Chemistry and Physics of Lipids 165 (2012) 261–269

Contents lists available at SciVerse ScienceDirect

Chemistry and Physics of Lipids

journal homepage: www.elsevier.com/locate/chemphyslip

Synthesis of the 3-sulfates of *S*-acyl glutathione conjugated bile acids and their biotransformation by a rat liver cytosolic fraction

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ARTICLE INFO

Article history:
Received 6 December 2011
Received in revised form 31 January 2012
Accepted 3 February 2012
Available online 10 February 2012

Keywords:
Bile acid sulfation
Bile acid–glutathione conjugate
Collision-induced dissociation
Selected reaction monitoring

ABSTRACT

The 3-sulfates of the *S*-acyl glutathione (GSH) conjugates of five natural bile acids (cholic, chenodeoxycholic, deoxycholic, ursodeoxycholic, and lithocholic) were synthesized as reference standards in order to investigate their possible formation by a rat liver cytosolic fraction. Their structures were confirmed by proton nuclear magnetic resonance, as well as by means of electrospray ionization-linear ion-trap mass spectrometry with negative-ion detection. Upon collision-induced dissociation, structurally informative product ions were observed. Using a triple-stage quadrupole instrument, selected reaction monitoring analyses by monitoring characteristic transition ions allowed the achievement of a highly sensitive and specific assay. This method was used to determine whether the 3-sulfates of the bile acid–GSH conjugates (BA–GSH) were formed when BA–GSH were incubated with a rat liver cytosolic fraction to which 3′-phosphoadenosine 5′-phosphosulfate had been added. The *S*-acyl linkage was rapidly hydrolyzed to form the unconjugated bile acid. A little sulfation of the GSH conjugates occurred, but greater sulfation at C-3 of the liberated bile acid occurred. Sulfation was proportional to the hydrophobicity of the unconjugated bile acid. Thus GSH conjugates of bile acids as well as their C-3 sulfates if formed in vivo are rapidly hydrolyzed by cytosolic enzymes.

1. Introduction

In mammals, bile acids (BAs) are synthesized in pericentral hepatocytes from cholesterol and then *N*-acylamidated (conjugated) with glycine or taurine in peroxisomes. BAs are then secreted into the biliary tract via canalicular BA transporters such as the bile salt export pump and multidrug resistance protein 2 (Alrefai and Gill, 2007). BAs are efficiently absorbed in the distal intestine with the result that a recycling pool of BA occurs.

BAs undergo bacterial deconjugation in the distal intestine, and a fraction of the unconjugated BAs thus formed are absorbed from the intestine and return to the liver where they are efficiently taken up by the hepatocyte by a variety of mechanisms (Hofmann et al., 2010; Ridlon et al., 2006). On entering the hepatocyte, the carboxyl group is activated by conversion to its acyl-adenylate derivative that in turn is converted to its acyl-coenzyme A (CoA) thioester derivative (Ikegawa et al., 1999; Kelley and Vessey, 1994; Mano et al., 2001). The acyl-CoA thioester then reacts with the amino group of taurine or glycine (in peroxisomes) to form conjugated (*N*-acylamidated) BAs which join the recycling conjugated BAs.

Based on analyses of bile samples from numerous species, conjugation of C_{24} BA is predominantly *N*-acylamidation with glycine or taurine in health (Hofmann et al., 2010). In healthy vertebrates, glucuronides [either at C-3 (ethereal) or C-24 (ester)], *N*-acetylglucosaminides, or *S*-acyl conjugates with glutathione (GSH) or *N*-acetylcysteine (NAC) of endogenous BAs are present in only trace proportions in bile. Nonetheless, our laboratory has identified trace concentrations of GSH conjugates of BA in rats (Mitamura et al., 2011a) and infants, but not in adults (Mitamura et al., 2011b). Sulfation, i.e. esterification with the sulfate moiety, is a major mode of conjugation for lithocholate in man, with the result that about half of the lithocholyl amidates in bile are sulfated (Rossi et al., 1987). Nonetheless, sulfated amidates have never been shown to be major biliary BAs in any species in health. In patients with cholestasis, however, sulfated (and amidated) BAs are formed

Abbreviations: BAs, bile acids; CoA, coenzyme A; GSH, glutathione; NAC, *N*-acetylcysteine; LC, liquid chromatography; ESI, electrospray ionization; MS, mass spectrometry; CA-GSH, *S*-(cholyl)glutathione; CDCA-GSH, *S*-(chenodeoxycholyl) glutathione; DCA-GSH, *S*-(deoxycholyl) glutathione; LCA-GSH, *S*-(lithocholyl)glutathione; UDCA-GSH, *S*-(ursodeoxycholyl)glutathione; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; UDCA, ursodeoxycholic acid; LCA, lithocholic acid; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; ^{1}H NMR, proton-nuclear magnetic resonance; CID, collision-induced dissociation; HPLC, high-performance liquid chromatography; RP, reversed-phase; SRM, selected reaction monitoring; IS, internal standard.

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doi:10.1016/j.chemphyslip.2012.02.001

in greatly increased amounts and are eliminated mostly in urine (Raedsch et al., 1981; Hedenborg et al., 1985).

In previous work from this laboratory, we have described the synthesis of *S*-acyl conjugates of BAs with NAC as well as their corresponding sulfates (Mitamura et al., 2009, 2011c). When these thioesters were incubated with a cytosolic fraction of rat liver, the NAC conjugates were hydrolyzed and sulfation of the liberated unconjugated BA occurred (Mitamura et al., 2011c).

In the present paper, we have pursued a similar experimental approach with GSH conjugates of the five BAs predominating in human bile (cholic, deoxycholic, chenodeoxycholic, ursodeoxycholic, lithocholic). The GSH conjugates of these BAs as well as their corresponding C-3 sulfates were synthesized. The biotransformation of the synthetic BA–GSH conjugates by a rat liver cytosolic fraction was assessed by liquid chromatography (LC)/electrospray ionization (ESI)-mass spectrometry (MS).

2. Experimental

2.1. Materials

S-(Cholyl)glutathione (CA-GSH), *S*-(chenodeoxycholyl)glutathione (CDCA-GSH), *S*-(deoxycholyl)glutathione (DCA-GSH), *S*-(ursodeoxycholyl)glutathione (UDCA-GSH), *S*-(lithocholyl)glutathione (LCA-GSH) and *S*-([2,2,4,4,-$^2H_4$]-deoxycholyl)glutathione (d_4-DCA-GSH) were synthesized in our laboratories by the method previously reported (Mitamura et al., 2007, 2011a). Cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), ursodeoxycholic acid (UDCA), lithocholic acid (LCA), and their 3-sulfates were stock samples in our laboratories. 3′-Phosphoadenosine-5′-phosphosulfate (PAPS, lithium salt hydrate) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and ammonium acetate of HPLC grade were purchased from Nacalai Tesque Inc. (Kyoto, Japan), and distilled water of HPLC grade was purchased from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). An Oasis® HLB cartridge (adsorbent weight, 60 mg and 1 g) was provided by Waters Co. (Milford, MA, USA) and was successively conditioned by washings with methanol and water prior to use. All other chemicals and solvents were analytical grade and obtained from Nacalai Tesque Inc. Water from a Millipore water filtration system (Milli Q UV Plus) was used to prepare the aqueous solutions described below.

2.2. Apparatus

Proton-nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a JNM-ECP700 (700.12 MHz, JEOL Ltd., Tokyo, Japan) or a JNM-ECA500 (500.16 MHz, JEOL Ltd.) with CD_3OD containing tetramethylsilane as the solvent; chemical shifts were expressed as δ ppm relative to tetramethylsilane. The following abbreviations are used: s, singlet; d, doublet; m, multiplet. Preparative high-performance liquid chromatography (HPLC) was carried out with a Shimadzu LC-10A VP (Shimadzu Co., Kyoto, Japan) equipped with a UV detector (SPD-20A; Shimadzu Co.).

