*O*-G: (CID of $MH^+$ ion [$^{35}$Cl] at *m/z* 472), *m/z* (%): *m/z* 454 ([M + H − $H_2O$]$^+$, 7%), *m/z* 296 ([diclofenac + H]$^+$, 100%), *m/z* 178 ([glucuronic acid]$^+$, 2%). The concentration of D-1-*O*-G in the stock solution was determined to be 1.3 mM from analysis of hydrolyzed (1 N NaOH treated) stock solution compared to a standard curve of similarly treated diclofenac-free acid. Quantification of D-1-*O*-G in these studies was performed

by HPLC analysis (UV, 275 nm, as above) using a linear standard curve generated from peak area measurements.

**In Vitro Reactivity of D-1-*O*-G with GSH.** Incubations (2 ml total volume) of D-1-*O*-G (100 $\mu$M) were performed in potassium phosphate buffer (0.05 M, pH 7.4) at 37°C in the presence or absence of GSH (10 mM). Incubations in the absence of GSH were performed to determine the chemical stability of the D-1-*O*-G metabolite. Aliquots (50-$\mu$l) of the incubation mixtures, performed in triplicate, were taken at 0.2, 10, 20, 30, 45, 60, 120, 240, and 360 min and added to a quench solution (50 $\mu$l) containing 3% formic acid in methanol and containing 10 $\mu$M CBZ internal standard for analysis of the amount of D-1-*O*-G remaining and D-SG formed in the incubation. The rate of D-SG formation was determined by LC-MS/MS with MRM analysis in the positive ion mode using the transitions *m/z* 585 > *m/z* 438 (for D-SG) and *m/z* 237 > *m/z* 194 (for CBZ). Concentrations of D-SG were determined from a linear standard curve of peak area ratios (D-SG/CBZ). The amount of D-1-*O*-G remaining, in addition to the amount of diclofenac acyl migration isomers formed, in the incubation was assayed by HPLC analysis with UV detection (275 nm) and the concentrations determined from a linear standard curve generated from peak area measurements of authentic D-1-*O*-G. When analyzed by HPLC with detection at 275 nm (as described above under *Instrumentation and Analytical Methods*), the retention time of D-1-*O*-G was 12.7 min, and the retention times of three detected $\beta$-glucuronidase-resistant diclofenac glucuronide isomers (as confirmed by LC/MS) were 11.8, 13.1, and 17.6 min (data not shown).

**In Vitro Reactivity of D-1-*O*-*G* and D-SG with NAC.** Experiments on the reactivity of 100 $\mu$M D-SG or 100 $\mu$M D-1-*O*-G with NAC were performed as described above for the reaction of D-1-*O*-G with GSH, except NAC (10 mM) was substituted for GSH. Aliquots (50-$\mu$l) of the incubations (5 ml total volume), performed in triplicate, were taken at 0.2, 10, 20, 30, 60, 120, 240, and 360 min of incubation and added directly to a quench solution containing CBZ (as above). The rate of D-SNAC formation was determined by LC-MS/MS with MRM analysis in the positive ion mode and using the transitions *m/z* 441 > *m/z* 278 (for D-SNAC) and *m/z* 237 > *m/z* 194 (for CBZ). Concentrations of D-SNAC were determined from a linear standard curve of peak area ratios (D-SNAC/CBZ).

**In Vivo Rat Bile Study.** A male Sprague-Dawley rat was given a dose of diclofenac (200 mg/kg, i.v.), and bile was collected over a 6-h time period (as above) at the 0-, 0.5-, 1-, 2-, 4-, and 6-h time points. Collected bile (100 $\mu$l) for each time point was added to 2 ml of a 50:50 mixture of 3% formic acid in methanol (containing 10 $\mu$M CBZ) and distilled water, followed by sonication (1 min) at room temperature. The resulting solution was centrifuged (14,000 rpm), and the supernatant was analyzed by LC-MS/MS. Quantification of D-SG in the bile was performed by MRM analysis and from a linear standard curve of D-SG as described above. Analysis of rat bile for diclofenac indolinone derivative was performed by adding aliquots (50-$\mu$l) of diclofenac-dosed rat bile to a quench solution of 10% methanol (with 3% formic acid and 10 $\mu$M CBZ) and 90% ACN, followed by vortex mixing, centrifugation (14,000 rpm), and analysis of the supernatant by LC-MS/MS [MRM analysis (CBZ) *m/z* 237 > *m/z* 194; (indolinone) *m/z* 278 > *m/z* 214]. Chromatography was performed as above, except the gradient used was 5 to 100% ACN over 13 min, and the retention time of the indolinone derivative was 11.9 min.

**Degradation of D-SG in Vitro.** The time-dependent degradation of D-SG (100 $\mu$M) was performed as above for the reaction of D-SG with GSH. Aliquots (200-$\mu$l) taken from the incubation (10 ml total volume) at the 0-, 0.5-, 1-, 2-, 3-, and 4-h time points were added to quench solution (200 $\mu$l) containing internal standard CBZ and then analyzed by LC/MS in the positive ion scan mode. The concentration of D-SG remaining and the amounts of diclofenac and the indolinone derivative formed were determined from linear standard curves of peak area ratios (peak area of analyte/peak area of CBZ) of extracted ions *m/z* 585, *m/z* 278, *m/z* 296, and *m/z* 237, for D-SG, indolinone derivative, diclofenac, and CBZ, respectively.

