Identification of unusual 7-oxygenated bile acid sulfates in a patient with Niemann-Pick disease, type C[1]

Gunvor Alvelius,* Ola Hjalmarson,§ William J. Griffiths, Ingemar Björkhem,† and Jan Sjövall[2,]****

Clinical Research Center* and Division of Clinical Chemistry,† Karolinska Institutet, Huddinge University Hospital, SE-14186 Stockholm, Sweden; Department of Pediatrics,§ Sahlgrenska University Hospital, Göteborg University, SE-41345 Göteborg, Sweden; and Department of Medical Biochemistry and Biophysics,** Karolinska Institutet, SE-17177 Stockholm, Sweden

Abstract **Niemann-Pick disease, type C, was diagnosed in a 3-month-old boy with hepatosplenomegaly, mild signs of cholestasis, hepatic inflammation and extramedullary erythropoesis, together with chronic airway disease. He developed muscular hypotonia, psychomotor retardation, rickets, and signs of peripheral neuropathy. The patient was found to excrete abnormal amounts of unusual bile acids in urine at 3 and 5 months of age. These acids were shown to have a 3β-hydroxy-Δ^5 structure and to carry an oxo or hydroxy group at C-7. They were sulfated at C-3 and nonamidated or conjugated with glycine or taurine at C-24. Part of the 7-hydroxy acids, presumably the 7β-hydroxylated one, was also conjugated with *N*-acetylhexosamine, probably *N*-acetylglucosamine, at the 7-hydroxy group. Possible metabolic pathways for the formation of the 7-oxo and 7β-hydroxycholenoic acids are discussed. Based on previous data concerning the effects of 3β-hydroxy-Δ^5 bile acids on bile acid transport, it is suggested that the formation of such bile acids is responsible for the cholestasis in this patient.**—Alvelius, G., O. Hjalmarson, W. J. Griffiths, I. Björkhem, and J. Sjövall. **Identification of unusual 7-oxygenated bile acid sulfates in a patient with Niemann-Pick disease, type C. *J. Lipid Res.* 2001.** 42: **1571-1577.**

Supplementary key words bile acid biosynthesis • bile acid conjugates • inborn metabolic disease • urine • chromatography • mass spectrometry

Niemann-Pick disease, type C (NP-C) is a rare inherited lipid trafficking disorder characterized by disturbances in intracellular cholesterol homeostasis (1). NP-C is genetically heterogenous, comprising two complementation groups, with NP-C1 constituting >90% of the cases. In this group, the primary molecular defect lies in a specific *NPC1* gene coding for a protein containing a number of transmembrane domains and a sterol-sensing domain with homologies to PATCHED, HMG-CoA reductase, and sterol regulatory element binding protein cleavage-activating protein (SCAP) (1–3). The protein was recently shown to be a eukaryotic member of the resistance nodulation division (RND) permease family and probably functions to transport lipophilic molecules, but not cholesterol, out of the endosomal/lysosomal system (4). The minor complementation group, NP-C2, was recently found to be due to mutations in the *HE1* gene coding for a cholesterol-binding lysosomal protein (5). As a consequence of the mutation(s), endocytosed or LDL-derived cholesterol is sequestered in lysosomes, and the transport of it to the plasma membrane and the endoplasmic reticulum is retarded. The clinical manifestations of the disease are heterogenous, involving progressive neurological disease, hepatic and splenic enlargement, and sometimes pulmonary symptoms. Diagnosis is obtained by the demonstration of abnormal distribution of cholesterol after culture of fibroblasts in the presence of LDL (1). Patients with an early presentation of the disease show symptoms of liver disease, including neonatal cholestasis.

In the present work, we have studied a Somalian infant with NP-C. Before the diagnosis was established, the presence of intrahepatic cholestasis, the liver histology, and the laboratory data suggested the possibility of an inborn error in bile acid biosynthesis. The patterns of bile acids in urine and plasma were therefore analyzed in some detail by electrospray (ES) mass spectrometry and gas chromatography–mass spectrometry (GC/MS). The pattern was found to be abnormal, with an increased excretion of sulfated and additionally conjugated forms of 3β-hydroxy-, 3β-hydroxy-7-oxo-, and 3β,7α/β-dihydroxy-5-cholenoic acids. The presence of the 7-oxo acid in biological fluids has not been described previously.

MATERIALS AND METHODS

Patient and sample collections

The patient is the only boy in a kindred of four. The parents are both immigrants from Somalia but unrelated. The patient's sisters have no signs of disease. He was born after 37 weeks of gestation,

Abbreviations: CID, collision-induced dissociation; ES, electrospray; GC/MS, gas chromatography-mass spectrometry; GlcNAc, *N*-acetylglucosamine; NP-C, Niemann-Pick disease, type C; RI, retention index.

[1] Results from this study were presented at the 15th International Mass Spectrometry Conference, Barcelona, August 26–September 1, 2000 (6) and at the XIV International Bile Acid Meeting, Den Haag, October 12–13, 2000.

[2] To whom correspondence should be addressed.
e-mail: jan.sjovall@mbb.ki.se

birth weight 2,335 g. The neonatal period was uneventful. At 10 weeks of age, he acquired a severe respiratory infection caused by respiratory syncytial virus (RSV). A massive hepatosplenomegaly was found together with elevated serum transaminases and slight cholestasis (serum bilirubin 41 mmol/l, conjugated 29; normal <20, conjugated <15%). Plasma prothrombin was elevated to 1.7 INR (internationally normalized ratio, normal <1.2) but normalized after K-vitamin treatment. The serum levels of α_1-antitrypsin, lipids, and amino acids were normal. Sweat chloride and organic acids in urine were normal. Cultures and serology for infectious agents were negative. A percutaneous liver biopsy showed marked inflammation with giant cell formation and foci of hematopoesis. Assays for sphingomyelinase, glucosyl ceramidase, and acid lipase were also normal, but β-galactosidase activity in lymphocytes and fibroblasts was moderately reduced to 61 μkat/kg protein (normal 85–145) and 159 μkat/kg protein (normal 220–450), respectively. NP-C was diagnosed from the finding of accumulation of lysosomal cholesterol in fibroblasts. It is not yet known whether the patient belongs to complementation group 1 or 2.

