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Simple and quantitative analysis of urinary sulfated tauro- and glycodihydroxycholic acids in infant with cholestasis by electrospray ionization mass spectrometry☆

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Abstract

Here we report a simple, sensitive, and accurate method for detecting urinary sulfated tauro- and glyco-bile acids that uses electrospray ionization mass spectrometry. The sulfated tauro- and glycodihydroxycholic acids mainly generated $[M-2H]^{2-}$ negative ions at *m/z* 288.6 and *m/z* 263.6, respectively. These doubly charged ions appeared primarily in samples prepared from the urine of patients with cholestasis and were detected quantitatively. Cholestatic jaundice is the primary clinical sign of biliary atresia. The measurement of doubly charged negative ions, especially of sulfated taurodihydroxycholic acid (principally taurochenodeoxycholate-3-sulfate), is a useful screening modality for biliary atresia in neonates.

Keywords: Sulfated bile acids; ESIMS; Cholestasis; Biliary atresia

Biliary atresia (BA) can cause severe liver dysfunction in neonates, and the success of surgical correction is clearly higher when BA is diagnosed before 60 days of age. Therefore, early detection of BA is important. The detection of cholestasis is the first step towards a diagnosis of BA. The urinary excretion of sulfated bile acid (USBA) increases with cholestasis, due to the activation of an alternate metabolic pathway for bile acid catabolism. Thus, the measurement of USBA is useful in selective screens for BA and neonatal hepatitis syndrome. As a rapid method for detecting cholestatic jaundice, a direct enzyme assay for USBA (3α-sulfated bile acids) has been reported [1,2]. Here, we introduce an even simpler selective method for measuring USBAs (tauro- and glycodihydroxycholate-3-sulfate), using electrospray ionization mass spectrometry (ESIMS).

1. Experimental

1.1. Reagents

Taurine-conjugated forms of lithocholic acid and lithocholic acid-3-sulfate were obtained from Sigma–Aldrich (Saint Louis, MO, USA). Bond Elut C_{18} (100 mg, 1 ml) was purchased from VARIAN (Palo Alto, CA, USA).

1.2. Urine samples

Urine was collected from 10 children with normal liver function as control samples, and from 16 children with hepatic insufficiency. Urine was also collected from 17 children with cholestasis or obstructive jaundice, including four children with citrin deficiency [3,4]. The age of these patients was between 1 month and 1 year. The urine from one biliary atresia patient (1 year and 2 months old) who had been treated with ursodeoxycholate was analyzed to examine the stabilities of tauro- and glycodihydroxycholate-3-sulfate using taurolithocholate (2 nmol) as an internal standard. To examine the variation in USBA excretion over time, urine samples from another cholestasis patient were collected at age 3, 4, 5, 7, 9, and

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18 months old. The urine specimens were kept frozen at −20 °C until analysis.

Authentic sodium taurolithocholate and sodium taurolitocholate-3-sulfate were dissolved in distilled water (0.05 nmol/ml).

1.3. Bile acid analysis

Sulfated bile acids were extracted from the urine samples by a modification of the method previously described by Nichols et al. [5]. Twenty microliters of urine was diluted with 0.5 ml distilled water, and 2 nmol of taurolithocholate was added to this diluted sample as an internal standard. Lithocholate is one of the common secondary bile acids, but little is found in neonatal urine, prompting us to choose taurolithocholate as the internal standard [6]. The sample mixture was applied to a Bond Elut C_{18} cartridge that was preconditioned with 1 ml of methanol and 1 ml of 0.1 M NaOH. The column was then washed with 1 ml of distilled water, and the USBA fraction was eluted with 1 ml of methanol. The eluate was evaporated under reduced pressure and dried under N_2. The sample was re-dissolved in 0.5 ml of a 50% acetonitrile/water solution, and 10 μl of this solution was injected using a Shimadzu LC 10 LC system (Kyoto, Japan) connected to an API 300 LCMS/MS system (PE SCIEX, Foster City, CA, USA). The LC system was used only for injecting the sample and was operated isocratically with a flow rate of 40 μl/minute for the mobile phase (50% acetonitrile/water), at room temperature.