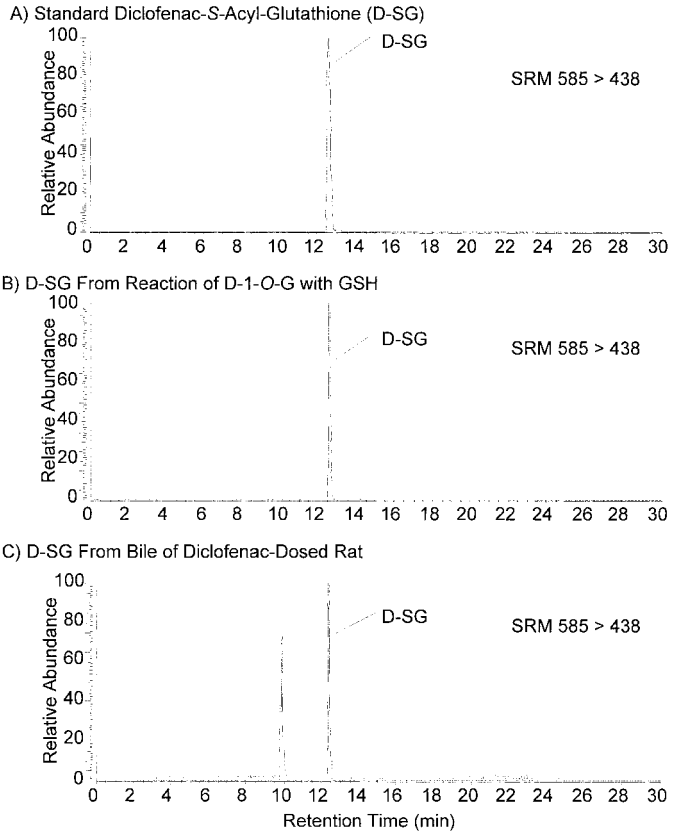


FIG. 2. *Representative reverse-phase gradient LC-MS/MS SRM chromatograms of authentic D-SG standard (A), extract from the reaction of D-1-O-G (100 $\mu$M) with GSH (10 mM) at pH 7.4 and 37°C after 120 min of incubation (B), and extracts from the bile of a diclofenac-dosed rat (200 mg/kg, i.p.) (C).*

The transition used for the SRM analysis was *m/z* 585 > *m/z* 438.

## Results

**Identification of D-SG in Bile of Diclofenac-Dosed Rats.** By using sensitive and selective LC-MS/MS MRM techniques, the identification of D-SG formed in vivo in rats and excreted in bile (Fig. 2) was facilitated. The transition used for these analyses was *m/z* 585 > *m/z* 438, which was chosen because of it being the major fragmentation pathway for the glutathione conjugate as assessed by positive ion LC-MS/MS CID of the $MH^+$ ion at *m/z* 585 of authentic D-SG (Fig. 3). Reverse-phase LC-MS/MS analysis showed the presence of D-SG in vivo in bile of diclofenac-dosed rats (200 mg/kg), which coeluted with authentic D-SG standard at a retention time of 12.6 min (Fig. 2). The unknown substance eluting at 10 min (Fig. 2C) was also detected in extracts from predose bile. Tandem LC-MS/MS analysis of biologically formed D-SG provided enough sensitivity to obtain a product ion spectrum that showed product ions that were consistent with the mass spectrum of authentic D-SG and also consistent with its structure (Fig. 3). Together, these results provide strong evidence for the in vivo formation of D-SG in rats dosed with diclofenac. The time-dependent elimination of D-SG into the bile of diclofenac-dosed rats showed the most rapid elimination of the glutathione thioester

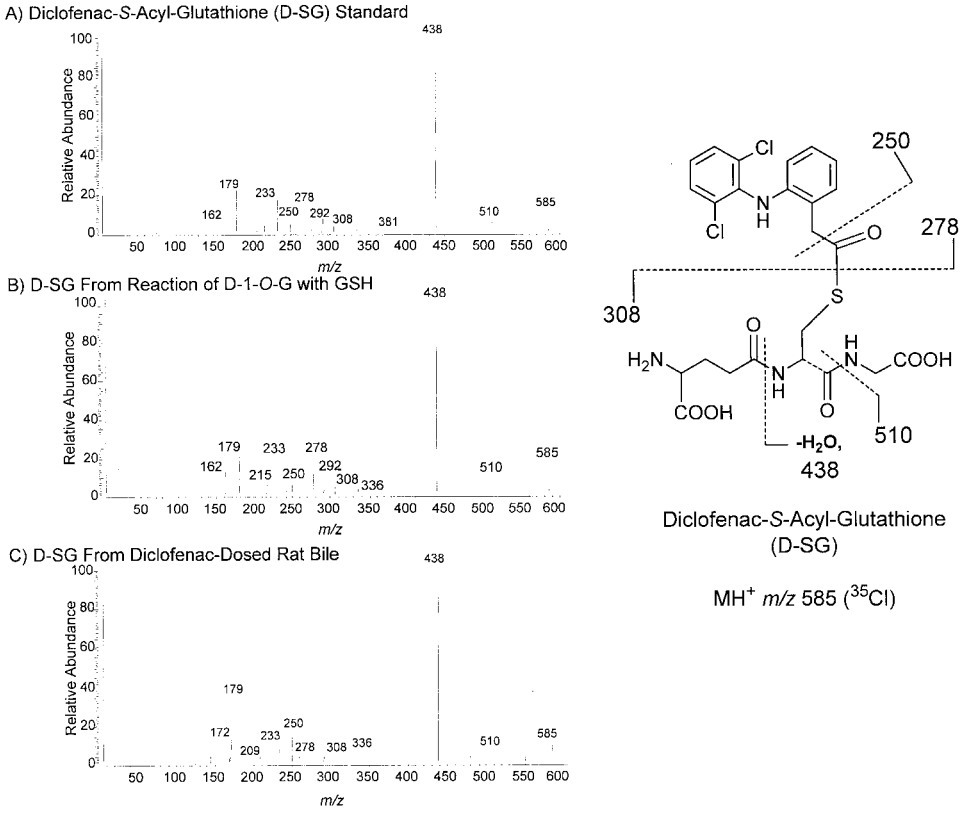


FIG. 3. *LC-MS/MS tandem mass spectra of authentic D-SG standard (A), extract from the reaction of D-1-O-G (100 μM) with GSH (10 mM) at pH 7.4 and 37°C after 120 min of incubation (B), and extracts from the bile of a diclofenac-dosed (200 mg/kg, i.v.) rat (C).*