The samples analyzed in the present study were collected, with informed consent of the parents, at the ages of 3 months (urine and serum) and 5 months (urine). Unfortunately, a bile sample could not be obtained.

At 4 months of age, clinical rachitis appeared with malleolar swelling, craniotabes, and typical radiological bone changes. Serum levels of alkaline phosphatase and parathyroid hormone were elevated, serum calcium and phosphate were low, and 25-hydroxy-vitamin D_3 was not detectable in serum. The bone disease resolved after treatment with alphacalcidol.

The respiratory signs were very prominent, with a productive cough over the first year of life. They subsided very slowly and responded little to symptomatic therapy. Repeated chest X-ray showed central interstitial changes of varying intensity and a moderately increased heart size up to 33 months of age, when the picture became normal.

Treatment with ursodeoxycholic acid (10–20 mg/kg/day) was started at 5.5 months of age and continues in an attempt to improve bile secretion.

At 36 months of age, the patient has a more moderate hepatomegaly than during the first 6 months of life, but the edge of the spleen reaches the iliac crest. Serum aspartate aminotransferase is 1.6–2.2 μkat/l (normal <0.7), but other transaminases, bilirubin, albumin, and prothrombin are normal. His psychomotor development is retarded. He uses one or two words but understands "everything," according to the parents. His fine motor function is more advanced than his gross motor function. He began to stand and walk with support at 34 months of age. His neurological development is also abnormal, with a moderate muscular hypotonia as a dominant sign. This has tended to improve. From 30 to 36 months of age, signs of peripheral neuropathy have developed, with muscular atrophy and diminishing tendon reflexes in the lower extremities. No signs of ophthalmoplegia and no attacks with convulsions have appeared.

Synthesis of 3β-hydroxy-7-oxo-5-cholenoic acid

This compound was prepared from the methyl ester of 3β-acetoxy-5-cholenoic acid by allylic oxidation with chromic acid according to Fieser (7). The product was hydrolyzed with 5% potassium carbonate in methanol at room temperature overnight. The methyl ester trimethylsilyl ether derivative gave the expected mass spectrum with prominent peaks at *m/z* 474 (M^+), 384 (M^+-90), 369 ($M^+-90-15$) and 129 (C-1–C-3 with the trimethylsiloxy group).

Sample preparation for bile acid analysis

Bile acids were isolated from the urine samples and separated into groups essentially as described in previous studies (8–10). In brief, 10-ml portions of urine were extracted on beds (0.3 g) of octadecylsilane-bonded silica (Sep-Pak C_{18}; Waters, Milford, MA). After a wash with distilled water, the conjugated and free bile acids were eluted with 5 ml methanol. Water (2 mL) was added, and the solution was passed through a bed (70 × 4 mm) of the lipophilic anion exchanger Lipidex-DEAP (Packard Bioscience B.V., Groningen, The Netherlands). Neutral compounds were removed by a wash with 70% ethanol, "free" bile acids were collected by elution with 7 ml 0.1 M acetic acid in 70% ethanol, nonsulfated glycine-, and taurine-conjugated bile acids were collected by elution with 6 ml acetic acid/ammonium hydroxide, 0.15 M acetate, pH 6.6, in 70% ethanol, and sulfated bile acids were collected by elution with 10 ml of acetic acid/ammonium hydroxide, 0.5 M acetate, pH 9.6, in 70% ethanol.

Bile acids in serum were extracted as described for urine after dilution of the sample (0.6 ml) with an equal volume of 0.5 M aqueous triethylamine sulfate, pH 7.0 (9, 10).

ES mass spectrometry

About 10 μL of the original urine extract and of the fractions from the anion exchanger (after desalting with Sep-Pak C_{18}) were analyzed with ES mass spectrometry. Initial screening was performed on a Micromass Quattro 1 instrument (Micromass, Manchester, UK) at unit mass resolution. Optimal interface conditions for recording of negative ion spectra were established with a solution containing a mixture of conjugated bile acids. The samples were injected in a stream of 50% aqueous methanol at a flow rate of 10 μl/min. The *m/z* range 200–800 was scanned at a rate of 10 s/scan for 2 min. To search for ketonic bile acids, the urine extract and the sulfate fraction were reacted with methoxyammonium chloride (Sigma, St Louis, MO) in pyridine (11) and then reanalyzed by ES mass spectrometry.

The original extract was also analyzed by nano-ES mass spectrometry on an AutoSpec-OATOFFPD hybrid tandem mass spectrometer (Micromass). The sample in 50% aqueous methanol (2–5 μl with adjusted concentrations of individual bile acids in the 10 ng–10 pg/μl range) was loaded into a gold-coated capillary (Protana AS, Odense, Denmark) whose tip was cracked to give a spraying orifice of ~5 μm. The capillary was then installed into the nano-ES probe and inserted into the ES interface. The voltages on the capillary cone and skimmer were ~−5.3, −4.3, and −4.3 kV, respectively. The accelerating potential was 4 kV and the resolution ~1,500 (10% valley definition). Collision-induced dissociation (CID) spectra were also recorded using this instrument. Monoisotopic $[M-H]^-$ or $[M-2H]^{2-}$ precursor ions were selected by the double focusing sectors, decelerated to 400 eV, and focused into the fourth field-free region collision cell. Xenon was used as the collision gas at a pressure sufficient to give ~75% attenuation of the selected ion. The resulting fragments and undissociated precursor ions were mass measured by the TOF (time-of-flight) analyzer.

