Tandem Mass Spectrometric Determination of Atypical 3β-Hydroxy-Δ^5-Bile Acids in Patients with 3β-Hydroxy-Δ^5-C_{27}-Steroid Oxidoreductase Deficiency: Application to Diagnosis and Monitoring of Bile Acid Therapeutic Response

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BACKGROUND: 3β-Hydroxy-Δ^5-C_{27}-steroid oxidoreductase (HSD3B7) deficiency, a progressive cholestatic liver disease, is the most common genetic defect in bile acid synthesis. Early diagnosis is important because patients respond to oral primary bile acid therapy, which targets the negative feedback regulation for bile acid synthesis to reduce the production of hepatotoxic 3β-hydroxy-Δ^5-bile acids. These atypical bile acids are highly labile and difficult to accurately measure, yet a method for accurate determination of 3β-hydroxy-Δ^5-bile acid sulfates is critical for dose titration and monitoring response to therapy.

METHODS: We describe a electrospray ionization LC-MS/MS method for the direct measurement of atypical 3β-hydroxy-Δ^5-bile acid sulfates in urine from patients with HSD3B7 deficiency that overcomes the deficiencies of previously used GC-MS methods.

RESULTS: Separation of sulfated 3β-hydroxy-Δ^5-bile acids was achieved by reversed-phase HPLC in a 12-min analytical run. The mean (SE) urinary concentration of the total 3β-sulfated-Δ^5-cholenoic acids in patients with HSD3B7 deficiency was 4650 (1711) μmol/L, approximately 1000-fold higher than in noncholestatic and cholestatic patients with intact primary bile acid synthesis. GC-MS was not reliable for measuring 3β-hydroxy-Δ^5-bile acid sulfates; however, direct analysis of urine by fast atom bombardment mass spectrometry yielded meaningful semiquantitative assessment of urinary excretion.

CONCLUSIONS: The tandem mass spectrometry method described here for the measurement of 3β-hydroxy-Δ^5-bile acid sulfates in urine can be applied to the diagnosis and accurate monitoring of responses to primary bile acid therapy in HSD3B7 patients.

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Inborn errors in bile acid synthesis are a well-recognized category of metabolic liver disease (1). These autosomal recessive genetic defects manifest as a broad phenotype presenting with an overlapping spectrum of variable degrees of neonatal cholestasis, fat-soluble vitamin malabsorption, and neuropathies. 3β-Hydroxy-Δ^5-C_{27}-steroid oxidoreductase (HSD3B7) deficiency is the most common of the 9 known defects in the cholesterol–bile acid biosynthetic pathway (2) and is often the cause of idopathic forms of late-onset chronic cholestasis in children and adolescents. HSD3B7 deficiency was even recently identified in a 32-year-old adult (3). Early diagnosis of these genetic defects is crucial to prognosis, because if undiagnosed or untreated, the liver disease, which is a progressive form of intrahepatic cholestasis, leads to fibrosis, cirrhosis, and end-stage disease. Treatment options include liver transplantation (4, 5) or preferably

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5 Nonstandard abbreviations: HSD3B7, 3β-hydroxy-Δ^5-C_{27}-steroid oxidoreductase; MS, mass spectrometry; FAB, fast atom bombardment; MS/MS, tandem MS; LC, liquid chromatography; ESI, electrospray ionization; SHCA, 3β-sulfooxy-7α-hydroxy-chol-5-en-24-oic acid; SDHCA, 3β-sulfooxy-7α,12α-dihydroxy-chol-5-en-24-oic acid; GSHCA, glycine conjugate of SHCA; GSDHCA, glycine conjugate of SDHCA; UDCA-7S, ursodeoxycholic acid-7-sulfate; MRM, multiple reaction monitoring; S/N, signal-to-noise ratio; SIR, single ion recording.
oral bile acid therapy with the primary bile acids, cholic or chenodeoxycholic acids (1, 6 –12).