Product ion spectra were obtained by CID of the protonated molecular ion $MH^+$ at *m/z* 585. The origins of the characteristic fragments are as shown.

occurring by 1 h postadministration of the drug (~143 ng eliminated at 1 h), and by the 6-h time point, a total of ~311 ng had been excreted into bile (Fig. 4).

**Time Course of D-SG Formation in Reactions of D-1-*O*-G with GSH.** When D-1-*O*-G (100 μM) was incubated with GSH (10 mM) in phosphate buffer at pH 7.4 and 37°C, the formation of D-SG was indicated by LC-MS/MS SRM (*m/z* 585 > *m/z* 438) analysis (Fig. 2) and by coeluting with authentic D-SG standard. As with the D-SG detected in bile, the product ion spectrum of the D-SG in vitro reaction product was identical to the authentic standard (Fig. 3B). The time course of the reaction of D-1-*O*-G to form D-SG was linear up to 60 min of incubation (3 μM/h), reaching a maximum of 3.7 μM after 2 h of incubation. The major reaction that occurred was acyl migration of D-1-*O*-G ($t_{1/2}$, 54.4 min) to less reactive isomers. After the 120-min time point, the D-SG formed degraded to ~1 μM after 360 min of incubation. The major reaction products in the incubation mixture at the 360-min time point were the acyl migration isomers of D-1-*O*-G (Fig. 5). The presence of GSH in the incubation mixture had no effect on the half-life of D-1-*O*-G, in that the degradation rate of D-1-*O*-G in similar incubations performed in the absence of 10 mM GSH was not significantly different (data not shown). In the present experiments, the rate of D-SG formation was probably underestimated because of the instability of the thioester product, which undergoes degradation primarily to the indolinone derivative (see below). The ability of glutathione *S*-transferases to catalyze the reaction of GSH with the 1-*O*-acyl glucuronide of clofibric acid forming clofibryl-*S*-acyl-glutathione in vitro in buffer has been shown (Shore et al., 1995), but glutathione *S*-transferase-mediated catalysis, although it might occur, was not investigated for similar reactions of D-1-*O*-G with GSH in the present work.

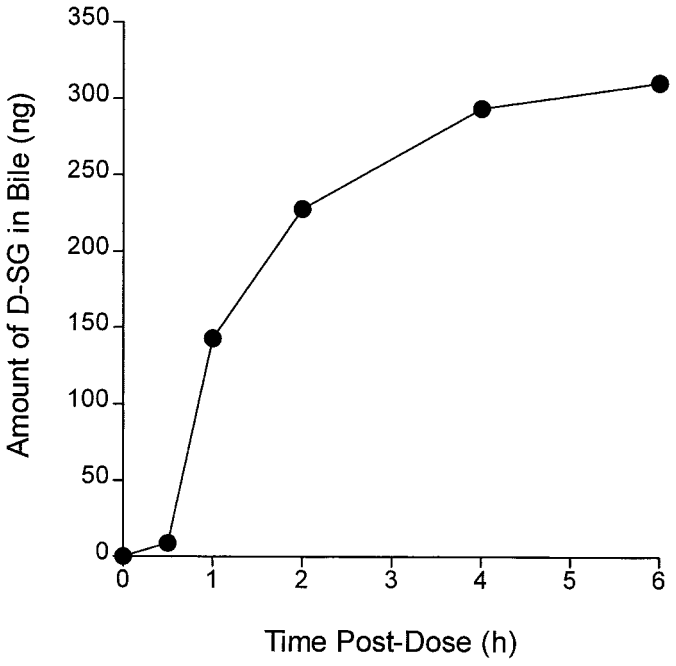


FIG. 4. *Cumulative excretion of D-SG into bile after a single dose of diclofenac (200 mg/kg, i.p.) to a male Sprague-Dawley rat (N = 1).*

**Degradation of D-SG in Buffer.** When D-SG (100 μM) was incubated in phosphate buffer (pH 7.4, 37°C), the thioester was shown to degrade in a linear fashion over a 4-h period of incubation (~10