The negative ion mass spectrum of the eluate was recorded in the mass range of 200–600 amu (ion spray voltage, −3500 V; orifice voltage, −50 V; ring voltage, −380 V; ion source temperature of the turbo ion spray, 200 °C). The USBAs were quantified by measuring the $[M-2H]^{2-}$ ions at *m/z* 288.6 (taurodihydroxycholate-3-sulfate), *m/z* 263.6 (glycodihydroxycholate-3-sulfate), and *m/z* 280.6 (taurolithocholate-3-sulfate) in MRM (multiple reaction monitoring, collision energy, 20 eV) mode, respectively. The $[M-H]^{1-}$ ion of taurolithocholate (*m/z* 482.2) was used as an internal standard. No fragment ions were generated from these molecules by ESI-MS/MS (product scan mode) under the measurement conditions used. Therefore, only one ion ($[M-2H]^{2-}$ or $[M-H]^{1-}$) corresponding to each compound was selected for the MRM scan. The peak intensity ratio of these ions (ratio = ion intensity at 288.6, 263.6, or 280.6/ion intensity at 482.2) was used as the quantitative value (ratio/μmol creatinine).

2. Results and discussion

Bile acids are normally excreted into the urine mainly in a taurine- or glycine-conjugated form. The level of sulfated bile acid in urine is a highly sensitive indicator of cholestasis in children and adults [2,7]. Sulfate conjugation mainly occurs on the 3-OH group of bile acids [8]. There are three possible sulfated forms of bile acids: 3-sulfated bile acid, or 3-sulfated taurine- or glycine-conjugated forms. Although, there are several reports of direct enzymatic assays for USBA [9,10], these assays do not determined which bile acid molecules show increased sulfation. There are also several reports on the measurement of USBAs using electrospray ionization mass spectrometry and tandem mass spectrometry, but in general, these analyses have focused only on the $[M-H]^{1-}$ negative ions in the mass ion range higher than *m/z* 350 [11,12].

We used ESIMS to analyze directly some of the USBAs in patient with cholestasis. Fig. 1 shows a typical ESIMS spectrum of the urinary bile acids in a patient with biliary atresia who had been treated with ursodeoxycholate (secondary dihydroxycholate), obtained by analysis with the negative ion scan mode. Because the sulfated bile acids in the urine are mostly in the glycine- or taurine-conjugated form, we sought to detect the $[M-H]^{1-}$ ions of these sulfated bile acids. Yousef et al. reported that taurotetrahydroxycholate was the main urinary bile acid in a patient with chronic liver disease, but did not mention about sulfated bile acids [13]. We found several intense ion peaks in the urine of this patient that corresponded to the conjugated bile acids. Ions at *m/z* 530, 514, and 464 corresponded to the $[M-H]^{1-}$ of taurotetrahydroxycholate, taurocholate, and glycocholate, respectively. Ions corresponding to the $[M-H]^{1-}$ of the sulfated dihydroxycholic acid (ursodeoxycholate) were not detected in the patient's urine under our analytical conditions. Usually, in the ESIMS analyses of bile acids, researchers do not include the low-mass ion range below *m/z* 300, yet we found very intense peaks in this region (*m/z* 263.6 and 288.6). These ions were missing or present at low levels in the urines of a normal liver function and a patient with non-cholestatic liver disease. They correspond to the doubly charged negative ions $[M-2H]^{2-}$ of the sulfates of glycine-conjugated and taurine-conjugated dihydroxylcholate, respectively. However, we did not confirm whether the main component of the dihydroxycholate was ursodeoxycholate or chenodeoxycholate in this patient.

Lithocholate is one of the common secondary bile acids, but its level in infant urine is usually very low, and in fact, taurolithocholate (*m/z* 482) was not detected in our patient's urine (Fig. 1). The negative ESIMS spectrum of authentic taurolithocholate-3-sulfate and taurolithocholate are shown in

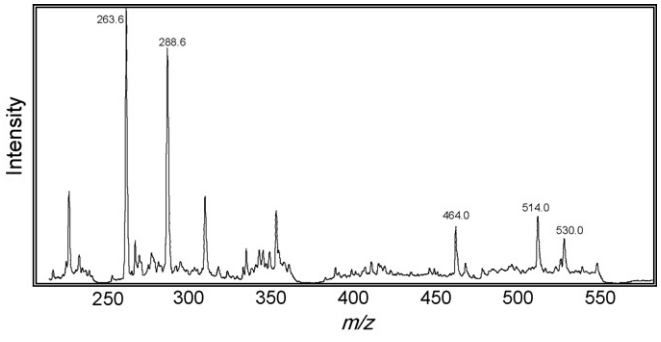

Fig. 1. Urinary conjugated bile acid ESIMS spectrum (negative ion scan mode) showing the full scan of a sample collected from a patient with biliary atresia who had been treated with ursodeoxycholic acid. The ions at *m/z* 530, 514, and 464 correspond to the $[M-H]^{1-}$ of taurotetrahydroxycholate, taurocholate, and glycocholte, respectively. The ions at *m/z* 263.5 and 288.6 correspond to the $[M-2H]^{2-}$ of glycodihydroxycholate-3-sulfate and taurodihydroxycholate-3-sulfate, respectively.

