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Tandem mass spectrometric characterization of bile acids and steroid conjugates based on low-energy collision-induced dissociation

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ABSTRACT

We examined the characteristics of several bile acids and some steroid conjugates under low-energy-collision-induced dissociation conditions using a triple quadrupole tandem mass spectrometer. According to conjugation types, we observed characteristic product ions and/or neutral losses in the product ion spectra. Amino acid conjugates afforded specific product ions. For example, glycine-conjugated metabolites routinely produced a product ion at *m/z* 74, and taurine-conjugated metabolites produced product ions at *m/z* 124, 107, and 80. When a strong peak appeared at *m/z* 97, the molecule contained a sulfate group. In contrast to amino acid conjugates, carbohydrate conjugates required a combination of product ions and neutral losses for identification. We could discriminate a glucoside from an acyl galactoside according to the presence or absence of a product ion at *m/z* 161 and a neutral loss of 180 Da. Discrimination among esters, aliphatic ethers, and phenolic ether types of glucuronides was based upon differences in the intensities of a product ion at *m/z* 175 and a neutral loss of 176 Da. Furthermore, *N*-acetylglucosamine conjugates showed a characteristic product ion at *m/z* 202 and a neutral loss of 203 Da, and the appearance of a product ion at *m/z* 202 revealed the existence of *N*-acetylglucosamine conjugated to an aliphatic hydroxyl group without a double bond in the immediate vicinity.

Together, the data presented here will help to enable the identification of unknown conjugated cholesterol metabolites by using low-energy collision-induced dissociation.

1. Introduction

Cholesterol is involved in the stabilization of biomembranes, and is a precursor for the biosynthesis of small biomolecules, including bile acids, steroid hormones, and vitamin D. Humans produce cholesterol from acetyl-CoA via mevalonic acid, and also ingest cholesterol in the diet. It has a simple chemical structure possessing only a hydroxyl group at the C-3 position as a characteristic functional group. Natural cholesterol metabolites, including bile acids, steroid hormones, and vitamin D, possess hydroxyl, carbonyl, and carboxyl groups, which are often conjugated to other small molecules, particularly when detected in the urine [1–4]. For example, the reactive chemical handles may be conjugated to amino acids [5], sulfuric acid [6], glucuronic acid [7], glucose [8], galactose [9], or *N*-acetylglucosamine (GlcNAc) [10]. These conjugations result in the addition of very polar functional groups onto the cholesterol metabolites; therefore, electrospray ionization (ESI) is a suitable ionization method for their mass spectrometric analysis [11–14].

Bile acids are quantitatively major metabolites of cholesterol, and all of the above-mentioned conjugations have been found in the human body. Sulfation is the most commonly observed modification made during the body's elimination of bile acids; sulfated bile acids constitute approximately 40% of total urinary bile acids [15]. Glucuronidation is known as an important detoxification route for low molecular weight compounds including drugs [16–19]. Since Back et al. [7,20] found bile acid glucuronides in the urine of cholestasis patients, the glucuronidation of bile acids had been thought to occur almost exclusively as an ether modifying a hydroxyl group at the C-3 position. However, it is now known that the main component of bile acid glucuronides are esters linked through a carboxyl group at the C-24 position [21]. In the urine of ursodeoxycholic acid (UDCA)-treated patients with primary biliary cirrhosis, a hydroxyl group at the C-7β position

Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; CID, collision-induced dissociation; DCA, deoxycholic acid; ESI, electrospray ionization; GlcNAc, *N*-acetylglucosamine; LC, liquid chromatography; LCA, lithocholic acid; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NPC, Niemann–Pick disease type C1; SNAG-Δ^5-CA, 3β-sulfo-7β-*N*-acetylglucosaminyl-5-cholen-24-oic acid; SNAG-Δ^5-CG, glycine-conjugated 3β-sulfo-7β-*N*-acetylglucosaminyl-5-cholen-24-oic acid; SNAG-Δ^5-CT, taurine-conjugated 3β-sulfo-7β-*N*-acetylglucosaminyl-5-cholen-24-oic acid; UDCA, ursodeoxycholic acid.