Diagnosis of HSD3B7 deficiency is based on the detection of increased concentrations of atypical 3β-hydroxy-Δ5-bile acids that accumulate in urine (1, 2) as a result of a lack of enzyme activity caused by mutations in the gene encoding the 3β-hydroxy-Δ5-C27-steroid oxidoreductase enzyme (13, 14). These atypical bile acids are preferentially sulfated (15) at the C-3 position and conjugated in the side-chain before elimination in the urine (1, 2). When this genetic defect was discovered, these conjugated bile acids were detected in the urine by liquid secondary ionization mass spectrometry (MS) with fast atom bombardment ionization (FAB) (16 –18), now an obsolete although still useful technique. Although we continue to use FAB-MS for diagnosis and monitoring of response to therapy (1), more recently tandem mass spectrometry (MS/MS) has been applied to screening for bile acid synthesis defects (19 –22). Accurate quantification of the concentrations of 3β-hydroxy-Δ5-bile acids in urine has proven difficult because of the lack of authentic standards. Historically, quantification has been performed with GC-MS after solvolysis and hydrolysis of the conjugate groups (2, 8, 11); however, the presence of the 7α-hydroxyl group in these atypical bile acids generates an allylic structure in the B-ring of the steroid nucleus that is highly labile and readily degrades under acidic conditions and, in the presence of methanol, yields a series of methoxylated derivatives as artifacts (2, 23). This approach to quantifying 3β-hydroxy-Δ5-bile acids cannot be used to monitor response to primary bile acid therapy in patients with HSD3B7 deficiency, the goal of which is to demonstrate a reduction or disappearance of these hepatotoxic atypical bile acids in the urine. Direct and accurate measurement of these conjugated 3β-hydroxy-Δ5-bile acids has until now not been possible because of the lack of reference standards.

We recently described a novel chemical synthesis for producing the sulfate and glyco-sulfate forms of 3β,7α-dihydroxy-chol-5-en-24-oic and 3β,7α,12α-trihydroxy-chol-5-en-24-oic acids (24) that provided the reference standards for direct quantification of these conjugated atypical bile acids in urine without having to resort to degradative solvolytic/hydrolytic steps. Here we describe an electrospray ionization (ESI) LC-MS/MS method for their quantification, compare this with the currently used FAB-MS method, and document the concentrations of these conjugated 3β-hydroxy-Δ5-bile acids in the urine of patients with HSD3B7 deficiency and patients with intact primary bile acid synthesis. Our findings indicate a large underestimation of the concentrations of these 3β-hydroxy-Δ5-bile acids when GC-MS is used for their measurement, which invalidates GC-MS for monitoring responses to bile acid therapy (8, 11) and indicates the importance of direct quantification of these atypical bile acids.

Materials and Methods

CHEMICALS AND HUMAN SAMPLES
We obtained the reference compounds 3β-sulfooxy-7α-hydroxy-chol-5-en-24-oic acid (SHCA), 3β-sulfooxy-7α,12α-dihydroxy-chol-5-en-24-oic acid (SDHCA), glycine conjugate of SHCA (GSHCA), and glycine conjugate of SDHCA (GSDHCA) by a recently described method (24). Ursodeoxycholic acid 7-sulfate (UDCA-7S) was prepared as described in US Patent # 5763435 and used as an internal standard for the assay. We obtained all other solvents and chemicals from Sigma-Aldrich and Thermo Fisher Scientific. Urine samples were obtained from those submitted for routine screening and investigation for possible genetic bile acid synthetic defects. These patients were infants/children with idiopathic cholestasis and increased serum transaminases found to be synthesizing increased concentrations of primary bile acids (n = 5), infants/children with undefined liver dysfunction but with healthy bile acid synthesis and excretion (n = 7), patients with biochemical and genetically confirmed HSD3B7 deficiency (n = 10), and healthy children 6 –10 years old (n = 6). Patients with a confirmed HSD3B7 deficiency were treated with informed consent under an Investigational New Drug application (45470) approved by the US Food and Drug Administration for oral cholic acid therapy (10 –15 mg/kg per day, n = 10) according to a protocol approved by the Cincinnati Children’s Hospital Medical Center Investigational Review Board (#2011–2448). Samples were blinded and deidentified for analysis.