GC/MS

For further analysis and identification of individual bile acids by GC/MS, the conjugated fractions were subjected to enzyme hydrolysis [cholylglycine hydrolase (Sigma) to remove glycine and taurine (9)] and solvolysis (10). The liberated bile acids [purified by passage through Lipidex-DEAP (9)] were methylated using a slight modification of the method used by Hayamizu et al. for methylation of dansyl amino acids (12). The dried samples were dissolved in 0.1 ml methanol; 0.4 ml toluene was then added, followed by 25 μl of a 2.0 M solution of trimethylsilyldiazomethane in hexane (Aldrich, Milwaukee, WI). The solution was left for 5 min at room temperature and then taken to dryness under a stream of nitrogen at 40°C. The samples were then trimethylsilylated (9) or converted into *O*-methyloxime-trimethylsilyl

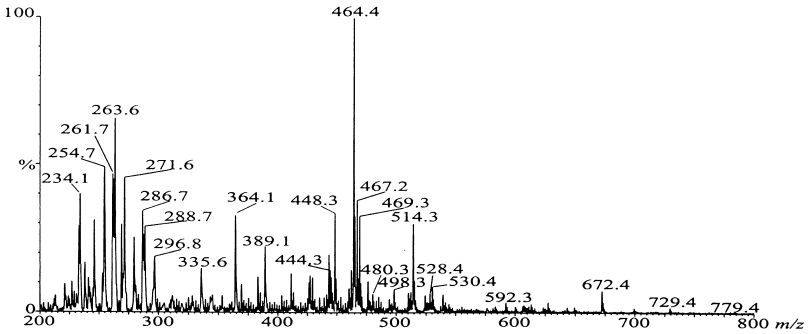

Fig. 1. Electrospray mass spectrum of the extract of urine collected at 5 months of age (Quattro 1 instrument).

ether derivatives (11). The derivatives were analyzed under temperature-programmed conditions using a fused-silica capillary column coated with cross-linked methyl silicone (25 m × 0.25 mm, 0.25-μm film thickness; J & W Scientific Inc., Folsom, CA) in a Hewlett-Packard 2890 gas chromatograph connected to a Nermag R10-10H quadrupole mass spectrometer with an electron-impact ion source. Retention indices (RI) of the bile acid derivatives were calculated from the retention times of a series of normal hydrocarbons (9).

RESULTS

Analysis of urine

The intense peaks in the ES mass spectra of the urine extracts (**Figs. 1** and **2**) showed that the bile acid excretion in urine was elevated and that the bile acid pattern differed from the patterns seen in other conditions of neonatal cholestasis (13–20). The spectra of urine collected at 3 and 5 months of age were very similar and showed a series of doubly charged ions whose *m/z* values were compatible with the presence of sulfated glycine and taurine conjugates of bile acids deficient in two and four hydrogens compared with normal saturated bile acids (**Table 1**). Singly charged ions of the sulfated glycine conjugates and of sulfated nonamidated bile acids were also present. The ES mass spectra of the fractions from Lipidex-DEAP showed all of these compounds to be eluted as expected of bile acid sulfates (data not shown).

After reaction with methoxyammonium chloride, the ions deficient in four hydrogens were shifted by 29 Da (data not shown), whereas the ions of the assumed monounsaturated bile acids were not shifted. This indicated that the doubly unsaturated bile acids had one keto group and one double bond.

The urine extract and sulfate fraction also contained three minor compounds giving singly and doubly charged ions indicative of a sulfated unsaturated dihydroxy bile acid conjugated with *N*-acetylhexosamine, probably *N*-acetylglucosamine (GlcNAc) (*m/z* 672), and with glycine (*m/z* 729) or taurine (*m/z* 779) (Figs. 1 and 2, Table 1). The ES spectrum of urine also showed a peak at *m/z* 592, compatible with a GlcNAc conjugate of the dihydroxycholenoate, previously identified in urine from healthy subjects (21). As expected, this compound appeared in the fraction of "free" bile acids from the Lipidex-DEAP column.

After enzymatic removal of glycine and taurine and solvolytic removal of sulfate, the bile acids in the sulfate fraction were analyzed by GC/MS of their methyl ester trimethylsilyl ether derivatives. Three major peaks of bile acids were detected (**Fig. 3**). Their mass spectra (**Fig. 4**) were compared with previously recorded spectra of reference compounds (22) and the spectrum of the synthetic 7-oxo acid (Materials and Methods). The bile acids were identified as 3β-hydroxy-7-oxo-5-cholenoic acid (Fig. 4, RI 3,452), 3β,7β-dihydroxy-5-cholenoic acid (Fig. 4, RI 3,319), and a mixture of 3β-hydroxy-5-cholenoic and 3β,7α-dihydroxy-5-cholenoic acids (Fig. 4, RI 3,189). No 12-hydroxylated analogs of these acids were detected. Unexpectedly, peaks of di- and trihydroxy bile acids in the GC/MS analysis of the sulfate fraction were small. Examination of appropriate ion current chromatograms showed the presence of cholate (RI 3,221), chenodeoxycholate (RI 3,207), and an isomer of the latter (RI 3,201). This is in contrast to the intensities of corresponding peaks of deprotonated mole-

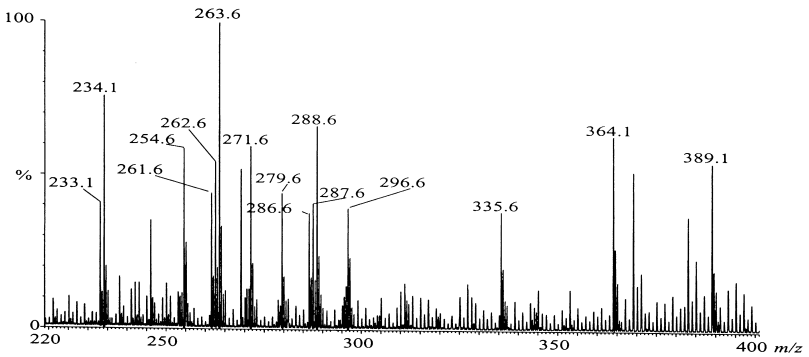

Fig. 2. The region of doubly charged ions in the ES mass spectrum of the same urine extract as that shown in Fig. 1 (AutoSpec-OATOFFPD instrument).

