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Simultaneous characterization of bile acids and their sulfate metabolites in mouse liver, plasma, bile, and urine using LC–MS/MS

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ABSTRACT

Sulfation is a major metabolic pathway involved in the elimination and detoxification of bile acids (BAs). Several lines of evidence are available to support the role of sulfation as a defensive mechanism to attenuate the toxicity of accumulated BAs during hepatobiliary diseases. Individual BAs and their sulfate metabolites vary markedly in their physiological roles as well as their toxicities. Therefore, analytical techniques are required for the quantification of individual BAs and BA-sulfates in biological fluids and tissues. Here we report a simple, sensitive, and validated LC–MS/MS method for the simultaneous quantification of major BAs and BA-sulfates in mouse liver, plasma, bile, and urine. One-step sample preparation using solid-phase extraction (for bile and urine) or protein precipitation (for liver and plasma) was used to extract BAs and BA-sulfates. Base-line separation of all analytes (unsulfated- and sulfated BAs) was achieved in 25 min with a limit of quantification of 1 ng/ml. This LC–MS/MS method was applied to simultaneously quantify BAs and BA-sulfates in both male and female mouse tissues and fluids. Less than 3% of total BAs are present in the sulfate form in the mouse liver, plasma, and bile, which provides strong evidence that sulfation is a minor metabolic pathway of BA elimination and detoxification in mice. Furthermore, we report that the marked female-predominant expression of Sult2a1 is not reflected into a female-predominant pattern of BA-sulfation.

1. Introduction

Bile acids (BAs), the major components of bile, are synthesized in hepatocytes from cholesterol, conjugated with glycine or taurine, and secreted into the small intestine via bile. Cholic acid (CA) and chenodeoxycholic acid (CDCA) are primary BAs in humans, whereas muricholic acid (MCA) and ursodeoxycholic acid (UDCA) are primary BAs in rodents and bears, respectively. The secondary BAs, deoxycholic acid (DCA) and lithocholic acid (LCA), are produced in the intestine by bacterial metabolism of the primary BAs. Most BAs are reabsorbed in the intestine and they undergo efficient enterohepatic recycling.

BAs play important physiological functions in the absorption of fat and fat-soluble vitamins. In addition, BAs are signaling molecules with diverse paracrine and endocrine functions [1]. BAs are ligands for the G-protein coupled bile acid receptor (TGR5) [2], and several nuclear receptors, such as the farnesoid-X-receptor (FXR) [3], pregnane-X-receptor (PXR) [4], constitutive androstane receptor (CAR), and vitamin D receptor (VDR) [5]. However, BAs are also cytotoxic and cancer promoters [6]. A variety of pathologic changes induced by BAs, including cholestasis, bile duct infarction, liver fibrosis, liver cirrhosis, liver and colon cancer were demonstrated in previous studies [6,7].

The physiological and pathophysiological activities of individual BAs vary markedly. For example, the monohydroxyl BA (LCA) is the most toxic BA and a potent PXR ligand [4], whereas CDCA is less toxic and is a potent FXR ligand [8]. Therefore, BA homeostasis and the composition of the BA pool must be tightly controlled to prevent the accumulation of toxic levels, and yet maintain physiological levels of BAs in the liver and extrahepatic tissues.

Sulfation by sulfotransferase 2A1 (SULT2A1) is the dominant metabolic pathway that facilitates BA detoxification and elimination in humans [9], whereas hydroxylation seems to play a major role in BA metabolism in rodents [10]. Sulfation increases BA water solubility, decreases their intestinal absorption, enhances their fecal and urinary excretion, and directly decreases their cytotoxicity by increasing their critical micellar concentrations (CMC). Several lines of evidence provided by

Abbreviations: UPLC, ultra-performance liquid chromatography; LC–MS/MS, liquid chromatography–tandem mass spectrometry; BAs, bile acids; G-BAs, glycine conjugated bile acids; T-BAs, taurine conjugated bile acids; MCA, muricholic acid; CA, cholic acid; UDCA, ursodeoxycholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; LCA, lithocholic acid; IS, internal standard; MeOH, methanol; ACN, acetonitrile; QC, quality control.

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animal and human data suggest that sulfation of BAs is upregulated during hepatobiliary diseases as a compensatory pathway to detoxify and eliminate the accumulated BAs [9]. The role of the alterations in the composition of the BA profile, including BA sulfation, in the progression of hepatobiliary diseases is unknown, largely because of the lack of analytical techniques to selectively quantify individual BAs and their sulfates. Traditionally, enzymatic methods, HPLC-UV, HPLC-fluorescence (HPLC-FL), gas chromatography (GC), GC-mass spectrometry (GC-MS), LC–MS, and LC–MS/MS assays were applied for the qualitative and quantitative analysis of unsulfated BAs [11–16]. Quantitative and qualitative analysis of unsulfated BAs have been recently reviewed [17].

The simultaneous analysis of individual BAs and BA-sulfates in biological samples has always presented technical difficulties due to their structural similarity, their relatively low concentrations, and the presence of isomeric forms. Older methods for the quantification of BA-sulfates required the isolation of BA-sulfate fractions and desulfation of BA-sulfates enzymatically by sulfatases or chemically by solvolysis under acidic or basic conditions, followed by the quantification of the resulting unsulfated BAs using enzymatic methods, HPLC-UV, HPLC-FL, GC, or GC-MS. The enzymatic method of assaying total BA-sulfates by desulfating BA-sulfates using a sulfatase enzyme, followed by colorimetric detection of the resulting unsulfated BAs using 3-β-hydroxysteroid dehydrogenase (β-HSD) is a widely used method, which is commercially available as a kit. All these methods provided valuable data, which deepened our understanding of the various roles of BAs in biological systems. However, every one of these techniques had limitations including: indirect rather than direct analyses are performed, total rather than individual BA-sulfates are quantified, and contamination and selectivity issues associated with the fractionation and desulfation steps are encountered.

Because of the disadvantages associated with the indirect analysis of BA-sulfates, HPLC-UV methods, which bypassed the isolation of sulfated BA fractions and BA-sulfate deconjugation, was developed for direct BA and BA-sulfate analysis [18]. However, HPLC-UV does not provide the required sensitivity and selectivity to detect the trace amount of some BAs or BA-sulfates in biological matrices. The persistent need for rapid and sensitive methods has motivated the efforts to exploit the high sensitivity, specificity, and simple sample preparation requirements of HPLC–mass spectrometry (LC–MS and LC–MS/MS) for the direct analysis of BA-sulfates. Several methods for the direct quantification of BA-sulfates were developed using HPLC-single stage-mass spectrometry (LC–MS) [16]. These methods provide high sensitivity compared to other UV-based methods, but they do not take advantage of the even higher sensitivity and selectivity provided by tandem mass spectrometry (MS/MS). A LC–MS/MS method was recently developed to quantify BA-sulfates directly in human urine [19]. This method, however, was not designed for the simultaneous quantification of individual BAs and BA-sulfates. As a result, a second chromatographic run is required to quantify unsulfated BAs. Therefore, here we report the first validated LC–MS/MS method for the simultaneous quantification of individual BAs and their sulfate conjugates in mouse tissues and fluids.