2.3. LC/ESI-MS

The LC/ESI-MS analyses were carried out using two analytical systems. The first consisted of a Finnigan LTQ linear ion-trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an ESI source and coupled to a Paradigm MS4 pump (Michrom Bioresources Inc., Auburn, CA, USA) and an autosampler (HTC PAL, CTC Analytics, Zwingen, Switzerland). The second was a Finnigan TSQ Quantum Access triple-stage quadrupole mass spectrometer equipped with a LC-2010CHT system (Shimadzu Co.). For the first system, the ionization conditions for verifying the structures of BA–GSH 3-sulfates were as follows: ion source voltage, −4 kV; capillary temperature, 270 °C; capillary voltage, −20 V; sheath gas (nitrogen gas) flow rate, 50 arbitrary units; auxiliary gas (nitrogen gas) flow rate, 5 arbitrary units; tube lens offset voltage, −100 V. For tandem MS (MS/MS) analysis, helium gas was used as the collision gas and the normalized collision energy was set at 35%. The ionization conditions of the second system for the analytical studies on the sulfation of BA–GSH were as follows: spray voltage, −3 kV; sheath gas (nitrogen gas) pressure, 35 arbitrary units; auxiliary gas flow rate, 5 arbitrary units; capillary temperature, 350 °C. For collision-induced dissociation (CID), argon gas was used as the collision gas at a pressure of 1.5 mTorr. The LC separations were conducted on a reversed-phase (RP) semi-micro column, TSKgel ODS-100 V (5 μm, 150 mm × 2.0 mm I.D.) from Tosoh Co. (Tokyo, Japan) by a linear gradient elution: 30% solvent B (acetonitrile used for first system and acetonitrile–methanol, 3:7, v/v used for second system) to 80% B against solvent A (5 mM ammonium acetate buffer, pH 6.0) over 30 min at a flow rate of 200 μL/min.

2.4. Synthesis of CA-, CDCA-, DCA-, UDCA-, and LCA-GSH 3-sulfates (**1a**–**1e**)

2.4.1. General method

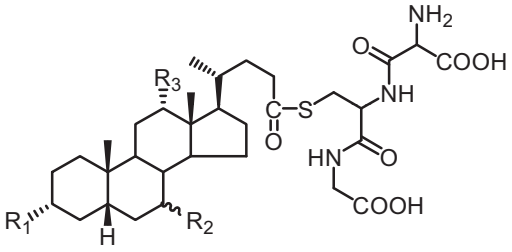
To a solution of 3-sulfates of CA, CDCA, DCA, UDCA, LCA (120–280 mg) in dioxane (2 mL) was added two equimolar amounts of *p*-nitrophenol (2 equimolar) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, and the mixture was stirred at room temperature for 6–8 h. After dilution of the reaction mixture with water, the resulting solution was loaded onto an Oasis® HLB cartridge (adsorbent weight, 1 g). Water (20 mL) was used to elute nonhydrophobic reaction constituents, after which the *p*-nitrophenyl esters were eluted with methanol (20 mL). After evaporation of methanol in vacuo, the crude products which without purification were treated with two equimolar amounts of GSH (reduced form) and trimethylamine (200 μL) in *N*,*N*′-dimethylformamide (10 mL) with continuous stirring for 6–8 h at room temperature under a stream of N_2 gas. After dilution of the reaction mixture with water (20 mL), the resulting solution was loaded onto an Oasis® HLB cartridge (1 g of solid phase). After washing with water (20 mL), GSH conjugate was eluted with methanol (20 mL) and the solvent was evaporated in vacuo. The products were purified by preparative RP-HPLC with UV detection at 215 nm on a Cosmosil 5C_{18}-AR-II column (5 μm, 250 mm × 20 mm I.D.; Nacalai Tesque Inc.) by a linear gradient elution: 30% solvent B (acetonitrile) to 100% B against solvent A (0.1% trifluoroacetic acid) over 30 min at a flow rate of 4 mL/min. The fraction containing desirable product was collected and lyophilized to dryness.

2.4.2. *S*-(3-Sulfooxycholyl)glutathione (CA-GSH 3-sulfate; **1a**)

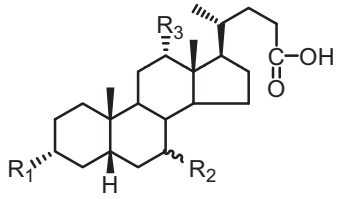
This compound **1a** was obtained from CA 3-sulfate (120 mg) as colorless amorphous solids with isolated yield 5.5 mg (3.2%). ^{1}H NMR δ: 0.70 (3H, s, 18-CH_3), 0.91 (3H, s, 19-CH_3), 1.05 (3H, d, J = 6.8 Hz, 21-CH_3), 2.19–2.35 (2H, m, Glu-β,β′-H_2), 2.47–2.55 (3H, m, Glu-γ,γ′-H_2 and 23-H), 2.72–2.77 (1H, m, 23-H′), 3.23–3.26 and 3.42–3.45 (2H, m, Cys-β,β′-H_2), 3.84–3.87 (1H, m, 7β-H), 3.94–3.96 (3H, m, Gly-α,α′-H_2 and 12β-H), 4.01–4.04 (1H, m, Glu-α-H), 4.18–4.22 (1H, m, 3β-H), 4.40–4.70 (1H, m, Cys-α-H). ESI–MS: m/z 778.3 $[M+H]^+$, m/z 776.3 $[M-H]^-$, m/z 387.8 $[M-2H]^{2-}$.

2.4.3. *S*-(3-Sulfooxychenodeoxycholyl)glutathione (CDCA-GSH 3-sulfate; **1b**)

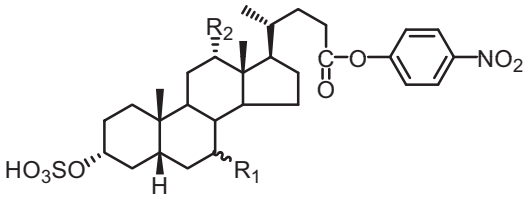
This compound **1b** was obtained from CDCA 3-sulfate (180 mg) as colorless amorphous solids with isolated yield 7.5 mg (2.8%). ^{1}H NMR δ: 0.68 (3H, s, 18-CH_3), 0.92 (3H, s, 19-CH_3), 0.95 (3H, d, J = 6.7 Hz, 21-CH_3), 2.14–2.22 (2H, m, Glu-β,β′-H_2), 2.49–2.55



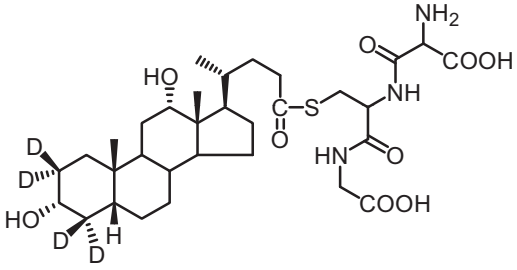
CA-GSH 3-sulfate (**1a**): R_1 = OSO_3H, R_2 = α-OH, R_3 = OH
CDCA-GSH 3-sulfate (**1b**): R_1 = OSO_3H, R_2 = α-OH, R_3 = H
DCA-GSH 3-sulfate (**1c**): R_1= OSO_3H, R_2 =H, R_3 = OH
UDCA-GSH 3-sulfate (**1d**): R_1 = OSO_3H, R_2 = β-OH, R_3 = H
LCA-GSH 3-sulfate (**1e**): R_1 = OSO_3H, R_2 = R_3 = H
CA-GSH: R_1 = OH, R_2 = α-OH, R_3 = OH
CDCA-GSH: R_1= OH, R_2 = α-OH, R_3 = H
DCA-GSH: R_1 = OH, R_2=H, R_3 = OH
UDCA-GSH: R_1 = OH, R_2 = β-OH, R_3 = H
LCA-GSH: R_1 = OH, R_2 = R_3 = H