PREPARATION OF CALIBRATORS AND SAMPLES FOR LC-MS/MS ANALYSIS
We prepared calibrators and QC samples by adding the reference compounds at different concentrations to pooled urine collected from healthy individuals. We prepared calibrators at concentrations of 0, 0.1, 0.2, 0.5, 1.0, 2.5, 5.0, and 10.0 μg/mL and 4 QC samples at concentrations of 0.2, 1.0, 3.0, and 6.0 μg/mL to correspond to the low, medium, and high portions of the calibration curve. All calibrators and QC samples were stored at −20 °C until analyzed. The internal standard, UDCA-7S (10 μL of a 10 μg/mL methanolic solution) was added to urine (100 μL) and also to the calibrators and QC urine samples. The samples were diluted with distilled H2O (1 mL), and bile acids were extracted by solid-phase extraction on octadecylsilane-bonded silica cartridges (C18-E, Phenomenex) as described previously (25, 26). After the cartridge was washed with water (2 mL), bile acids were desorbed from the cartridge with methanol (2 mL). The dried methanol extract was redis-
solved in 200 µL of the reconstitution solution (30% acetonitrile in H2O), and a 10 µL volume of this sample extract was injected onto the column for analysis by LC-MS/MS.

ANALYSIS OF 3β-SULFATED-Δ5-CHOLENOIC ACIDS BY LC-MS/MS

We carried out quantitative analysis of 3β-sulfated-Δ5-cholenoic acids with a Waters Premier API triple quadrupole mass spectrometer interfaced with an Alliance 2690 HPLC system. Optimized parameters, i.e., cone voltage and collision energy for sulfated 3β-hydroxy-Δ5-cholenoic acids, were determined by prior infusion of the individual compounds interfaced with LC flow (see Supplemental Table 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol61/issue7). For quantification, the ESI-MS/MS was operated under negative ion mode and with multiple reaction monitoring (MRM), with detection of the transitions of the individual deprotonated parent ions and their common daughter ion m/z 96.6. The following transition ions were monitored in negative ion mode: m/z 469.2 > 96.6 for SHCA, m/z 485.2 > 96.6 for SDHCA; m/z 526.2 > 96.6 for GSHCA; and m/z 542.2 > 96.6 for GSDHCA. The internal standard UDCA-7-sulfate was monitored from the m/z 471.2 > 96.6 transition. The individual bile acids were separated by HPLC on a C18 Kinetex XB-C18 column (2.6 µm, 100 × 3.0 mm internal diameter, Phenomenex) with isocratic elution in a mobile phase of 10 mmol/L aqueous ammonium formate (pH 3.5)/acetonitrile (70:30, vol/vol), and the total run time was 12 min.

HYDROLYSIS OF 3β-SULFOOXY-Δ5-CHOLEN-24-OIC ACIDS

We investigated the efficiency of enzymatic hydrolysis of the sulfate moiety of SHCA, SDHCA, GSHCA, and GSDHCA with 2 different sulfatase enzymes under 2 different pH conditions. We used the following enzyme preparations: (a) Helix pomatia digestive juice, a mixed β-glucuronidase/sulfatase enzyme (Sigma G0876), and (b) sulfatase from Helix pomatia (Sigma S9751). In each case, 1 µg of each bile acid sulfate was incubated with 10 mM sodium acetate buffer (0.05 M, pH 4.6 or 6.2) and enzyme (0.1 mL) at 37°C overnight in a shaking water bath. The bile acids were then extracted by solid-phase extraction on a C18 cartridge as described above. The dried methanolic extract was redissolved in 1.0 mL reconstitution solution (30% acetonitrile in H2O) and analyzed by LC-ESI-MS/MS.

FAB-MS ANALYSIS OF URINE

For comparison of this quantitative LC-ESI-MS/MS method with FAB-MS, urine samples previously analyzed by FAB-MS were also analyzed by LC-ESI-MS/MS. The conditions used for FAB-MS analysis have been described (18, 27, 28). Quantification by FAB-MS was based on measuring the relative intensity of the ions in the negative ion mass spectrum for atypical bile acids and assigning arbitrary scores for the relative magnitude of excretion: 0 = ions either not detectable or present only in traces, 1 = mild elevation [signal-to-noise ratio (S/N) <5], 2 = moderate elevation (S/N ratio >5), and 3 = marked increase (S/N >20).

