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Chemical synthesis of *N*-acetylcysteine conjugates of bile acids and *in vivo* formation in cholestatic rats as shown by liquid chromatography/electrospray ionization-linear ion trap mass spectrometry☆

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ABSTRACT

N-Acetylcysteine (NAC) conjugates of the five major bile acids occurring in man were synthesized in order to investigate the possible formation *in vivo* of these conjugates. Upon collision-induced dissociation, structurally informative daughter ions were observed. The transformation of cholyl-adenylate and cholyl-CoA thioester into a *N*-acetyl-*S*-(cholyl)cysteine by rat hepatic glutathione *S*-transferase was confirmed by liquid chromatography/electrospray ionization-linear ion trap mass spectrometry (LC/ESI-MS^2). Lithocholic acid was administered orally to bile duct-ligated rats that also received NAC intraperitoneally. The NAC conjugate of lithocholic acid was identified in urine by means of LC/ESI-MS^2. Rapid hydrolysis of the BA-NAC conjugates by rabbit liver carboxylesterase was found, demonstrating the possible labile nature of the NAC conjugates formed in the liver.

1. Introduction

Recently, considerable attention has been focused on the role of bile acids (BAs) in the pathogenesis of disorders such as cholestatic liver injury. Although the mechanism of BA toxicity remains elusive, one hypothesis has been developed, focusing on the metabolic activation of BAs. One possible pathway implicates the reactive acyl-glucuronides of BAs as a potential causative agent for the BA-associated toxicity. In this regard, it has been shown that the glucuronide conjugation involving the 24-carboxyl group of BAs is catalyzed by a microsomal enzyme present in rat liver [1–3] and human uridine 5′-diphosphoglucuronosyltransferase 1A3 [4,5]; in addition, it has been shown that the resultant acyl-glucuronides are excreted into human urine [6] and are capable of covalent modification of cellular proteins [7]. The second proposed mechanism for BA toxicity is based on the finding of BA-protein adducts in tissue [8] and histones [9] under pathological conditions such as cholestatic liver injury as well as the *in vitro* formation of BA-protein adducts in incubations of reactive metabolites of BAs, acyl-adenylates of lithocholic acid (LCA) and deoxycholic acid (DCA) with lysozyme and histones [10,11]. In light of these findings, we recently identified Rab-3, Rab-12, Rab-16, and M-Ras as proteins covalently linked with LCA in the liver of bile duct-ligated rats [12]. In addition, we showed that cholyl-CoA thioester (CA-CoA) and its reactive intermediate choly-adenylate (CA-AMP) are converted into glutathione (GSH, γ-L-glutamyl-L-cysteinylglycine) conjugates and then excreted into the bile of the rat [13,14], thereby providing a detoxification pathway for CoA derivatives of bile acids.

GSH conjugation is a prominent pathway of metabolism of many xenobiotics, particularly the ones that are bioactivated to reactive electrophilic intermediates via oxidative metabolism [15]. The GSH conjugates may either be excreted intact in bile or may undergo further processing via the so-called “mercapturic acid pathway”. The

Abbreviations: BA, bile acid; CA, cholic acid; UDCA, ursodeoxycholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; NAC, *N*-acetylcysteine; GSH, glutathione; GST, glutathione *S*-transferase; CA-AMP, cholyl-adenylate; CA-CoA, cholyl-CoA thioester; AAP, acetaminophen; NAPQI, *N*-acetyl-*p*-benzoquinoneimine; LC, liquid chromatography; MALDI, matrix-assisted laser desorption ionization; TOFMS, time-of-flight mass spectrometry; ESI, electrospray ionization; MS, mass spectrometry; CID, collision-induced dissociation.

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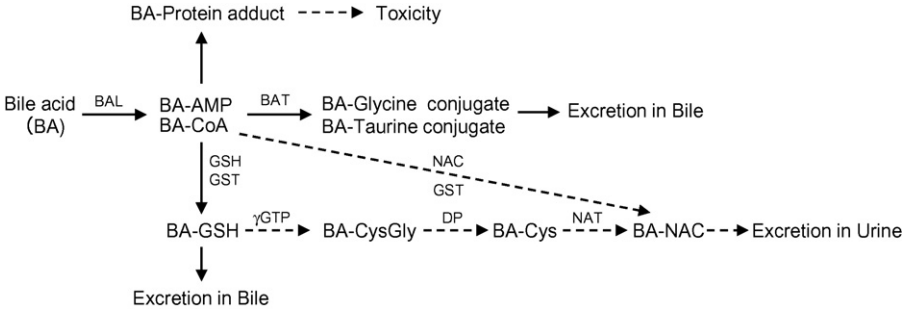

Fig. 1. Proposed scheme for the metabolic activation of bile acids followed by reaction of these activated bile acids with GSH and the pathway in the degradation of BA-GSH *in vivo*. BAL, bile acid:CoA ligase; BAT, bile acid:amino acid transferase; γGTP, γ-glutamyltranspeptidase; DP, dipeptidase; NAT, *N*-acetyltransferase.

latter involves sequential hydrolysis by γ-glutamyltranspeptidase and cysteinylglycine dipeptidase to form the corresponding cysteinylglycine and cysteine *S*-derivative [14,15]. *N*-Acetylation of the latter species leads to the formation of *N*-acetylcysteine (NAC) conjugates, which are usually excreted in urine as end products of GSH conjugates [15] (Fig. 1).

NAC is used for the clinical treatment for acetaminophen (AAP, 4-hydroxyacetanilide) overdose. It is well recognized that hepatic GSH depletion by the generation of *N*-acetyl-*p*-benzoquinoneimine (NAPQI) is a crucial component in AAP hepatotoxicity, resulting in elevated protein arylation by NAPQI [16]. NAC provides cysteine for GSH synthesis and thereby increases hepatic GSH levels, that in turn reduces AAP hepatic toxicity [17–19]. In clinical treatments, NAC is administered after the occurrence of an AAP overdose, and it is most effective when administered to patients within 10–16 h after AAP ingestion [20]. NAC is a small molecule containing a thiol group, which has antioxidant properties, and it is freely filterable with a ready access to intracellular compartments [21,22]. The diversity of the pharmacological applications of NAC is attributed to the chemical properties of the cysteinyl group of its molecule, because the ability of the reduced thiol group to scavenge oxygen-free radicals is well established [23–26]. Because of these properties, NAC is widely used in clinical practice as an antioxidant in which reactive intermediates can often be conjugated with NAC to form stable adducts, thereby preventing covalent binding and cell injury. Taking into account the reactivities of reactive electrophilic intermediates of BAs toward the nucleophiles, we speculated that the NAC adducts would be formed by the trans-acylation reaction of acyl-adenylate and acyl-CoA of BAs with NAC in a manner similar to that observed for GSH [12,13] (Fig. 1).

