Focusing Review

Development of highly sensitive methods for the determination of activated carboxylic acids in biological specimens

Takaaki Goto

Graduate School of Pharmaceutical Sciences, Tohoku University, Aobayama, Aoba−ku, Sendai 980−8578, Japan

Received for review December 26, 2006. Accepted December 29, 2006

Abstract

Carboxylic acids are biologically important compounds in living systems, and their activated forms play important roles in a variety of biochemical processes, including energy transduction, biosynthesis, and metabolism. In general, activated carboxylic acids under physiological conditions are highly chemically reactive, and can form covalently bound protein adducts, which may cause hypersensitive reactions. Accordingly, considerable attention has been focused on the dynamics of the formation and degradation of activated carboxylic acids in relation to the toxic side effects of drugs. Therefore, reliable analytical methods to quantify activated carboxylic acids are essential. In this study, we focused on acyl glycosides, one of the activated forms of carboxylic acids, and developed a highly sensitive method to determine these substances in biological specimens.

Keywords: carboxylic acid, acyl glycoside, glucuronide, galactoside, bile acid, 2-aryl propionic acid, liquid chromatography/mass spectrometry

Introduction

In the human body, endogenous biologically active substances and xenobiotics that contain a carboxy group are known to transform various metabolites. Commonly, these carboxylic acids are activated to form the corresponding intermediates, such as coenzyme A (CoA) thioesters, key intermediates in reactions that result in the transfer of an acyl group, including conjugation with amino acids, chiral inversion of 2-methyl branched carboxylic acids, and β-oxidation and elongation of fatty acids. During the formation of CoA thioesters, acyl adenylates have also been identified as intermediates. These activated forms of carboxylic acid generally have high chemical reactivities and play important roles in a variety of biochemical processes, such as energy transduction, biosynthesis, and metabolism.

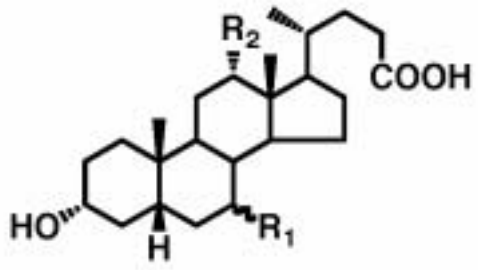
On the other hand, conjugation with glucuronic acid takes place through carboxy groups as well as hydroxy groups, amino groups, and mercapto groups. Glucuronidation, which converts lipophilic substances into water-soluble forms, plays a significant role in the metabolism and disposition of xenobiotics and endogenous compounds. This conjugation process, which is catalyzed by UDP-glucuronosyltransferase (UGT), has long been considered to be an important detoxification mechanism. Recent observations, however, indicate that certain carboxylic acid substrates of UGTs become more toxic upon glucuronidation [1]. Furthermore, recent evidence indicates that ester-type glucuronides, namely acyl glucuronides, react with nucleophiles, such as amino groups on some proteins, to produce covalently bound adducts [2, 3]. These reactions involve the displacement of the glucuronic acid moiety with the nucleophile. Alternatively, such adducts also form after the migration of an acyl group on the sugar moiety and tautomerization for conversion into the aldose form, which is readily condensed with nucleophiles [4, 5]. The structures of these adducts are similar to those of hapten-carrier protein conjugates, which are used for immunizations with small molecules. The formation of these adducts has attracted attention as a possible explanation for hypersensitivity and adverse reactions to carboxylic acids [6, 7].

Bile acids are endogenous carboxylic acid derivatives with a steroid nucleus and a side chain at the C-17β position. The com-

Tel: +81−22−795−6819
Fax: +81−22−795−6816
E−mail: t−goto@mail.pharm.tohoku.ac.jp

mon bile acids have hydroxy groups at the 3α, 7α, 7β and/or 12α positions, and a carboxy group at the C-24 position, the terminus of the side chain. The human body usually contains five different bile acids: cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), ursodeoxycholic acid (UDCA), and lithocholic acid (LCA) (Fig. 1). Bile acids are the main metabolites of cholesterol and the primary bile acids CA and CDCA are biosynthesized in the liver by hepatic enzymes. The 7α-hydroxylation of cholesterol is the first and the rate-limiting step in bile acid biosynthesis. The 7α-hydroxycholesterol then undergoes modification of the A and B rings and oxidative cleavage of the side chain, resulting in the generation of CA and CDCA. Bile acids are excreted into the small intestine via the bile duct as their glycine and taurine conjugates. In the intestinal lumen, bile acids assist in lipolysis and the absorption of fats by forming mixed micelles. Additionally, intestinal bacteria mediate the deconjugation and dehydroxylation of the 7α-hydroxy groups, leading to the formation of the secondary bile acids DCA, LCA, and UDCA. Bile acids are then reabsorbed from the ileum−proximal colon and returned to the liver via the portal vein. Although the size of the bile acid pool in humans is 3−4 g, the concentrations of bile acids in the peripheral blood are low because of efficient hepatic uptake of these compounds. Both primary and secondary bile acids in the liver undergo various phase II reactions, such as amino acid conjugation, sulfation, and glycosidation, and most of the bile acids are present as these conjugates in body fluids. In patients with hepatobiliary diseases, however, the levels and metabolic profiles of bile acids in biological fluids are significantly changed.

In this article, we present our recent studies on acyl glycosides, one of the activated forms of carboxylic acids.



Compound	R_1	R_2
Cholic acid (CA)	α-OH	OH
Chenodeoxycholic acid (CDCA)	α-OH	H
Deoxycholic acid (DCA)	H	OH
Lithocholic acid (LCA)	H	H
Ursodeoxycholic acid (UDCA)	β-OH	H

Figure 1. Chemical structures of common bile acids in humans.