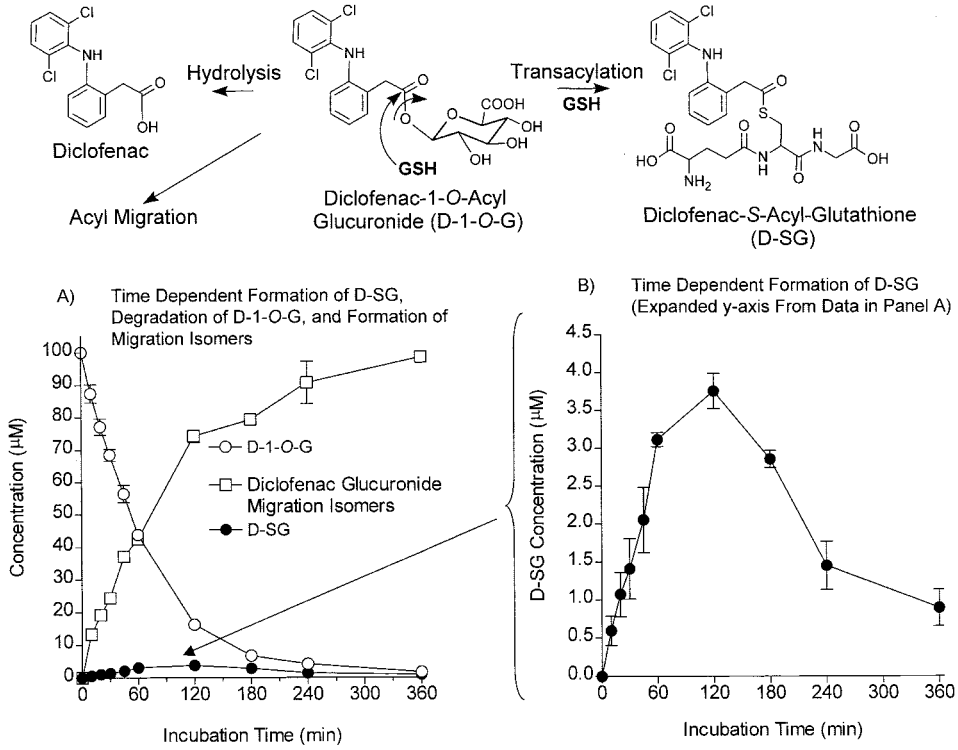


FIG. 5. *Panel A, Time course for the reaction of D-1-O-G (100 μM) with GSH (10 mM) forming D-SG and D-1-O-G acyl migration products in buffer (0.1 M potassium phosphate, pH 7.4, 37°C); panel B, the time course plot for the formation of D-SG (from the data shown in panel A), where the y-axis has been expanded.*

Values are expressed as the mean ± S.E. of triplicate incubations.

μM/h; Fig. 6). Analysis of the products formed showed that hydrolysis of the thioester to diclofenac and GSH was minor (~1.2 μM/h), whereas intramolecular cyclization to the indolinone-type product (1-[2,6-dichlorophenyl]indolin-2-one; ~9 μM/h) was the major degradation route. Formation of the indolinone derivative was confirmed by LC/MS (Fig. 7), which provided a mass spectrum of the derivative (retention time, 12.6 min) consistent with the protonated molecular weight of the synthetic indolinone standard ($MH^+$ *m/z* 278). In addition to having identical retention times (data not shown), the product ion spectrum, from CID of the $MH^+$ ion at *m/z* 278, showed fragment ions that were identical to the mass spectrum of authentic indolinone derivative and consistent with its structure (Fig. 8A). In addition, 1-[2,6-dichlorophenyl]indolin-2-one was also found in diclofenac-dosed rat bile. Figure 8B shows the tandem mass spectra of the peak coeluting with the synthetic indolinone standard (Fig. 9); the spectra also show the major product ions corresponding to the indolinone derivative. The time-dependent elimination of 1-[2,6-dichlorophenyl]indolin-2-one into the bile of diclofenac-dosed rats showed the most rapid elimination of the indolinone derivative occurring by 1 h postadministration of 200 mg/kg dose of the drug (~51 μg eliminated at 1 h), and by the 6-h time point a total of ~86 μg (0.04% of dose) had been excreted into bile (Fig. 9D).

**Comparison of the Chemical Reactivity of D-SG and D-1-*O*-G with NAC in Vitro.** When D-SG (100 μM) was incubated with NAC (10 mM) in phosphate buffer at pH 7.4 and 37°C, the formation of D-SNAC was rapid and reached ~94 μM D-SNAC after 20 min of incubation (Fig. 10). By contrast, incubation of D-1-*O*-G under identical conditions resulted in only ~0.8 μM D-SNAC formation at the 20-min time point. The formation of D-SNAC in the reaction of NAC with the glucuronide reached a maximum concentration of 2.6 μM after 240 min of incubation, the time point after which most of the D-1-*O*-G has degraded to acyl migration isomers less able to acylate GSH. The formation of D-SNAC from reactions of both D-SG and D-1-*O*-G with NAC was confirmed by LC-MS/MS MRM analysis and by coelution with authentic D-SNAC standard (retention time, 12.0 min; data not shown) and by having identical product ion spectra from CID of the $MH^+$ ion at *m/z* 441 to the D-SNAC synthetic standard (Fig. 11). Together, these data show the superior acylating ability of the D-SG thioester derivative and indicate that the detoxification of D-1-*O*-G by transacylation reactions with GSH-forming D-SG may result in thioester derivatives that are more able to acylate protein nucleophiles in vivo.

## Discussion

Chemically reactive intermediates have been implicated in the mechanisms of toxicity of varied xenobiotics (Nelson and Pearson, 1990), and it has been proposed that reactive intermediates of diclofenac may be mediating the idiosyncratic toxicity associated with its clinical use. In the present study, D-1-*O*-G was determined to be reactive in a transacylation-type fashion with GSH leading to the formation of D-SG thioester (Figs. 2 and 3). The reaction was time-dependent and showed that once the D-1-*O*-G conjugate underwent acyl migration to the less reactive isomers, the rate of D-SG formation decreased substantially (Fig. 5). These results are in agreement with similar studies performed with clofibryl-1-*O*-acyl glucuronide (Shore et al., 1995) and 2-phenylpropionyl-1-*O*-acyl glucuronide (Li et al., 2002), where the superior transacylation-type reactivity of the 1-*O*-acyl isomers versus the acyl migration glucuronide isomers was shown. Based on results from the present study, we proposed that transacylation reactions may be occurring in vivo, but that the competing intramolecular acyl migration might be functioning to make the 1-*O*-acyl glucuronide less able to acylate biological nucleophiles (Grillo and Benet, 2002). When we characterized the apparent degradation half-life of D-1-*O*-G in the presence of human serum albumin