In summary, there is currently no simple, sensitive, direct, and valid method available to simultaneously quantify individual BAs and their sulfates in various tissues and fluids. In this study, we present a LC–MS/MS method for the simultaneous quantification of 32 unsulfated and sulfated BAs in mouse liver, plasma, bile, and urine. This sensitive and reliable method was validated to have high precision and accuracy (<15%) and was applied to simultaneously quantify unsulfated and sulfated BAs in mouse fluids and tissues.

2. Experimental

2.1. Chemicals and reagents

Cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), ursodeoxycholic acid (UDCA), tauro-cholic acid (T-CA), tauro-chenodeoxycholic acid (T-CDCA), tauro-deoxycholic acid (T-DCA), tauro-lithocholic acid (T-LCA), tauro-ursodeoxycholic acid (T-UDCA), glyco-cholic acid (G-CA), glyco-chenodeoxycholic acid (G-CDCA), glyco-deoxycholic acid (G-DCA), glyco-lithocholic acid (G-LCA), and glyco-ursodeoxycholic acid (G-UDCA) were purchased from Sigma–Aldrich (St. Louis, MO). β-Muricholic acid (MCA) and tauro-β-muricholic acid (T-MCA) were purchased from Steraloids, Inc. (Newport, Rhode Island). LCA-sulfate and activated charcoal were also obtained from Sigma–Aldrich. UDCA-sulfate, CDCA-sulfate, DCA-sulfate, CA-sulfate, their G- and T-conjugates, as well as G-LCA-sulfate and T-LCA-sulfate were generously provided by Dr. Junichi Goto, Tohoku University, Aoba-ku, Japan. $^{2}H_{4}$-G-CDCA was purchased from C/D/N Isotopes, Inc. (Pointe-Claire, Quebec, Canada). HPLC-grade methanol, acetonitrile, water, ammonium acetate, ammonium formate, ammonium hydroxide, formic acid, and acetic acid were obtained from Fisher Scientific (Fair Lawn, NJ).

2.2. Instrumentation

A Waters ACQUITY ultra performance liquid chromatography (UPLC) system (Waters, Milford, MA) coupled to an Applied Biosystem 4000 Q TRAP® quadrupole linear ion trap hybrid mass spectrometer with an electrospray ionization (ESI) source (Applied Biosystems, MDS Sciex, Foster City, CA) was used throughout. The UPLC and MS systems are controlled by Empower Pro 6.0 and Analyst 1.4.2 software, respectively. All chromatographic separations were performed with an ACQUITY UPLC®BEH Phenyl column (1.7 μm, 150 mm × 2.1 mm) equipped with an ACQUITY UPLC C_{18} guard column (Waters, Milford, MA).

2.3. Liquid chromatographic and mass spectrometric conditions

The mobile phase consisted of methanol (MeOH) (mobile phase A) and 7.5 mM ammonium acetate, adjusted to pH 7.0 using 10 M ammonium hydroxide (mobile phase B), at a total flow rate of 0.3 ml/min. The gradient profile was held at 52.5% mobile phase A for 12.75 min, increased linearly to 68% mobile phase A in 0.25 min, held at 68% for 9 min, and brought back to 52.5% in 0.25 min followed by 3-min re-equilibration. The injection volume of all samples was 10 μl.

The mass spectrometer parameters, such as temperature, voltage, gas pressure, etc., were optimized by infusing each analyte and the internal standard (IS) using a 10 μg/ml solution in 50% MeOH via a Harvard '22' standard infusion syringe pump (Harvard Apparatus, South Natick, MA, USA). All BAs and their respective sulfates were detected in the negative ionization mode with the following mass spectrometer source settings: ion spray voltage, −4000 V; source temperature, 600 °C, curtain gas, 20 AU; gas-1, 35 AU, gas-2, 35 AU, collision gas pressure, high; Q1/Q3 resolution, high; and interface heater, on. The multiple reaction monitoring (MRM) transitions for each analyte and IS, as well as their respective optimum MS parameters, such as declustering potential (DP), collision energy (CE), and cell exit potential (CXP), are shown in Table 1.

2.4. Preparation of standard solutions and calibration curves

The stock solutions of individual unsulfated BAs and IS were prepared in MeOH at a concentration of 10 mg/ml, whereas

Table 1
MRM transitions and MS parameters for the BA and BA-sulfate LC–MS/MS analysis.

Analyte	MRM transition	Declustering potential (V)	Collision energy (eV)	Cell exit potential (V)
LCA-S	455.2 → 96.8	−120	−90	−15
UDCA-S	471.2 → 96.8	−100	−90	−15
CDCA-S	471.2 → 96.8	−100	−90	−15
DCA-S	471.2 → 96.8	−100	−90	−15
CA-S	487.2 → 96.8	−120	−100	−15
G-LCA-S	512.2 → 432.0	−95	−42	−10
G-UDCA-S	528.2 → 448.0	−95	−42	−10
G-CDCA-S	528.2 → 448.0	−95	−42	−10
G-DCA-S	528.2 → 448.0	−95	−42	−10
G-CA-S	544.2 → 464.0	−95	−42	−11
T-LCA-S	280.6 → 96.8	−70	−42	−14
T-UDCA-S	288.6 → 96.8	−70	−42	−14
T-CDCA-S	288.6 → 96.8	−70	−42	−14
T-DCA-S	288.6 → 96.8	−70	−42	−14
T-CA-S	296.6 → 96.8	−70	−42	−7
LCA	375.2 → 375.2	−130	−30	−7
UDCA	391.2 → 391.2	−130	−30	−7
CDCA	391.2 → 391.2	−130	−30	−7
DCA	391.2 → 391.2	−130	−30	−7
CA	407.2 → 407.2	−130	−30	−1
MCA	407.2 → 407.2	−130	−30	−1
G-LCA	432.2 → 74.0	−120	−68	−4
G-UDCA	448.2 → 74.0	−115	−70	−1
G-CDCA	448.2 → 74.0	−115	−70	−1
G-DCA	448.2 → 74.0	−115	−70	−1
G-CA	464.2 → 74.0	−120	−77	−1
G-MCA	464.2 → 74.0	−120	−77	−1
T-LCA	482.2 → 79.8	−165	−125	−1
T-UDCA	498.2 → 79.8	−165	−125	−1
T-CDCA	498.2 → 79.8	−165	−125	−1
T-DCA	498.2 → 79.8	−165	−125	−1
T-CA	514.2 → 79.8	−165	−125	−1
T-MCA	514.2 → 79.8	−165	−125	−1
IS	452.2 → 74.0	−65	−70	−1

1 mg/ml stock solutions of individual sulfated BAs were prepared in water:MeOH (1:1).