1. Inhibition of the formation of acyl glucuronides by bile acids

The levels of sulfated bile acids in the urine of patients with some hepatobiliary diseases significantly increase, whereas those of bile acid glucuronides are only slightly higher. The conjugation of bile acids with glucuronic acid involves the hydroxy group at the C-3 position of the steroid nucleus of the bile acid. Recently, we demonstrated that bile acid acyl glucuronides that are conjugated through the carboxy group at C-24 are present in human urine (Fig. 2) [8]. The bile acid 24-glucuronides (24-GlcUs) were detected at low levels in human urine obtained from both healthy subjects and from patients with hepatobiliary disease, whereas these 24-GlcUs were preferentially biosynthesized in rat hepatic microsomal preparations [9, 10]. Although the preferential biosynthesis of the 24-GlcUs is known to occur, the formation of 24-GlcUs by hepatic UGT has not yet been clearly elucidated. We therefore attempted further characterization of this enzyme, for which a reliable method to quantify the resulting 24-GlcUs was needed. Acyl glucuronides, however, are chemically unstable, are easily hydrolyzed, and undergo ester exchange in the presence of alcohol. Therefore, liquid chromatography/electrospray-mass spectrometry (LC/ESI-MS) in the negative ion detection mode was employed, because glucuronides have an anionic group in the sugar moiety, allowing this technique to be performed with high specificity and selectivity.

To investigate the substrate specificity of hepatic UGT, five common bile acids were incubated with rat liver microsomal preparations [11]. Of these bile acids, monohydroxylated LCA, which is the most lipophilic of the common bile acids, was most effectively glucuronidated to its acyl glucuronide. Dihydroxylated CDCA, DCA, and UDCA were moderately metabolized into their corresponding 24-GlcUs. In contrast to these bile acids, the formation of the 24-GlcU of trihydroxylated CA, which is more water soluble than the other common bile acids, was barely detected. No substrate-saturation curve was obtained for LCA, and the initial velocity of the glucuronidation of LCA decreased with increasing concentrations of the substrates. This result strongly suggested that LCA has an inhibitory activity for bile acid acyl glucuronidation. In human body fluids, most bile acids exist as glycine or taurine conjugates, which are analogues of the substrates. We thus investigated the inhibitory effects of glycine- and taurine-conjugated CDCA on the formation of CDCA 24-GlcU. Both the glycine and taurine conjugates inhibited the metabolism of CDCA into its 24-GlcU by 20−25% (Fig. 3 A). The inhibition of CDCA 24-GlcU formation by UDCA derivatives with a 7β-hydroxy group was also examined. As with conjugated CDCA, equimolar amounts of glycine- and taurine-conjugated UDCA inhibited the formation of CDCA 24-GlcU by 20−25% (Fig. 3 B). Additionally, unconjugated

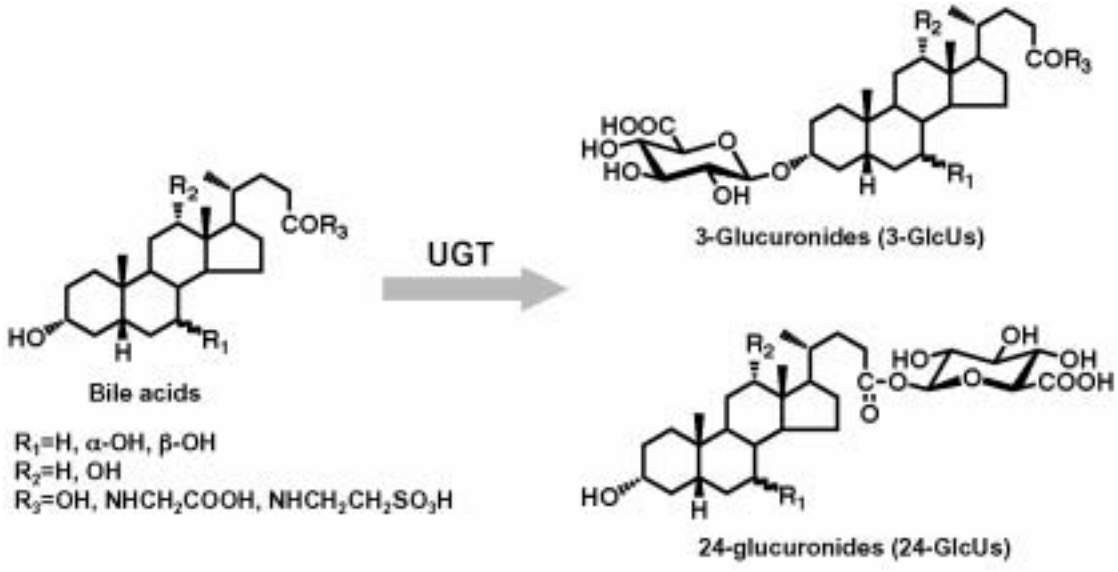


Figure 2 Glucuronidation of bile acids.

UDCA inhibited the glucuronidation of CDCA at twice the level of the inhibition produced by the conjugated UDCAs. The bile acid 24-GlcU product of the enzymatic reaction is a derivative of the substrate. LCA 24-GlcU was therefore added as an inhibitor in the incubation mixture, which also included CDCA and rat liver microsomal preparations. The addition of an equimolar amount of LCA 24-GlcU into the incubation mixture resulted in a 40% inhibition of the acyl glucuronidation of CDCA. In addition, the formation of CDCA 24-GlcU was reduced by 25% in the presence of a five-fold molar excess of LCA 24-GlcU. UDCA 24-GlcU also inhibited the acyl glucuronidation of CDCA; the level of inhibition was similar to those observed with glycine- and taurine-conjugated UDCA.

The results demonstrate that LCA as well as amino-acid-conjugated bile acids inhibit the formation of 24-GlcUs catalyzed by the acyl glucuronosyltransferase, a phenomenon referred to as substrate inhibition. Inhibition by the 24-GlcU products was also confirmed. Glycine and taurine conjugates of the bile acids also inhibit the acyl glucuronidation of bile acids. In the body, amino-acid -conjugated bile acids exist at higher concentrations, which may underlie the low concentrations of bile acid 24-GlcUs in human urine; these inhibitory effects may result in a reduction in the formation of bile acid 24-GlcUs in the body.