TABLE 1. Peaks of deprotonated bile acid molecules in the mass spectrum of the urine extract

Structure	Mass/Charge Ratio (Th)		Structure	Mass/Charge Ratio (Th)	
	−H	*−2H*		*−H*	*−2H*
B$^{\Delta}$-ol-one-G	444.3		B$^{\Delta}$-ol-one-G-S	524.4	261.7
B-diol-G	448.3		B$^{\Delta}$-diol-G-S	526.2	262.6
B$^{\Delta}$-diol-one-G	460.4		B-diol-G-S	528.4	263.6
B-diol-one-G	462.2		B-tetrol-T	530.4	
B-triol-G	464.2		B-triol-G-S		271.6
B$^{\Delta}$-ol-one-S	467.2	233.1	B$^{\Delta}$-ol-T-S		279.6
B$^{\Delta}$-diol-S	469.3	234.1	B$^{\Delta}$-ol-one-T-S		286.7
B-tetrol-G	480.4		B$^{\Delta}$-diol-T-S		287.7
B$^{\Delta}$-ol-one-T	494.3		B-diol-T-S		288.7
B-diol-T	498.3		B$^{\Delta}$-diol-GlcNAc	592.3	
B$^{\Delta}$-diol-one-T	510.3		B-triol-T-S		296.8
B$^{\Delta}$-ol-G-S	510.3	254.7	B$^{\Delta}$-diol-GlcNAc-S	672.4	335.6
B-diol-one-T	512.3		B$^{\Delta}$-diol-G-GlcNAc-S	729.4	364.1
B-triol-T	514.3		B$^{\Delta}$-diol-T-GlcNAc-S	779.4	389.1

B, cholanoic acid; $^{\Delta}$, double bond; G, glycine; T, taurine; S, sulfate; GlcNAc, *N*-acetylglucosamine; -H, singly charged ions; -2H, doubly charged ions.

cules of conjugated saturated bile acid sulfates in the ES mass spectra of the urine extract (Fig. 1) and the sulfate fraction. This apparent discrepancy may be explained by structure-dependent differences in the ion yields or fragmentation of the bile acids in the ES interface (e.g., greater formation of sulfate ions from the 3β-hydroxy-Δ^5 bile acid sulfates) and by the presence of low levels of isobaric isomers of saturated bile acids that separate in the GC/MS analyses**.** The possibility that unknown bile acids are lost can never be excluded, but because saturated bile acids are more stable than the unsaturated ones found, this seems an unlikely explanation.

Some of the conjugated bile acid sulfates were also analyzed by CID of their deprotonated molecules produced by ES ionization. The spectra of the unsaturated 7-oxo and 7-hydroxy bile acids showed that their 3β-hydroxy group was sulfated (data not shown). The CID spectrum of the sulfated *N*-acetylhexosamine conjugate (*m/z* 672) is shown in **Fig. 5**. The fragment ions show that the sulfate group is at C-3 and the *N*-acetylhexosamine group on a hydroxyl group in the B- or C-ring (see inset in Fig. 5). On the basis of this spectrum, the previous demonstration of selective conjugation of 7β-hydroxylated bile acids with GlcNAc (23), and the previous identification of a GlcNAc conjugate of 3β,7β-dihydroxy-5-cholenoic acid in human urine (21), we assume that the *N*-acetylhexosamine conjugates found in this patient represent different conjugated forms of 3β,7β-dihydroxy-5-cholenoic acid conjugated with GlcNAc.

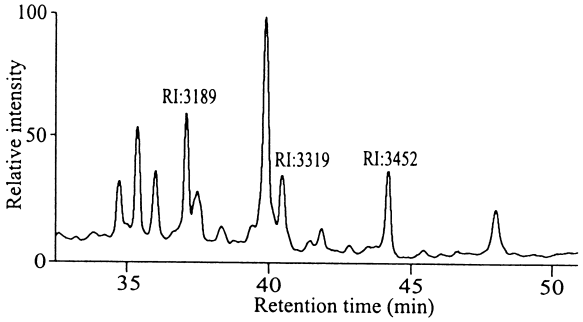

Fig. 3. Total ion chromatogram obtained in the GC/MS analysis of bile acid derivatives prepared from the sulfate fraction after chromatography of the urine extract on Lipidex-DEAP. Peaks of Δ^5 bile acid derivatives are labeled with their retention indices. The peak at 40 min represents an unknown compound; minor peaks are due to derivatives of dihydroxycholanoates, cholesterol and other contaminants, and n-hydrocarbons added as standards.

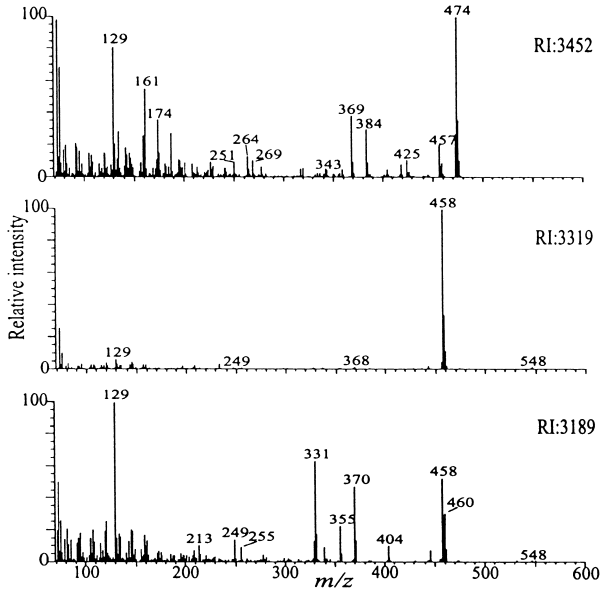

Fig. 4. Mass spectra of the methyl ester trimethylsilyl ether derivatives of 3β-hydroxy-7-oxo-5-cholenoic acid (RI 3,452), 3β,7β-dihydroxy-5-cholenoic acid (RI 3,319), and the mixture of 3β-hydroxy- and 3β,7α-dihydroxy-5-cholenoic acids (RI 3,189) recorded in the GC/MS analysis of the urinary bile acid sulfate fraction (cf. Fig. 3).