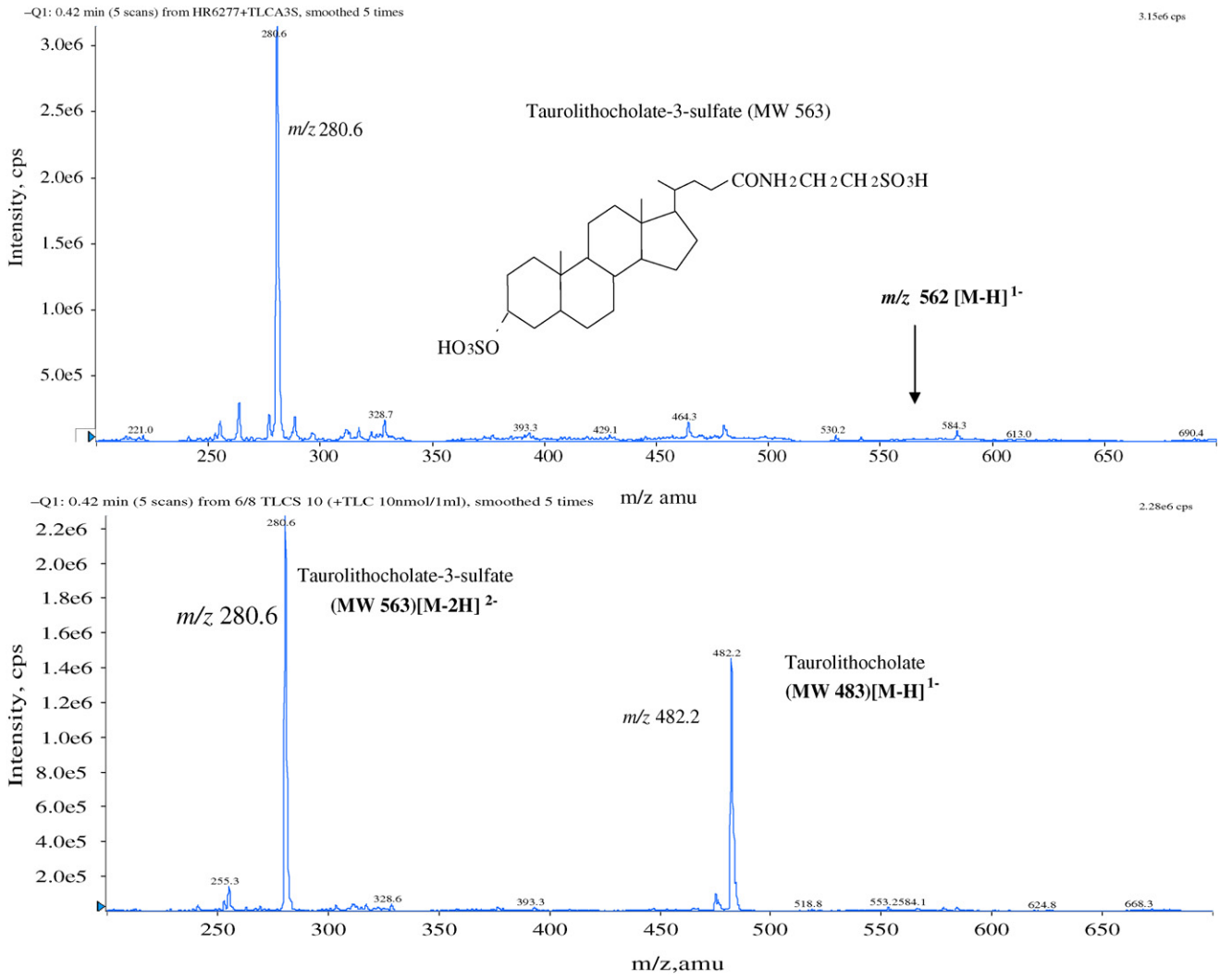

Fig. 2. Negative ESIMS spectra of authentic taurolithocholate (M.W. 429) and taurolithocholate-3-sulfate (M.W. 563). The ion at *m/z* 482.2 corresponds to the $[M-H]^{1-}$ of taurolithocholate and at *m/z* 280.6 corresponds to the $[M-2H]^{2-}$ of taurolithocholate-3-sulfate. Two nanomoles of authentic sample were used.