Liver, plasma, bile, and urine were collected and each pooled from 6 untreated mice. Livers were homogenized in deionized water (1:2, w/v) and bile was 100-fold diluted using deionized water. Homogenized liver, plasma, diluted bile, and urine were incubated with 100 mg/ml activated charcoal for 2 h to strip these matrices of endogenous BAs. Mixtures were centrifuged at 13,000 × *g* for 10 min, and the supernatants were filtered. The filtrates of these stripped matrices were used to construct the calibration curves, each in the corresponding biological matrix to be analyzed. Fixed volumes of these stripped matrices were spiked with 10 μl of the appropriate standard solution containing IS to construct a calibration curve with the range of 1–1000 ng/ml for all unsulfated and sulfated BAs. The concentration of the $^{2}H_{4}$-G-CDCA internal standard (IS) was 500 ng/ml.

2.5. Sample preparation

The sample preparation procedure was based on our previous method for the analysis of unsulfated BAs with slight modification [15]. For plasma samples, 1 ml of ice-cold alkaline ACN (5% NH_4OH in ACN) was added to 100 μl plasma-spiked with 10 μl IS, vortexed, and centrifuged at 16,000 × *g* for 10 min. The supernatant was aspirated, evaporated under vacuum, and reconstituted in 100 μl of 50% MeOH. For liver samples, approximately 100 mg of liver was homogenized in 2 volumes of H_2O. A 100 μl of liver homogenate was spiked with 10 μl IS and 2 ml of ice-cold alkaline ACN was added. Samples were vortexed and shaked continuously for 30 min, and then centrifuged at 16,000 × *g* for 10 min. The supernatant was aspirated and the pellet was extracted with another 1 ml of ice-cold alkaline ACN. Supernatants from the 2 extraction steps were pooled, evaporated, and reconstituted in a 100 μl of 50% MeOH.

For bile samples, Supelclean™ LC-18 SPE cartridges (Sigma–Aldrich, St. Louis, MO) were used for sample extraction. Bile samples were diluted 20- and 2000-fold with deionized water, a 100 μl of diluted bile samples was spiked with 10 μl IS, vortexed, and loaded onto SPE cartridges pre-conditioned with 2 ml MeOH, followed by 2 ml H_2O. Loaded cartridges were washed with 2 ml H_2O and eluted with 4 ml MeOH. Urine samples (500 μl) were spiked with 10 μl of IS and prepared similarly to bile samples. For all samples, the elute was evaporated under vacuum at room temperature and reconstituted in 100 μl of 50% MeOH.

Extraction recoveries were determined for each quality control (QC) point in each matrix from the ratio of the analyte peak area in samples spiked before extraction compared to the corresponding peak area in untreated samples prepared in neat solution.

2.6. Method validation

All calibration curves were prepared in matrices striped from endogenous BAs by treatment with activated charcoal and $1/x^2$ weighting scheme was used for all calibration curves. The method was validated using 5 QC points for each calibration curve and the concentrations of the QC points were 1, 2, 50, 500, and 1000 ng/ml for all unsulfated and sulfated BAs. Five replicates of each QC point were analyzed each day to determine the intra- and inter-day accuracy and precision. This process was repeated 3 times over 3 days in order to determine the inter-day accuracy and precision using freshly prepared calibration curves. Intra-day accuracy and precision were calculated from the % bias [% (Measured − Theoretical)/Measured concentrations] and relative standard deviation [% RSD = % Standard Deviation/Mean], respectively, for the 5 replicates of each QC point. Inter-day accuracy and precision were calculated similarly using the 15 replicates of each QC point from the three validation runs.

2.7. Animal studies

Eight-week-old C57BL/6 male and female mice were purchased from Charles River Laboratories Inc (Wilmington, MA). Mice were fed Laboratory Rodent Chow W (Harlan Teklad, Madison, WI) ad libitum, and housed in laboratory animal facility at the University of Kansas Medical Center according to the American Animal Association Laboratory Animal Care guidance. All protocols and procedures were approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center. To obtain urine samples, 6 male and 6 female mice were placed in metabolic cages and urine was collected for 24 h. The same set of mice was anesthetized using i.p. ketamine (100 mg/kg)/midazolam (5 mg/kg) and the common bile duct was cannulated with a 30-gauge needle attached to PE-10 tubing. Bile was collected from the cannula for 90 min at 15-min intervals. Another 6 male and 6 female mice were anesthetized, a PE-10 cannula was placed in the carotid artery, and approximately 700 μl of blood was collected into heparanized tubes from each mouse. Blood samples were centrifuged at 1000 × *g* for 5 min, and plasma was collected. Livers were harvested from the same animals, gallbladders were removed, and livers were washed with saline, dried, frozen in liquid nitrogen, and stored at −80 °C until analysis.

2.8. Statistical analysis

Data are presented as mean ± standard error of the mean (S.E.M). Statistical differences between male and female mice were determined by a two-tailed Student's *t* test ($P < 0.05$).

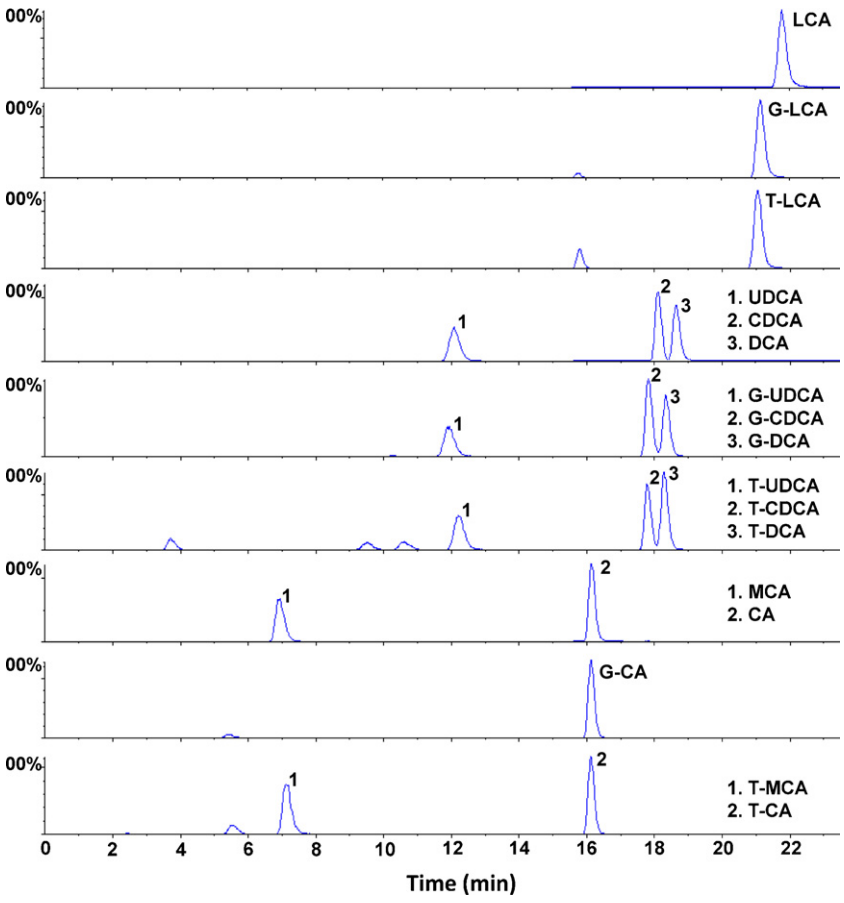

Fig. 1. Representative LC–MS/MS chromatogram of the authentic standards of 17 BAs under the final chromatography and detection conditions.