The fraction of nonsulfated glycine- and taurine-conjugated bile acids was also analyzed by GC/MS after enzymatic hydrolysis and derivatization. Cholic acid (RI 3,225) was the predominant bile acid in this fraction, in agreement with the intense peaks at *m/z* 464 and 514 in the ES mass spectrum of the urine extract (Fig. 1). The derivative of its 5α-isomer, allocholic acid (RI 3,196), was also detected. The derivatives of chenodeoxycholic (RI 3,206), allochenodeoxycholic (RI 3,166, about half the peak height of RI 3,206), and ursodeoxycholic (RI 3,253) acids were all present in this fraction. The ratio of trihydroxy to dihydroxy acids calculated from GC/MS peak areas was roughly the same as the ratios of *m/z* 514 to *m/z* 498 and *m/z* 464 to *m/z* 448 in the ES spectrum of the urine extract (see Fig. 1). The GC/MS analyses showed that the tetrahydroxy acids indicated by *m/z* 530 in the ES spectrum consisted predominantly of 1β-hydroxycholic acid. Minor amounts of 7α,12α-dihydroxy-3-oxo-4-cholenoic acid were found, whereas 7α-hydroxy-3-oxo-4-cholenoic acid (or its degradation product 3-oxo-4,6-choladienoic acid), of prognostic significance in liver disease (24, 25), was not detected.

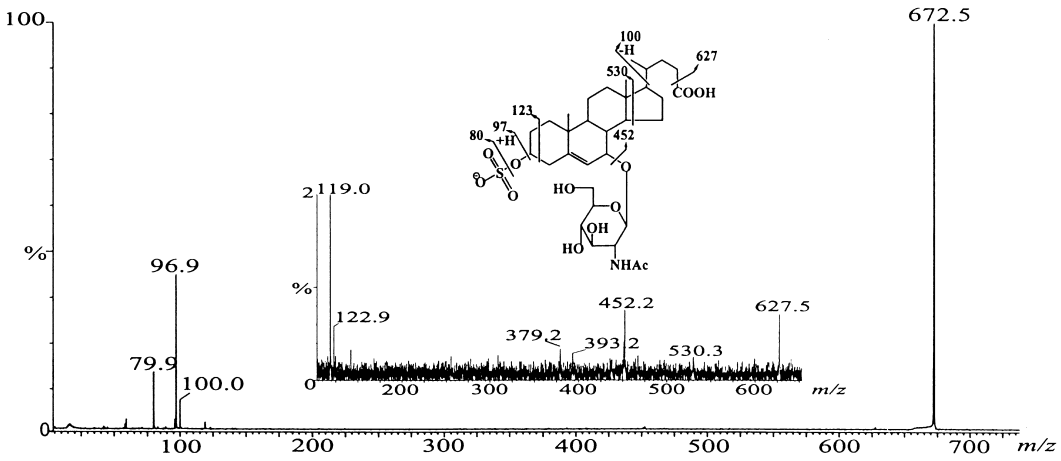

Fig. 5. CID spectrum of *m/z* 672.5, representing the GlcNAc conjugate of a dihydroxycholenoic acid sulfate. The spectrum was recorded on the AutoSpec-OATOFFPD instrument (see Materials and Methods). Bond cleavages are indicated on the inserted formula of the proposed structure.

The proportions of different groups of bile acids in the samples collected at 3 and 5 months of age were evaluated from the relative intensities of the peaks in the ES spectra. ES mass spectrometry is not a quantitative method, but the samples were analyzed under identical conditions and so an approximate comparison should be possible. The percentage of 5,6-unsaturated bile acids was the same or slightly higher at 5 months (56%) than at 3 months (47%). Saturated trihydroxycholanoate(s) (24 and 27% of total bile acids, mainly nonsulfated) and 3β,7α/β-dihydroxy-5-cholenoates (28 and 19%, sulfated, 35–45% of which were also conjugated with GlcNAc) were the predominant individual bile acids. The 7-oxo acid constituted ~20% of the total bile acids.

Analysis of serum

The ES spectrum of the extract of serum collected at 3 months of age showed a number of the same peaks as the urine extract. The major peaks of deprotonated common conjugated bile acids were at *m/z* 448.4, 464.4, 498.5, and 514.5. Their intensities were much higher than in spectra of normal serum. Amidated and sulfated bile acids also gave singly and doubly charged ions corresponding to double conjugates of di- and trihydroxycholanoates [e.g., *m/z* 263.7, 528.4, 288.7, 271.7, 544.4, cf. Table 1)]. Peaks of sulfated and doubly conjugated mono- and dihydroxycholenoates were seen, e.g., at *m/z* 453.4, 469.4, 279.6, 576.4, cf. Table 1). Different forms of the unsaturated hydroxy-oxo bile acid were also present, as shown by a comparison between ES spectra taken before and after reaction with methoxyammonium chloride [e.g., *m/z* 467.3 and 524.3 shifted to *m/z* 496.3 and 553.4, respectively (cf. Table 1)]. Small peaks of the GlcNAc conjugates mentioned above were also seen at *m/z* 592.5, 649.5 (glycine conjugated), and 672.4 (sulfated). There were no peaks at masses corresponding to C_{27} or C_{29} bile acids or their conjugates. The patterns of bile acid peaks in the spectra of urine and serum were not sufficiently different to motivate further analyses of the serum bile acids by GC/MS.

DISCUSSION

The ES mass spectra of the urine extract showed intense peaks corresponding to deprotonated molecules of the common biliary bile acids. The spectrum reflected a cholestatic state, and GC/MS analyses showed that cholic acid was the major normal bile acid. Other nonspecific characteristics of the cholestatic condition were the excretion of 1β-hydroxycholic acid (26) and 7α,12α-dihydroxy-3-oxo-4-cholenoic acid. These bile acids were present as glycine or taurine conjugates or in an unconjugated form. 7α-Hydroxy-3-oxo-4-cholenoic acid, present in severe cases of intrahepatic cholestasis (14, 17, 24, 25), could not be detected.