CA: R_1 = OH, R_2 = α-OH, R_3 = OH
CDCA: R_1= OH, R_2 = α-OH, R_3 = H
DCA: R_1 = OH, R_2=H, R_3 = OH
UDCA: R_1 = OH, R_2 = β-OH, R_3 = H
LCA: R_1 =OH, R_2 = R_3 = H
CA 3-sulfate: R_1 = OSO_3H, R_2 = α-OH, R_3 = OH
CDCA 3-sulfate: R_1= OSO_3H, R_2 = α-OH, R_3 = H
DCA 3-sulfate: R_1= OSO_3H, R_2 =H, R_3 = OH
UDCA 3-sulfate: R_1 = OSO_3H, R_2 = β-OH, R_3 = H
LCA 3-sulfate: R_1 = OSO_3H, R_2 = R_3 = H



CA-PNP 3-sulfate: R_1 = α-OH, R_2 = OH
CDCA-PNP 3-sulfate: R_1 = α-OH, R_2 = H
DCA-PNP 3-sulfate: R_1 =H, R_2 = OH
UDCA-PNP 3-sulfate: R_1 = β-OH, R_2 = H
LCA-PNP 3-sulfate: R_1 = R_2 = H



[2,2,4,4-d_4]-DCA-GSH (IS)

Fig. 1. Chemical structures of BA–GSH 3-sulfates and their related compounds.

(3H, m, Glu-γ,γ′-H_2 and 23-H), 2.60–2.65 (1H, m, 23-H′), 3.11–3.15 and 3.41–3.44 (2H, m, Cys-β,β′-H_2), 3.77–3.80 (1H, m, 7β-H), 3.89–3.93 (3H, m, Gly-α,α′-H_2 and Glu-α-H), 4.11–4.15 (1H, m, 3β-H), 4.57–4.60 (1H, m, Cys-α-H). ESI–MS: *m*/*z* 762.3 $[M+H]^+$, *m*/*z* 760.3 $[M-H]^-$, *m*/*z* 379.9 $[M-2H]^{2-}$.

2.4.4. S-(3-Sulfooxydeoxycholyl)glutathione (DCA-GSH 3-sulfate; **1c**)

This compound **1c** was obtained from DCA 3-sulfate (230 mg) as colorless amorphous solids with isolated yield 7.4 mg (2.2%). ^{1}H NMR δ: 0.70 (3H, s, 18-CH_3), 0.92 (3H, s, 19-CH_3), 1.01 (3H, d, *J* = 6.8 Hz, 21-CH_3), 2.19–2.28 (2H, m, Glu-β,β′-H_2), 2.52–2.58 (3H, m, Glu-γ,γ′-H_2 and 23-H), 2.64–2.68 (1H, m, 23-H′), 3.17–3.20 and 3.34–3.40 (2H, m, Cys-β,β′-H_2), 3.63–3.66 (1H, m, 12β-H), 3.95–3.98 (3H, m, Gly-α,α′-H_2 and Glu-α-H), 4.31–4.37 (1H, m, 3β-H), 4.59–4.64 (1H, m, Cys-α-H). ESI–MS: *m*/*z* 762.3 $[M+H]^+$, *m*/*z* 760.3 $[M-H]^-$, *m*/*z* 379.9 $[M-2H]^{2-}$.

2.4.5. S-(3-Sulfooxyursodeoxycholyl)glutathione (UDCA-GSH 3-sulfate; **1d**)

This compound **1d** was obtained from UDCA 3-sulfate (180 mg) as colorless amorphous solids with isolated yield 54.8 mg (24.0%). ^{1}H NMR δ: 0.69 (3H, s, 18-CH_3), 0.94 (3H, s, 19-CH_3), 0.94 (3H, d, *J* = 6.6 Hz, 21-CH_3), 2.19–2.25 (2H, m, Glu-β,β′-H_2), 2.49–2.55 (3H, m, Glu-γ,γ′-H_2 and 23-H), 2.60–2.65 (1H, m, 23-H′), 3.12–3.16 and 3.40–3.43 (2H, m, Cys-β,β′-H_2), 3.46–3.50 (1H, m, 7α-H), 3.91 (2H, s, Gly-α,α′-H_2), 4.02 (1H, t, *J* = 6.3 Hz, Glu-α-H), 4.20–4.25 (1H, m, 3β-H), 4.58–4.60 (1H, m, Cys-α-H). ESI–MS: *m*/*z* 762.3 $[M+H]^+$, *m*/*z* 760.3 $[M-H]^-$, *m*/*z* 379.9 $[M-2H]^{2-}$.

2.4.6. S-(3-Sulfooxylithocholyl)glutathione (LCA-GSH 3-sulfate; **1e**)

This compound **1e** was obtained from LCA 3-sulfate (200 mg) as colorless amorphous solids with isolated yield 23.7 mg (8.9%). ^{1}H NMR δ: 0.67 (3H, s, 18-CH_3), 0.94 (3H, s, 19-CH_3), 0.94 (3H, d, *J* = 6.4 Hz, 21-CH_3), 2.12–2.21 (2H, m, Glu-β,β′-H_2), 2.49–2.54 (3H, m, Glu-γ,γ′-H_2 and 23-H), 2.59–2.64 (1H, m, 23-H′), 3.11–3.15 and 3.39–3.44 (2H, m, Cys-β,β′-H_2), 3.91 (2H, s, Gly-α,α′-H_2), 3.96 (1H, t, *J* = 6.4 Hz, Glu-α-H), 4.24–4.30 (1H, m, 3β-H), 4.57–4.60 (1H, m, Cys-α-H). ESI–MS: *m*/*z* 746.3 $[M+H]^+$, *m*/*z* 744.3 $[M-H]^-$, *m*/*z* 371.9 $[M-2H]^{2-}$.

2.5. Preparation of rat liver cytosolic fraction

Animal studies were approved by the Institutional Animal Care and Use Committee of Kinki University. Male Wistar rats (230–250 g), given a commercial pellet diet and water ad libitum, were used. Animals were fasted overnight and then killed. The liver (10 g wet weight) was minced and homogenized in 3 volumes of ice-cold 10 mM phosphate buffered saline (pH 7.4) by 10