3. Results and discussion

3.1. Method development

Common techniques including enzymatic methods and chromatographic techniques have been used for the assay of BA-sulfates in biological matrices. However, this method has limited sensitivity and is linear only in the range of 2.5–100 μM and total BAs, rather than individual BAs, is quantified. In addition, this method is not selective for the detection of BA-sulfates because the sulfatase enzyme used has affinity to other hydroxyl steroid-sulfates such as eipandrosterone-3-sulfate. Therefore, chromatographic techniques with UV, FL, or MS detection were developed to quantify individual BAs and BA-sulfates. Individual BA-sulfates could be quantified indirectly using these methods after desulfation by solvolysis under acidic or basic conditions with heat. BA-sulfate concentration is then indirectly calculated from the difference in the concentration of individual BAs before and after solvolysis. In addition, individual BA-sulfates could be determined by isolating a sulfate fraction during sample preparation using ion exchange solid phase extraction or thin-layer chromatography (TLC) [20]. The most polar fraction, which primarily contained BA-sulfates, were then separated and quantified using HPLC-UV, HPLC-FL or GC-MS. A major drawback associated with fraction collection is that the collected BA-sulfate fractions were shown to be contaminated with other BA-species such as glucuronidated and unsulfated BAs. Furthermore, degradation of BAs and incomplete desulfation of BA-sulfates during the solvolysis step were demonstrated [21].

As discussed earlier, LC–MS/MS provides the required sensitivity and selectivity for the quantification of BAs and BA-sulfates in biological samples. Therefore, we report the first validated LC–MS/MS method for the simultaneous and direct quantification of individual BAs and their sulfate conjugates in mouse tissues and fluids. Table 1 summarizes the precursor and the product m/z values, as well as the MS parameters for each analyte. All BAs and BA-sulfates were analyzed using the negative ionization mode. The fragmentation pattern of BA and BA-sulfates was consistent with previous reports [15,19]. The steroid backbone of BAs is very resistant to collision induced dissociation (CID), therefore the same mass was monitored for both precursor and product ions of nonamidated BAs [22]. SO_3^- (m/z = 79.8) and $C_2H_4NO_2^-$ (m/z = 74.0) were the major fragments for T-BAs and G-BAs, respectively. HSO_4^- (m/z = 96.8) was the major product ion of nonamidated BA-sulfates and T-BA-sulfates, whereas ions resulting from the loss of 80 $[M-HSO_3]^-$ were the most abundant product ions of G-BA-sulfates. For G- and T-BA-sulfates, both the doubly charged precursor ions $[M-2H]^{-2}$ and the singly charged precursor ions $[M-H]^-$ were detected. The doubly charged ions had one-eighth the intensity of the singly charged ions for G-BA-sulfates, whereas the intensity of the doubly charged ions for T-BA-sulfates was 1.5-fold higher than that of the singly charged ions. Therefore, the singly charged and doubly charged ions were selected as the precursor ions of G-BA-sulfates and T-BA-sulfates, respectively. The ratio between doubly charged ions and singly charged ions was highly dependent on the pH of the mobile phase. For T-BA-sulfates, the ratio of doubly/singly charged ions increased with increasing the mobile phase pH. The corresponding ratios for G-BA-sulfates were insensitive to changes in the mobile phase pH in the range of 3–7, whereas the ratio decreased with further increases of mobile phase pH. These results are consistent with findings reported in a previous study [19].

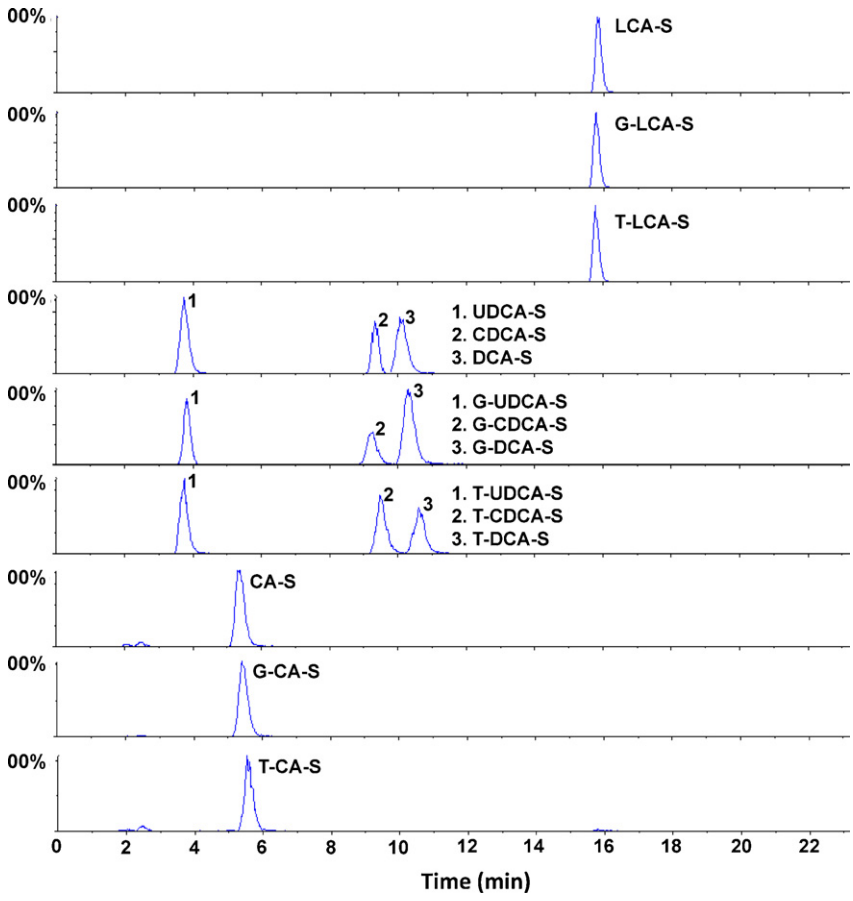

Fig. 2. Representative LC–MS/MS chromatogram of the authentic standards of 15 BA-sulfates under the final chromatography and detection conditions.