Generally, glucuronidation is believed to be a very important detoxification mechanism. Moreover, recent papers have shown that the acyl glucuronide conjugates of drugs produce covalently bound adducts with proteins, which may be responsible for hypersensitivity reactions to acidic compounds [6, 7]. 2-Aryl-propionic-acid derivatives are a widely used family of nonsteroidal anti-inflammatory drugs. These drugs are transformed into several metabolites through the actions of hepatic enzymes. As the enantiomer of flurbiprofen (FP), (±)-2-(2-fluoro-4-biphenylyl) propionic acid does not act as a substrate of acyl CoA thioester ligases, indicating that acyl glucuronidation is a critical phase II reaction for producing FP amino acid conjugates. Accordingly, we developed a rapid, accurate, and reproducible analytical method for the separation and detemination of flurbiprofen glucuronides (FP-GlcUs) in incubations containing hepatic microsomal preparations using high-performance liquid chromatography (HPLC).

Due to the instability of acyl glucuronides, solid-phase extraction with an octadecylsilanized (ODS) silica gel cartridge or a reversed-phase polymer cartridge, which is a commonly used extraction procedure, was not effective for the quantitative extraction of FP-GlcU produced in the incubation mixture. Therefore, a simple column-switching system attached to a trapping column for deproteinization was developed [12]. The glucuronides were separated on an ODS column and monitored with a UV detector at 246 nm. The detection limit for the FP-GlcUs was 600 fmol/injection with a signal-to-noise ratio of 10. A typical chromatogram obtained by injection of an incubation mixture spiked with the standard flurbiprofen glucuronides, an internal standard (FP-β-alanine conjugate), and substrate is shown in Figure 4, in which well-resolved peaks of the target compounds can be seen without any interference. This method should be very useful for the characterization the activity of hepatic UGT for FP.

As mentioned above, bile acid derivatives inhibit the acyl glucuronidation of bile acids. Acyl glucuronidation of both bile acids and FP are catalyzed by the same isozyme of UGT, suggesting that FP glucuronidation may be inhibited by bile acids derivatives. To investigate the inhibition of FP glucuronidation by bile acid derivatives, we tested several bile acids as inhibitors in incubation mixtures containing FP and rat liver microsomal proteins [13]. First,

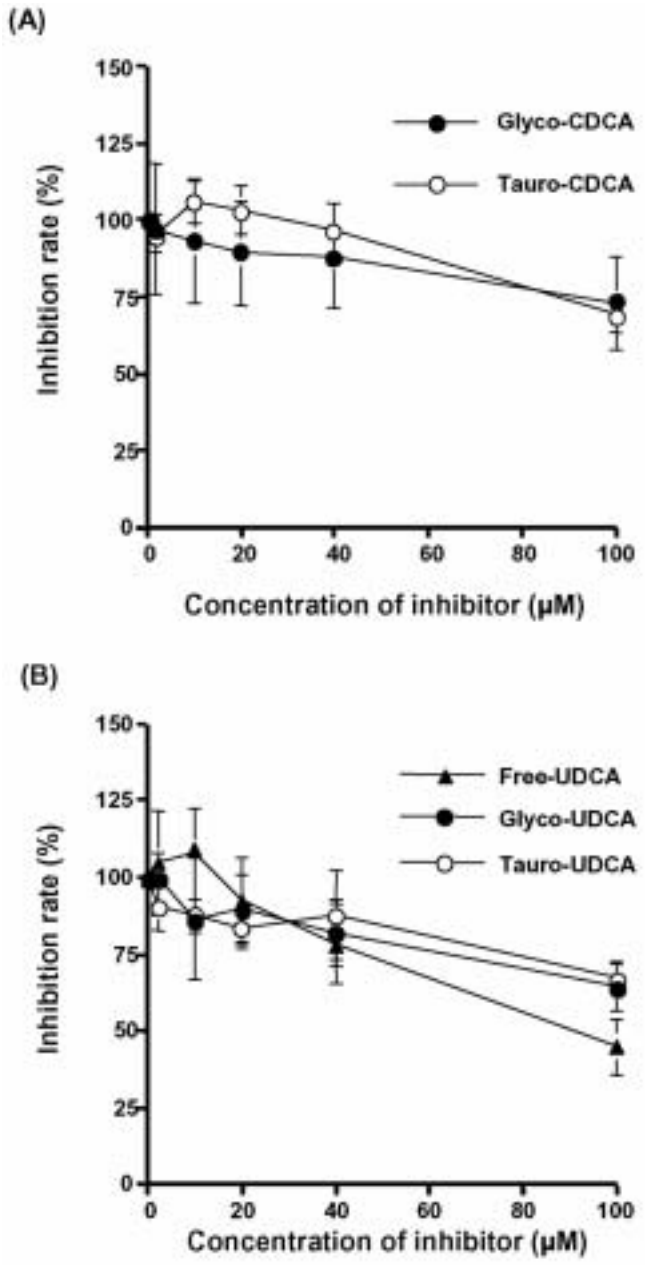


Figure 3 Effect of (A) amino-acid-conjugated CDCA and (B) UDCA on the formation of CDCA acyl glucuronide. Conditions: 20 μM CDCA was incubated with microsomal preparations (400 μg protein/mL) in the presence of various amounts of (A) glycine-conjugated CDCA and taurine-conjugated CDCA; or (B) UDCA, glycine-conjugated UDCA, and taurine-conjugated UDCA at 37 ℃ for 10 min.