Results and Discussion

OPTIMIZATION OF A QUANTITATIVE LC-ESI-MS/MS METHOD

The ESI negative ion mass spectra of SHCA and SDHCA, which are published elsewhere (24), reveal intense, predominantly singly charged, deprotonated ions [M-H]− at m/z 469.2 and 485.2, respectively, with little fragmentation. In this regard, these spectra are similar to those generated under FAB ionization conditions (2, 18). The corresponding glyco-sulfate conjugates, GSHCA and GSDHCA, also yield singly charged, deprotonated ions at m/z 526.2 and 542.2, but unlike FAB-MS, doubly charged ions at m/z 262.8 and 270.8 were also generated. These arose because of the presence of the 2 acidic groups in the molecule (the C-3 sulfate, pKa <1, and the carboxylic acid of the glycine moiety, pKa approximately 4.5), and the relative intensities were influenced by the cone energy. With high cone energies, the formation of singly charged ions was favored, whereas lower cone energies promoted the formation of doubly charged ions. The cone energy was therefore optimized at 60 eV to generate mainly singly charged ions. With collision-induced dissociation of the parent ion, all of the 3β-hydroxy-Δ5-bile acid sulfates yielded a common intense base peak at m/z 96.6 resulting from loss of the sulfate group. Consequently, the deprotonated molecular ion and its [HSO4−]− fragment were selected as the appropriate mass transition pairs for monitoring and quantifying all the 3β-hydroxy-Δ5-bile acid sulfates by LC-ESI-MS/MS (Fig. 1A). The ESI negative ion mass spectra for the 3β-hydroxy-Δ5-bile acids that were desulfated, i.e., 3β,7α-dihydroxy-5-cholen-24-oic and 3β,7α-dihydroxy-5-cholen-24-oic acids, had intense deprotonated molecular ions at m/z 389.3 and 405.3, respectively, and their corresponding glycine conjugates gave deprotonated molecular ions at m/z 446.2 and 462.3 (data not shown); these ions were selected for monitoring in single ion recording (SIR) mode for experiments designed to evaluate the efficiency of enzymatic hydrolysis used in previous GC-MS methods to cleave the sulfate group (2, 8, 23).

UDCA-7S was used as an internal standard for quantification because isotopically labeled 3β-hydroxy-Δ5-bile acid sulfates are not available and because this structural analog does not exist endogenously in human urine. Furthermore, no other components in the patient
Fig. 1. Chemical structures of the major atypical 3β-hydroxy-Δ5-bile acid sulfates synthesized by patients with liver disease caused by HSD3B7 deficiency compared with the internal standard used in the assay.

Negative ion LC-ESI-MS/MS mass chromatograms comparing the separation of a mixture of pure standards (3.0 μg/mL) of sulfated 3β-hydroxy-Δ5-bile acids and the internal standard that was added to human urine (A); urine from a patient with a confirmed genetic defect in bile acid synthesis caused by HSD3B7 deficiency (the internal standard was omitted to show no competing coeluting peaks) (B); urine from a patient with a confirmed genetic defect in bile acid synthesis caused by HSD3B7 deficiency with added internal standard (C); and urine from a healthy individual (D).
urine coeluted at the same retention time as this internal standard (Fig. 1B). Under ESI conditions, UDCA-7S yielded a deprotonated molecular ion at \( m/z \) 471.4, and under collision-induced dissociation, the loss of the sulfate moiety resulted in the same \( m/z \) 96.6 ion fragment, permitting the MRM mass transition \( m/z \) 471.4 > 96.6 to be used for its monitoring.

HPLC separation of all the sulfated bile acids was achieved with an isocratic mobile phase comprising 10 mmol/L ammonium formate (pH 3.5):acetonitrile (70:30) in a total run time of 12 min. Fig. 1 compares the MRM mass chromatograms for a mixture of the \( \beta \)-sulfooxy-\( \Delta^5 \)-cholenoic acids standards spiked into human urine (Fig. 1A), urine from a patient with HSD3B7 deficiency confirmed by FAB-MS analysis with omission of the internal standard (Fig. 1B), urine from a patient with HSD3B7 deficiency confirmed by FAB-MS analysis with added internal standard (Fig. 1C), and a healthy individual (Fig. 1D).