In this article, we describe the chemical synthesis of NAC conjugates of the five major BAs occurring in man (Fig. 2) and their characterization by means of liquid chromatography (LC)/electrospray ionization (ESI)-linear ion trap mass spectrometry (MS). In addition, we present evidence for *in vivo* and *in vitro* formations of putative NAC conjugates. We also show that such NAC conjugates may be rapidly hydrolyzed by carboxylesterase.

2. Experimental

2.1. Materials and reagents

Cholic acid (CA), chenodeoxycholic acid (CDCA), and DCA were purchased from Nacalai Tesque Inc. (Kyoto, Japan). LCA was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and ursodeoxycholic acid (UDCA) was donated by Mitsubishi Tanabe Pharma Co. (Osaka, Japan). CA-AMP, CA-CoA [27], and 12-oxolithocholic acid (12-oxoLCA) [28] were synthesized in our laboratory by using the method previously reported. NAC, GST (E.C. 2.5.1.18, 18 units/mg solid) isolated from rat liver, and carboxylesterase (E.C. 3.1.1.1, 80 units/mg solid) isolated from rabbit liver were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). An Oasis HLB cartridge (60 mg, 3 mL) was provided by Waters Co. (Milford, MA, USA) and successively conditioned with methanol (1 mL) and water (1 mL) prior to use. Acetonitrile and ammonium acetate of HPLC grade were purchased from Nacalai Tesque, Inc.

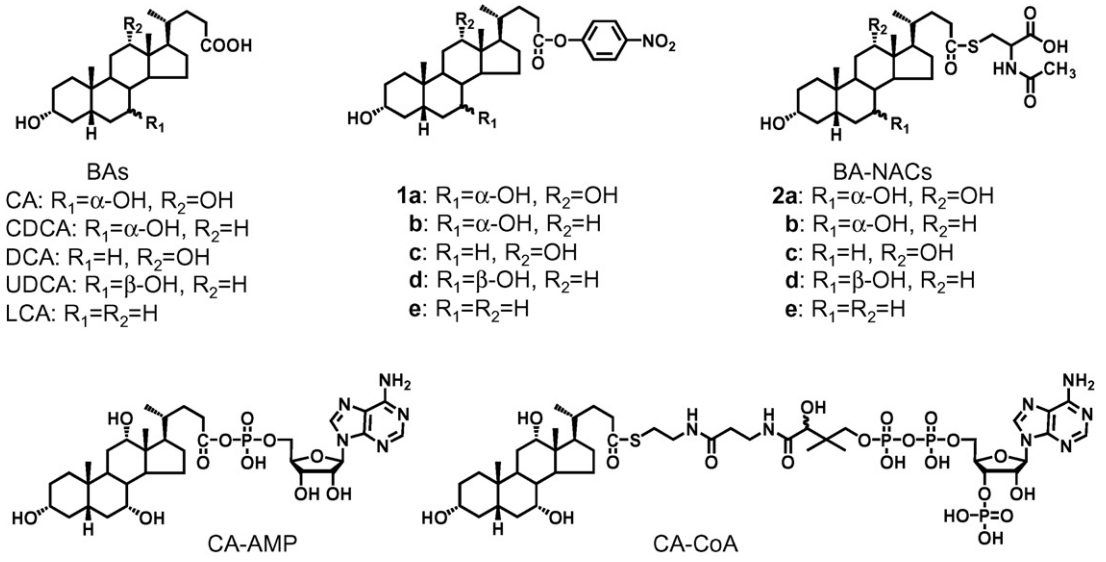

Fig. 2. Chemical structures of the five major bile acids present in human bile and their derivatives.

(Tokyo, Japan), and distilled water of HPLC grade was purchased from Wako Pure Chemical Industries, Ltd. All the other chemicals and solvents were of 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-ECX400 (399.65 MHz, JEOL Ltd., Tokyo, Japan) by using tetramethylsilane as an internal standard (s, singlet; d, doublet; t, triplet; m, multiplet). Matrix-assisted laser desorption ionization (MALDI) time-of-flight mass spectrometry (TOFMS) was carried out by using a Kratos Kompact MALDI4 (Shimadzu Co., Kyoto, Japan). The samples were loaded onto the MALDI target plate by mixing 0.5 μL of each methanolic solution (1 mg/mL) with the same volume of a matrix solution prepared by dissolving 10 mg/mL of 2,5-dihydroxybenzoic acid in 50% acetonitrile containing 0.1% of trifluoroacetic acid (TFA). Calibration was accomplished by using angiotensin II (Mr. 1047.20) as an internal standard.

2.3. LC/ESI-MS

The LC/MS analyses were carried out by using a Finnigan LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific Inc. Waltham, MA, USA) equipped with an ESI source and coupled to a Paradigm MS4 chromatograph (Michrom Bioresources, Inc., Auburn, CA, USA) and an autosampler (HTC PAL, CTC Analytics, Zwingen, Switzerland). The ionization conditions for verifying the structures of BA-NACs 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 (arb. units); auxiliary gas (nitrogen gas) flow rate, 5 arb. units; tube lens offset voltage, ±100 V. For tandem MS (MS^2) analysis, helium gas was used as the collision gas and the normalized collision energy was set at 35%. 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) 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. Under these conditions, NAC conjugates of CA, UDCA, CDCA, DCA, and LCA were eluted at 6.1, 6.4, 8.6, 8.9, and 12.3 min, respectively.

2.4. Synthesis of NAC conjugates of BAs (BA-NACs)

2.4.1. General method for the preparation of BA-NACs (**2a**–**2e**)

To a solution of each BA (1 g) in dioxane (8 mL), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1 g) and *p*-nitrophenol (two equimolar amount) were added, and the mixture was stirred at room temperature (r.t.) for 3 h. After evaporation of the solvent, the residue dissolved in AcOEt was washed with H_2O. The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to yield *p*-nitrophenyl esters (**1a**–**1e**), which were used for the subsequent reaction without further purification. To a solution of the *p*-nitrophenyl ester in *N*,*N*′-dimethylformamide (10 mL), NAC (two equimolar amount) and triethylamine (two equimolar amount) were added, and the mixture was stirred for 3 h at r.t. under N_2. After evaporation of the solvent *in vacuo*, the residue redissolved in AcOEt was washed with 5% HCl and H_2O. The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to yield the desired compounds, which were purified by column chromatography on a silica gel (30 g) by using $CHCl_3$–MeOH (30:1, v/v) as the eluant.

*2.4.1.1. N-Acetyl-S-(cholyl)cysteine (**2a**).* The compound **2a** was obtained from **1a** (870 mg) as colorless semi-solid: yield 556 mg (58.3%). ^{1}H NMR (CD_3OD) δ: 0.61 (3H, s, 18-H), 0.82 (3H, s, 19-H), 0.91 (3H, d, J = 6.4 Hz, 21-H), 1.86 (3H, s, –$COCH_3$), 2.44 and 2.52 (each 1H, m, 23-H), 3.05 and 3.45 (each 1H, m, Cys-β,β′-H), 3.25 (1H, m, 3β-H), 3.70 (1H, m, 7β-H), 3.84 (1H, m, 12β-H), 4.33 (1H, m, Cys-α-H). MALDI-TOFMS Found: m/z 554.6 $[M+H]^+$ (calcd. for $C_{29}H_{48}NO_7S$: 554.3); 576.6 $[M+Na]^+$ (calcd. for $C_{29}H_{47}NNaO_7S$: 576.3).