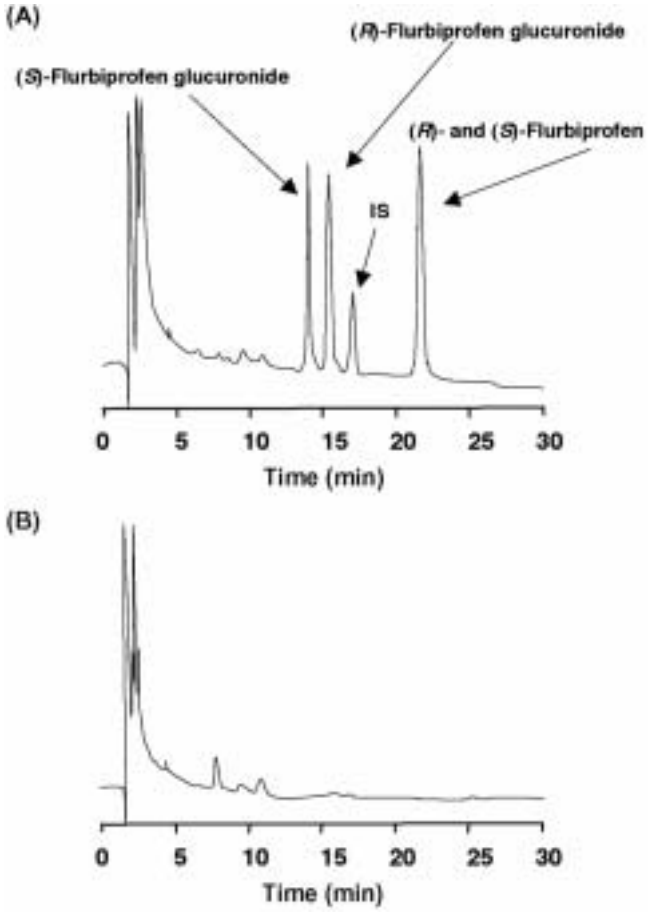


Figure 4 Typical chromatogram for the incubation mixture spiked with (A) and without (B) authentic specimens. Conditions: trapping column, MAYI-ODS column (5 μm, 4.0 mm I. D.×10 mm); analytical column, TSKgel ODS-80 Ts (5 μm, 2.0 mm I. D.×150 mm); mobile phase for trapping, 20 mM ammonium acetate buffer (pH 4.0) at a flow rate of 1.0 mL/min; mobile phase for analysis, 20 mM ammonium acetate buffer (pH 5.6)/ acetonitrile/ethanol (20:7:2, v/v/v) at a flow rate of 0.2 mL/min; detection, UV 246 nm.

the formation of FP-GlcUs in incubation mixtures containing rat liver microsomal preparations was investigated. The concentration of FP-GlcU produced from each enantiomers increased in a time-dependent manner, and no chiral inversion occurred under these incubation conditions. The ratio of the resulting level of the R-FP glucuronide to that of the S-FP glucuronide was 1.19. Moreover, the formation of FP-GlcU was inhibited in the presence of bile acid derivatives, including amino acid conjugates and carboxy-linked glucuronides. Equimolar concentrations of UDCA or CDCA in the incubation mixture reduced R-FP glucuronide production by 50%. Furthermore, the addition of equimolar DCA, LCA, GCDCA, CDCA 24-GlcU, or DCA 24-GlcU resulted in approximately 40−45% inhibition. The acyl glucuronidation of S-FP was also inhibited by bile acid derivatives. Bile acid acyl glucuronides, which are carboxy-linked glucuronides similar to FP glucuronides, are also strong inhibitors of FP glucuronidation.

These inhibitory effects of bile acids and their conjugates on acyl glucuronidation may suppress the formation of protein-bile acid adducts. The liver concentrations of bile acids and FP are significantly different. These facts suggest that bile acids and their

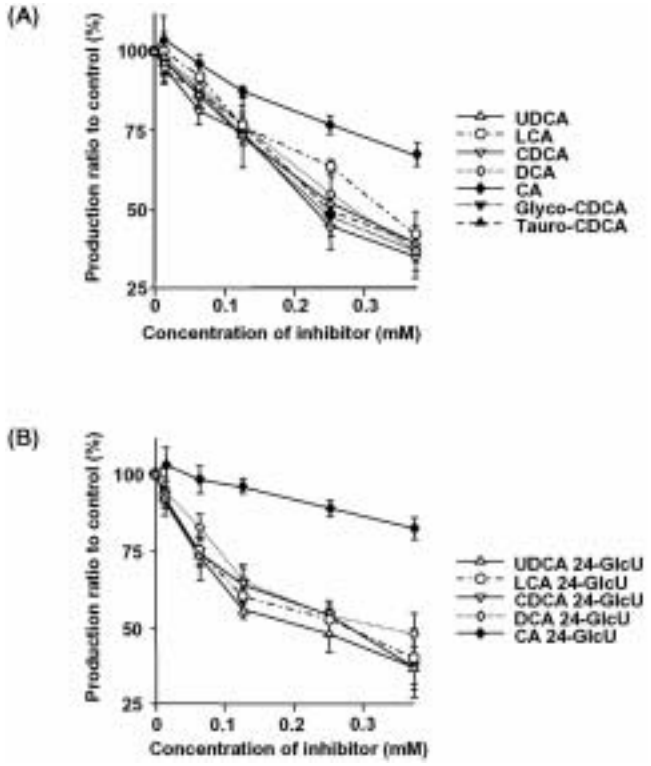
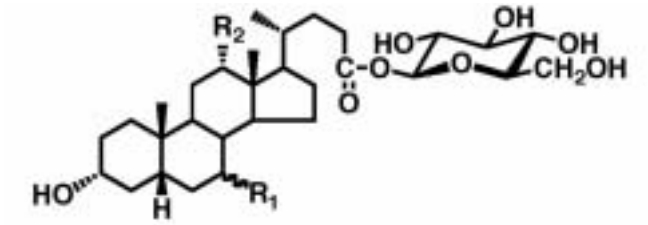


Figure 5 Inhibition of (*R*)-flurbiprofen acyl glucuronidation by bile acids.
Conditions: 250 μM *R*-FP was incubated with microsomal preparations (500 μg protein/mL) in the presence of various amounts of bile acid derivatives at 37 ℃ for 10 min.