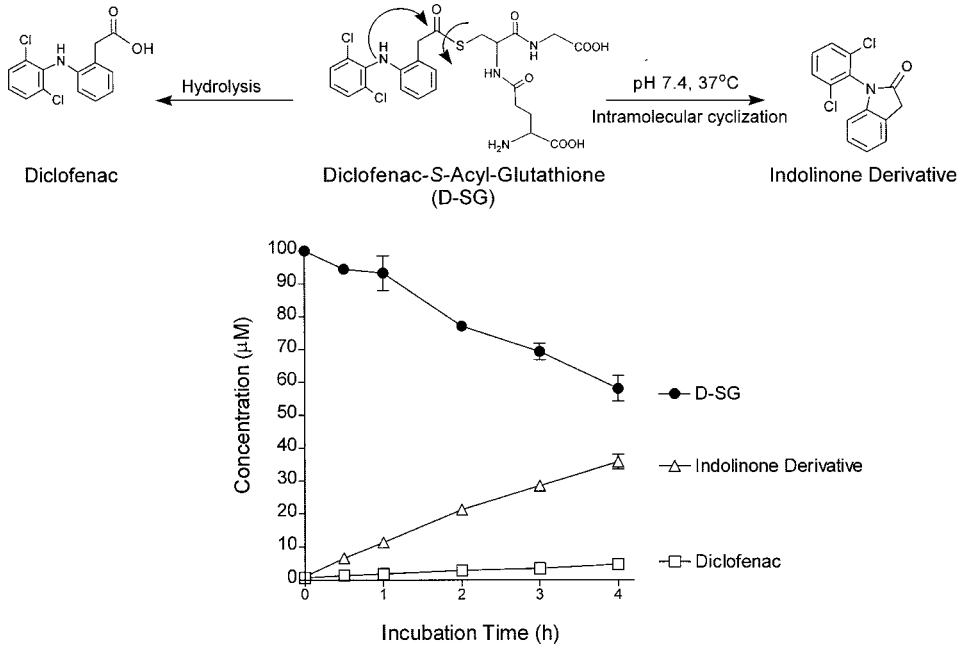


FIG. 6. *Time course for the degradation of D-SG (100 μM) resulting in the time-dependent formation of an indolinone-type product and diclofenac in buffer (0.1 M potassium phosphate, pH 7.4, 37°C).*

Values are expressed as the mean ± S.E. of triplicate incubations.

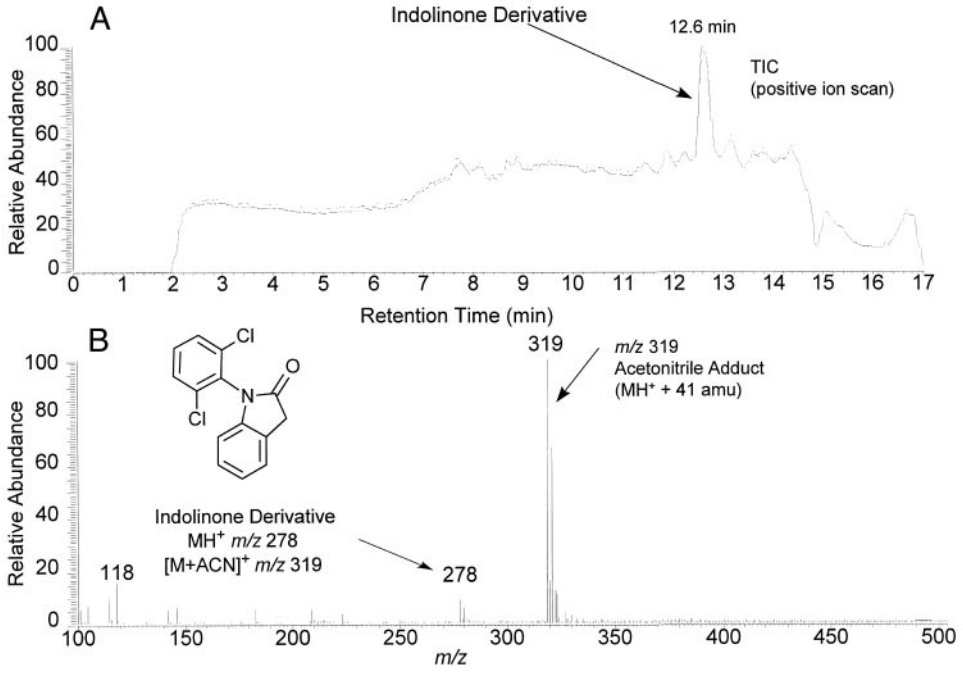


FIG. 7. *Representative reverse-phase gradient LC/MS analysis of the extract from the degradation of D-SG (100 μM) in buffer (0.1 M potassium phosphate, pH 7.4, 37°C) after 24 h of incubation.*

Analysis was performed in the positive ion scan mode and shows the total ion chromatogram (A) and the mass spectrum of the indolinone product eluting at 12.6 min (B).

(40 mg/ml; data not shown), we found the half-life to decrease from 54.4 to 6.2 min. The primary degradation products were the acyl migration isomers, which would be less able to acylate protein. The measurement of D-SG formation in the presence of HSA was not determined in the present study, but we propose that its rate of formation would be dramatically reduced due to the rapid degradation of the reactive D-1-*O*-G conjugate.