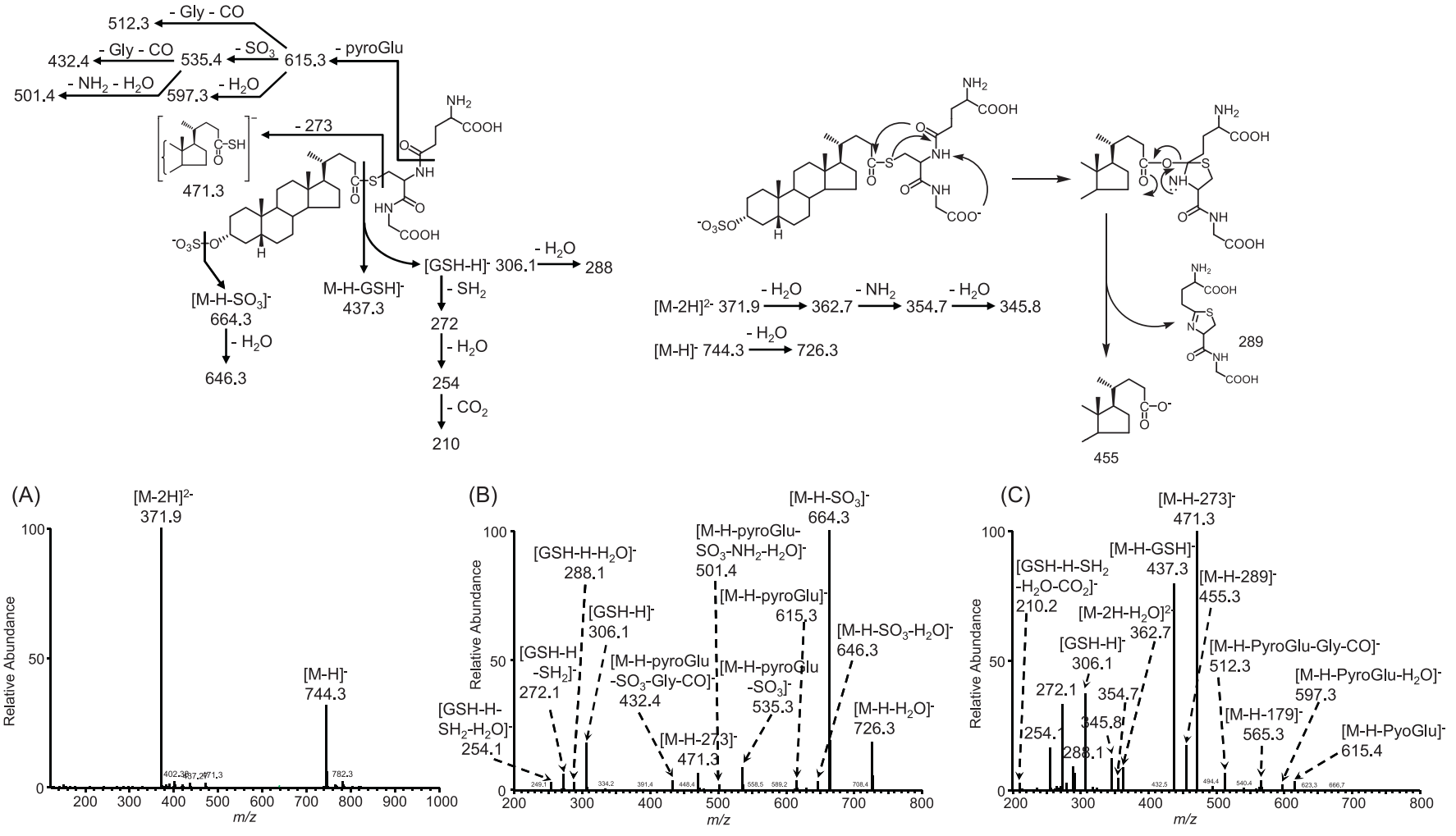


Fig. 2. Negative-ion (A) ESI-MS spectrum of LCA-GSH 3-sulfate and its MS/MS spectra obtained by CID of (B) $[M-H]^-$ and (C) $[M-2H]^{2-}$ ions. Proposed major m/z values of fragment ions are depicted.

Table 1
Observed ions of BA–GSH 3-sulfates on negative-ion ESI-MS^n.

	m/z				
	CA-GSH 3-sulfate	CDCA-GSH 3-sulfate	DCA-GSH 3-sulfate	UDCA-GSH 3-sulfate	LCA-GSH 3-sulfate
MS					
$[M-H]^-$	776.3 (63)	760.3 (50)	760.3 (45)	760.3 (34)	744.3 (29)
$[M-2H]^{2-}$	387.9 (100)	379.9 (100)	379.9 (100)	379.9 (100)	371.8 (100)
MS/MS[a]					
$[M-H-H_2O]^-$	758.3 (7)	742.3 (9)	742.3 (10)	742.4 (17)	726.3 (18)
$[M-H-SO_3]^-$	696.3 (100)	680.3 (100)	680.3 (100)	680.4 (100)	664.3 (100)
$[M-H-SO_3-H_2O]^-$	678.3 (4)	662.3 (3)	662.4 (3)	662.4 (3)	646.3 (2)
$[M-H-SO_3-NH_2-H_2O]^-$	N.D.	646.3 (<1)	646.6 (<1)	N.D.	N.D.
$[M-H-pyroGlu]^-$	647.3 (2)	631.3 (4)	631.4 (2)	631.4 (6)	615.4 (3)
$[M-H-pyroGlu-SO_3]^-$	567.4 (4)	551.3 (6)	551.4 (5)	551.4 (9)	535.4 (9)
$[M-H-pyroGlu-SO_3-NH_2-H_2O]^-$	N.D.	517.4 (<1)	517.4 (<1)	517.4 (1)	501.5 (2)
$[M-H-pyroGlu-SO_3-Gly-CO]^-$	464.3 (<1)	448.4 (2)	448.4 (1)	448.4 (3)	432.4 (3)
$[GSH-H]^-$	306.1 (5)	306.1 (10)	306.1 (8)	306.2 (17)	306.1 (18)
$[GSH-H-H_2O]^-$	288.0 (<1)	288.1 (1)	288.1 (<1)	288.2 (3)	288.1 (2)
$[GSH-H-SH_2]^-$	272.1 (2)	272.1 (3)	272.2 (2)	272.2 (5)	272.1 (5)
$[GSH-H-SH_2-H_2O]^-$	254.1 (<1)	254.1 (2)	254.1 (1)	254.1 (3)	254.1 (4)
$[M-H-273]^-$	503.3 (3)	487.3 (4)	487.3 (4)	487.4 (6)	471.3 (6)
MS/MS[b]					
$[M-H-pyroGlu]^-$	N.D.	631.3 (3)	631.3 (3)	631.4 (3)	615.3 (3)
$[M-H-pyroGlu-H_2O]^-$	629.4 (2)	613.3 (2)	N.D.	613.4 (2)	597.3 (3)
$[M-H-pyroGlu-Gly-CO]^-$	544.3 (5)	528.3 (5)	528.4 (5)	528.4 (6)	512.3 (5)
$[M-H-GSH]^-$	469.3 (71)	453.3 (76)	453.3 (73)	453.3 (78)	437.3 (79)
$[M-2H-H_2O]^{2-}$	378.7 (6)	370.8 (7)	370.8 (7)	370.8 (7)	362.7 (9)
$[M-2H-NH_2-H_2O]^{2-}$	370.7 (5)	362.8 (3)	362.7 (4)	362.8 (4)	354.7 (5)
$[M-2H-NH_2-2H_2O]^{2-}$	361.8 (8)	353.7 (9)	353.8 (10)	353.8 (10)	345.8 (11)
$[M-2H-pyroGlu]^{2-}$	323.2 (13)	315.2 (14)	315.2 (15)	315.2 (16)	N.D.
$[GSH-H]^-$	306.1 (5)	306.1 (33)	306.1 (33)	306.1 (34)	306.1 (36)
$[GSH-H-H_2O]^-$	288.1 (8)	288.1 (8)	288.1 (8)	288.2 (9)	288.1 (8)
$[GSH-H-SH_2]^-$	272.1 (31)	272.1 (30)	272.2 (32)	272.1 (31)	272.1 (32)
$[GSH-H-SH_2-H_2O]^-$	254.1 (14)	254.1 (14)	254.1 (15)	254.1 (16)	254.1 (15)
$[GSH-H-SH_2-H_2O-CO_2]^-$	210.0 (3)	210.1 (3)	210.1 (2)	210.1 (2)	210.1 (2)
$[GSH-2H-H_2O]^{2-}$	143.0 (2)	143.0 (3)	N.D.	N.D.	N.D.
$[M-H-pyroGlu-NH_2-H_2O]^-$	N.D.	298.2 (6)	298.2 (6)	298.3 (7)	N.D.
$[M-H-179]^-$	N.D.	581.4 (3)	N.D.	581.4 (3)	565.3 (3)
$[M-H-273]^-$	503.0 (100)	487.3 (100)	487.3 (100)	487.3 (100)	471.3 (100)
$[M-H-289]^-$	N.D.	471.3 (16)	471.3 (17)	471.4 (16)	455.3 (17)

Values in parenthesis represent relative intensity. N.D., not detectable.
[a] $[M-H]^-$ ions of each BA–GSH 3-sulfate were used as precursor ion.
[b] $[M-2H]^{2-}$ ions of each BA–GSH 3-sulfate were used as precursor ion.