**ASSAY PERFORMANCE**

A full validation of the assay including linearity, intra- and interassay imprecision and bias, freeze–thaw cycles, dilution integrity, and short- and long-term stability was performed and confirmed the assay to be robust. Calibration curves for all \( \beta \)-sulfated-\( \Delta^5 \)-cholenoic acids were linear over the concentration range 0.1–10.0 \( \mu \)g/mL. The limits of detection, defined as S/N > 3, were 5, 2, 5, and 2 pg on column for SHCA, SDHCA, GSHCA, and GSDHCA, respectively. The lowest limit of quantification, defined as the lowest concentration measurable in urine with CV < 20%, was 0.2 \( \mu \)g/mL. The analytical recoveries of \( \beta \)-sulfated-\( \Delta^5 \)-cholenoic acids spiked in pooled human urine ranged from 82.6% to 86.8%. Four different concentrations (0.2, 1.0, 3.0, and 6.0 \( \mu \)g/mL) of QC samples of human urine were included within each batch of samples assayed. The intraassay imprecision, expressed as CV for the lowest QC sample (lowest limit of quantification) for SHCA, SDHCA, GSHCA, and GSDHCA, was 5.3%, 8.6%, 8.7%, and 6.4%, respectively, whereas the bias was 10.8%, 2.5%, −6.7%, and 4.2% (see online Supplemental Table 2). The corresponding interassay imprecision on the basis of 5 separate batches of assays for SHCA, SDHCA, GSHCA, and GSDHCA was 5.4%, 6.4%, 7.8%, and 9.4%, respectively, whereas the bias was 6.3%, −0.3%, −4.5%, and −0.2%.

We evaluated the stability of the \( \beta \)-sulfated-\( \Delta^5 \)-cholenoic acids from analysis of human urine samples that were spiked with known concentrations (1.0 and 6.0 \( \mu \)g/mL) of the reference standards and then subjected to 3 freeze–thaw cycles. The differences between the measured and expected concentrations for these 2 samples were −7.30% to 1.08% and −5.05% to 1.40%, respectively, when stored at −20 °C. The compounds were also stable for 6 h at ambient temperature, 24 h in the autosampler, and 1 and 3 months at −20 °C (data not shown). The long-term stability of the \( \beta \)-sulfated-\( \Delta^5 \)-cholenoic acids was further demonstrated by repeat analysis of the same urine sample from a patient with HSD3B7 deficiency collected in 1993 that had been stored at −20 °C and reanalyzed 19 years later (Fig. 2). The 2 spectra were virtually identical, and the diagnostically significant ions at \( m/z \) 469, 485, 526, and 542 that established this genetic defect were the prominent ions in both mass spectra. The relative intensities and S/N were also similar.

**CLINICAL APPLICATION OF LC-ESI-MS/MS METHOD**

\( \beta \)-Hydroxy-\( \Delta^5 \)-cholenoic acids, the major bile acids synthesized in the liver of patients with the HSD3B7 deficiency, are excreted predominantly as sulfate and glyco-sulfate conjugates (2, 23). Diagnosis of this genetic defect has been previously accomplished by direct analysis of urine with FAB-MS, which yields definitive mass spectra highlighted by intense deprotonated molecular ions and no fragmentation (1). FAB-MS, however, is not an accepted technique for accurate quantification but does provide a semiquantitative assessment of the relative concentrations of the atypical bile acids excreted in patients with genetic defects in bile acid synthesis. The assessment was based on the relative S/N of the ions for the atypical bile acids and the fundamental principle that the intensity of any ion generated in the ion source is proportional to the mass of compound ionized, and thus its concentration. The ability to accurately quantify concentrations of these atypical bile acids, however, is crucial to the evaluation of oral primary bile acid therapy, in which therapeutic efficacy is contingent on suppressing endogenous bile acid synthesis to effect a reduction in the synthesis and urinary excretion of these hepatotoxic bile acids (1, 7, 10, 12).

