Familial Giant Cell Hepatitis Associated with Synthesis of 3β,7α-Dihydroxy- and 3β,7α,12α-Trihydroxy-5-Cholenoic Acids

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Abstract

Urinary bile acids from a 3-mo-old boy with cholestatic jaundice were analyzed by ion exchange chromatography and gas chromatography–mass spectrometry (GC-MS). This suggested the presence of labile sulfated cholenoic acids with an allylic hydroxyl group, a conclusion supported by analysis using fast atom bombardment mass spectrometry (FAB-MS). The compounds detected by FAB-MS were separated by thin layer chromatography and high performance liquid chromatography. The sulfated bile acids could be solvolyzed in acidified tetrahydrofuran, and glycine conjugates were partially hydrolyzed by cholylglycine hydrolase. Following solvolysis, deconjugation, and methylation with diazomethane, the bile acids were identified by GC-MS of trimethylsilyl derivatives. The major bile acids in the urine were 3β,7α-dihydroxy-5-cholenoic acid 3-sulfate, 3β,7α,12α-trihydroxy-5-cholenoic acid monosulfate, and their glycine conjugates. Chenodeoxycholic acid and cholic acid were undetectable in urine and plasma. The family pedigree suggested that abnormal bile acid synthesis was an autosomal recessive condition leading to cirrhosis in early childhood.

Introduction

Production of bile by the liver is largely dependent on the synthesis and secretion of bile salts (1). The primary bile acids, chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanoic acid) and cholic acid (3α,7α,12α-trihydroxy-5β-cholanoic acid), are synthesized from cholesterol. There are several pathways by which the syntheses can be accomplished (2).

Regardless of the order of reactions, hydroxylations occur at C-7 and C-12, the 3β-hydroxy-Δ^5 structure[1] is transformed to a 3α-hydroxy-5β(H) configuration via a 3-oxo-Δ^4 intermediate, and the side chain is shortened by oxidation. The inborn errors of bile acid synthesis described to date all affect side-chain oxidation (2–8), eg., cerebrotendinous xanthomatosis (2–5), Zellweger syndrome, infantile Refsum's disease, neonatal adrenoleukodystrophy (2, 6), and pseudo-Zellweger syndrome (7).

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1. Δ^4 and Δ^5 are used to indicate the position of a double bond in bile acids, sterols, and steroids, except in formal chemical names.

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This report describes an infant with cholestatic jaundice whose urine contained unusual labile sulfated C_{24} bile acids with two or three hydroxyl groups and a double bond. Application of mild isolation procedures and mass spectrometry permitted the identification of 3β,7α-dihydroxy-5-cholenoic acid and 3β,7α,12α-trihydroxy-5-cholenoic acid. The presence of these compounds in the urine and the absence of chenodeoxycholic acid and cholic acid from the urine and plasma suggested a defect of the 3β-hydroxy-Δ^5 steroid dehydrogenase/isomerase involved in bile acid synthesis.

Methods

Case histories

The propositus, MU2, was the fifth child of Saudi Arabian parents who were first cousins. He was the third to be affected by progressive liver disease starting in the neonatal period.

JU. The first affected child, a girl, was jaundiced from the first week of life with pale stools and dark urine. Her stools were loose and offensive on normal infant formula (as opposed to low fat) feeds. She never developed pruritus. Her growth and mental development in infancy were normal. At 18 mo a liver biopsy showed an aggressive hepatitis with giant cells and bridging fibrosis; normal intrahepatic bile ducts could be identified. She died following a gastrointestinal hemorrhage at 19 mo.

MU1. This boy's early course resembled his sister's. A liver biopsy at 6 wk showed a giant cell hepatitis. The number of interlobular bile ducts was reduced. He remained jaundiced but had no pruritus and only mild steatorrhea. His developmental progress was normal. At the age of 3 yr, 9 mo he had a second biopsy. The liver cells were swollen and granular. The parenchyma was divided into irregular nodules by bands of fibrous tissue linking the portal tracts. The latter were infiltrated by small round cells and showed some bile duct proliferation. Cholestasis was evident from the presence of canalicular bile plugs and intracellular bile pigment. Thus the biopsy showed cholestasis, inflammatory changes, and a micronodular cirrhosis. MU1 died from hemorrhage following the biopsy.