conjugates may act as inhibitors of FP acyl glucuronidation in hepatocytes. According to inhibition studies, CDCA and GCDCA can bind to the free enzyme, the R: or SFP:enzyme complex, and the UDPGA:enzyme complex, and inhibit the enzyme activity through multiple mechanisms. CDCA 24-GlcU also can bind to each of these enzyme forms, and it can compete with UDPGA for binding to the active site. These data indicate that excess bile acids may block FP acyl glucuronidation in rat hepatocytes. Among the biologically important carboxylic acids, the covalently bound protein adducts formed through the active ingredient of acyl glucuronide-conjugated drugs can promote immunogenicity and cause allergic disease. Little evidence exists, however, linking allergic disease to drugs with carboxy groups, even though a wide variety of drugs with carboxylic acid groups are used clinically. It is reported that decreasing the production of protein-bound adducts by adding inhibitors of UGT is also effective for reducing the toxicity of drugs [14]. Therefore, our study suggests that the inhibitory effects of bile acids and their conjugates on the acyl glucuronidation of drugs may be responsible for inhibiting the toxicity of the drugs.



Compound	R_1	R_2
Cholic acid 24-Glucoside (CA 24-Glc)	α-OH	OH
Chenodeoxycholic acid 24-Glucoside (CDCA 24-Glc)	α-OH	H
Deoxycholic acid 24-Glucoside (DCA 24-Glc)	H	OH
Lithocholic acid 24-Glucoside (LCA 24-Glc)	H	H
Ursodeoxycholic acid 24-Glucoside (UDCA 24-Glc)	β-OH	H

Figure 6 Chemical structures of bile acid 24-glucosides.

2. A sensitive method for the determination of neutral acyl glycosides

Bile acid glucosides have been found in biological fluids and the conjugated site of the sugar moiety has been identified as the 3 α-hydroxyl group on the steroid nucleus. These previous findings led us to predict the existence in human biological fluids of bile acid acyl glucosides, in which the 24-carboxylic acid group of the bile acid is bound to the anomeric hydroxy group of glucose. Detection of the bile acid 24-glucosides (24-Glcs), however, was very difficult, because they contain neither a chromophore nor an ionic functional group (Fig. 6). Therefore, their existence and the dynamics of their formation and degradation in humans were not clearly known. We therefore chemically synthesized five authentic bile acid 24-Glcs as potential phase II metabolites [15], and developed a highly sensitive method to detect these glucosides [16].

For the measurement of phase II metabolites of bile acids in biological fluids, negative-ion ESI-MS is very useful, because most of the metabolites have an anionic group and such compounds easily produce deprotonated molecules ($[M-H]^-$) in the negative ion detection mode under ESI processes. Because bile acid 24-Glcs are typical neutral compounds, the ionization efficiencies of these molecules using ESI are very low, and this mode seems to be undesirable for a highly sensitive detection method. The atmospheric-pressure chemical-ionization (APCI) technique is more suitable for the ionization of low-molecular-weight compounds with relatively weak polar properties, and can be easily coupled with conventional HPLC. Bile acid 24-Glcs, however, have multiple hydroxyl groups, making it difficult to produce molecular-related ions with high efficiency using APCI. Fortunately, these neutral compounds are sometimes released into the gas-phase as adduct ions, and the existence of additives in the mobile phase increases adduct ion formation, thereby increasing the reproducibility of the quantitative

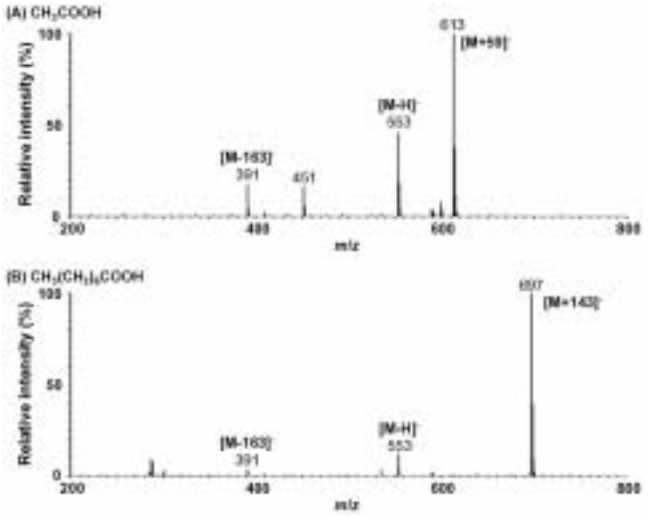


Figure 7 Negative atmospheric-pressure chemical-ionization mass spectra of CDCA 24-glucoside.
Conditions: mobile phase, (A): 20 mM ammonium acetate (pH 7.0)/acetonitrile (8:5, v/v); (B): 20 mM octanoic acid and ammonia in water/acetonitrile (8:5, v/v); injection amount, 50 ng for CDCA 24-Glc; flow rate, 1.0 mL/min; needle voltage, −2.5 kV; orifice-1 voltage, 0 V; ring lens voltage, −70 V.

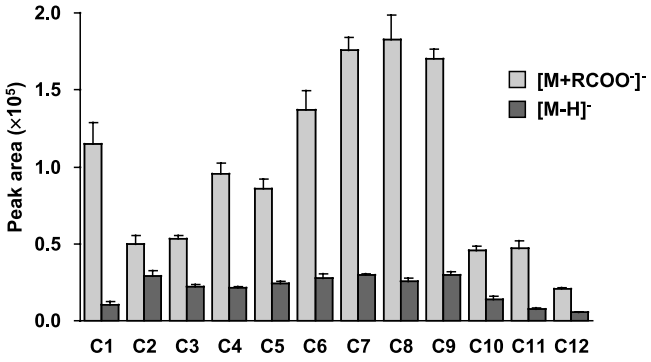


Figure 8 Effects of the number of carbon atoms in an organic acid as a mobile phase additive on the adduct formation of CDCA 24-glucoside.
Conditions: mobile phase, water−acetonitrile (8:5, v/v) containing 20 mM various organic acids and ammonia; flow rate, 1.0 mL/min; injection amount, 5 ng for CDCA 24-Glc; needle voltage, −5.0 kV; orifice-1 voltage, 0 V; ring lens voltage, −60 V.

analysis. Organic acids readily produce deprotonated molecules during the APCI process, and they often generate a corresponding gas-phase ion in combination with neutral compounds after they are added to a spray liquid. A typical mass spectrum of CDCA 24-Glc using a mixture of 20 mM ammonium acetate solution and acetonitrile as the mobile phase is shown in Figure 7 A. Although the adduct ion (*m/z*: 613) with the acetate was observed as the base peak with the deprotonated molecule (*m/z*: 553) and a fragment ion (*m/z*: 391) formed by removing the glucose moiety, the intensities of the peaks were insufficient for highly sensitive detection of this glucoside.