Table 2
MS/MS optimized conditions for quantitative determination of target analytes by the triple-quadrupole analyzer.

Analyte	Tube lens offset voltage (V)	Collision energy (V)	Transition ions for SRM	
			Precursor ion (*m/z*)	Product Ion (*m/z*)
CA-GSH 3-sulfate	−90	35	$[M-H]^-$ (776.3)	$[GSH-H]^-$ (305.9)
	−115	19	$[M-2H]^{2-}$ (387.8)	$[M-H-GSH]^-$ (469.1)
CDCA-GSH 3-sulfate	−87	30	$[M-H]^-$ (760.3)	$[M-H-SO_4]^-$ (680.3)
	−114	21	$[M-2H]^{2-}$ (379.9)	$[M-H-273]^-$ (486.7)
DCA-GSH 3-sulfate	−126	38	$[M-H]^-$ (760.3)	$[GSH-H]^-$ (306.0)
	−92	19	$[M-2H]^{2-}$ (379.8)	$[M-H-273]^-$ (487.1)
UDCA-GSH 3-sulfate	−125	36	$[M-H]^-$ (760.0)	$[GSH-H]^-$ (305.7)
	−92	20	$[M-2H]^{2-}$ (379.9)	$[M-H-273]^-$ (487.1)
LCA-GSH 3-sulfate	−118	39	$[M-H]^-$ (744.3)	$[GSH-H]^-$ (305.9)
	−91	18	$[M-2H]^{2-}$ (371.7)	$[M-H-273]^-$ (470.9)
CA-GSH	−106	31	$[M-H]^-$ (696.3)	$[GSH-H]^-$ (306.0)
CDCA-GSH	−107	29	$[M-H]^-$ (680.3)	$[GSH-H]^-$ (305.6)
DCA-GSH	−113	30	$[M-H]^-$ (680.3)	$[GSH-H]^-$ (305.7)
UDCA-GSH	−110	29	$[M-H]^-$ (680.3)	$[GSH-H]^-$ (306.0)
LCA-GSH	−113	30	$[M-H]^-$ (664.3)	$[GSH-H]^-$ (305.9)
CA 3-sulfate	−120	42	$[M-H]^-$ (487.0)	$[HSO_4]^-$ (97.0)
CDCA 3-sulfate	−115	47	$[M-H]^-$ (471.3)	$[HSO_4]^-$ (97.0)
DCA 3-sulfate	−124	41	$[M-H]^-$ (471.3)	$[HSO_4]^-$ (97.0)
UDCA 3-sulfate	−120	41	$[M-H]^-$ (471.3)	$[HSO_4]^-$ (97.0)
LCA 3-sulfate	−120	44	$[M-H]^-$ (455.3)	$[HSO_4]^-$ (97.0)
CA	−70	25	$[M-H+CH_3COOH]^-$ (467.3)	$[M-H]^-$ (406.8)
CDCA	−75	27	$[M-H+CH_3COOH]^-$ (451.3)	$[M-H]^-$ (390.7)
DCA	−117	35	$[M-H]^-$ (391.3)	$[M-H-CO_2]^-$ (344.8)
UDCA	−70	26	$[M-H+CH_3COOH]^-$ (451.3)	$[M-H]^-$ (390.7)
LCA	−78	26	$[M-H+CH_3COOH]^-$ (435.3)	$[M-H]^-$ (375.3)
d_4-DCA-GSH	−112	29	$[M-H]^-$ (684.3)	$[GSH-H]^-$ (306.1)

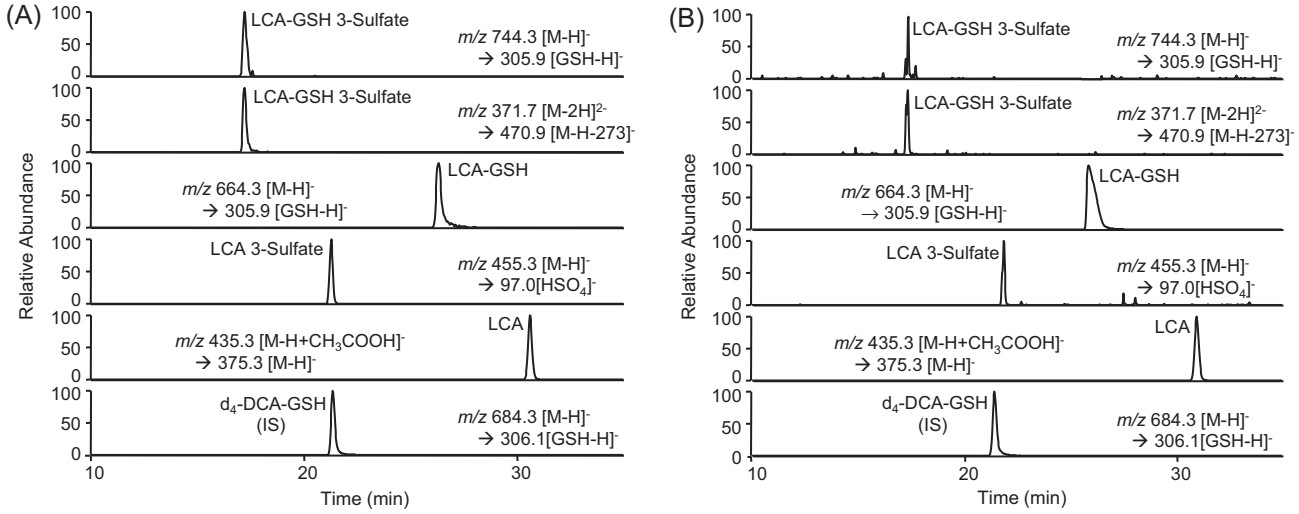


Fig. 3. Typical extracted ion chromatograms of (A) reference LA-GSH 3-sulfate, LCA-GSH, LCA 3-sulfate, and LCA and (B) incubation mixture obtained at 0.5 min incubation of LCA-GSH with a rat liver cytosolic fraction by means of SRM mode of TSQ mass spectrometer. Chromatographic and mass spectrometric conditions are detailed in Section 2.

rapid strokes using a motor-driven Teflon-glass homogenizer. The homogenate was centrifuged for 10 min at 600 × *g* in the R19A rotor of a Hitachi himac CR 21 centrifuge (Hitachi, Tokyo, Japan), followed by further centrifugation of the supernatant at 9000 × *g* for 10 min. The supernatant was centrifuged at 105,000 × *g* for 1 h. The resulting supernatant was used as a cytosolic fraction. The whole procedure was carried out at 0–4 °C.

The protein concentration was determined by the method of Smith et al. (1985) with a Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific) using bovine serum albumin as standard.