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was specifically conjugated with GlcNAc [22], as was also found to be the case with other bile acid-related substances [23]. Similarly, bile acids modified with glucoside at the C-3 position [23,24], and galactoside at the C-24 position [9] have been found in human urine. Some of these conjugations are also found on steroid hormones [25]. In addition, Alvelius et al. [26] found the highly concentrated abnormal cholesterol metabolites, 3β-sulfo-7β-*N*-acetylglucosaminyl-5-cholen-24-oic acid (SNAG-Δ^5-CA) and its amino acid conjugates, in urine from a Niemann–Pick disease type C1 (NPC) patient. Recently, we pointed out the possibility of their metabolites as new diagnostic biomarkers of the disease [27]. Importantly, we can provide information relevant to modifications on all conjugated cholesterol metabolites by investigating the characteristics of bile acid conjugates and some steroid conjugates (Fig. 1).

Accordingly, we investigated the mass spectrometric characteristics of all known conjugations using ESI-mass spectrometry (MS) and ESI-tandem mass spectrometry (MS/MS) with low-energy collision-induced dissociation (CID). The knowledge obtained here will help to enhance databases used for the identification of known conjugated cholesterol metabolites in human urine.

2. Experimental

2.1. Chemicals

Cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), and UDCA were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). The following compounds were synthesized in our laboratories using previously reported methods: glycine- and taurine-conjugated bile acids [28], bile acid 3-sulfates [29], bile acid 3-glucuronides [30], bile acid 24-glucuronides [31], bile acid 7β-*N*-acetylglucosamine conjugates [32], bile acid 3-glucosides [33], bile acid 24-galactosides [34], SNAG-Δ^5-CA and its glycine and taurine conjugates (SNAG-Δ^5-CG, SNAG-Δ^5-CT) [35]. 17β-Estradiol 3-sulfate, 3-glucuronide, and 17-glucuronide were purchased from Steraloids, Inc. (Newport, RI, USA). All conjugated standard specimens were dissolved at 2.0 μmol/L in water/ethanol (1:1, v/v), and unconjugated standard specimens were dissolved at 20 μmol/L in water/ethanol (1:1, v/v). Ultrapure water was prepared using a PURELAB ultra apparatus (Organo, Tokyo, Japan). All other chemicals and solvents were analytical grade.

2.2. Measurement of product ion spectra

ESI-MS/MS was performed using an API 5000 mass spectrometer (AB SCIEX, Framingham, MA, USA). The ion spray voltage, declustering potential, and Turbo V source heaters were set at −4500, −80 V, and 700 °C, respectively. Nitrogen was used for Curtain Gas, Gas1, Gas2, and Collision Gas, with flow rates of 25, 40, 60, and 6 units, respectively. The spray solvent was a mixture of 20 mmol/L ammonium acetate solution (pH 7.0) and methanol (3:7, v/v). Analytes were introduced into the ESI probe through an inline filter and a short ODS column (Shim pack MAYI-ODS [36], 2.0 mm i.d. × 10 mm, Shimadzu Corp., Kyoto, Japan) at a flow rate of 0.2 mL/min. Product ion spectra were measured with a scan range of *m/z* 5 –900 and a scan time of 1 s. The collision voltage was set from −5 to −130 V. Unit mass resolution was selected in both Q1 and Q3. Doubly charged deprotonated molecules ($[M-2H]^{2-}$) were selected as precursor ions for glycine- and taurine-conjugated bile acid 3-sulfates, SNAG-Δ^5-CG, and SNAG-Δ^5-CT. Singly charged deprotonated molecules ($[M-H]^-$) were used for all other compounds. Data were collected and processed using Analyst 1.4.1 data collection and integration software.