MU2. The propositus was born following a normal pregnancy and weighed 4 kg. Jaundice was noted at 60 h and was associated with pale stools and dark urine. He was referred at the age of 3 mo. His length, weight, and head circumference were between the 10th and 50th centiles. He had moderate jaundice and hepatomegaly (liver span, 6 cm). The spleen tip was palpable but there was no ascites. He was not observed to scratch himself. Developmental assessment was normal and there were no dysmorphic features. Investigation results are shown in Tables I and II. The parents refused to consent to a liver biopsy or duodenal intubation. They returned to Saudi Arabia with MU2 and could not be contacted subsequently.

Bile acid analyses

URINE

A 24-h collection of urine (500 ml) was obtained from MU2, each sample being frozen within 20 min. Similar samples were obtained from normal infants and infants with cholestasis of known cause. The bile acids from 25–50 ml of urine were extracted using 6-ml cartridges of octadecylsilane-bonded silica (Analytichem International, Inc., Harbor City, CA) (9). (Similar C_{18} column extractions were also used to extract bile acids from aqueous reaction mixtures.)

Table I. Results of Investigations Performed on Plasma/Serum of MU2 at the Age of 3 Mo: Evidence for Cholestasis, Hepatocellular Damage, and Fat-Soluble Vitamin Malabsorption

Investigation	Result	Normal range or control value
Total bilirubin	162 μM	<20
Conjugated bilirubin	132 μM	<20
Alanine aminotransferase	158 U/liter	<40
Aspartate aminotransferase	157 U/liter	<50
Alkaline phosphatase	1,710 U/liter	<1,000
Total protein	58 g/liter	50–75
Albumin	39 g/liter	30–50
Vitamin A	0.39 μM	0.4–2.1
25-Hydroxy-vitamin D	<1.0 μg/liter	3–30
Vitamin E	1.3 μM	11.5–35
Prothrombin time	16 s	13 s
Partial thromboplastin time	45 s	35 s
Cholesterol	3.9 mM	3.0–5.5

Table II. Investigation of Patient MU2 at the Age of 3 Mo: Results of Screening Tests for Known Causes of Hepatocellular Damage and Cholestasis

Investigation	Result (normal range)
Plasma amino acids	Normal
Urinary succinyl acetone	Normal
Urine reducing substances	Negative
Urine organic acids, phenolic acids	Normal
Urine amino acids	Normal
Serum α_1-antitrypsin	Normal
Blood count (+ white cell histochemistry)	Normal
Plasma copper	9.3 μM (12.6–26.8)
Plasma ceruloplasmin	0.9 μM (1.3–2.9)
Hepatitis B surface antigen	Negative
Antibodies to toxoplasma, rubella, cytomegalovirus, and Treponema pallidum	Negative
Urine culture and microscopy	Negative
Abdominal ultrasound	Hepatomegaly only Gall bladder not seen Portal vein normal
Abdominal X-ray	Normal
Chest X-ray	Normal

Analysis by gas-liquid chromatography–mass spectrometry (GC-MS). The initial analysis of urinary bile acids was performed as described previously (10) with some modifications. A urine extract (from 50 ml of urine) was passed through SP-Sephadex C-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) in 70% methanol. The acids in the effluent were fractionated on Lipidex-DEAP (Packard Instrument Co., Downers Grove, IL) into neutral derivatives, unconjugated, glycine-conjugated, taurine-conjugated and sulfated bile acids (10). Following enzyme hydrolysis of glycine and taurine conjugates and solvolysis of sulfates in ethyl acetate followed by alkaline hydrolysis (10), the liberated bile acids were methylated on a column of SP-LH-20 in H^+-form in methanol (11). They were then analyzed as trimethylsilyl (TMS)[2] ethers by gas-liquid chromatography (GLC) and GC-MS. Another sample was analyzed for bile alcohols (12). The glucuronide fraction was hydrolyzed with *Helix pomatia* intestinal juice, the mono- and disulfate fractions (13) in acidified tetrahydrofuran (THF) (14).

GLC was performed with a Carlo Erba HRGC 5300 gas chromatograph. The column was a 25 m × 0.32 mm i.d. fused silica capillary coated with cross-linked methyl silicone (0.25 μ film thickness; Quadrex Corp., New Haven, CT). Helium was used as carrier gas at 75 kPa. Samples were injected on-column in 1 μl hexane at 60°C and the temperature was then taken directly to 200°C and after 5 min to 280°C at 30°C/min.

GC-MS was carried out on a VG 7070E mass spectrometer connected to a Dani 3800 gas chromatograph and a DS 2350 data system (VG Analytical, Manchester, UK). The capillary column (25 m × 0.32 mm, coated with a 0.15 μ layer of cross-linked SE-30 [Orion Analytica, Espoo, Finland]) was kept at 250°C with the outlet end extending into the ion source at 260°C. An all-glass falling needle injection system was used and the samples were injected in 2 μl of hexane. The ionization energy was 22.5 eV and the trap current 200 μA. 70 eV spectra were recorded on a Finnigan MAT-112 with a SS200 data system.