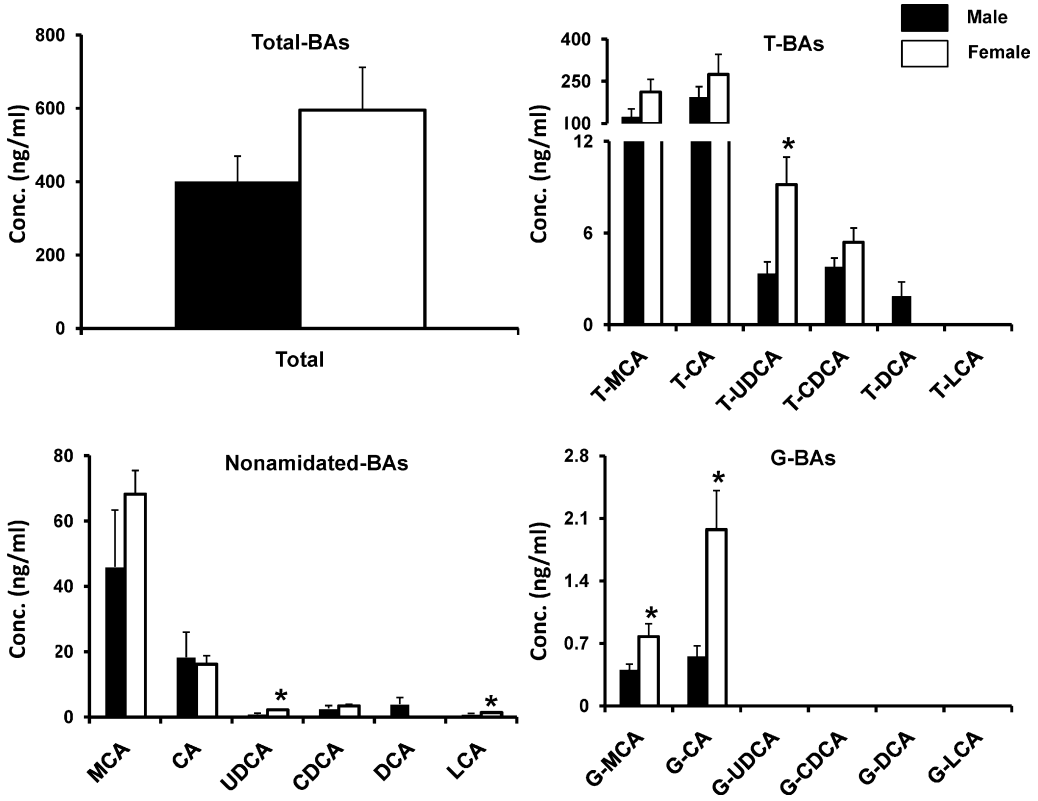

Fig. 3. BA concentration in male and female mouse plasma. The results are shown as mean ± S.E.M ($N = 6$). (*) Statistically significant differences between male and female groups ($P < 0.05$).

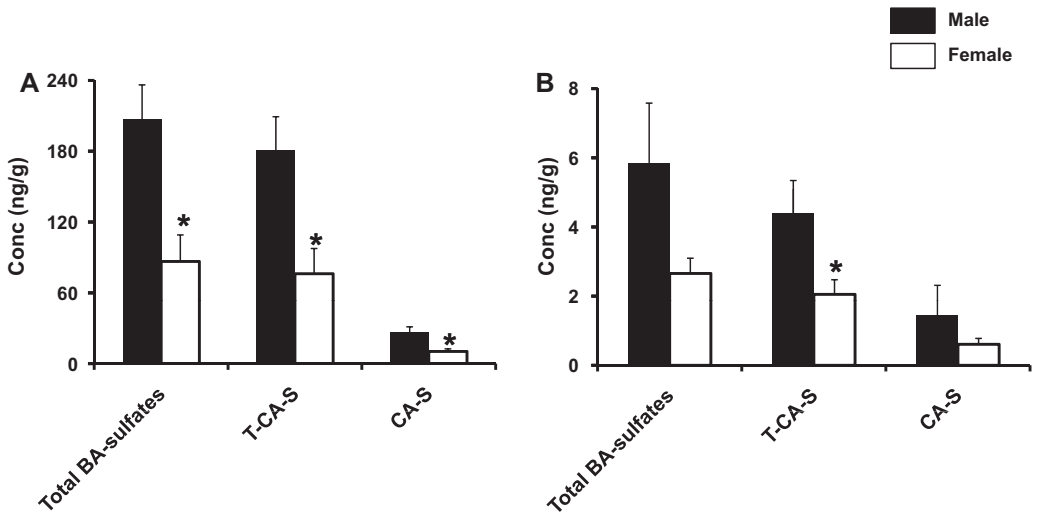

Fig. 4. BA-sulfate concentration in male and female mouse (A) liver and (B) plasma. The results are shown as mean ± S.E.M ($N = 6$). (*) Statistically significant differences between male and female groups ($P < 0.05$).

We investigated several chromatographic conditions, which were previously used for the separate quantification of BAs or BA-sulfates [15,19]. However, none of these conditions was able to simultaneously resolve all the isomeric BA and BA-sulfate forms in all four analyzed matrices. This might be due to the complex nature of these matrices such as liver tissues, which requires different chromatographic conditions than those used for the analysis of other common biological matrices such as plasma or urine. Using our chromatographic conditions, a baseline separation of all isomeric BAs and BA-sulfates in all four matrices was achieved in 25 min (Figs. 1 and 2). α, β and ω isomers of MCA and T-MCA could be separated using less organic in the mobile phase, but the run time becomes longer than 40 min (data not shown). Therefore, total concentration of three isomers for MCA and T-MCA was quantified instead of individual concentration of α, β and ω isomers. All BA-sulfates eluted earlier than the corresponding unsulfated BAs due to their higher hydrophilicity. BA-sulfates eluted in the same order as their corresponding unsulfated BAs. The retention behavior of the unsulfated BAs has been discussed in our previous publication [15].

Table 2
Extraction recoveries of BAs and their sulfates at 50 ng/ml in mouse fluids and tissues.