*2.4.1.2. N-Acetyl-S-(chenodeoxycholyl)cysteine (**2b**).* The compound **2b** was obtained from **1b** (686 mg) as colorless solid: yield 478 mg (63.5%). ^{1}H NMR (CD_3OD), δ: 0.59 (3H, s, 18-H), 0.83 (3H, s, 19-H), 0.85 (3H, d, J = 6.0 Hz, 21-H), 1.86 (3H, s, –$COCH_3$), 2.41 and 2.51 (each 1H, m, 23-H), 3.05 and 3.45 (each 1H, m, Cys-β,β′-H), 3.27 (1H, m, 3β-H), 3.70 (1H, m, 7β-H), 4.33 (1H, m, Cys-α-H). MALDI-TOFMS Found: m/z 599.7 $[M-H+Na+K]^+$ (calcd. for $C_{29}H_{47}KNNaO_6S$: 599.3).

*2.4.1.3. N-Acetyl-S-(deoxycholyl)cysteine (**2c**).* The compound **2c** was obtained from **1c** (769 mg) as colorless solid: yield 448 mg (63.5%). ^{1}H NMR (CD_3OD) δ: 0.60 (3H, s, 18-H), 0.83 (3H, s, 19-H), 0.90 (3H, d, J = 6.4 Hz, 21-H), 1.86 (3H, s, –$COCH_3$), 2.42 and 2.52 (each 1H, m, 23-H), 3.04 and 3.43 (each 1H, m, Cys-β,β′-H), 3.40 (1H, m, 3β-H), 3.84 (1H, m, 12β-H), 4.41 (1H, m, Cys-α-H). MALDI-TOFMS Found: m/z 560.8 $[M+Na]^+$ (calcd. for $C_{29}H_{47}NNaO_6S$: 560.3).

*2.4.1.4. N-Acetyl-S-(ursodeoxycholyl)cysteine (**2d**).* The compound **2d** was obtained from **1d** (779 mg) as colorless solid: yield 448 mg (64.3%). ^{1}H NMR (CD_3OD) δ: 0.61 (3H, s, 18-H), 0.86 (3H, d, J = 6.4 Hz, 21-H), 0.87 (3H, s, 19-H), 1.86 (3H, s, –$COCH_3$), 2.40 and 2.48 (each 1H, m, 23-H), 3.06 and 3.45 (each 1H, m, Cys-β,β′-H), 3.38 (2H, m, 3β-H and 7α-H), 4.36 (1H, m, Cys-α-H). MALDI-TOFMS Found: m/z 560.5 $[M+Na]^+$ (calcd. for $C_{29}H_{47}NNaO_6S$: 560.3); 576.5 $[M+K]^+$ (calcd. for $C_{29}H_{47}KNO_6S$: 576.3); 582.5 $[M-H+2Na]^+$ (calcd. for $C_{29}H_{46}NNa_2O_6S$: 582.3).

*2.4.1.5. N-Acetyl-S-(lithocholyl)cysteine (**2e**).* The compound **2e** was obtained from **1e** (1.02 g) as colorless solid: yield 618 mg (54.8%). ^{1}H NMR (CD_3OD) δ: 0.58 (3H, s, 18-H), 0.84 (3H, s, 19-H), 0.84 (3H, d, J = 5.2 Hz, 21-H), 1.86 (3H, s, –$COCH_3$), 2.40 and 2.51 (each 1H, m, 23-H), 3.05 and 3.44 (each 1H, m, Cys-β,β′-H), 3.39 (1H, m, 3β-H), 4.38 (1H, m, Cys-α-H). MALDI-TOFMS Found: m/z 544.2 $[M+Na]^+$ (calcd. for $C_{29}H_{47}NNaO_5S$: 544.3), 566.1 $[M-H+2Na]^+$ (calcd. for $C_{29}H_{46}NNa_2O_5S$: 566.3).

2.5. Incubation of CA-AMP and CA-CoA with NAC and GST

A duplicate solution of CA-AMP (1 nmol) or CA-CoA (1 nmol) in 10 mM Tris–HCl buffer (pH 7.4) (200 μL) was incubated at 37 °C with NAC (100 nmol), with or without GST (1.8 units/0.1 mg) being isolated from rat liver. After cooling the incubation mixture in an ice bath, the resultant mixture was loaded onto an Oasis HLB cartridge. The cartridge was washed with water (1 mL), and then BAs and their metabolites were eluted with methanol (1 mL) under the condition of atmospheric pressure. After the addition of CDCA-NAC (20 ng in 10 μL of methanol) as an internal standard (IS), the solvent was dried under a stream of N_2. The residue was redissolved in 30% (v/v) acetonitrile (30 μL), and an aliquot was subjected to the LC/ESI-MS^2 analysis in the negative-ion mode at a normalized collision energy of 35%. The corresponding deprotonated molecules ($[M-H]^-$) of each analyte were selected as the precursor ions at m/z 552.3 for CA-NAC, m/z 736.3 for CA-AMP, m/z 1156.3 for CA-CoA and m/z 536.3 for CDCA-NAC (IS), and full scan acquisitions were recorded. To obtain the mass chromatograms, the most abundant product ions at m/z 407.3 for CA-NAC, m/z 346.0 for CA-AMP, m/z

Table 1
Observed ions of BA-NACs on negative-ion ESI-MS^n.

		m/z				
		CA-NAC	CDCA-NAC	DCA-NAC	UDCA-NAC	LCA-NAC
MS	$[M-H+CH_3CO_2+K]^-$	650.3 (3)	634.2 (2)	634.2 (3)	634.1 (3)	618.2 (3)
	$[M-H+CH_3CO_2+Na]^-$	634.2 (3)	618.3 (4)	618.3 (4)	618.2 (4)	602.3 (4)
	$[M-H]^-$	552.2 (100)	536.3 (100)	536.2 (100)	536.2 (100)	520.2 (100)
	[M−H−*N*-acetylalanine]$^-$	N.D.	407.3 (2)	N.D.	407.3 (2)	391.2 (3)
MS^2[a]	$[M-H-CH_2{=}C{=}O]^-$	510.2 (4)	494.3 (4)	494.3 (4)	494.3 (4)	478.3 (5)
	$[M-H-CH_2{=}C{=}O-H_2O]^-$	492.2 (13)	476.3 (11)	476.3 (13)	476.2 (11)	460.3 (12)
	$[M-H-CH_3CONH-2H_2O]^-$	458.3 (3)	442.4 (6)	442.3 (4)	442.3 (6)	426.3 (7)
	[M−H−*N*-acetylalanine]$^-$	423.2 (42)	407.3 (66)	407.2 (34)	407.3 (66)	391.3 (50)
	[M−H−NAC+O]$^-$	407.3 (100)	391.3 (100)	391.3 (100)	391.3 (100)	375.3 (100)
	$[NAC-H]^-$	162.0 (7)	162.0 (8)	162.0 (8)	162.0 (10)	162.0 (5)

Values in parenthesis represent relative intensity. N.D.: not detected.
[a] $[M-H]^-$ ion of each BA-NAC was used as precursor ion.