2.6. Incubation of BA–GSH with rat liver cytosolic fraction

Rat liver cytosolic fraction (0.5 mg protein) was dispersed in 0.05 M phosphate buffer (pH 5.5) (980 μL) containing $MgCl_2$ (2 μmol) and PAPS (200 nmol) and pre-incubated at 37 °C for 5 min. The reaction was started by the addition of a solution of CA-GSH, CDCA-GSH, DCA-GSH, UDCA-GSH, and LCA-GSH (each 200 nmol) in methanol (20 μL). After 0.5, 5, 10, 15, 30, and 60 min of incubation, a 100-μL aliquot was collected from the incubation mixture and the reaction(s) was terminated by the addition of acetic acid (4 μL) and acetonitrile (200 μL). After addition of d_4-DCA-GSH (100 ng) in methanol (10 μL) as an internal standard (IS) and dilution with water (1 mL), the resulting mixture was passed through an Oasis® HLB (adsorbent weight, 60 mg) cartridge. After washing with water (1.5 mL), BAs were then eluted with methanol (1.5 mL), followed by evaporation of the solvent under a gentle stream of N_2 gas at room temperature, 10-μL aliquot of the re-dissolved solution of 30% acetonitrile (100 μL) were subjected to an LC/ESI–MS/MS analysis in the negative-ion mode. The transitions (precursor and product ions) for each analyte were monitored as shown in Table 2.

3. Results and discussion

3.1. Chemical synthesis of BA–GSH 3-sulfates

Our initial efforts were directed to the chemical synthesis of the 3-sulfates of BA–GSH as reference compounds (Fig. 1), as synthetic reference compounds are necessary to develop a sensitive selected reaction monitoring (SRM) method that would be valid for complex biological matrices. The synthetic method for preparation of BA 3-sulfates in the literature involved the protection of 7- and 12-hydroxy groups with a formyloxy group, sulfation of the C-3 hydroxy group, followed by final deprotection of the protected group with alkali (Goto et al., 1979). This method is not adequate for obtaining our target compounds because of the sensitivity to alkali of the thioester bond present in the BA–GSH conjugates. Accordingly, coupling the carboxyl group of BA 3-sulfates with the thiol group of GSH by the activated ester method was attempted. Each BA 3-sulfate was converted to the corresponding *p*-nitrophenyl ester, and the activated ester thus obtained was used to form the GSH conjugate. As the results, pure samples of the BA–GSH 3-sulfates were obtained after purification by preparative HPLC.

3.2. Structure confirmation of BA–GSH 3-sulfates

The structure of the products was confirmed by means of ^1H NMR and LC/ESI-MS. In the ^1H NMR spectra, the following proton signals were assigned: angular H_3-18, H_3-19 (each s) and H_3-21 methyl protons in a higher field region of 0.67–1.05 ppm, H_2-23 methylene protons (each m) at 2.47–2.77 ppm, and H-3β, H-7 and/or H-12β methyne protons (occurring at 3.46–4.37 ppm) geminally attached to oxygen-containing functions in the aglycone moieties, and H_2-β,β′ in glutamic acid moieties at 2.14–2.35 ppm (m), H_2-γ,γ′ in glutamic acid moieties at 2.47–2.58 ppm (m), H_2-β,β′ in cysteine moieties at 3.11–3.26 and 3.34–3.45 ppm (each m), H_2-α,α′ in glycine moieties (each s) at 3.89–3.98 ppm (m), H-α in glutamic acid moieties at 3.89–4.04 ppm (m), and H-α in cysteine moieties at 4.40–4.70 ppm (m).

The formation of major product ions of the sulfates under negative-ion ESI–MS/MS on the LTQ mass spectrometer was also interpreted, and fragmentation pathways are proposed for those BA–GSH 3-sulfates. The typical negative-ion ESI–MS and CID spectra of the double conjugate of LCA are shown in Fig. 2. In the full-scan mode, the spectra of all the conjugates showed doubly charged $[M-2H]^{2-}$ ion as the base peak along with a moderate deprotonated molecule $[M-H]^-$, indicating the covalently condensed product of BA 3-sulfate and GSH. The delocalization of the negative charge onto the carboxylic acid and sulfuric acid groups is responsible for stability of the $[M-2H]^{2-}$ ion. Further MS/MS experiments at the normalized collision energy of 35% were performed. Under the CID condition, singly charged $[M-H]^-$ ion underwent characteristic fragmentation patterns: the fragmentation of the sulfates afforded the same type of ions (Table 1). Thus, the sulfates showed the $[M-H-SO_3]^-$ ion as the base peak with weak $[M-H-H_2O]^-$,

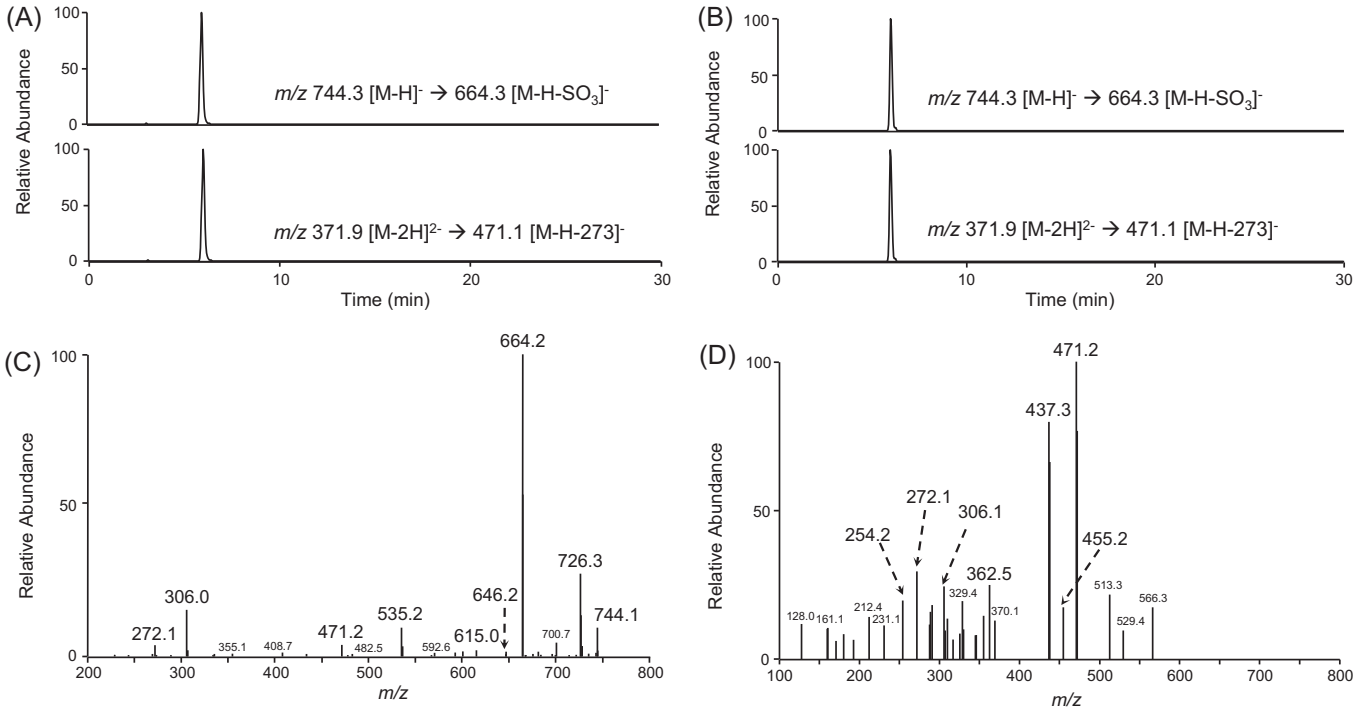


Fig. 4. LC/ESI-MS/MS analysis of (A) reference LCA-GSH 3-sulfate and (B) the incubation mixture obtained at 0.5 min incubation of LCA-GSH with rat liver cytsolic fraction by means of linear ion-trap mass spectrometer. Extracted ion chromatograms were obtained by monitoring the characteristic transitions of $[M-H]^-$ at m/z 744.3 → $[M-H-SO_3]^-$ at 664.3 (upper) and $[M-2H]^{2-}$ at m/z 371.9 → $[M-H-273]^-$ at m/z 471.1 (lower). Product ion spectra were obtained by CID of (C) $[M-H]^-$ ion at m/z 744.3 and (D) $[M-2H]^{2-}$ ion at m/z 371.9 of the peak associated with retention time at 6 min. Mobile phase used was 30% solvent B (acetonitrile) to 80% B against solvent A (5 mM ammonium acetate buffer, pH 6.0) over 30 min at a flow rate of 200 μL/min.