In addition to the peaks corresponding to the bile acids commonly found in the urine of patients with intrahepatic cholestasis, the ES mass spectrum also showed unusual peaks compatible with the presence of sulfated, unsaturated oxo bile acids. The simultaneous presence of a double bond and an oxo group in these bile acids was confirmed by comparison of spectra recorded before and after reaction with methoxyammonium chloride and suggested a previously unknown abnormality in bile acid synthesis or metabolism.

Sulfated bile acids and alcohols with a 3β-hydroxy-Δ^5 structure are predominant cholesterol metabolites in infants with 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase deficiency (10, 13), and nonsulfated bile acids having a 3-oxo-Δ^4 structure are excreted in the urine of cholestatic infants with inherited or acquired deficiency of Δ^4-3-oxosteroid 5β-reductase (14, 17, 25). However, to our knowledge ketonic bile acids being both unsaturated and sulfated have not previously been found in humans. This finding indicated a new form of neonatal cholestasis.

The sulfated and unsaturated bile acids identified in the urine and their different conjugated forms are summarized in **Fig. 6**. From the ES mass spectra, these bile acids were estimated to constitute about half of the urinary bile acids. The unsaturated ketonic bile acid was identified as 3β-hydroxy-7-oxo-5-cholenoic acid, a bile acid not previously found in humans. It was accompanied

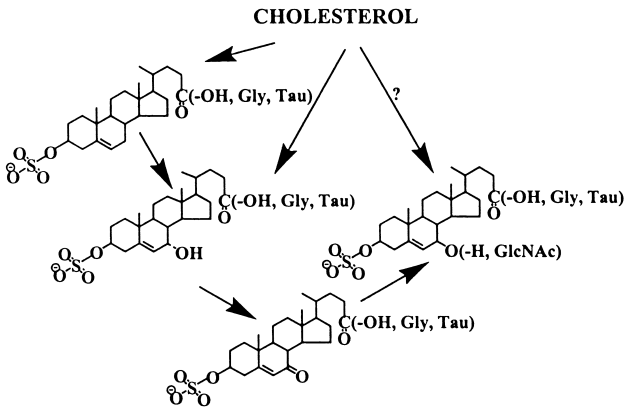

Fig. 6. Structures of unsaturated bile acid sulfates and their conjugates excreted in the urine of the patient. Arrows indicate potential metabolic relationships (reactions are likely to occur on the nonsulfated level).

by sulfated forms of 3β-hydroxy-, 3β,7α-dihydroxy-, and 3β,7β-dihydroxy-5-cholenoic acids. It is notable that, whereas cholic acid was the predominant normal bile acid, constituting ~25% of the total urinary bile acids, the 12α-hydroxylated analogs of these acids were not present. This is in contrast to the findings in patients with 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase deficiency, who excrete 3β,7α-dihydroxy-Δ^5 bile acids both without and with a 12α-hydroxy group (10, 13). The latter patients do not excrete 3β,7β-dihydroxy-Δ^5 bile acids.

The excretion of the Δ^5 bile acids in the present patient could indicate a partial deficiency of 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase specifically affecting a mitochondrial [see below and (27)] pathway in the synthesis of chenodeoxycholic acid, perhaps involving 3β-hydroxy-5-cholenoic acid as intermediate (28). However, existing data do not indicate the existence of multiple forms of the enzyme (29–31). Another possibility, without experimental support, would be an increased rate of sulfation resulting in selective trapping of some 3β-hydroxy-Δ^5 intermediates.

Previous studies have shown that 3β-hydroxy-5-cholenoic acid induces cholestasis in rats (32, 33), also in the sulfated form (33). This acid as well as the 7α-hydroxylated analog inhibit hepatic bile acid transport in in vitro systems (34). This inhibition has been proposed to be the mechanism for the cholestasis in infants with 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase deficiency (34). It is reasonable to assume that the 3β-hydroxy-Δ^5 bile acids formed in the present patient are also responsible for his intrahepatic cholestasis.

Another feature of the urinary bile acid pattern, resulting in characteristic peaks in the ES mass spectra of urine, was the presence of *N*-acetylhexosamine conjugates of sulfated dihydroxycholenoate(s) and its conjugates with glycine or taurine. Judging from the ES spectra, such sugar conjugates constituted ~10% of the total bile acids. Nonsulfated 3β,7β-dihydroxy-5-cholenoic acid has previously been identified as a 7-GlcNAc conjugate and shown to be a minor component in the urine of healthy subjects (21, 23). Its metabolic origin is not known, but it is tempting to suggest that it is an epimerization product of a 7α-hydroxylated precursor, as supported by the occurrence of the 7-oxo intermediate in this patient. An analogous epimerization of 7α-hydroxylated Δ^5 intermediates in bile acid biosynthesis has previously been demonstrated with human liver mitochondria (27). It may be mentioned that the 7β epimer is not a substrate of the 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase, at least not in pigs (29).

The relationship between the formation of abnormal bile acids and the metabolic defect in NP-C is not known. One case of NP-C1 has previously been reported in which the peroxisomal β-oxidation of branched-chain substrates including C_{27} bile acids was defective, resulting in elevated levels of these precursors of C_{24} bile acids (35). Such changes were not observed in our patient. Bile acid synthesis from cholesterol can occur via several pathways, all of which involve a 7α-hydroxylation. Cholesterol is primarily 7α-hydroxylated in the endoplasmic reticulum, whereas it may be 27-hydroxylated in the mitochondria and subsequently 7α-hydroxylated in mitochondria or the endoplasmic reticulum by a separate oxysterol 7α-hydroxylase. Other reactions in bile acid biosynthesis take place in a number of subcellular compartments, requiring extensive trafficking of intermediates between organelles and cytosol. In the pig, the 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase is remarkably found in the endoplasmic reticulum of the bile duct epithelium (30). An altered subcellular distribution of cholesterol such as that present in NP-C could possibly influence the channeling into different pathways in bile acid synthesis, resulting in the appearance of abnormal intermediates. The similarity of the urinary bile acid profiles at 3 and 5 months indicated that the biosynthetic pathway did not change during this time [cf. (18)]. Given the present state of knowledge, it is not possible to exclude the possibility that the NPC1 or HE1 proteins are involved in the traffic of intermediates in the conversion of cholesterol into bile acids. Because 7β-hydroxycholesterol and 7-oxocholesterol are major products in connection with autoxidation of cholesterol, the possibility must also be considered that the formation of 7-oxo- and 7β-hydroxy bile acids in our patient may be a consequence of extensive lipid peroxidation. An argument against this hypothesis is that the pattern of oxysterols in plasma, including the autoxidative products of cholesterol, was found to be normal in our patient (unpublished observations).