809.3 for CA-CoA and *m/z* 391.3 for CDCA-NAC (IS) were monitored, respectively.

The standard solution of CA-NAC (10–100 ng) in 10 mM Tris–HCl buffer (pH 7.4) was treated in the same way as sample of incubation mixture and the standard curve for quantification was constructed by plotting the peak area ratio of CA-NAC to IS versus the concentration of CA-NAC over the range of 2–20 ng/μL with $r^2 > 0.99$. The results are presented as mean values obtained with multiple incubations.

2.6. Animal studies

Male Wistar rats weighing 230–250 g (8-week-old, Japan SLC Inc., Hamamatsu, Japan) were used with approval from the Kinki University Committee for the Care and Use of Laboratory Animals, which conforms to the NIH guidelines. A rat was housed in regular cages in a controlled housing environment with 12-h cycles of lightness and darkness. A rat was allowed free access to food and water. For surgery, a rat was anesthetized with pentobarbital (50 mg/kg), and the bile duct was ligated. A suspension of LCA (3 mg) in 0.5 mL of olive oil was orally administered, and then a solution of NAC (40 mg) in H_2O (1 mL) was intraperitoneally injected. Administration of NAC was repeated a second time later that day. Twenty-four hours after the final administration, urine was collected. The urine of intact rat was also collected as the control urine, and both urine samples were stored at −20 °C until the analysis.

2.7. Analysis of NAC conjugates in urine of the bile duct-ligated rat

Urine (0.6 mL) was diluted with isotonic saline (0.9 mL) and the resultant solution was applied to a solid-phase extraction cartridge, Oasis HLB. After washing with water (2 mL), the BAs were eluted with methanol (1 mL) followed by evaporation of the solvent under a stream of N_2. The residue was dissolved in 30% (v/v) acetonitrile, and an aliquot (10 μL) was subjected to LC/ESI-MS^2 analysis in the negative-ion mode in which $[M-H]^-$ ion of LCA-NAC (*m/z* 520.3) was selected as a precursor ion and full scan acquisition was recorded. To obtain the mass chromatogram, the characteristic transition from $[M-H]^-$ ion at *m/z* 520.3 to [M−H−NAC+O]$^-$ ion at *m/z* 375.3 of LCA-NAC was monitored (Table 1).

2.8. Hydrolysis of BA-NACs by using carboxylesterase

A mixed solution of the synthetically prepared standards of the NAC conjugates of CA, CDCA, DCA, UDCA, and LCA (each 1 nmol) was incubated with partially purified carboxylesterase (2 unit) from rabbit liver in 10 mM Tris–HCl buffer (pH 7.4) (500 μL) at 37 °C. A portion (50 μL) of the incubation mixture at 5, 10, 30, 60, 120, 360, and 1440 min after incubation was transferred into another tube and was cooled on ice in order to terminate the reaction. After the addition of 12-oxoLCA (0.1 nmol) dissolved in methanol (10 μL) as an IS, the resultant mixture was diluted with 10 mM Tris–HCl buffer (pH 7.4) (50 μL) and then applied to an Oasis HLB cartridge. After washing with water (1 mL), the BAs were eluted with methanol (1 mL), followed by evaporation of the solvent under a stream of N_2. A 10-μL aliquot of redissolved solution in 30% (v/v) acetonitrile (50 μL) was subjected to LC/ESI-MS^2 analysis in the negative-ion mode in which BA-NACs were monitored on the basis of the transition (from the $[M-H]^-$ ions to [M−H−NAC+O]$^-$ ions) as follows: CA-NAC, *m/z* 552.3 → 407.3; CDCA-, DCA-, and UDCA-NAC, *m/z* 536.3 → 391.3; LCA-NAC, *m/z* 520.3 → 375.3 (Table 1). The liberated unconjugated BAs were monitored on the basis of the transition: $[M+CH_3CO_2]^-$ ions to $[M-H]^-$ ions for CA, *m/z* 467.3 → 407.3; CDCA and UDCA, *m/z* 451.3 → 391.3; LCA, *m/z* 435.3 → 375.3, and 12-oxoLCA, *m/z* 449.3 → 389.3, and $[M-H]^-$ ion to $[M-H-H_2O-CO]^-$ ion for DCA, *m/z* 391.3 → 345.3. The amounts of remaining NAC conjugates and liberated BAs in the incubation mixture were calculated from the peak area ratios of the analytes to IS of the incubation mixture versus those of the standard mixture of BA-NACs and BAs (each 0.1 nmol), which were extracted by using the Oasis HLB cartridge.

3. Results and discussion

3.1. Synthesis of BA-NAC conjugates

For the elucidation of biochemical pathway using mass spectrometry, reference compounds are essential. Therefore, our initial effort was directed toward the chemical synthesis of NAC conjugates of BAs (Fig. 2). The synthetic method employed for obtaining the target compound required the selective condensation of the carboxyl group of BAs with the thiol group of NAC, which possess two reactive functional moieties, i.e., the carboxyl and thiol groups. Therefore, we wanted to use the activated ester that could easily be condensed with the thiol group under basic conditions. In this study, we used *p*-nitrophenyl esters of BAs, which could easily be prepared from BAs. Each BA was converted into the corresponding *p*-nitrophenyl ester, and the activated ester obtained was then coupled with the free thiol of NAC under basic conditions to yield the desirable BA-NAC. The structure of the product was confirmed by means of ^1H NMR. The proton assignments were carried out by means of ^1H NMR spectra, and 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 0.58–0.91 ppm, acetyl protons (each s) at 1.86–1.96 ppm, H_2-23 methylene protons (each m) at 2.46–2.52 ppm, and H-3β, H-7 and/or H-12β methyne protons (occurring at 3.25–3.84 ppm) geminally attached to oxygen-containing functions in the aglycone moieties, and H_2-β,β′ in cysteine moieties at 3.05–3.45 ppm (m), H-α in cysteine