$[M-H-SO_3-H_2O]^-$, $[M-H-SO_3-NH_2-H_2O]^-$ ions. The ions $[M-H$–pyroglutamic acid$]^-$, $[M-H$–pyroglutamic acid–$SO_3]^-$, $[M-H$–pyroglutamic acid–$SO_3-NH_2-H_2O]^-$, $[M-H$–pyroglutamic acid–SO_3–Glycine–$CO]^-$, present in all the CID spectra, are formed by the loss of GSH and sulfuric acid groups. Similarly, the CID spectra of $[M-H]^-$ ion is characterized by a cleavage of the S–CH_2 bond, leading to a loss of 273 Da and the formation of corresponding negative ion at m/z 471, 487, and 503. The weak $[GSH-H]^-$ ion at m/z 306 is formed by the loss of steroid moiety. As a result of the localization of the negative charge at the GSH moiety, the frequently occurring fragments (m/z 288, 272, 254) are formed by the loss of H_2O, H_2S, and CO_2 molecules in various combinations from the GSH fragment ion.

CID of doubly charged $[M-2H]^{2-}$ ion generated $[M-H-273]^-$ ion as the base peak with moderate $[M-H-GSH]^-$ ion and without ion characteristic of sulfated conjugate, which give rise to the ion formed by the loss of SO_3 (80 Da). The fragmentation pattern showed the ions formed by the loss of glutamic acid and glycine, ions derived from the GSH, and ions by the neutral loss of 289 Da (Table 1). The latter ions are mostly charge-induced fragmentation from the GSH moiety. These fragment ions are common in tandem mass spectra of GSH conjugates of low molecular weight substances (Dieckhaus et al., 2005). These spectral data were consistent with the structures of BA–GSH 3-sulfates. In addition to negative-ion mode, the positive ESI was also investigated, but the obtained results were found less satisfying than in the negative-ion mode (data not shown).

3.3. Sulfation of BA–GSH with rat liver cytosolic fraction

In order to investigate the sulfation of BA–GSH, the amount of remaining BA–GSH and the BA–GSH 3-sulfates that were formed were determined by means of SRM mode of triple-stage quadrupole (TSQ) mass spectrometer. Unconjugated BAs and BA 3-sulfates were also determined, because the thioester bond of the GSH conjugate might be hydrolyzed, generating the unconjugated BA moiety (and its sulfate) during the incubation. Tube lens offset voltage and collision energy of each BA and their conjugates under negative-ion ESI-MS and MS/MS were optimized by directly injecting the standard solution into the mass spectrometer. BA–GSH 3-sulfates

Table 3
Biotransformation of LCA-GSH and DCA-GSH by a rat liver cytosol containing PAPS. Results shown as percent of the sum of substrate and its metabolites.

Compound	%					
	0.5 min	5 min	10 min	15 min	30 min	60 min
LCA-GSH	86.98	11.47	7.72	5.56	2.66	1.23
LCA-GSH 3-sulfate	0.02	N.D.	N.D.	N.D.	N.D.	N.D.
LCA	13.0	88.45	92.15	94.25	96.94	98.03
LCA 3-sulfate	0.00	0.08	0.13	0.19	0.40	0.74
DCA-GSH	88.66	0.19	0.02	N.D	N.D.	N.D.
DCA-GSH 3-sulfate	0.01	N.D.	N.D.	N.D.	N.D.	N.D.
DCA	11.31	99.62	99.65	99.56	99.12	98.32
DCA 3-sulfate	0.02	0.19	0.33	0.44	0.88	1.68

N.D., not detectable.

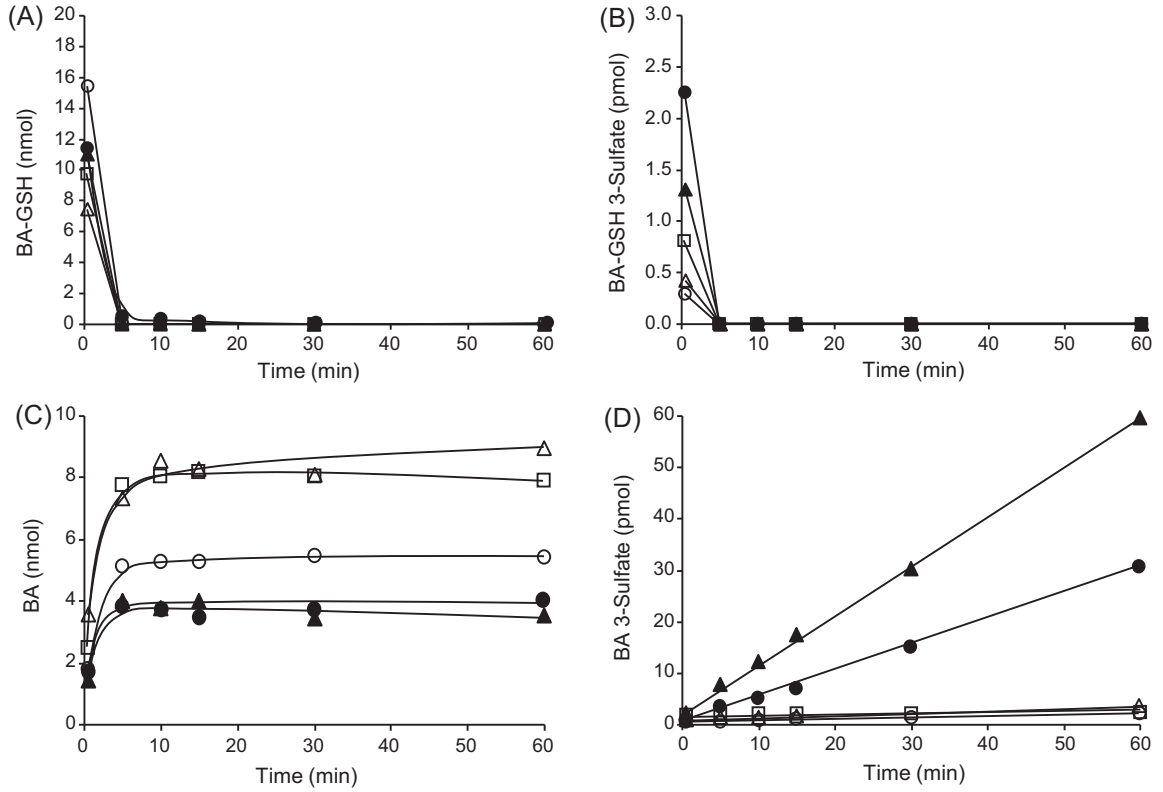
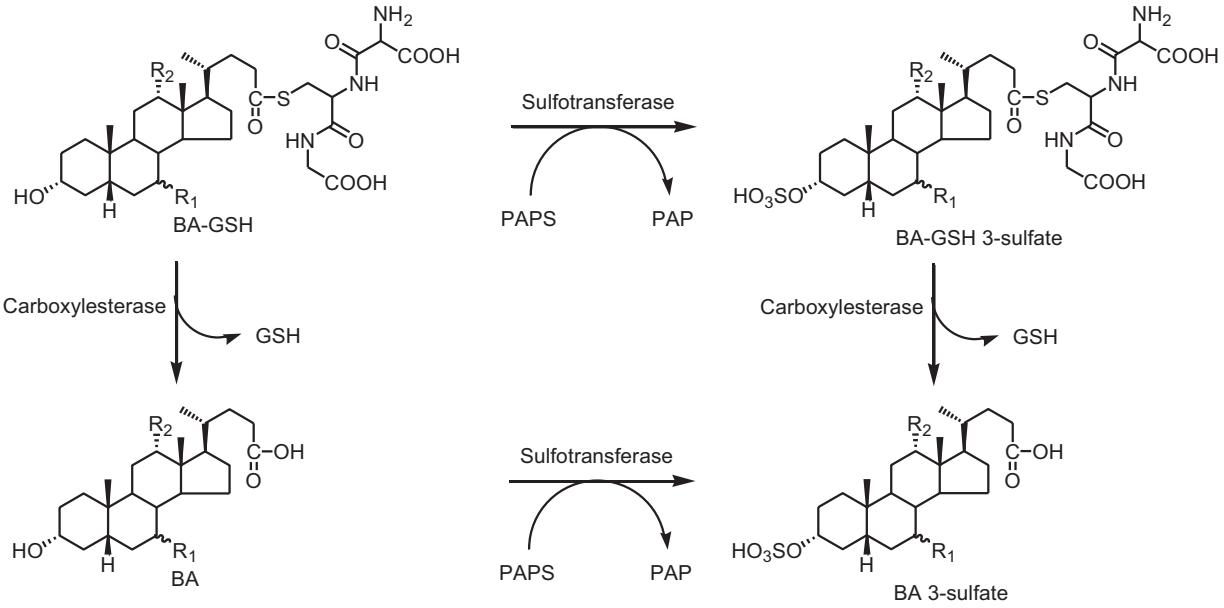