Further studies will show whether the abnormalities in bile acid synthesis is an isolated finding in this patient or restricted to one form of NP-C, and whether ES mass spectra of urinary bile acids may be of diagnostic use.

Dr. Jan-Eric Månsson, Department of Neurochemistry at the Sahlgrenska University Hospital, Mölndal, Sweden is gratefully acknowledged for neurochemical analyses, including diagnostic work on NP-C. This work was supported by grants from the Swedish Medical Research Council (03X-12551 and 03X-3141) and Karolinska Institutet.

Manuscript received 22 February 2001 and in revised form 17 May 2001.

REFERENCES

1. Patterson, M. C., M. T. Vanier, K. Suzuki, J. A. Morris, E. D. Carstea, E. B. Neufield, J. E. Blanchette-Mackie, and P. G. Pentchev. 2001. Niemann-Pick disease type C: a lipid trafficking disorder. *In* Metabolic Basis of Inherited Disease. C. R. Scriver, A. L. Baudet, W. S. Sly, and D. Valle, editors. McGraw-Hill, New York. 3611–3633.
2. Carstea, E. D., J. A. Morris, K. G. Coleman, S. K. Loftus, D. Zhang, C. Cummings, J. Gu, M. A. Rosenfeld, W. J. Pavan, D. B. Krizman, J. Nagle, M. H. Polymeropoulos, S. L. Sturley, Y. A. Ioannou, M. E. Higgins, M. Comley, A. Cooney, A. Brown, C. R. Kanesky, E. J. Blanchette-Mackie, N. K. Dwyer, E. B. Neufeld, T. Y. Chang, and L. Liscum. 1997. Niemann-Pick C1 disease gene: homology to mediators of cholesterol homeostasis. *Science.* **277:** 228–231.
3. Greer, W. L., D. C. Riddell, T. L. Gillan, G. S. Girouard, S. M. Sparrow, D. M. Byers, M. J. Dobson, and P. E. Neumann. 1998. The Nova Scotia (type D) form of Niemann-Pick disease is caused by a G3097 to T transversion in NPC1. *Am. J. Hum. Genet.* **63:** 52–54.
4. Davies, J. P., F. W. Chen, and Y. A. Ioannou. 2000. Transmembrane molecular pump activity of Niemann-Pick C1 protein. *Science.* **290:** 2295–2298.
5. Naureckiene, S., D. E. Sleat, H. Lackland, A. Fensom, M. T. Vanier, R. Wattiaux, M. Jadot, and P. Lobel. 2000. Identification of *HE1* as the second gene of Niemann-Pick C disease. *Science.* **290:** 2298–2301.
6. Alvelius, G., O. Hjalmarson, W. J. Griffiths, I. Björkhem, and J. Sjövall. 2001. Identification of unusual 7-oxygenated bile acid sulfates in a patient with Niemann-Pick disease, type C (Abstract). *In* Proceedings of the 15th International Mass Spectrometry Conference. John Wiley and Sons Ltd., Chichester, UK. In press.
7. Fieser, L. L. 1953. Oxidation of cholesterol. *J. Am. Chem. Soc.* **75:** 4386–4393.
8. Almé, B., A. Bremmelgaard, J. Sjövall, and P. Thomassen. 1977. Analysis of metabolic profiles of bile acids in urine using a lipophilic anion exchanger and computerized gas–liquid chromatography–mass spectrometry. *J. Lipid Res.* **18:** 339–362.
9. Meng, L. J., and J. Sjövall. 1997. Method for combined analysis of profiles of conjugated progesterone metabolites and bile acids in serum and urine of pregnant women. *J. Chromatogr. Biomed. Sci. Appl.* **688:** 11–26.
10. Ichimiya, H., B. Egestad, H. Nazer, E. S. Baginski, P. T. Clayton, and J. Sjövall. 1991. Bile acids and bile alcohols in a child with hepatic 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase deficiency: effects of chenodeoxycholic acid treatment. *J. Lipid Res.* **32:** 829–841.
11. Thenot, J-P., and E. C. Horning. 1972. MO–TMS derivatives of human urinary steroids for GC and GC–MS studies. *Anal. Lett.* **5:** 21–33.
12. Hayamizu, T., S. Kudoh, and H. Nakamura. 1998. Methylated *N*ε-dansyl-L-lysine as a fluorogenic reagent for the chiral separation of carboxylic acids. *J. Chromatogr. B.* **710:** 211–218.
13. Clayton, P. T., J. V. Leonard, A. M. Lawson, K. D. R. Setchell, S. Andersson, B. Egestad, and J. Sjövall. 1987. Familial giant cell hepatitis associated with synthesis of 3β,7α-dihydroxy- and 3β,7α,12α-trihydroxy-5-cholenoic acids. *J. Clin. Invest.* **79:** 1031–1038.
14. Clayton, P. T., E. Patel, A. M. Lawson, R. A. Carruthers, M. S. Tanner, B. Strandvik, B. Egestad, and J. Sjövall. 1988. 3-Oxo-Δ^4- bile acids in liver disease. *Lancet.* **ii:** 1283–1284.
15. Yang, Y., W. J. Griffiths, H. Nazer, and J. Sjövall. 1997. Analysis of bile acids and bile alcohols in urine by capillary column liquid chromatography–mass spectrometry using fast atom bombardment or electrospray ionization and collision-induced dissociation. *Biomed. Chromatogr.* **11:** 240–255.
16. Meng, L. J., W. J. Griffiths, H. Nazer, Y. Yang, and J. Sjövall. 1997. High levels of (24S)-24-hydroxycholesterol 3-sulfate, 24-glucuronide in the serum and urine of children with severe cholestatic liver disease. *J. Lipid Res.* **38:** 926–934.