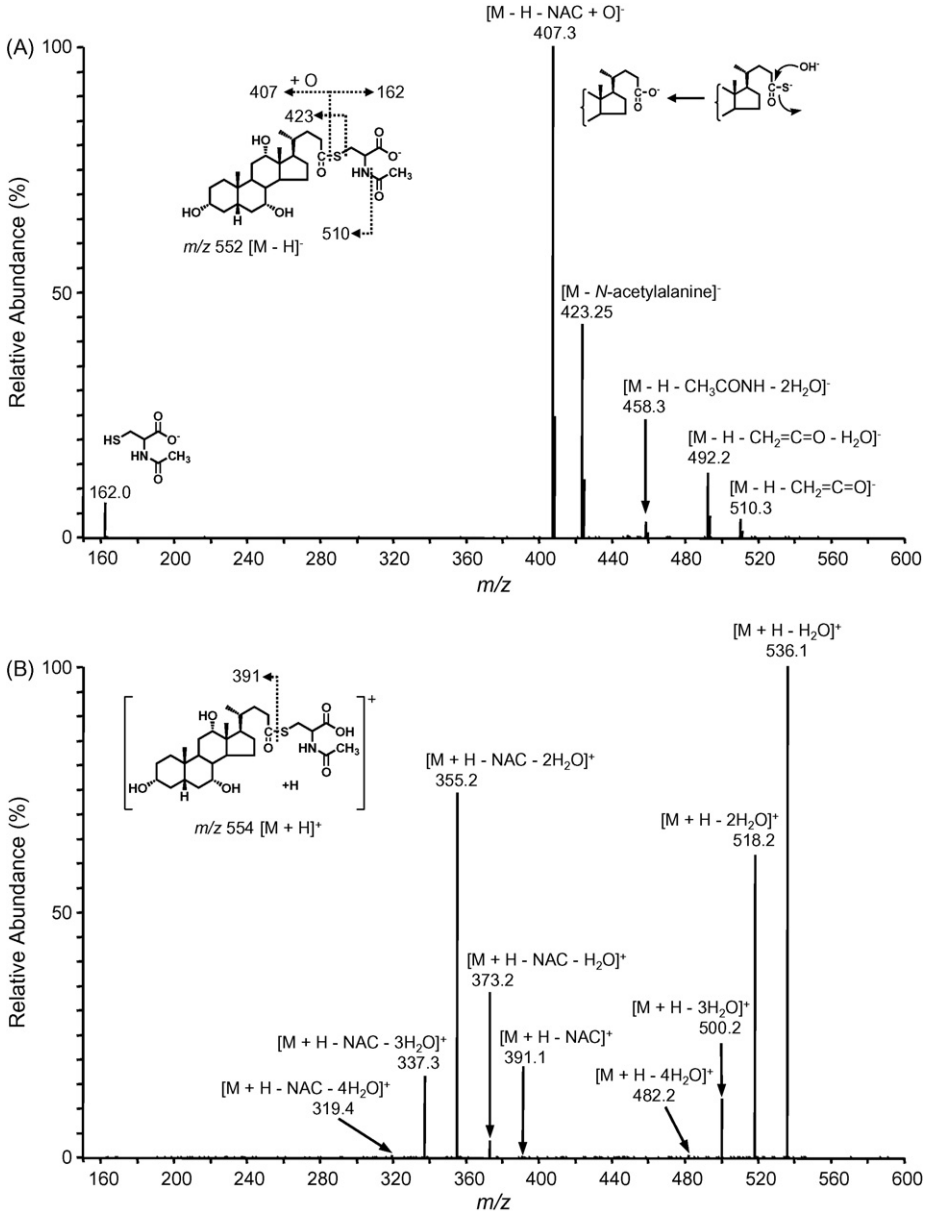

Fig. 3. Product ion spectra of CA-NAC obtained by CID of (A) $[M-H]^-$ and (B) $[M+H]^+$ ion. Proposed structures of major fragments are depicted.

moieties at 4.33–4.38 ppm (m). These spectral data were consistent with the structures of BA-NACs.

3.2. Mass spectrometric behaviors of the NAC conjugated BAs

The typical negative- and positive-ion CID spectra of the NAC conjugates are shown in Fig. 3. In the full-scan negative-ion mode, the spectra of all BA-NACs formed a deprotonated molecule $[M-H]^-$ as the base peak with scarce $[M-H+CH_3COO+K]^-$, $[M-H+CH_3COO+Na]^-$, and $[M-H-$*N*-acetylalanine$]^-$ ions, indicating the covalently condensed product of BA and NAC. Under CID conditions, the NAC conjugates exhibited characteristic fragmentation patterns: the fragmentation of the conjugates in negative CID showed the same type of ions. As one of two typical fragmentation processes, either the BA–S bond or the S–CH_2 bond on the NAC side of the parent molecule was cleaved. The cleavage of the NAC residue led to a dominant loss of 145 Da, producing fragment $[M-H-NAC+O]^-$ ions: these ions are considered to be generated by the hydrolytic cleavage of thioester bond. The corresponding fragment $[NAC-H]^-$ ion was observed at *m*/*z* 162.0. As a second common process, the combined neutral losses of ketene (42 Da, $CH_2=C=O$) and water molecules were observed, representing the $[M-H-CH_2=C=O]^-$, $[M-H-CH_2=C=O-H_2O]^-$ and $[M-H-CH_2=C=O-2H_2O]^-$ ions, which allows rapid identification of the acetylated molecule (Table 1).

In contrast to negative-ion spectra, the positive-ion spectra of all BA-NACs showed the corresponding ammonium adduct ion $[M+NH_4]^+$ as the base peak with scarce $[M+K]^+$, $[M+H]^+$, $[M+H-H_2O]^+$, and $[M+H-2H_2O]^+$ ions (Table 2). When the $[M+NH_4]^+$ ion was used as the precursor ion, the protonated molecular $[M+H]^+$ was observed as the base peak with its dehydrated ion (<13%). However, no other characteristic product ions were observed in the CID spectra (data not shown). On the other hand, the skimmer CID (SID) [29,30] with 20 V of collision energy eliminated the ammonium adduct to produce the intense $[M+H]^+$ ion as the most abundant ions. The CID of $[M+H]^+$ ions showed extensive fragmentation in which the several dehydrated ions were obtained with sequential losses of H_2O depending on the number

Table 2
Observed ions of BA-NACs on positive-ion ESI-MS^n.