	Liver	Bile	Plasma	Urine
BAs				
G-CA	83.4 ± 7.1	87.6 ± 8.3	83.9 ± 5.6	93.8 ± 3.9
G-UDCA	90.1 ± 3.4	84.3 ± 10.3	86.6 ± 5.4	87.7 ± 5.1
G-CDCA	82.4 ± 7.5	87.1 ± 9.8	82.7 ± 6.9	89.6 ± 4.5
G-DCA	82.2 ± 5.3	82.8 ± 8.8	92.7 ± 9.3	84.4 ± 6.6
G-LCA	89.8 ± 9.9	79.5 ± 6.0	90.8 ± 12.0	83.0 ± 4.8
T-MCA	83.0 ± 6.5	86.7 ± 6.0	82.5 ± 15.6	86.9 ± 5.2
T-CA	86.0 ± 9.0	84.9 ± 9.2	91.9 ± 15.7	87.3 ± 4.8
T-UDCA	87.1 ± 11.8	83.0 ± 10.2	88.7 ± 4.7	83.3 ± 6.1
T-CDCA	90.3 ± 14.2	76.1 ± 8.7	93.1 ± 3.7	80.9 ± 4.7
T-DCA	81.5 ± 5.2	83.5 ± 6.1	87.3 ± 3.3	83.9 ± 5.5
T-LCA	85.5 ± 8.5	87.6 ± 6.8	94.1 ± 12.6	81.1 ± 11.9
MCA	90.3 ± 11.7	88.1 ± 7.1	87.5 ± 3.8	91.3 ± 4.4
CA	96.5 ± 8.1	84.7 ± 8.5	90.5 ± 7.1	94.4 ± 3.9
UDCA	93.0 ± 12.6	87.1 ± 6.4	91.5 ± 9.4	88.2 ± 9.3
CDCA	88.8 ± 6.0	90.0 ± 10.5	96.1 ± 7.8	84.8 ± 5.7
DCA	89.1 ± 11.4	85.6 ± 6.6	86.3 ± 4.7	92.8 ± 4.2
LCA	88.2 ± 3.9	87.0 ± 11.1	79.9 ± 13.6	82.3 ± 9.5
BA-S				
G-CA-S	80.8 ± 4.4	78.8 ± 5.7	88.0 ± 5.0	85.3 ± 7.3
G-UDCA-S	81.9 ± 6.2	80.8 ± 6.2	85.3 ± 10.3	80.4 ± 6.1
G-CDCA-S	82.0 ± 6.6	81.3 ± 6.6	81.5 ± 9.1	76.3 ± 5.4
G-DCA-S	91.0 ± 5.6	78.0 ± 8.7	85.4 ± 4.5	84.4 ± 6.6
G-LCA-S	88.5 ± 14.8	84.1 ± 5.4	83.6 ± 12.1	82.5 ± 5.1
T-CA-S	81.2 ± 12.5	81.0 ± 8.2	82.0 ± 10.3	83.2 ± 6.7
T-UDCA-S	83.9 ± 14.6	85.4 ± 6.5	79.0 ± 7.2	86.6 ± 15.8
T-CDCA-S	83.4 ± 8.9	77.6 ± 5.9	80.2 ± 11.3	83.3 ± 5.9
T-DCA-S	87.5 ± 7.8	84.6 ± 7.5	79.9 ± 11.8	88.1 ± 6.9
T-LCA-S	87.6 ± 10.7	83.4 ± 9.0	81.1 ± 6.1	82.0 ± 9.5
CA-S	87.1 ± 4.6	86.0 ± 8.0	84.6 ± 5.2	82.0 ± 11.7
UDCA-S	88.6 ± 5.5	86.2 ± 4.8	81.2 ± 7.9	82.4 ± 10.2
CDCA-S	85.8 ± 7.5	88.3 ± 11.4	82.7 ± 11.4	81.3 ± 5.9
DCA-S	86.7 ± 6.5	85.0 ± 10.2	90.0 ± 12.4	81.7 ± 8.7
LCA-S	99.8 ± 9.2	89.0 ± 7.5	86.2 ± 8.2	86.4 ± 3.1

We have previously reported the effect of mobile phase pH and composition on the chromatographic behavior of unsulfated BAs [15]. The chromatographic behavior of sulfated BAs in this study was affected similarly. Briefly, decreasing the pH of the mobile phase markedly increased the retention of G- and non-amidated BA-sulfates, whereas the retention of T-BA-sulfates was marginally affected by changes in pH of the mobile phase. In addition, ionization efficiency, and therefore signal intensity, of BAs and BA-sulfates were also strongly dependent on mobile phase pH. Ammonium acetate buffer at pH 4 and pH 8 yielded the highest MS signal intensity for BAs and BA-sulfates, respectively. At optimum pH for BA-sulfates detection, pH 8, the signal intensity was markedly compromised for unsulfated BAs. In addition, chromatographic separation was not achieved for all analytes at basic pHs. Therefore, ammonium acetate buffer at pH 7 was selected, which provided the required resolution to separate all analytes of interest without a significant compromise of the signal intensity of either sulfated or unsulfated BAs.

Several SPE, protein-precipitation, and liquid–liquid extraction techniques were investigated to maximize extraction recovery and minimize suppression effect from the various matrices. Compared to our previous study, relatively high recoveries of most BA and BA-sulfate species in bile and urine matrices were achieved using Supelclean LC-18 SPE cartridge. In contrast, protein precipitation using alkaline-ACN for liver and plasma samples yielded the highest recovery of both BAs and BA-sulfates in these matrices [15]. Table 2 shows the extraction recoveries of all BAs and BA-sulfates at 50 ng/ml in mouse liver, bile, plasma, and urine. Extraction recoveries were consistent between the 5 QC concentrations (data only shown at 50 ng/ml) and were higher than 70% for all analytes in all four matrices.

Table 3
Summary of the inter-day accuracy and precision for BAs and BA-sulfates (BA-S) in mouse plasma.

Nominal Conc.	QC1		QC2		QC3		QC4		QC5	
	1 (ng/ml)	%R.S.D.	2 (ng/ml)	%R.S.D.	50 (ng/ml)	%R.S.D.	500 (ng/ml)	%R.S.D.	1000 (ng/ml)	%R.S.D.
BAs										
G-CA	1.02	10.89	2.06	5.36	49.56	1.44	494.4	2.11	987.8	2.27
G-UDCA	0.99	4.61	1.97	2.72	49.62	1.29	489.0	2.92	999.2	1.64
G-CDCA	1.06	6.40	2.14	5.99	50.86	1.06	484.0	0.99	938.4	1.13
G-DCA	1.06	6.32	2.12	5.34	49.98	2.21	500.2	1.43	996.2	2.00
G-LCA	1.04	2.76	1.94	2.53	51.76	1.93	515.6	2.55	993.0	1.57
T-MCA	0.99	9.85	1.88	5.09	47.82	2.39	483.4	1.92	1054.0	2.56
T-CA	0.91	10.02	1.79	5.55	46.40	2.49	504.8	1.89	1102.0	1.18
T-UDCA	1.00	4.12	1.85	3.29	48.38	1.72	497.6	2.68	1076.0	2.51
T-CDCA	1.08	3.53	1.93	3.23	46.74	1.61	492.0	1.29	1056.0	1.44
T-DCA	1.02	2.53	1.94	2.40	47.54	1.69	510.2	1.80	1102.0	2.07
T-LCA	1.06	4.03	1.93	2.50	48.42	1.69	506.8	2.02	1072.0	2.91
MCA	1.01	4.53	1.91	4.99	51.30	2.06	494.8	2.49	976.0	1.93
CA	1.00	5.11	1.89	1.33	52.78	1.56	507.4	2.41	954.2	1.53
UDCA	1.06	5.58	2.00	3.99	50.98	2.33	508.6	2.33	990.4	1.99
CDCA	1.01	6.35	2.04	5.96	49.32	3.86	546.6	2.49	984.6	1.48
DCA	1.14	11.29	2.16	8.37	52.26	4.26	493.2	1.31	926.2	1.95
LCA	1.02	1.28	2.08	7.17	45.36	1.78	566.4	2.15	1094.0	2.38
BA-S										
G-CA-S	1.02	5.41	1.90	3.23	51.92	4.75	509.2	2.75	1004.0	2.60
G-UDCA-S	1.01	7.28	1.93	4.71	50.06	3.54	504.8	2.21	1025.0	2.07
G-CDCA-S	0.96	3.32	1.96	7.30	49.66	3.57	503.0	2.20	1022.6	2.24
G-DCA-S	1.04	3.79	1.95	3.25	49.98	1.20	519.6	3.42	1118.0	2.32
G-LCA-S	1.02	7.44	2.02	2.65	46.86	2.25	489.8	6.65	1015.4	6.78
T-CA-S	0.97	5.32	2.00	8.66	52.76	3.03	488.2	6.22	970.8	1.96
T-UDCA-S	1.00	2.91	1.94	6.77	51.70	6.99	489.2	7.22	1058.0	4.30
T-CDCA-S	1.01	6.95	2.07	7.37	50.50	5.31	445.2	10.58	934.0	4.51
T-DCA-S	0.95	7.73	2.01	6.38	55.14	1.98	449.0	7.04	855.6	7.04
T-LCA-S	0.98	3.65	2.07	7.99	47.60	3.00	490.8	4.24	1017.4	4.15
CA-S	1.03	6.42	2.02	8.80	50.68	2.91	512.4	2.44	1030.0	2.48
UDCA-S	1.03	4.22	2.09	5.81	48.66	0.88	494.8	0.77	1060.0	1.63
CDCA-S	1.09	7.95	2.15	4.25	49.46	0.97	506.8	4.05	1032.0	1.59
DCA-S	1.09	5.96	2.11	3.22	49.70	1.00	509.0	4.07	1036.0	2.00
LCA-S	0.96	7.23	1.93	4.67	49.00	2.47	517.8	3.80	1020.6	2.31