Quantification of these atypical bile acids has previously relied on GC-MS analysis after solvolysis and enzymic hydrolysis of the conjugate groups and conversion of the resulting unconjugated \( \beta \)-hydroxy-\( \Delta^5 \)-bile acids to their volatile methyl ester-trimethylsilyl ether derivatives (7, 8, 11). The major drawback of this approach is that the unconjugated species, once formed, are extremely labile under acidic hydrolysis procedures and rapidly degrade (2, 23). Hydrolysis of the sulfate group by sulfatase enzymes, which potentially could offer an alternative to overcome the limitations of chemical solvolysis (8), proved ineffective at releasing the unconjugated species. When the 4 synthesized \( \beta \)-sulfooxy-\( \Delta^5 \)-cholenoic acids were incubated with 2 different commercial sulfatase preparations under different pH conditions and LC-ESI-SIR was used to monitor the formation of desulfated products at \( m/z \) 389.3, 405.3, 462.3, and 446.2 (see online Supplemental Fig. 1), only traces of the desulfated species were detected. On the
basis of peak areas, we estimated that only 1%–3% of the 3β-sulfooxy-Δ5-cholen-24-oic acids were hydrolyzed by these sulfatase enzymes. Consequently, measurement by GC-MS of 3β-hydroxy-Δ5 bile acids after enzyme hydrolysis is not feasible. The LC-ESI-MS/MS methodology described here circumvents these problems by enabling the direct determination of 3β-sulfooxy-Δ5-cholen-24-oic acids without the need for prior hydrolytic steps.

With our LC-ESI-MS/MS method, we report here the concentrations of the individual and total 3β-sulfated-Δ5-cholenoic acids in the urine of patients with confirmed HSD3B7 deficiency. The mean (SE) urinary concentration of the total 3β-sulfated-Δ5-cholenoic acids in 10 patients with cholestasis caused by HSD3B7 deficiency was 4650 (1711) μmol/L (Fig. 3), and these were predominantly excreted as double conjugates with glycine conjugated in the side-chain. These concentrations were approximately 1000-fold higher than the urinary excretion of 3β-sulfated-Δ5-bile acids in noncholestatic liver disease patients with healthy primary bile acid synthesis [5.9 (0.2) μmol/L, n = 7], idiopathic cholestatic liver disease patients with increased primary bile acid synthesis [7.8 (2.3) μmol/L, n = 5], and healthy children [0.13 (0.12) μmol/L, n = 6].

Interestingly, these findings are at variance with those of Gonzales et al. (8), where concentrations of atypical 3β-hydroxy-Δ5-bile acids in 13 patients with HSD3B7 deficiency measured by GC-MS were reported to be only 38.0 (5.7) μmol/L. This discrepancy is explained by the limitations described above, where destruction and loss of >97% of the labile 3β-hydroxy-Δ5-bile acids has occurred in the sample preparation. This is confirmed from repeat urine analysis in Cincinnati with this newly validated LC-ESI-MS/MS method of 5 of the patients with HSD3B7 deficiency measured by urine FAB-MS analysis as reported by Gonzales et al. (8) (Table 1). The total urinary excretion of 3β-sulfated-Δ5-cholenoic acids in these 5 patients was 6916 (1387) μmol/L at the time of diagnosis, and a comparison of the individual values obtained by LC-ESI-MS/MS and those reported with GC-MS (8) is shown in Table 1. The concentrations of 3β-sulfated-Δ5-cholenoic acids reported in these 5 patients by Gonzales et al. (8) were only 2%–11% of those measured by LC-ESI-MS/MS; moreover, these values were not consistent in rank order of concentration. These data support the
lack of validity of GC-MS methods for the measurement of 3β-Hydroxy-Δ⁵-bile acids involving pretreatment of the urine by procedures that fail to hydrolyze the sulfate conjugate moiety and/or are destructive to the allylic 3β,7α-dihydroxy-Δ⁵ structure. Unless alternative non-destructive methods of hydrolysis are used, GC-MS cannot be reliably used to monitor changes in the urinary excretion of 3β-sulfated-Δ⁵-cholenoic acids in patients undergoing cholic acid therapy, in whom reductions in the concentrations of atypical bile acids represent one of the main biochemical endpoints for efficacy. Destruction of 3β-sulfated-Δ⁵-cholenoic acids will lead to a false impression of the biochemical effectiveness of any therapy (8) and make it impossible to make therapeutic dose adjustments, which rely on a reduction in hepatic synthesis of 3β-sulfated-Δ⁵-cholenoic acids.