The apparent degradation half-life of D-1-*O*-G in buffer at pH 7.4 was shown to be 54.4 min in the present experiments, but under identical conditions, Ebner et al. (1999) determined the first order degradation half-life to be ~30 min. This $t_{1/2}$ of D-1-*O*-G is similar to that reported for other 1-*O*-acyl glucuronides of the phenylacetic acid-type, such as zomepirac- and tolmetin-1-*O*-acyl glucuronides, being 39 and 34 min, respectively (Benet et al., 1993). It is not known

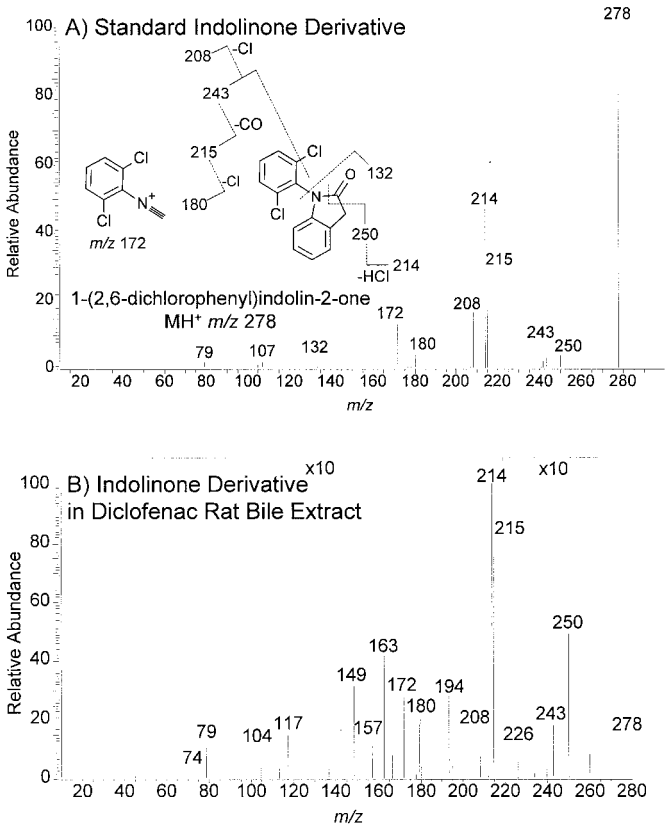


FIG. 8. *LC-MS/MS tandem mass spectra of the indolinone derivative obtained from the in vitro degradation of D-SG (A), and extracts from the bile of a diclofenac-dosed rat (1- to 2-h collection, 200 mg/kg, i.v.) (B).*

Spectra were obtained by CID of the protonated molecular ion ($MH^+$) at *m/z* 278. The origins of the characteristic fragments are as shown.

at this time the reason for the ~1.8-fold difference in degradation rates measured for D-1-*O*-G in the present study versus that reported by Ebner et al. (1999), but the difference may highlight the importance of performing control incubations with acyl glucuronide derivatives having known degradation half-life values to compare data between laboratories performing these types of studies. In a recent study by Kumar et al. (2002), it was shown that incubation of D-1-*O*-G (2 $\mu$M) with human liver microsomes resulted in a $t_{1/2}$ of ~50 min, which is similar to that determined in buffer in the present work. Since the primary focus of the present study was on the reactivity of D-1-*O*-G with GSH and not the determination of the half-life of D-1-*O*-G, we did not characterize the degradation rate of D-1-*O*-G further, with respect to other 1-*O*-acyl glucuronides with known degradation rates.

Before these studies, three GSH adducts, namely 5-hydroxy-4-(glutathion-*S*-yl)diclofenac, 4′-hydroxy-3′-(glutathion-*S*-yl)diclofenac, and 5-hydroxy-6-(glutathion-*S*-yl)diclofenac, were identified by LC-MS/MS analysis of bile from diclofenac-dosed rats (Tang et al., 1999). The formation of the GSH adducts was proposed to occur from reactions of GSH with cytochrome P450-mediated benzoquinone imine-reactive intermediates. The GSH adducts in that study were measured from rat bile that had been quenched with 10% trifluoroacetic acid, and then the protein precipitates were removed by centrifugation. From our experience with D-SG, unlike thioether-linked GSH adducts, the thioester derivative is not soluble in acidic aqueous mixtures but readily precipitates out of solution. This property of D-SG was used advantageously during its chemical synthesis and purification, where product D-SG was purified from excess GSH and buffer salts by washing the D-SG precipitate with acidic water (Grillo and Benet, 2002). Therefore, we believe that the inability to detect the thioester conjugate in the study by Tang et al. (1999) may have been because of the acidic work-up used, which led eventually to the precipitation of the D-SG adduct. In the present study, we were successful in extracting the conjugate from bile by using a 3% formic acid methanol quench, in which D-SG was shown to have high solubility (i.e., up to at least 10 mM D-SG at room temperature; data not shown). Another explanation for the lack of detection of the D-SG adduct by Tang et al. (1999) could be that the LC-MS/MS method used in their analyses, which employed constant neutral loss scanning for the loss of the 129-Da loss of the $\gamma$-glutamyl group (Baillie and Davis, 1993), did not allow for the detection of the D-SG adduct. LC-MS/MS analysis of D-SG in the present study did not afford the neutral loss of the 129-Da fragment ion (*m/z* 456), but instead the 456-Da fragment underwent a loss of water to provide the base peak *m/z* 438 fragment ion (Fig. 3). Therefore, analysis of bile extracts by constant neutral loss scanning for the loss of 129 Da did not lead to the detection of D-SG in the present experiments and under the mass spectrometer parameters used.