Therefore, we investigated whether the addition of organic acids to the APCI spray liquid improved the detection sensitivity for neutral bile acid 24-Glcs. The effect of the number of carbon atoms in the organic acid used as an additive on the formation of CDCA 24-Glc adduct ions was investigated under the flow-injection mode. Figure 8 shows the peak intensities of the $[M-H]^-$ deprotonated molecules and the $[M+RCOO^-]^-$ adduct ions plotted against the number of carbon atoms in the additive. In this experiment, the peak areas of the adduct ions were 1.7−11.2-fold larger than those of the deprotonated molecules. The highest peak intensity was observed using octanoic acid (C 8) as the mobile phase additive, and the ratio of the peak area of the adduct ion to that of the deprotonated molecule was greater than 7.2. The peak intensity of the organic anion adducts increased with increased carbon chain length up to eight carbon atoms. Further increase of the alkyl chain length resulted in a decrease in the signal intensity for the adduct ions. For bile acids with a 5 β-structure, hydrocarbons containing more than 10 carbon atoms may be too large to effectively interact with the hydrophobic areas of the B, C, and D rings of the steroid nucleus, resulting in the reduction in the peak intensity of the adduct ion. A typical mass spectrum of CDCA 24-Glc obtained under the optimized conditions is shown in Figure 7 B. The adduct ion (*m/z*: 697) with the octanoate anion resulted in the base peak. The addition of octanoic acid improved the detection limit due to the effective production of the adduct ion, and the mass shift of the monitoring ions to a higher mass region, which caused a reduction of the background noise levels. Typical selected ion recordings for the authentic bile acid 24-Glcs are shown in Figure 9 A. The detection limit in selected ion monitoring analysis of CDCA 24-Glc was 25 pg/injection with a signal-to-noise ratio of 5, indicating that the sensitivity was 10-fold higher than that obtained for these glucosides without octanoic acid as a mobile phase additive. Good linearities were observed for a 100-fold dynamic range of all of the bile acid 24-Glcs. These results demonstrated the utility of organic acids as spray liquid additives to improve the detection sensitivity of the APCI technique for hydrophobic and neutral compounds such as bile acid 24-Glcs.

These newly established MS conditions were then used to search for bile acid 24-Glcs in human urine [17]. When we analyzed the urine of a healthy volunteer, no peaks corresponding to acyl glucosides were detected on the chromatogram. As shown in Figure 9 B, however, we observed two intense peaks at *m/z* 697 and 713, corresponding to dihydroxylated bile acid acyl glycoside and trihydroxylated bile acid acyl glycoside, respectively, with different retention times than those of authentic specimens of the bile