Although FAB-MS remains a valuable and rapid tool for diagnosis of inborn errors of bile acid synthesis (1), direct-flow ESI-MS has more recently been used to detect a number of bile acid synthesis defects in a neonatal screen (19–22). Because of the limitations of GC-MS for quantification of 3β-sulfooxy-Δ⁵-cholen-24-oic acids, we have used FAB-MS for the last 20-plus years to evaluate responses to cholic acid therapy and assist in dose adjustments. To evaluate the validity of this approach, in a blinded manner, we analyzed 26 randomly selected urine samples from patients with HSD3B7 deficiency (n = 10) before and during cholic acid therapy and several healthy controls (n = 6) and compared the concentrations determined by LC-ESI-MS/MS with the previously reported scores assigned from the original FAB-MS spectra (Fig. 4). This FAB-MS semiquantitative method correlated well with the accurate concentrations determined by LC-ESI-MS/MS. Samples with a FAB-MS score of 3 had a urinary concentration of total atypical 3β-sulfooxy-Δ⁵-cholen-24-oic acids of 5747 (1962) μmol/L (n = 8), whereas the concentrations in samples with FAB-MS scores of 0, 1, and 2 were for 4.13 (1.78) μmol/L (n = 11), 6.69 (1.48) μmol/L (n = 11), and 48.5 (10.2) μmol/L (n = 11), respectively. The results are shown in Table 1.

### Table 1. Reported values for 3β-hydroxy-Δ⁵ bile acid concentrations in 5 patients at the time of diagnosis.

<table>
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<tr>
<th>Identifier</th>
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<td>Mean (SE)</td>
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</table>

* Measured in Paris by GC-MS methodology (8) and in Cincinnati by LC-ESI-MS/MS, providing evidence for destruction of 3β-Hydroxy-Δ⁵ bile acids by GC-MS analysis.

* Numbers refer to patients previously described in Gonzales et al. Supplemental Table 1 (8).
μmol/L (n = 2), and 164.4 (86.2) μmol/L (n = 5), respectively. However, the LC-ESI-MS/MS method described permitted accurate assignment of the concentrations of 3-β-sulfooxy-Δ5-cholen-24-oic acids in urine and provided an improved approach for evaluating the responsiveness to bile acid therapy. Fig. 5 shows a typical therapeutic response to oral administration of cholic acid (10–15 mg/kg per day) in a 3-year-old patient with a biochemically and genetically confirmed HSD3B7 deficiency. Therapeutic efficacy is illustrated from the marked reduction in urinary concentrations of all the 3-β-sulfooxy-Δ5-cholen-24-oic acids that was sustained throughout the duration of therapy.

In summary, we describe a validated LC-ESI-MS/MS methodology for the determination of 3-β-sulfooxy-Δ5-cholen-24-oic acids in the urine of patients with the genetic defect of HSD3B7 deficiency. Application of this methodology highlighted deficiencies of methods based on GC-MS for the measurement of these atypical bile acids, while validating the traditionally used FAB-MS method as a rapid semiquantitative means of assessing excretion. This LC-ESI-MS/MS methodology is applicable to the diagnosis of HSD3B7 deficiency and accurate monitoring of the biochemical response to primary bile acid therapy, the goal of which is to downregulate the production of these hepatoxic atypical 3-β-hydroxy-Δ5-bile acids by negative feedback inhibition on hepatic synthesis.

**References**


6. Daugherty CC, Setchell KDR, Heubi JE, Balistreri WF. Resolution of liver biopsy alterations in three siblings with bile acid treatment of an inborn error of bile acid