The D-SG thioester derivative was shown to be unstable in buffer leading to intramolecular cyclization affording the indolinone-type derivative, 1-[2,6-dichlorophenyl]indolin-2-one (Fig. 6). Indolinone derivatives of hydroxylated diclofenac metabolites have been detected in biological fluids of diclofenac-dosed rat, dog, baboon, and man, but these derivatives were proposed to have been artifacts formed during acidic work-up of the biological samples (Stierlin et al., 1979). Here we show that the D-SG thioester readily cyclized to form the indolinone derivative, but diclofenac under similar incubation conditions and acidic work-up showed no indolinone product (data not shown). Therefore, as shown in Fig. 6, we propose that an intramolecular transacylation reaction between the aromatic-amine group and the carbonyl-carbon of the thioester linkage occurs to form the stable lactam product. In addition, similar in vitro degradation experiments were performed with D-1-*O*-G (10 $\mu$M) in buffer, but results did not show the formation of the indolinone derivative (data not shown). In that experiment, the D-1-*O*-G conjugate was shown to primarily undergo intramolecular acyl migration to chemically stable acyl glucuronide isomers. Hence, the carbonyl-carbon of the acyl glucuronide linkage reacted preferentially with the adjacent hydroxyl (2-position) of the glucuronic acid moiety rather than with the amino group of diclofenac. These results are in agreement with the data (Fig. 5) showing that the primary products formed in the reaction of D-1-*O*-G with GSH were the acyl migration isomers of D-1-*O*-G. Studies have shown that the formation of the indolinone derivative from diclofenac-free acid in aqueous solutions is temperature- and pH-dependent, occurring at increased rates with increasing temperature and decreasing pH conditions (Roy et al., 2001). Since the indolinone derivative in the present study was not detected from acidic work-up of diclofenac nor from the cyclization of D-1-*O*-G, but instead was detected from D-SG thioester cyclization, we propose that the indolinone derivatives detected by Stierlin et al. (1979) may have been formed in vivo from unstable thioester metabolites of diclofenac and/or its hydroxylated metabolites. For instance, presumably D-SCoA thioester, if formed in vivo, would also cyclize in a similar fashion to the intramolecular cyclization of D-SG thioester, affording the indolinone derivative. No direct evidence exists for the formation of D-SCoA to date, but indirect evidence comes from studies showing a urinary metabolite of diclofenac to be the taurine amide conjugate, an acyl-CoA mediated process (Stierlin et al., 1979; Caldwell, 1984). Toward this hypothesis, the indolinone derivative of diclofenac, which was not detected by Stierlin et al. (1979), was detected in the present study in the bile of a diclofenac-dosed rat (Figs. 8 and 9). The indolinone derivative detected in the bile accumulated to approximately

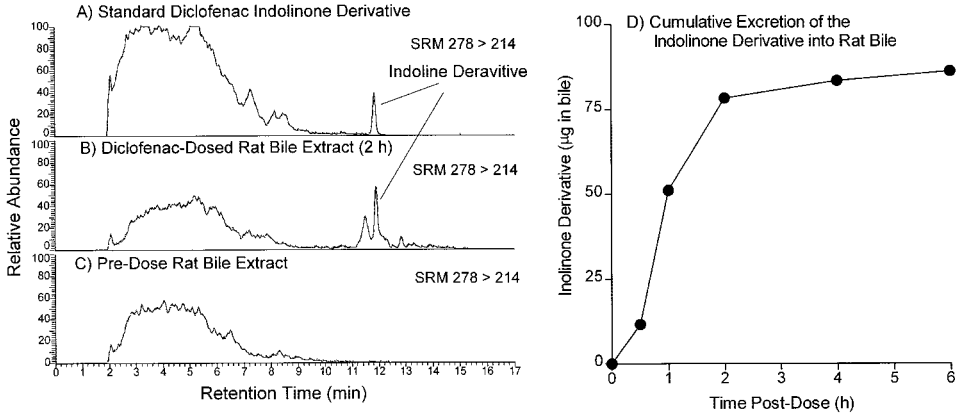


FIG. 9. *Representative reverse-phase gradient LC-MS/MS SRM chromatograms.*

Representative reverse-phase gradient LC-MS/MS SRM chromatograms of diclofenac indolinone derivative synthetic standard (A), extracts from the bile of a diclofenac-dosed rat (1–2 h collection, 200 mg/kg, i.v.) (B), and extracts from predose rat bile (C). The transition used for the SRM analysis was *m/z* 278 > *m/z* 214. Also shown is the cumulative excretion of diclofenac indolinone derivative into bile after a single dose of diclofenac (200 mg/kg, i.v.) to a male Sprague-Dawley rat ($N = 1$) (D).