Fig. 5. Time course for (A) hydrolysis of BA–GSH, (B) formation of BA–GSH 3-sulfates, (C) liberation of unconjugated BA, and (D) appearance of BA 3-sulfates by incubation with rat liver cytosolic fraction. A 100 nmol each of BA–GSH was separately incubated with rat liver cytosolic fraction (0.5 mg protein) in the presence of PAPS (100 nmol) at 37 °C. Values represent the average of triplicate incubations. CA and its derivatives, open circle; CDCA and its derivatives, open triangle; DCA and its derivatives, filled triangle; UDCA and its derivatives, open square; LCA and its derivatives, filled circle.

generated similar ions using linear ion-trap mass spectrometer. But the most abundant product ions were different by mass spectrometer. The most abundant transitions that could be used for the monitoring ion are listed in Table 2. The chromatographic conditions, especially the composition of mobile phase, were optimized through several trials. When using a TSKgel ODS-100V column and a linear gradient elution of 30% solvent B (acetonitrile-methanol, 3:7, v/v) to 80% B against solvent A (5 mM ammonium acetate–acetic acid buffer, pH 6.0) over 30 min at a flow rate of 200 μL/min, satisfactory chromatographic separation of



CA, R_1=α-OH, R_2=OH; CDCA, R_1=α-OH, R_2=H; DCA, R_1=H, R_2=OH; UDCA, R_1=β-OH, R_2=H; LCA, R_1=R_2=H

Fig. 6. Sulfation of LCA-GSH by a rat liver cytosolic fraction.

BA–GSH, BA–GSH 3-sulfate, BA 3-sulfate, and unconjugated BA was achieved.

Fig. 3A shows a typical extracted ion chromatogram of LCA-GSH and its related compounds. Calibration graphs were then constructed by plotting the peak-area ratio of each compound to that of [2,2,4,4-d_4]-DCA-GSH (IS) versus the weights of each BA. The response was linear in the range of 0.05–100 ng.

To investigate the substrate specificity, individual BA–GSH (100 nmol, [10 μM]) were incubated with a rat liver cytosolic fraction (0.5 mg protein) at 37 °C for 60 min. A 100-μL aliquot was collected from the incubation mixture at appropriate times. After the addition of IS and solid phase extraction, BA–GSH, BA–GSH 3-sulfates, along with unconjugated BAs and BA 3-sulfates were determined by the method described above. Fig. 3B shows a typical extracted ion chromatogram of the incubation mixture at 0.5 min incubation of LCA-GSH. The chromatogram clearly revealed the appearance of LCA-GSH 3-sulfate, LCA, and LCA 3-sulfate, and remaining LCA-GSH. The identification of the peak of LCA-GSH 3-sulfate was carried out by comparison of retention time and CID spectrum with those of reference compound by means of linear ion-trap mass spectrometer. Although coexistence of several ions originating from endogenous compounds were observed in the product ion spectra (Fig. 4C and 4D) obtained by CID of $[M-H]^-$ and $[M-2H]^{2-}$ of the peak, the CID spectra were identical with those of the reference compounds (Fig. 2) with respect to the ion species and the relative abundances of the product ion. As shown in Table 3 and Fig. 5A, the BA–GSH proportion decreased rapidly with complete biotransformation after 30 min incubation. In contrast, the enzymatic production of BA–GSH 3-sulfates was extremely limited; it reached a peak in 0.5 min and then decreased in a time dependent manner (Fig. 5B). LCA, which is a monohydroxy BA and therefore the most lipophilic of the BAs examined, was most effectively sulfated to form LCA-GSH 3-sulfate with the conversion rate of 0.02% at 0.5 min. CDCA-GSH, DCA-GSH, and UDCA-GSH were metabolized into their corresponding 3-sulfates at a more moderate rate. The trihydroxy CA-GSH, which is more hydrophilic than the other BA–GSH tested, showed feeble formation of its 3-sulfate.

In a previous paper (Mitamura et al., 2009), we demonstrated that BA-NACs were easily hydrolyzed to form unconjugated BAs by carboxyl esterases, which are widely distributed throughout the body, with high levels occurring in the liver, kidneys, testes, lungs, and plasma. Furthermore, we showed that BA-NAC 3-sulfates were formed from BA-NAC conjugates when incubated with a rat liver cytosolic fraction, albeit to a very limited extent, and were labile, as the thioester bond of the NAC conjugates underwent rapid hydrolysis (Mitamura et al., 2011c). As shown in Fig. 5C and D, both unconjugated BAs and BA 3-sulfates were generated during the incubation. The BA–GSH conjugates underwent sulfation at C-3, but the fraction formed was small and transient. Unconjugated BAs were generated, and these in turn were sulfated. The BA 3-sulfates could be formed by either hydrolysis of the thioester bond of the sulfated GSH conjugates or by sulfation of the unconjugated BAs (Fig. 6). The latter route appears to be the dominant one. The sulfation of unconjugated BAs does not occur to any extent in the healthy vertebrate (other than lithocholic acid sulfation in man) because of the high K_m of sulfotransferase 2A1 as well as the low V_{max} for endogenous, hydrophilic BAs (Huang et al., 2010).

4. Conclusion

The 3-sulfates of the GSH conjugates of the five BAs present in human bile were synthesized and their MS and NMR properties defined. Sulfation of BA–GSH conjugates occurred when the BA–GSH conjugates were incubated with a rat liver cytosolic fraction, albeit to a very limited extent. Moreover, the BA–GSH conjugates were labile, as the thioester bond of the GSH conjugates underwent rapid hydrolysis. The liberated unconjugated BAs then underwent sulfation at C-3 with hydrophobic BAs undergoing greater sulfation than hydrophilic BAs (Fig. 5). Further work is required to define whether the 3-sulfates of the GSH conjugates of BAs are present in rodent urine and bile and whether such formation of the sulfate conjugates of BA–GSH is an important excretion route for BA–GSH conjugates.

Acknowledgements

The authors acknowledge support for this work by a Grant-in-Aid for Scientific Research (C) for 2009–2011 (To S.I., Grant 21590184) and 2011–2013 (To. K.M., Grant 23590209) from the Japan Society for the Promotion of Science, and Culture of Japan, and High-Tech Research Center Project for Private Universities: matching fund subsidy from the Ministry of Education, Culture, Sports, Science and Technology (2007–2011).

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