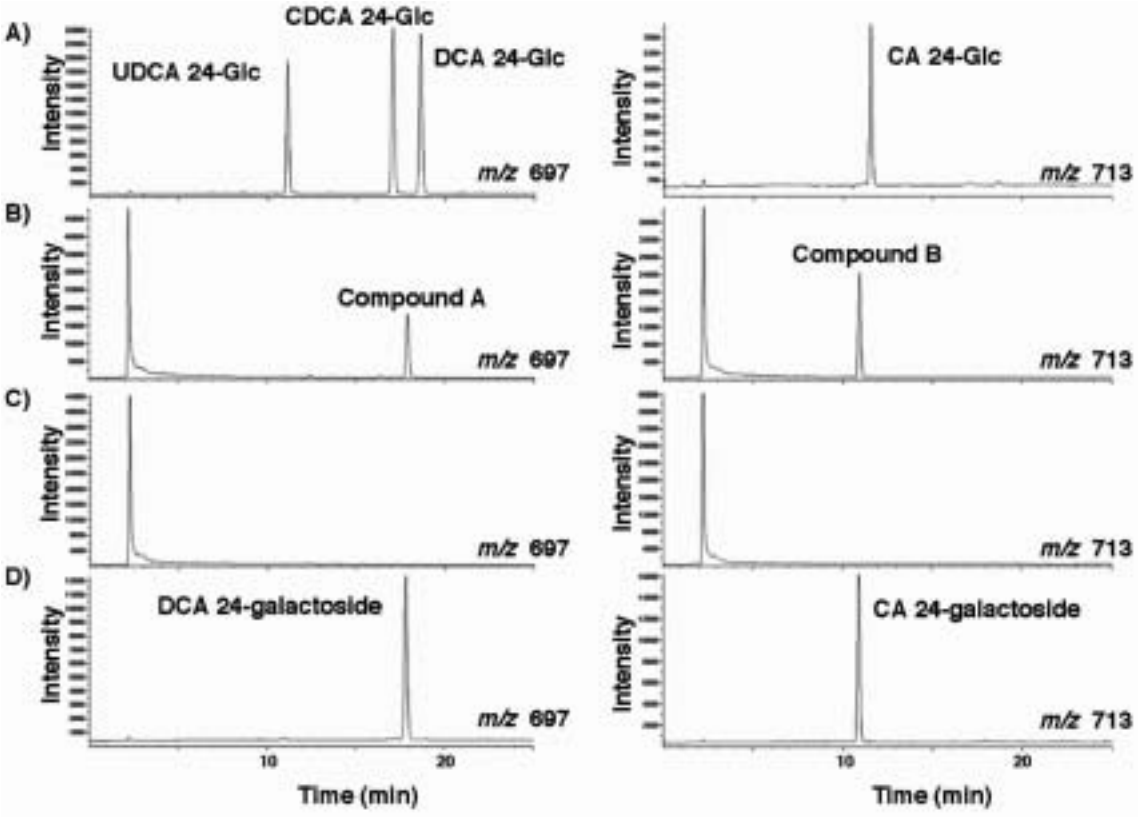


Figure 9 Liquid chromatography/atmospheric-pressure chemical-ionization mass spectrometry of bile acid glycosides.
(A): authentic specimens of bile acid 24-glucosides (24-Glcs); (B): human urine extract; (C): human urine extract following alkaline hydrolysis; (D): authentic specimens of bile acid 24-galactosides (24-Gals).
Conditions: column, Symmetry C 18 (5 μm, 150 mm x 4.6 mm I. D.); mobile phase, 1 mM ammonium acetate (pH 7.0)/acetonitrile (v/v from 5:2 to 5:6, 0 to 30 min) at a flow rate of 0.9 mL/min; solution for post-column addition, 200 mM octanoic acid and ammonia in water/acetonitrile (8:5, v/v) at a flow rate of 0.1 mL/min; needle voltage, −2.5 kV; orifice-1 voltage, 0 V; ring lens voltage, −70 V.

acid acyl glucosides (Fig. 9 A). We identified these unknown compounds by means of LC/MS and tandem mass spectrometry (MS/MS) analysis coupled with microchemical and derivatization reactions. The unknown compounds corresponding to the two major peaks on the chromatogram were named compound A (*m/z*: 697) and compound B (*m/z*: 713) (Fig. 9 B). These compounds resulted in ions with base peaks on the mass spectrum at *m/z* 697 and *m/z* 713 and fragment ions that produced peaks at *m/z* 391 and 407, corresponding to dihydroxylated and trihydroxylated bile acids, respectively. The intensities of the fragment ion peaks varied as the voltages of orifice 1 and the ring lens were changed; a similar phenomenon was also observed for the bile acid 24-Glcs. Under the soft ionization modes, the ester bond is more easily cleaved than the corresponding ether bond to yield fragment ions. When the urine extract was subjected to mild alkaline hydrolysis, as shown in Figure 9 C, the peaks corresponding to compounds A and B disappeared. Acyl glycosides are readily hydrolyzed under alkaline conditions; therefore, it is reasonable to suppose that compounds A and B were bile acid acyl glycosides conjugated through the carboxylic acid group located on the side chain. Thus, these observations strongly implied that compounds A and B were acyl glycosides consisting of dihydroxylated and trihydroxylated bile acids with a glycosidic residue, which had the same molecular weight as glucose.

The eluate fractions corresponding to peaks A and B on the chromatogram were collected and subjected to alkaline hydrolysis, followed by LC/ESI-MS analysis. The hydrolysis of the fractions containing compounds A and B produced peaks with the same retention times as DCA (*m/z*: 391) and CA (*m/z*: 407), respectively. These compounds were named compound A' and compound B', respectively. The chromatographic behavior of bile acids is dependent on the number and positions of the hydroxy groups on the steroid nucleus, and this behavior can be used to identify trace amounts of bile acids in biological fluids. Thus, the fractions corresponding to the peaks for compounds A' and B' were collected and subjected to LC/ESI-MS analysis with four mobile phases at different pHs. The relative *k* values of A' and B' with respect to those of the internal standards (stable isotope-labeled DCA and CA) were

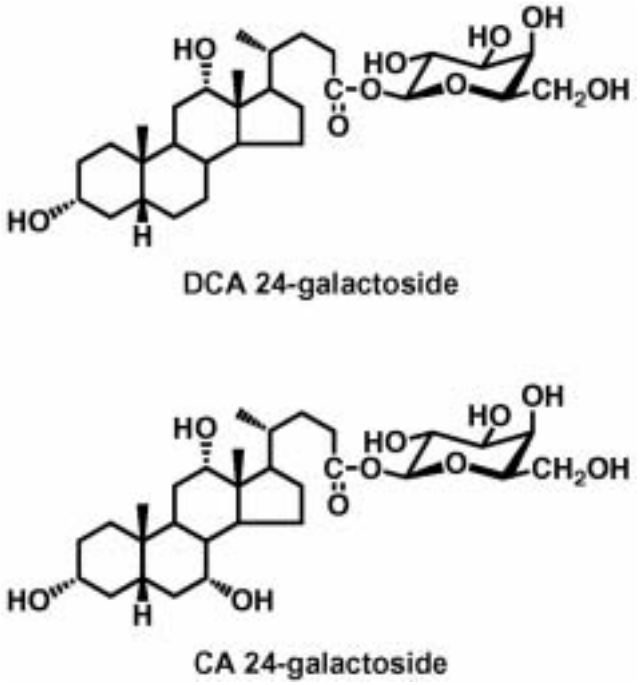


Figure 10 The structures of deoxycholic acid 24-galactoside and cholic acid 24-galactoside.

identical to those of authentic DCA and CA. According to these results, compounds A' and B' were clearly identified as DCA and CA, respectively.