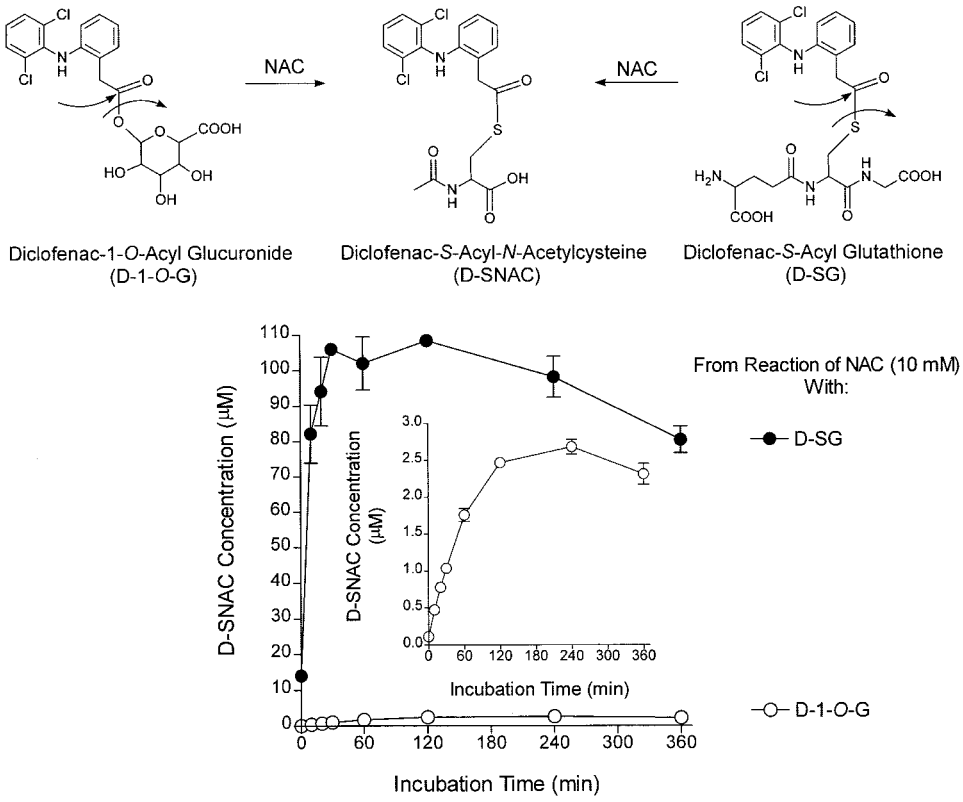


FIG. 10. *Time course for the reaction of D-1-O-G (100 μM) and D-SG (100 μM), in separate incubations, with NAC (10 mM) forming D-SNAC in buffer (0.1 M potassium phosphate, pH 7.4, 37°C).*

Values are expressed as the mean ± S.E. of triplicate incubations. The inset shows the expanded time course plot for the formation of D-SNAC from reactions with D-1-*O*-G.

86 μg 6 h postadministration, which corresponds to a minor percentage of the dose (~0.04%). Therefore, we propose that formation of the indolinone derivative may have occurred via the degradation of D-SG in vivo, ex vivo in bile, or from the intramolecular cyclization of D-SCoA in vivo.

Comparing the reactivity of D-SG with D-1-*O*-G in transacylation-type reactions with NAC, we determined the D-SG thioester to be nearly 200-fold more reactive than the glucuronide under the in vitro conditions used (Fig. 10). From these results, we propose that the D-SG excreted into bile may also contribute to the covalent binding to extracellular canalicular membrane proteins (Sallustio and Holbrook, 2001). In fact, one of these proteins, γ-glutamyltranspeptidase, was shown to be affected by diclofenac. We propose that since γ-glutamyltranspeptidase can accept glutathione thioesters as substrates (Tate, 1975; Grillo and Benet, 2001), that D-SG might be reacting with the protein covalently in the active site of the enzyme to alter its function in vivo rather than by the nonsubstrate D-1-*O*-G conjugate.

Results from the present study provide clear evidence that D-1-*O*-G

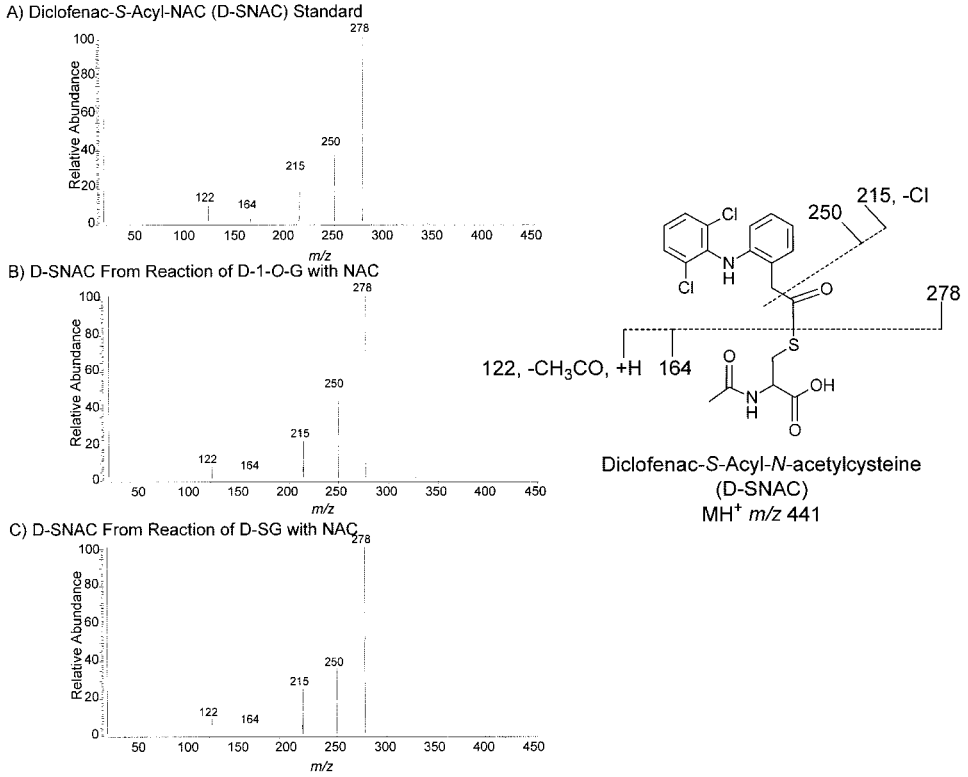


FIG. 11. *LC-MS/MS tandem mass spectra of authentic D-SNAC standard (A), extract from the reaction of D-1-O-G (100 μM) with NAC (10 mM) (B), and extract from the reaction of D-SG with NAC (10 mM) at pH 7.4 and 37°C after 120 min of incubation (C).*

Product ion spectra were obtained by CID of the protonated molecular ion ($MH^+$) at *m/z* 441. The origins of the characteristic fragments are as shown.

is a reactive metabolite of diclofenac that is able to transacylate the nucleophilic cysteinyl-thiol of glutathione-forming D-SG. However, because of its rapid acyl migration, we propose that D-1-*O*-G may not be the sole reactive metabolite of diclofenac that acylates GSH, as well as protein nucleophiles, in vivo.

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