Similar to our approach for the analysis of unsulfated BAs, we have constructed all analyte calibration curves in matrices treated with activated charcoal. Endogenous BAs and BA-sulfates are adsorbed to the activated charcoal particles producing analyte-free matrices, which can be used to prepare calibration curves in the same matrices to be analyzed.

3.2. Method validation

To ensure the method reliability and reproducibility for BA and BA-sulfate analysis, intra-day and inter-day accuracy and precision were determined using 5 QC concentrations distributed throughout the calibration range for each analyte in each matrix. The intra-day accuracy and precision for all analytes in plasma was less than 11% at all concentration levels (data not shown). The inter-day accuracy and precision for all analytes in plasma were less than 12% (Table 3). Similarly, the intra- and inter-day accuracy and precision obtained in other matrices (liver, bile, and urine) were also less than 15% (data not shown). The assay was linear over the tested concentration range of 1–1000 ng/ml for all analytes in all four matrices, and the limit of detection for the individual unsulfated and sulfated BAs ranged from 0.2 to 0.5 ng/ml. The correlation coefficients for all the standard calibration curves of unsulfated and sulfated BAs were higher than 0.992. Furthermore, the stability of stock solutions under storage conditions and the stability of extracted biological samples in the autosampler were tested. BAs and their sulfates were stable for at least 3 months in the −20 °C freezer, and 36 hrs in the 4 °C autosampler (data not shown).

3.3. Mouse BA and BA-sulfate profiles

BA and BA-sulfate profiles in male and female mouse liver, bile, plasma, and urine were characterized using this LC–MS/MS method. In accordance with previous reports, BAs are mainly present in the T-amidated form in both male and female mice (91.4%, 99.9%, 84.9%, and 73.7% in liver, bile, plasma, and urine, respectively) [15]. In addition, no gender differences were observed in the percentage of BAs present in T-amidated BA form. In the liver, the concentration of unsulfated nonamidated BAs was higher in females than males livers, whereas no gender differences in the hepatic concentration of total, G-, and T-BAs were observed (data not shown). Total and individual unsulfated BA concentrations in male and female mouse plasma are shown in Fig. 3, which is in good agreement with data we reported previously [15]. Marked gender differences were observed in five individual BAs including T-UDCA, UDCA, LCA, G-MCA, and G-CA ($P < 0.05$). The concentration of T-UDCA, UDCA, LCA, G-MCA, and G-CA were 1.6- to 3.6-fold higher in female than male mice (Fig. 3).

Nonamidated CA-and T-CA-sulfates were the only sulfated BAs detected in the mouse liver and plasma and they both had a higher concentration in males than females (Fig. 4). In addition, the percentages of CA and T-CA present in the sulfated form in male liver (2.0% and 0.6%) and in male plasma (8.1% and 2.1%) were 3- to 4-fold higher than those in females (0.6% and 0.15%) in liver and (2.6% and 0.8%) in plasma. In bile, less than 1.2% and 0.3% of total BAs were sulfated in male and female mice, respectively. The biliary excretion profiles of BA-sulfates are shown in Fig. 5. The percentages of individual BAs excreted in the sulfated form in male and

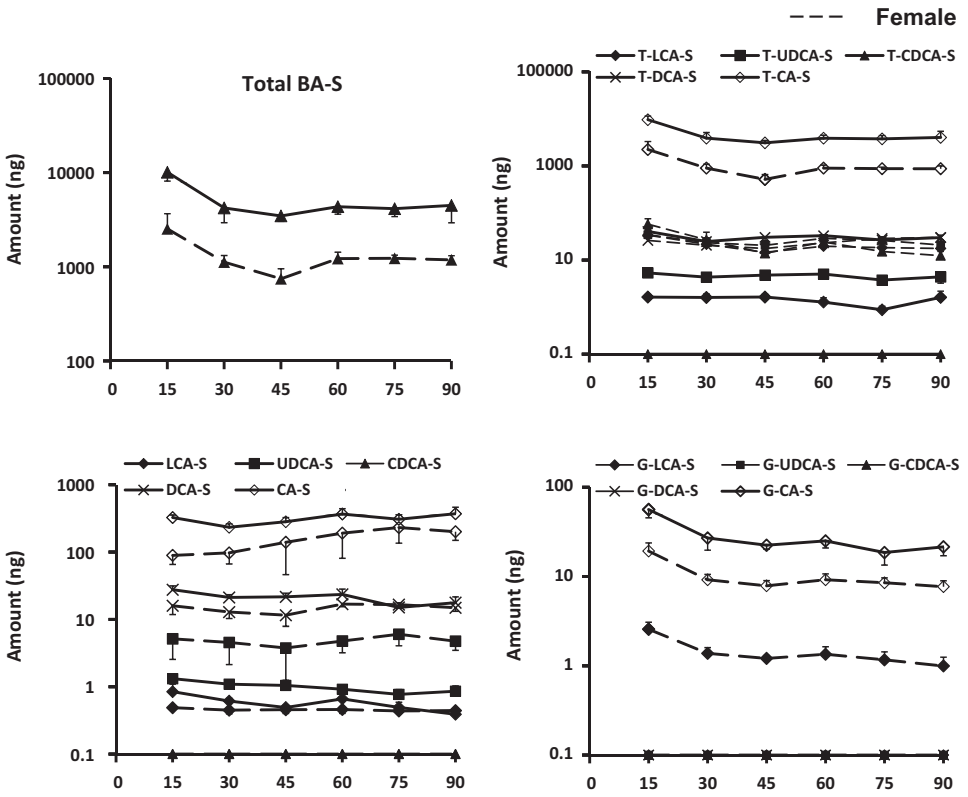

Fig. 5. BA-sulfate excretion in male and female mouse bile. The results are shown as mean ± S.E.M ($N = 6$).

female mouse bile and urine are summarized in Table 4. In bile, the percentages of CA and DCA sulfation are male predominant, whereas the percentages of UDCA and CDCA sulfation are female predominant. The percentage of BAs present in the sulfated form was inversely proportional to the number of hydroxyl groups of bile acids. The monohydroxy- and most toxic bile acid (LCA) was 10.0% and 16.1% sulfated in male and female mouse bile, respectively, followed by the dihydroxy- and toxic DCA (1.3% and 0.4% sulfated), whereas the trihydroxy- and least toxic bile acid (CA) was only1.2% and 0.2% sulfated. The marked differences in the extent to which individual BAs are sulfated can be explained by their different affinities to sulfation by SULT2A1, as we have recently shown [23].