17. Setchell, K. D. R., F. J. Suchy, M. B. Welsh, L. Zimmer-Nechemias, J. Heubi, and W. F. Balistreri. 1988. Δ^4-3-Oxosteroid 5β-reductase deficiency described in identical twins with neonatal hepatitis. *J. Clin. Invest.* **82:** 2148–2157.
18. Setchell K. D. R., M. Schwartz, N. C. O'Connell, E. Lund, D. L. Davis, R. Lathe, H. R. Thompson, R. W. Tyson, R. J. Sokol, and D. W. Russell. 1998. Identification of a new inborn error in bile acid synthesis: mutation of the oxysterol 7α-hydroxylase gene causes severe neonatal liver disease. *J. Clin. Invest.* **102:** 1690–1703.
19. Clayton, P. T. 1991. Inborn errors of bile acid metabolism. *J. Inherit. Metab. Dis.* **14:** 478–496.
20. Clayton, P. T., M. Casteels, G. Mieli-Vergani, and A. M. Lawson. 1995. Familial giant cell hepatitis with low bile acid concentrations and increased urinary excretion of specific bile alcohols: a new inborn error of bile acid synthesis? *Pediatr. Res.* **37:** 424–431.
21. Marschall, H-U., H. Matern, B. Wietholtz, S. Egestad, S. Matern, and J. Sjövall. 1992. Bile acid *N*-acetylglucosaminidation. In vivo and in vitro evidence for a selective conjugation reaction of 7β-hydroxylated bile acids in humans. *J. Clin. Invest.* **89:** 1981–1987.
22. Shoda, J., M. Axelson, and J. Sjövall. 1993. Synthesis of potential C_{27}-intermediates in bile acid biosynthesis and their deuterium-labeled analogs. *Steroids.* **58:** 119–125.
23. Marschall, H-U., W. J. Griffiths, J. Zhang, H. Wietholtz, H. Matern, S. Matern, and J. Sjövall. 1994. Positions of conjugation of bile acids with glucose and *N*-acetylglucosamine in vitro. *J. Lipid Res.* **35:** 1599–1610.
24. Sjövall, J. 1994. Mass spectrometry in studies of inherited and acquired diseases of bile acid synthesis and metabolism. *In* Advances in Chemical Diagnosis and Treatment of Metabolic Disorders. Vol 2. I. Matsumoto, T. Kuhara, O. A. Mamer, L. Sweetman, and R. G. Calderhead, editors. Kanazawa Medical University Press, Kanazawa, Japan. 107–122.
25. Kimura, A., M. Suzuki, T. Murai, T. Kurosawa, M. Tohma, M. Sata, T. Inoue, A. Hoshiyama, E. Nakashima, Y. Yamashita, T. Fujisawa, and H. Kato. 1998. Urinary 7α-hydroxy-3-oxochol-4-en-24-oic and 3-oxochola-4,6-dien-24-oic acids in infants with cholestasis. *J. Hepatol.* **28:** 270–279.
26. Bremmelgaard, A., and J. Sjövall. 1980. Hydroxylation of cholic, chenodeoxycholic, and deoxycholic acids in patients with intrahepatic cholestasis. *J. Lipid Res.* **21:** 1072–1081.
27. Shoda, J., A. Toll, M. Axelson, F. Pieper, K. Wikvall, and J. Sjövall. 1993. Formation of 7α- and 7β-hydroxylated bile acid precursors from 27-hydroxycholesterol in human liver microsomes and mitochondria. *Hepatology.* **17:** 395–403.
28. Javitt, N. B., E. Kok, F. Carubbi, T. Blizzard, M. Gut, and C. Y. Byon. 1986. Bile acid biosynthesis. Metabolism of 3β-hydroxy-5-cholenoic acid to chenodeoxycholic acid. *J. Biol. Chem.* **261:** 12486–12489.
29. Furster, C., J. Zhang, and A. Toll. 1996. Purification of a 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase from pig liver microsomes active in major and alternative pathways of bile acid biosynthesis. *J. Biol. Chem.* **271:** 20903–20907.
30. Furster, C. 1999. Hepatic and extrahepatic dehydrogenation/isomerization of 5-cholestene-3β,7α-diol: localization of 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase in pig tissues and subcellular fractions. *Biochim. Biophys. Acta.* **1436:** 343–353.
31. Schwarz, M., A. C. Wright, D. L. Davies, H. Nazer, I. Björkhem, and D. W. Russell. 2000. The bile acid synthetic gene 3β-hydroxy-Δ^5-C_{27}-steroid oxidoreductase is mutated in progressive intrahepatic cholestasis. *J. Clin. Invest.* **106:** 1175–1184.
32. Javitt, N. B., and S. Emerman. 1968. Effect of sodium taurolithocholate on bile flow and bile excretion. *J. Clin. Invest.* **47:** 1002–1014.
33. Mathis, U., G. Karlaganis, and R. Preisig. 1983. Monohydroxy bile salt sulfates: tauro-3β-hydroxy-5-cholenoate-3-sulfate induces intrahepatic cholestasis in rats. *Gastroenterology.* **85:** 674–681.
34. Stieger, B., J. Zhang, B. O'Neill, J. Sjövall, and P. J. Meier. 1997. Differential interaction of bile acids from patients with inborn errors of bile acid synthesis with hepatocellular bile acid transporters. *Eur. J. Biochem.* **244:** 39–44.
35. Sequeira, J. S. S., A. Vellodi, M. T. Vanier, and P. T. Clayton. 1999. Niemann–Pick disease type C and defective peroxisomal β-oxidation of branched-chain substrates. *J. Inher. Metab. Dis.* **21:** 149–154.