Next, we focused on identifying the glycosidic residue liberated by alkaline hydrolysis. Derivatization of the product of the alkaline hydrolysis was performed using 3-methyl-1-phenyl-5-pyrazolone (PMP), and the resulting derivative was subjected to LC/ESI-MS analysis. The retention time of the PMP derivative of the glycosidic residue liberated by alkaline hydrolysis of compounds A and B was identical to that of the PMP derivative of galactose. These results suggested that compounds A and B were bile acid acyl galactosides, which were conjugated by an ester bond between the bile acid and galactose. On the basis of these results, we synthesized authentic specimens of DCA 24-galactoside (24-Gal) and CA 24-Gal [18], and compared them with compounds A and B using LC/APCI-MS and APCI-MS/MS. The retention times of compounds A and B were identical to those of the authentic DCA 24-Gal and CA 24-Gal (Fig. 9 D). DCA 24-Gal and compound A were subjected to APCI-MS/MS analysis. The fragment ion peak at *m/z* 391 corresponding to the aglycone DCA represented a reduction of *m/z* 306, whereas the peak for the deprotonated molecule $[M-H]^-$ at *m/z* 553 represented a reduction of *m/z* 144. Similarly, in the case of CA 24-Gal and compound B, the fragment ion peak at *m/z* 407 corresponding to the aglycone represented a reduction of *m/z* 306, whereas the peak for the deprotonated molecule $[M-H]^-$ at *m/z* 569 represented a reduction of *m/z* 144. Considering these data, compounds A and B were identified as DCA 24-Gal and CA 24-Gal, respectively. It seems quite probable that these conjugates are commonly excreted in urine, because both conjugates were detected in all six urine specimens from healthy volunteers. A semiquantitative estimate indicated the concentrations of CA 24-Gal and DCA 24-Gal in urine were about 20−100 ng/mL. These concentrations are similar to the levels of bile acid 24-GlcUs in urine (28−156 ng/mL). Only three types of glycosides, namely, glucuronides, glucosides, and *N*-acetylglucosaminides, have been identified as phase II metabolites in human biological fluids. Therefore, we are interested in the physiological significance of these pathways, as well as the regions where these conjugates are synthesized, the enzymes that catalyze these conjugation reactions, and the conjugate dynamics.

Conclusion

Acyl glycosides have attracted attention as one of the activated forms of carboxylic acids. We developed a highly sensitive method for the determination of acyl glycosides, which are chemically unstable and easily hydrolyzed to produce aglycones, in biological specimens. Using this method, we revealed that bile acids and their derivatives inhibit hepatic bile acid acyl glucuronidation. These inhibitory mechanisms may be important as a detoxification system in the body. Moreover, we demonstrated the existence in human urine of a novel glycoside, which is conjugated by an ester bond between the bile acid and the galactose. Although, the dynamics of the formation and the degradation of the conjugates remain to be studied, elucidation of the physiological significances of the glycosides is a very attractive future direction for our research.

Acknowledgments

The author would like to acknowledge Professor Junichi Goto (Tohoku University) for his kind help and encouragement. The author is also very grateful to Associate Professor Nariyasu Mano (Tohoku University), Professor Takashi Iida (Nihon University), and all of his co-workers in this study. The author is very grateful to the Japan Society for Chromatographic Sciences for selecting him as the recipient of the Encouragement Award in 2006 and giving him an opportunity to publish this review paper. This work was supported in part by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

[1] Vore, M.; Durham, S.; Yeh, S.; Ganguly, T. *Biochem. Pharmacol.*, **1991**, *41*, 431−437.

[2] Smith, P. C.; Liu, J. H. *Xenobiotica*, **1993**, *23*, 337−348.

[3] Ikegawa, S.; Murao, N.; Nagata, M.; Ohba, S.; Goto, *J. Anal. Sci.*, **1999**, *15*, 213−215.

[4] Ding, A.; Ojingwa, J. C.; McDonagh, A. F.; Burlingame, A.

L.; Benet, L. Z. *Proc. Natl. Acad. Sci. USA*, **1993**, *90*, 3797−3801.

[5] Williams, A. M.; Dickinson, R. G. *Biochem. Pharmacol.*, **1994**, *47*, 457−467.

[6] Worrall, S.; Dickinson, R. G.; *Life Sci.*, **1995**, *56*, 1921−1930.

[7] Smith, P. C.; Liu, J. H. *Xenobiotica*, **1995**, *25*, 531−540.

[8] Ikegawa, S.; Okuyama, H.; Oohashi, J.; Murao, N.; Goto, J. *Anal. Sci.*, **1999**, *15*, 625−631.

[9] Ikegawa, S.; Murao, N.; Motoyama, T.; Yanagihara, T.; Niwa, T.; Goto, J. *Biomed. Chromatogr.*, **1996**, *10*, 313−317.

[10] Goto, J.; Murao, N.; Nakada, C.; Motoyama, T.; Oohashi, J.; Yanagihara, T.; Niwa, T.; Ikegawa, S. *Steroids*, **1998**, *63*, 186−192.

[11] Mano, N.; Nishimura, K.; Narui, T.; Ikegawa, S.; Goto, J. *Steroids*, **2002**, *67*, 257−262.

[12] Mano, N.; Nikaido, A.; Narui, T.; Yamasaki, D.; Goto, J. *J. chromatogr. B*, **2002**, *776*, 125−131.

[13] Mano, N.; Goto, T.; Nikaido, A.; Narui, T.; Goto, J. *J. Pharm. Sci.*, **2003**, *92*, 2098−2108.

[14] Kretz−Rommel, A.; Boelsterli, U. A. *Toxicol. Appl. Pharmacol.*, **1993**, *120*, 155−161.

[15] Iida, T.; Nakamori, R.; Yabuta, R.; Yada, S.; Takagi, Y.; Mano. N.; Ikegawa, S.; Goto, J.; Nambara, T. *Lipids*, **2002**, *37*, 101−110.

[16] Goto, T.; Shibata, A.; Iida, T.; Mano, N.; Goto, J. *Rapid Commun. Mass Spectrom.*, **2004**, *18*, 2360−2364.

[17] Goto, T.; Shibata, A.; Sasaki, D.; Suzuki, N.; Hishinuma, T.; Kakiyama, G.; Iida, T.; Mano N.; Goto, J. *Steroids*, **2005**, *70*, 185−192.

[18] Kakiyama, G.; Sadakiyo, S.; Iida, T.; Mushiake, K.; Goto, T.; Mano, N.; Goto, J.; Nambara, T. *Chem. Phys. Lipids*, **2005**, *134*, 141−150.