		m/z				
		CA-NAC	CDCA-NAC	DCA-NAC	UDCA-NAC	LCA-NAC
MS	$[M+K]^+$	592.3 (5)	576.3 (5)	576.3 (5)	576.3 (5)	560.3 (4)
	$[M+NH_4]^+$	571.1 (100)	555.0 (100)	555.1 (100)	555.0 (100)	539.0 (100)
	$[M+H]^+$	554.2 (12)	538.1 (29)	538.2 (12)	538.1 (32)	522.2 (33)
	$[M+H-H_2O]^+$	536.1 (1)	520.2 (2)	520.2 (1)	520.2 (3)	504.2 (1)
	$[M+H-2H_2O]^+$	518.2 (1)	502.2 (1)	502.2 (0.5)	N.D.	N.D.
	$[M+H-NAC]^+$	391.0 (3)	N.D.	N.D.	N.D.	N.D.
	$[M+H-NAC-H_2O]^+$	N.D.	N.D.	N.D.	N.D.	341.3 (1)
	$[M+H-NAC-2H_2O]^+$	N.D.	N.D.	339.3 (1)	N.D.	N.D.
MS[a]	$[M+K]^+$	592.3 (7)	576.3 (6)	576.3 (7)	576.3 (5)	560.3 (7)
	$[M+NH_4]^+$	571.0 (65)	555.0 (25)	555.1 (55)	555.0 (36)	539.0 (17)
	$[M+H]^+$	554.1 (100)	538.1 (100)	538.2 (100)	538.1 (100)	522.2 (100)
	$[M+H-H_2O]^+$	536.0 (9)	520.2 (12)	520.2 (12)	520.2 (44)	504.2 (6)
	$[M+H-2H_2O]^+$	518.3 (6)	502.2 (3)	N.D.	502.2 (8)	N.D.
	$[M+H-NAC]^+$	391.0 (10)	N.D.	N.D.	N.D.	N.D.
	$[M+H-NAC-H_2O]^+$	N.D.	N.D.	N.D.	N.D.	341.2 (4)
	$[M+H-NAC-2H_2O]^+$	N.D.	339.2 (4)	339.2 (4)	N.D.	N.D.
MS^2[b]	$[M+H-H_2O]^+$	536.1 (100)	520.1 (100)	520.1 (100)	520.1 (100)	504.1 (48)
	$[M+H-2H_2O]^+$	518.2 (62)	502.1 (9)	502.1 (17)	502.1 (8)	486.2 (1)
	$[M+H-3H_2O]^+$	500.2 (12)	484.2 (<1)	484.3 (1)	484.2 (<1)	N.D.
	$[M+H-4H_2O]^+$	482.2 (<1)	N.D.	N.D.	N.D.	N.D.
	$[M+H-NAC]^+$	391.1 (<1)	375.1 (1)	375.0 (1)	375.2 (<1)	N.D.
	$[M+H-NAC-H_2O]^+$	373.2 (3)	357.2 (1)	357.3 (11)	357.2 (1)	341.2 (100)
	$[M+H-NAC-2H_2O]^+$	355.2 (74)	339.3 (5)	339.2 (33)	339.3 (5)	323.3 (10)
	$[M+H-NAC-3H_2O]^+$	337.3 (16)	321.4 (<1)	321.3 (2)	321.3 (<1)	N.D.
	$[M+H-NAC-4H_2O]^+$	319.4 (<1)	N.D.	N.D.	N.D.	N.D.
	$[NAC+H]^+$	N.D.	164.0 (<1)	164.0 (1)	164.0 (<1)	164.0 (4)

Values in parenthesis represent relative intensity. N.D.: not detected.
[a] SID: 20 V.
[b] $[M+H]^+$ ion of each BA-NAC was used as precursor ion.

of hydroxyl groups on the steroidal moiety from the protonated molecule $[M+H]^+$ and fragment $[M+H-NAC]^+$ ion; losses of 1–4H_2O for CA-NAC having three hydroxyl groups, losses of 1–3H_2O for CDCA-, DCA-, and UDCA-NAC having two hydroxyl groups and losses of 1–2H_2O for LCA-NAC having one hydroxyl group occurred and thereby $[M+H-H_2O]^+$ gave rise to a prominent ion except for LCA-NAC, which gave rise to $[M+H-NAC-H_2O]^+$ ion as the moderate peak. In addition, the NAC conjugate underwent fragmentation

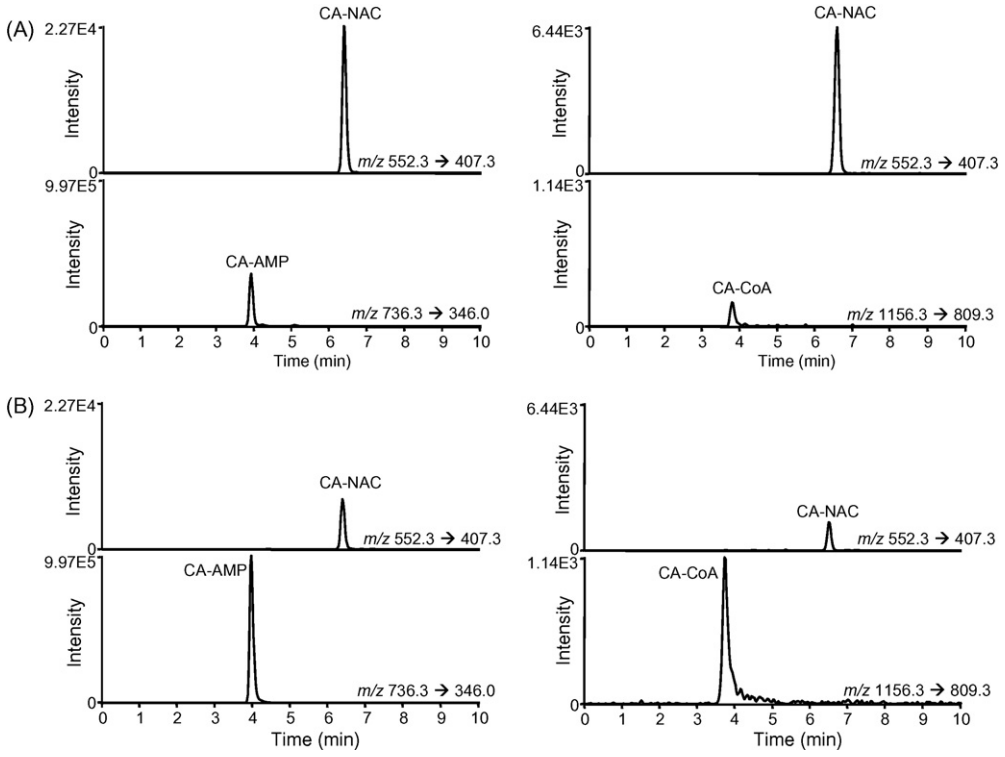

Fig. 4. Product ion mass chromatograms of the incubation mixture after 1 h incubation of CA-AMP (left) and CA-CoA (right) with NAC, with (A) and without (B) the GST. The transitions used for this LC/ESI-MS^2 analysis were *m/z* 736.3 → 346.0 for CA-AMP, *m/z* 1156.3 → 809.3 for CA-CoA, and *m/z* 552.3 → 407.3 for CA-NAC.