In our previous study we were not able to detect BAs in urine [15] and only trace amounts of total BAs were detected in mouse urine under normal liver conditions by others [16]. However, in the present study, urine samples were concentrated 5-fold, and therefore, we were able to detect seven BA and six BA-sulfate species in mouse urine. In contrast to the small proportions of BA present in the sulfate form in bile, liver, and plasma, the total concentration of BA-sulfates in male and female mouse urine is equal or higher than that of unsulfated BAs (Fig. 6). This indicates the efficiency

Table 4
The percentage of individual BAs excreted in the sulfated form in mouse bile and urine.

Bile								
BAs	Nonamidated		Glycine conjugated		Taurine conjugated		Total	
	Male	Female	Male	Female	Male	Female	Male	Female
CA	83.9 ± 3.7	74.2 ± 5.8	22.9 ± 2.5	8.5 ± 0.6*	1.1 ± 0.2	0.2 ± 0.03*	1.2 ± 0.2	0.2 ± 0.03*
UDCA	63.1 ± 4.9	82.0 ± 5.7*	–	–	0.1 ± 0.01	0.2 ± 0.03*	0.1 ± 0.01	0.3 ± 0.05*
CDCA	–	–	–	–	–	0.1 ± 0.03*	–	0.1 ± 0.03*
DCA	99.2 ± 0.6	100.0 ± 0.0	–	–	0.7 ± 0.1	0.2 ± 0.03*	1.3 ± 0.2	0.4 ± 0.05*
LCA	100.0 ± 0.0	100.0 ± 0.0	–	100.0 ± 0.0*	6.8 ± 1.5	14.3 ± 3.0	10.0 ± 2.1	16.1 ± 3.0
Total	84.6 ± 3.6	77.1 ± 5.3	21.0 ± 2.3	8.3 ± 0.5*	1.1 ± 0.2	0.2 ± 0.03*	1.2 ± 0.2	0.2 ± 0.03
Urine								
CA	81.7 ± 6.4	48.5 ± 9.7*	–	–	74.7 ± 4.6	45.9 ± 12.3	82.3 ± 2.2	46.9 ± 11.1*
UDCA	100.0 ± 0.0	100.0 ± 0.0	–	–	87.2 ± 3.4	–*	88.6 ± 3.2	52.4 ± 4.4*
CDCA	–	–	–	–	4.2 ± 0.6	3.0 ± 0.8	2.9 ± 0.5	2.5 ± 0.6
DCA	–	–	–	–	–	–	–	–
LCA	–	–	–	–	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Total	78.5 ± 7.7	45.9 ± 9.4*	–	–	70.1 ± 4.9	41.8 ± 12.0	79.5 ± 2.4	43.1 ± 10.8*

Value shown represent % sulfated of individual, nonamidated, glycine- and taurine-conjugated BAs in mouse bile and urine. Each value represents the average of twelve individual mice (means ± S.E.M). (*) Statistically significant difference between male and female groups ($P < 0.05$). –, BA-sulfate was below the lower limit of quantification.

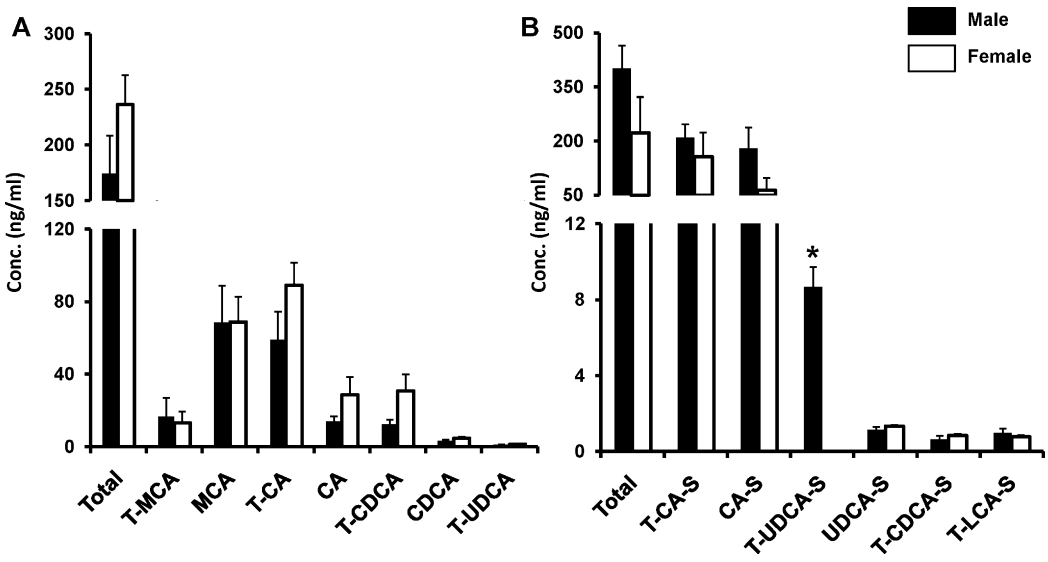

Fig. 6. BA (A) and BA-sulfate (B) concentration in male and female mouse urine. The results are shown as mean ± S.E.M ($N = 6$). (*) Statistically significant differences between male and female groups ($P < 0.05$).

of sulfation in increasing the urinary excretion of BAs. However, the percentage of sulfated BAs in mouse urine, is markedly lower than that detected in human urine [24]. Collectively, these data demonstrate that sulfation is a minor pathway of BA metabolism in mice.

Gender differences in the expression of Sults and in BA sulfation have been reported in several animal species [25–28]. Sult2a1 is female predominant in mice and rats [25,29]. However, conflicting data about the gender differences in BA-sulfates were reported in different animal species [26,27,30]. Despite the well-known female-predominant expression of Sult2a1 in mice, our data as well as others indicate that some BA-sulfates are more abundant in male than female tissues and fluids in mice [26].

4. Conclusions

This study reports the first validated LC–MS/MS method for the simultaneous and direct quantification of major BAs, and their sulfated metabolites in mouse liver, plasma, bile, and urine. This LC–MS/MS method provides high sensitivity and selectivity to perform quantitative profiling of 32 individual BA and BA-sulfate species in a relatively short run-time using a simple one-step sample preparation. The method was validated and applied for the quantification of BAs and BA-sulfates in mouse fluids and tissues. Less than 3% of BAs were detected in the sulfated form in the liver, plasma, and bile, whereas relatively high proportions (43–80%) of BAs were excreted in the sulfate form in male and female mouse urine. The low proportion of BAs present in the sulfated form in the mouse liver, plasma, and bile, provides strong evidence that, in contrast to humans, sulfation is a minor metabolic pathway of BA elimination and detoxification in mice. Finally, the marked female-predominant expression of Sult2a1 is not reflected into a female-predominant pattern of BA-sulfation.

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