Fig. 2. The $[M-H]^{1-}$ ions of these compounds are *m/z* 562 and *m/z* 482, respectively. The *m/z* 482 ion of taurolithocholate was detected at *m/z* 482.2, but the ion at *m/z* 562 was not detected. In addition, an intense ion peak was found at *m/z* 280.6, corresponding to the doubly charged negative ion ($[M-2H]^{2-}$) of taurolithocholate-3-sulfate. The pK_a value of the conjugated sulfonyl group (pK_a = 1.5 in taurocholic acids) is lower than that of the carboxy group of unconjugated bile acids [14]. The 50% acetonitrile/H_2O solution used for the mobile phase in the ESIMS analysis has an almost neutral pH, so the both sulfonyl groups of taurine-conjugated bile acid-3-sulfate remain in their ionized form. This is why the doubly charged negative ion of taurolithocholate-3-sulfate appeared in the spectrum. Most bile acids are excreted in the urine in a taurine- or glycine-conjugated form, prompting us to choose taurolithocholate as an internal standard.

We used 50% acetonitrile/H_2O solution as a mobile phase. The $[M-2H]^{2-}$ ion of taurolithocholate-3-sulfate appeared as a huge peak, but the $[M-H]^{1-}$ ion was below the detection limit under our analytical conditions (Fig. 2). The occurrence of doubly charged ions in taurine- or glycine-conjugated bile acid–3-sulfates was reported by Ikegawa et al. [15]. They showed that the relative abundance of $[M-2H]^{2-}$ with respect to $[M-H]^{1-}$ in taurine-conjugated ursodeoxycholic acid-3-sulfate decreased, along with a decrease in the pK_a of the acidic component in the buffer. It has been widely thought that a doubly charged ion is unstable and unsuitable as a quantitative target ion in liquid column chromatographic separation. Therefore, researchers use buffer solutions for ESIMS analysis that prevent the occurrence of doubly charged ions, and doubly charged ions had not been applied to the analysis of USBAs.

We examined the stability of the doubly charged ion under our experimental conditions using authentic taurolithocholate-3-sulfate. The doubly charged ion at *m/z* 280.6 was very stable under our measurement conditions. The within-day precision (CV%) of the method was 2.5%, as determined by replicate analyses of taurolithocholate-3-sulfate at 5 nmol (n = 5) using taurolithocholate (10 nmol, $[M-H]^{1-}$ = *m/z* 482.2) as an internal standard. The between-day precision (CV%) was 5.1%,

determined by replicate analyses of the same sample on 10 separate days. The calibration curve of taurolithocholate-3-sulfate showed good linearity in the concentration range of 0–5 nmol/ml ($y = 2.104x + 0.118$, $R^2 = 0.998$) using taurolithocholte (2 nmol) as an internal standard. The detection limit of this method was 0.1 nmol/ml (5 nmol/μmol creatinine). The analytical recoveries were also determined. The Bond Elut C_{18} column was preconditioned with 0.1 M NaOH to increase the absorption of sulfated cholic acids to the resin. The recovery of taurolithocholate-3-sulfate from the Bond Elut C_{18} separation was greater than 85%.

The stabilities of tauro- and glycodihydroxycholate-3-sulfate were also examined using the urine of a patient with biliary atresia who had been treated with ursodeoxycholate. We found huge ion peaks that corresponded to tauro- and glycodihydroxycholate-3-sulfate, but only a small amount of taurolithocholate in this patient's urine (Fig. 1). The secondary bile acids (lithocholate and deoxycholate) are known to be very minor components in neonate urine. Therefore, we decided to use taurolithocholate (2 nmol) as an internal standard in this experiment. The within-day precision (CV%) of taurodihydroxycholate-3-sulfate was 2.6% and of glycodihydroxycholate-3-sulfate was 2.7%. Taurochenodeoxycholate is a main bile acid in the urine of hepatitis patients [5], and taurine is the predominant conjugating moiety of bile acids in infants. Therefore, we mainly focused on detecting the ion of taurodihydroxycholate-3-sulfate (principally taurochenodeoxycholate-3-sulfate).