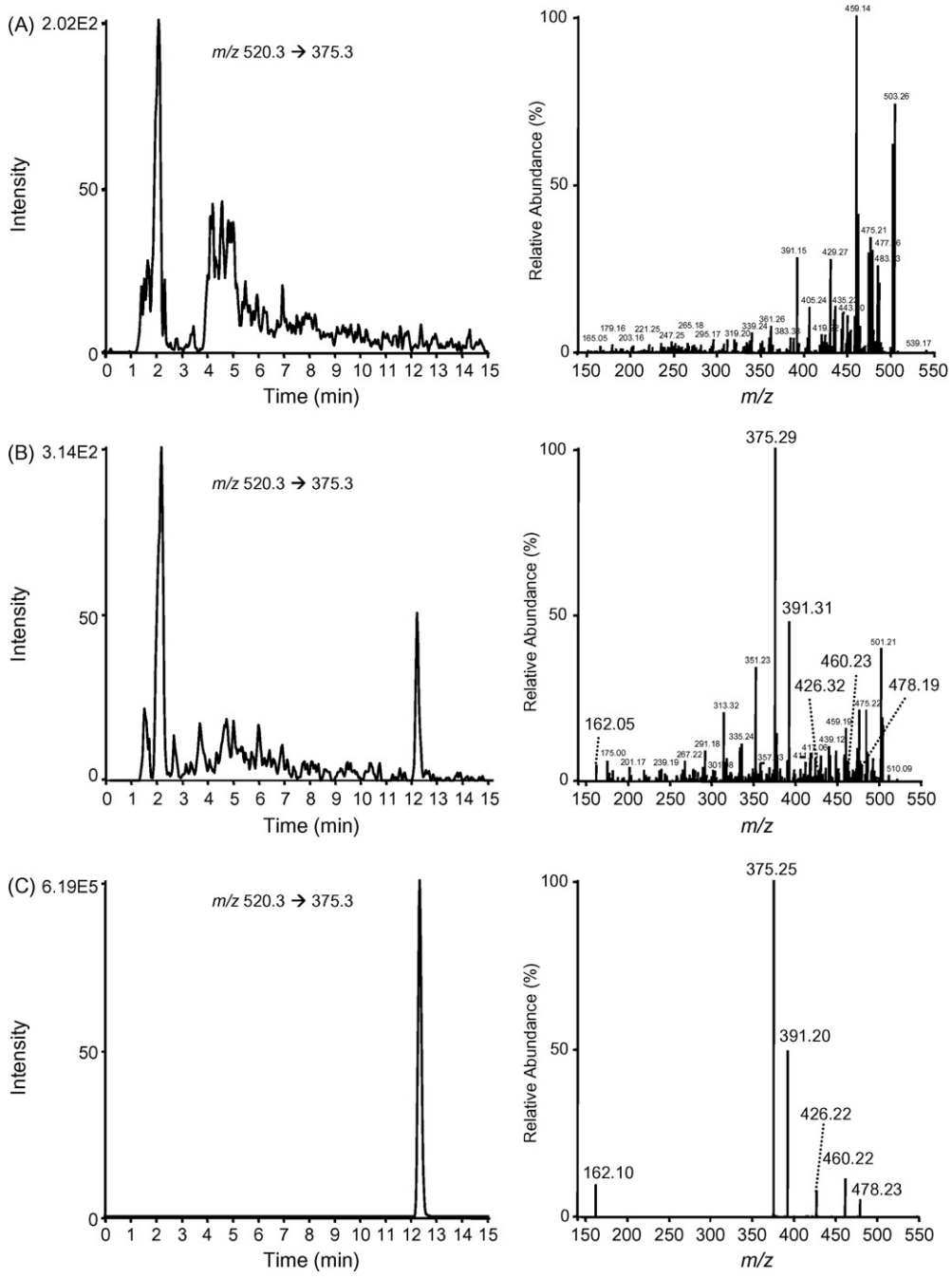

Fig. 5. Product ion mass chromatograms monitored at m/z 520.3 → 375.3 (left) and CID spectra (right) of the peaks eluted at 12.3 min of the extracts from urine of control rat (A), those of LCA- and NAC-dosed rat (B), and the reference standard LCA-NAC (20 ng) (C).

by direct cleavage of the thioester bond on the NAC side, resulting in the generation of weak $[M+H-NAC]^+$ ions. This aglycone ion in turn was similarly subjected to successive losses of water molecule depending on the number of hydroxyl group on the steroidal moiety.

Finally, the monitoring of precursor ions characteristic of each target parent compound (mainly pseudo-molecular ions) and intense fragment ions characteristic of each conjugate was expected to be a powerful analytical strategy for characterizing the metabolism of BA-NAC. When using a TSKgel ODS-100 V column and a linear gradient elution of 30% solvent B (acetonitrile) to 80% solvent B against solvent A (5 mM ammonium acetate buffer, pH 6.0) over 30 min, the satisfactory chromatographic separation of each NAC conjugate was achieved (data not shown). The detection limits (signal-to-noise ratio > 3) of BA-NAC in negative- and positive-ion mode were up to 10 and 200 pg, respectively. Therefore BA-NAC was monitored with negative-ion mode in the following experiments.

3.3. Transformation of CA-AMP and CA-CoA into thioester-linked NAC conjugates by rat liver GST

The rate of formation of CA-NAC when CA-AMP and CA-CoA were incubated with rat liver GST was investigated. We used a sensitive and selective negative-ion LC/ESI-MS^2 analytical method for the identification of CA-NAC. The transitions used for this analysis were m/z 736.3 → 346.0 for CA-AMP, m/z 1156.3 → 809.3 for CA-CoA, and m/z 552.3 → 407.3 for CA-NAC. The transition of CA-NAC was

selected because it is the major transition at which the compound undergoes fragmentation by CID of the deprotonated molecule, as assessed by negative-ion LC/ESI-MS^2. The analysis clearly revealed the presence of CA-NAC with a retention time of 6.5 min when both the substrates were incubated with the enzyme GST (Fig. 4). Although an appreciable amount of CA-AMP and CA-CoA remained, both substrates were mostly consumed in the reaction as judged by the peak intensity. In the absence of the enzyme, a small peak corresponding to CA-NAC was observed, indicating that these can undergo spontaneous trans-acylation with the nucleophilic cysteinyl thiol of NAC to form CA-NAC similar to that observed for CA-GSH [12,13]. The structures of the compounds corresponding to these peaks were confirmed by comparing the compounds with authentic samples in terms of their mass chromatograms and their CID spectra. CA-NAC was quantitatively analyzed and found a 2.7–2.9-fold increase in the rate of formation of CA-GSH when CA-AMP and CA-CoA were incubated with the enzyme. The results of these *in vitro* studies demonstrate that the conjugation of CA-AMP and CA-CoA with NAC can be mediated by GST but that a small amount of NAC conjugate is formed in the absence of the enzyme.

3.4. Detection of LCA-NAC in the urine of bile duct-ligated rats treated with LCA and NAC

The formation and urinary excretion of NAC conjugates of orally administered BAs was examined in the bile duct-ligated rat as an animal model simulating cholestatic liver disease. Animal was injected intraperitoneally injected with NAC and was gavaged with LCA, which is known as a cytotoxic BA [31]. The extracts of urine obtained by the solid phase extraction of BAs on an Oasis HLB cartridge were analyzed by using negative-ion LC/ESI-MS^2. As shown in Fig. 5A, the product ion chromatogram monitored with the transitions from $[M-H]^-$ at m/z 520.3 → $[M-H-NAC+O]^-$ at m/z 375.3 for LCA-NAC of the extracts obtained from the urine of control animal showed no peak that corresponded to LCA-NAC. On the other hand, extracts obtained from the LCA-dosed rat revealed an extremely sharp peak with a retention time of 12.3 min (Fig. 5B, left); the CID spectrum of the ion associated with the peak was identical to that of LCA-NAC with respect to the ion species and the relative abundances of the product ions (Fig. 5B, right). None of these ions were detected in blank control. These results confirm the biotransformation of LCA into the LCA-NAC conjugate in the liver and its excretion into urine in the bile duct-ligated rat. However, we have also found that NAC conjugates are relatively unstable in the clean-up procedure, i.e., the solid phase extraction on an Oasis HLB cartridge, suggesting incomplete recovery of the NAC conjugates. A method for obtaining complete recovery of the NAC conjugates and other urinary components including the glycine and taurine conjugates is currently being developed in our laboratory.