3. Results and discussion

3.1. Differences in low-energy CID patterns among bile acids and their amino acid conjugates

We have already analyzed unconjugated, glycine-, and taurine-conjugated bile acids using a Q-TOF instrument [37]; here, using a triple quadrupole instrument, we obtained similar product ion spectra for these conjugates, as shown in Fig. 2B, D, and F. The similarity of the spectra is not surprising because of a similarity of the ionization conditions. We performed product ion scan analysis of unconjugated, glycine-, and taurine-conjugated CDCA (Figs. 2B, D, and F). Only $[M-H-H_2O]^-$ was detected at *m/z* 373 using collision voltages from −30 to −60 V (Table 1). The intensity of that product ion was very low (Fig. 2A), indicating that the unconjugated bile acid was very stable under low-energy CID. Although we did not show a mass spectrum of CA, product ions with neutral losses consistent with formic acid, e.g., $[M-H-(H_2O+HCOOH)]^-$ at *m/z* 343 and $[M-H-(2H_2O+HCOOH)]^-$ at *m/z* 325, were detected at relatively high intensity using a collision voltage of −60 V (Table 1S). By contrast, we never observed product ions of LCA. Qiao et al. [38] investigated the fragmentation pathway of 18 species of bile acids predominantly comprising dihydroxylated bile acids and including amino acid conjugates, by tandem mass spectrometry using an ion trap/time-of-flight instrument operated in negative ion mode. They show very similar results, and in the product ion spectrum of CDCA, a dehydroxylated product ion appeared only at *m/z* 373. In addition, DCA possessing a 12α-hydroxyl group produced neutral loss of 44 Da (CO_2) and 46 Da (HCOOH) as with our data. These suggested that different instruments shared similar fragment pattern under low-energy CID conditions.

For glycine-conjugated CDCA (GCDCA), three product ions appeared at *m/z* 74, *m/z* 384, and *m/z* 386, as shown in Fig. 2D. The product ion observed at *m/z* 74 was $NH_2CH_2COO^-$, and the peaks at *m/z* 384 and *m/z* 386 were estimated to be $[M-H-(H_2O+HCOOH)]^-$ and $[M-H-(H_2O+CO_2)]^-$, respectively, which may be produced by the elimination of water and formic acid or carbon dioxide. Those three product ions were most intense at a collision voltage of −50 V. Although dehydrated product ions were found in the spectra of unconjugated bile acids, when analyzing glycine conjugates we found little dehydrated product ion (Table 1S). Instead, we observed that water was eliminated with CO_2 or HCOOH; this same fragmentation was also found in CA and DCA. Both CA and DCA have a 12α-hydroxyl group, which is known to bind with a carboxyl group at the end of the side chain through intra-molecular hydrogen bonding [39]. Similarly, the carboxyl group in glycine conjugates can bind to the 12α-hydroxyl and to the 7α- and 7β-hydroxyl groups. Therefore, glycine conjugates produced $[M-H-(H_2O+CO_2)]^-$ and $[M-H-(H_2O+HCOOH)]^-$, except for GLCA, which possesses only a 3α-hydroxyl group. $NH_2CH_2COO^-$ at *m/z* 74 was a common product ion in all glycine conjugates (Tables 1, 1S–3S, and 5S).

The product ion spectrum of taurine-conjugated CDCA (TCDCA) is shown in Fig. 2F. Specifically, the three product ions observed at *m/z* 80, 107, and 124 were all derived from the taurine moiety, and were identified as SO_3^-, $CH_2CHSO_3^-$, and $NH_2CH_2CH_2SO_3^-$, respectively. These three product ions were common in all taurine conjugates (Tables 1, 1S–3S, and 5S). Although all product ions appeared in the product ion spectrum at a collision voltage of −80 V, the use of higher collision voltages resulted in the convergence of product ions to SO_3^-, at *m/z* 80. Since taurine conjugates have no carboxyl group, they produced $[M-H-H_2O]^-$, at *m/z* 480, similar to unconjugated bile acids, but did not give rise to $[M-H-(H_2O+CO_2)]^-$ or $[M-H-(H_2O+HCOOH)]^-$. These facts also confirm the effect of intra-molecular hydrogen bonding between a hydroxyl group and a