Using this analytical technique, we measured taurodihydroxycholate-3-sulfate in the urine of 17 patients with cholestasis (4 of them had a citrin deficiency, for which cholestasis is one of the most important clinical symptoms) (Fig. 3). The levels of taurodihydroxycholate-3-sulfate in the urine of cholestatic patients ($n = 13$) was 30.5 ± 23.4 ratio/μmol creatinine (7.3–73.0 ratio/μmol creatinine) and 36.9 ± 32.2 ratio/μmol creatinine (14.3–83.6 ratio/μmol creatinine) in the citrin-deficient patients ($n = 4$), but it was very low in the patients with non-cholestatic hepatic insufficiency ($n = 16$)

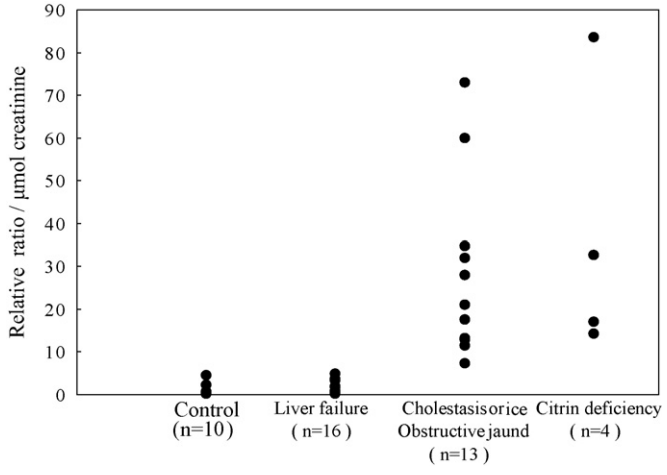

Fig. 3. Urinary taurodihydroxycholate-3-sulfate in patients with chronic liver disease or cholestasis patients measured by ESIMS.

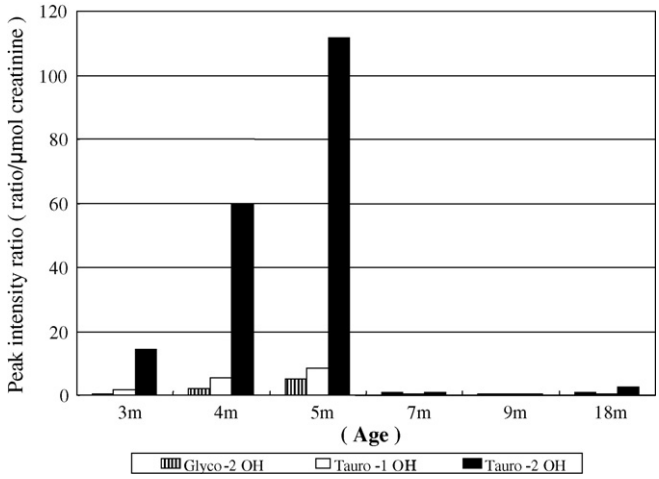

Fig. 4. Changes in the urinary excretion of taurodihydroxycholate-3-sulfate, taurolithocholate-3-sulfate, and glycodihydroxycholate-3-sulfate in one patient with cholestasis. Taurolithocholate (2 nmol) was used as the internal standard. Glyco-2OH, glycodihydroxycholate-3-sulfate; tauro-1OH, taurolithocholate-3-sulfate; tauro-2OH, taurodihydroxycholate-3-sulfate.

(1.3 ± 1.5, 0.1–4.9 ratio/μmol creatinine) and in controls with normal liver function ($n = 10$)(1.1 ± 1.3, 0.2–4.5 ratio/μmol creatinine).

We also investigated the excretion of tauro- and glycodihydroxycholate-3-sulfate in one cholestatic patient with an unknown liver disease, at several ages (Fig. 4). Taurodihydroxycholate-3-sulfate was the predominant bile acid in this patient's urine during cholestasis (at 3-, 4-, and 5-months of age). After recovery from cholestasis (measurements made when the patient was 7, 9, and 18 months old), this compound dramatically decreased. Glycodihydroxycholate-3-sulfate was also increased when this patient was cholestatic, but not as dramatically as the taurodihydroxycholestate-3-sulfate. Urinary taurodihydroxycholate-3-sulfate was always increased in the patient with cholestasis, but the excretion of glycodihydroxycholate-3-sulfate was not parallel with that of taurodihydroxycholate-3-sulfate in examined patients.

Here we found that taurolithocholate can be used as an internal standard for the detection of USBAs in the urine of infants. We also conclude that urinary taurodihydroxycholate-3-sulfate (principally taurochenodeoxycholate-3-sulfate) is an effective marker for cholestasis in infants, and that detection of the binary negative ion of this conjugated compound is very useful in screens for cholestasis and biliary atresia.

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