3.5. Hydrolysis of BA-NACs by using rabbit liver carboxylesterase

Carboxylesterase (E.C. 3.1.1.1) catalyzes the conversion of a carboxyl ester into alcohol and carboxylic acid. In addition to carboxylic acid hydrolysis, the enzyme catalyzes the hydrolysis of amides, thioesters, phosphoric acid ester, and acid anhydrides. Carboxylesterases are widely distributed throughout the body, with high levels occurring in the liver, kidneys, testes, lungs, and plasma, both in normal and tumor tissues. Liver microsomal carboxylesterase function in the hydrolysis of a wide variety of endogenous and xenobiotic compounds, and carboxylesterase play an important role in drug and lipid metabolism in mammalian species. Multiple isozymes of liver microsomal carboxylesterase exist in various animal species, and the enzyme is involved in the metabolic activation of ester- and amide-type prodrugs [32,33]. Therefore, we examined the hydrolysis of BA-NACs by rabbit carboxylesterase at 37 °C for 24 h. The remaining BA-NACs along with liberated BAs were quantitatively analyzed by a sensitive and selective negative-ion LC/ESI-MS^2. As shown in Fig. 6A, the disappearance of BA-NACs was dependent on the incubation time, and the degree of hydrolysis decreases with the number of hydroxyl groups. The monohydroxylated LCA-NAC was most effectively hydrolyzed with complete consumption after 6 h of incubation. CDCA-NAC, UDCA-NAC, and DCA-NAC, which have hydroxyl groups at C-3α,7α, C-3α,7β, and C-3α,12α, respectively, were moderately hydrolyzed, and these NAC conjugates were reduced to less than 10% at the 24-h incubation. In contrast to these NAC conjugates, the trihydroxylated CA-NAC showed feeble hydrolysis of its conjugate with around 40% of the remaining CA-NAC at the 24-h incubation. On the other hand, the appearances of the unconjugated BAs were not associated with the consumption of BA-NACs. UDCA was the most abundant among the unconjugated BAs liberated from BA-NACs (Fig. 6B). The remaining LCA-, CDCA-, and DCA-NAC were less than 20% at the 24-h incubation, and unconjugated BAs liberated from these NAC conjugates were less than 40%. The differences between the consumption of BA-NACs and appearance of liberated BAs may be attributed to the nature of the carboxylesterase used in this study and/or incomplete recovery of BAs in the clean-up step.

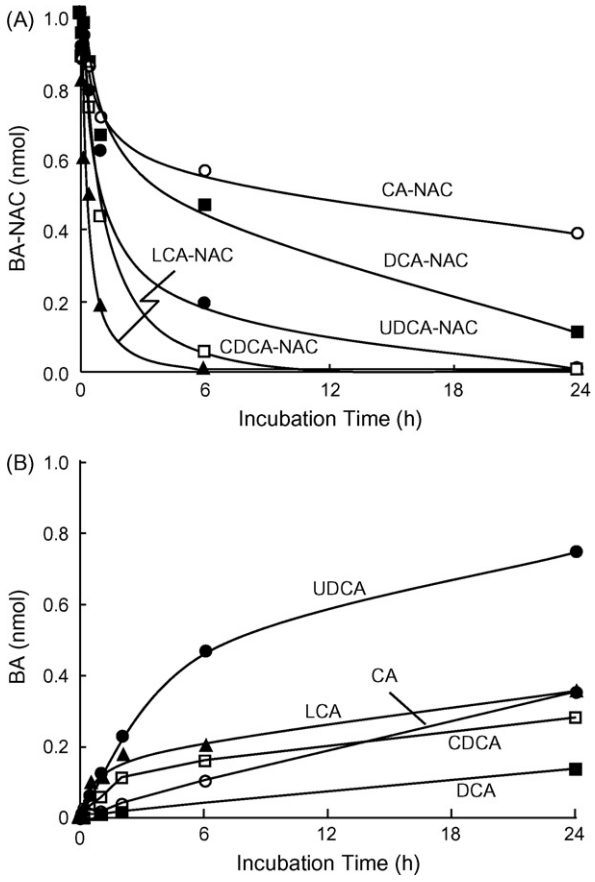

Fig. 6. Time course for hydrolysis of BA-NACs (A) and production of unconjugated BAs (B) by incubation with carboxylesterase isolated from rabbit liver. A mixture of 2 μM each of CA-NAC (○), CDCA-NAC (□), DCA-NAC (■), UDCA-NAC (●), and LCA-NAC (▲) was incubated with carboxylesterase (2 units) at 37 °C for 24 h. Values represent the average of duplicate incubations.

4. Conclusion

In this study, we synthesized the NAC thioesters of the five bile acids present in human bile, and then investigated whether CA-AMP and CA-CoA could be transformed into NAC conjugates. When

CA-AMP and CA-CoA were incubated with rat liver GST, a 2–3-fold increase in the rate of formation of the NAC conjugate was found (Fig. 4), indicating the capability of GST to catalyze the trans-acylation reaction of these reactive species with NAC. The higher reactivity of CA-AMP as compared to that of CA-CoA is likely to be caused by the differing electrophilicity of the carbonyl-carbon of the acyl-linkage.

These results demonstrate the presence of a new mode of BA conjugation, wherein BAs are transformed into NAC conjugates via intermediary metabolites formed by bile acid:CoA ligase (BAL). We also demonstrated that the NAC conjugate of LCA was excreted in the urine of LCA and NAC dosed rat with bile duct ligation. The conceivable pathways on the formation of NAC conjugates are considered as follows: BAs are metabolically activated by BAL to produce the corresponding acyl-AMPs or acyl-CoA thioesters followed by trans-acylation with NAC catalyzed by GST to afford the NAC conjugates. Alternatively, or in parallel, trans-acylation with GSH to give the GSH conjugates may occur, followed by loss of the glycine and glutamic acid residues mediated by γ-glutamyltranspeptidase and a dipeptidase; the result is the formation of a cysteine conjugate. The cysteine conjugate can then undergo acetylation by *N*-acetyltransferase to form a NAC conjugate [15]. Additional work is required to define the quantitative significance of the NAC conjugate formation as well as to elucidate the individual steps in NAC conjugate formation.

In addition, we found that the BA-NACs were easily hydrolyzed by carboxylesterase from rabbit liver. This result raised the possibility that NAC conjugates could undergo hydrolysis after their formation. *In vivo* hydrolysis of BA-NACs and liberation of unconjugated BA are under examination in our laboratories.

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