Analysis by fast atom bombardment mass spectrometry (FAB-MS). A sample of urine extract (≡ 25 μl urine) in 10 μl of methanol was applied under a stream of N_2 to the FAB target coated with glycerol matrix. Negative ion FAB spectra were recorded by computer from a Finnigan MAT-731 mass spectrometer fitted with a fast atom gun (M-scan Ltd., Ascot, Berkshire, UK) (9) or from the VG 7070E instrument with a FAB source and Ion Tech atom gun (15) operating with xenon at 8 keV.

The Lifschütz reaction. Lifschütz reagent, which produces a purple color with cholenoic acids with an allylic hydroxyl or alkoxyl substituent (16–19), was prepared by mixing 10 ml glacial acetic acid and 1 ml conc. H_2SO_4, 200 μl of reagent was added to the dried extract from 25 ml of MU2's urine. Similar urine extracts from 85 normal and 17 cholestatic controls were also tested. The cholestatic group included patients suffering from biliary atresia, idiopathic neonatal hepatitis, arteriohepatic dysplasia, nonsyndromatic paucity of interlobular bile ducts and Zellweger syndrome.

Chromatographic separation of compounds detected by FAB-MS. The above studies suggested that MU2's urine contained sulfated dihydroxy- and trihydroxycholenoic acids with an allylic hydroxyl group that could become methylated when exposed to methanol and acid. Thus it was necessary to devise schemes of analysis that avoided such artefacts.

(a) Separation using thin layer chromatography (TLC) was performed using silica gel G plates (20 × 20 × 0.25 cm) and the solvent system chloroform/methanol/7M NH_4OH (80:40:4 by vol) (20). The bile acid extract from 8 ml of urine was dissolved in methanol and applied to the plate. Reference bile acids (200 μg of each compound listed in Table III) were visualized using iodine vapor, and the allylic cholenoic acids were located by spraying with Lifschütz reagent (30 s at 20°C). By running two TLC plates and spraying one with Lifschütz reagent it was possible to scrape from the second plate the silica gel corresponding to each Lifschütz positive spot. Lifschütz-positive compounds were then eluted from the gel by shaking with 10 ml water-saturated ethyl acetate to which 10 ml of methanol was subsequently added. 1 ml of the supernatant was analyzed by FAB-MS.

(b) Separation using high performance liquid chromatography (HPLC) was performed. The column system comprised a Guard-Pak with a C_{18} cartridge (Waters Associates, Milford, MA) and a μBondapak C_{18} steel column, 3.9 mm × 30 cm (Waters Associates). The solvent system was 0.5% (wt/vol) aqueous ammonium carbonate/acetonitrile

2. *Abbreviations used in this paper:* C_{18}, octadecylsilane-bonded silica; FAB-MS, fast atom bombardment mass spectrometry; GC-MS, gas-liquid chromatography–mass spectrometry; GLC, gas-liquid chromatography; HPLC, high performance liquid chromatography; R_f, distance moved relative to solvent front; RI, Kovats retention index; THF, tetrahydrofuran; TLC, thin layer chromatography; TMS, trimethylsilyl.

Table III. Separation of Lifschütz-Positive Compounds in Urine of MU2 by Thin Layer Chromatography

Lifschütz +ve spot	R_f value	Strength of color	Major peaks on FAB-MS	Proposed identities
1	0.09	+++++	485, 542	Trihydroxycholenoic acid sulfate and its glycine conjugate
2	0.13	+++++	469, 526	Dihydroxycholenoic acid sulfate and its glycine conjugate
3	0.29	++	405, 462	Trihydroxycholenoic acid and its glycine conjugate
4	0.31	++	389, 446	Dihydroxycholenoic acid and its glycine conjugate
5	0.38	++++	529	Monosulfated cholestenepentol
6	0.47	+	513	Monosulfated cholestenetetrol

R_f values of reference compounds (Lifschütz negative): lithocholic acid 3-sulfate, 0.25; glycocholate, 0.26; cholic acid, 0.27; glycochenodeoxycholate, 0.31; chenodeoxycholic acid, 0.33; taurocholate, 0.38; taurochenodeoxycholate, 0.42; 3β-hydroxy-5-cholenoic acid, 0.59; 3-oxo-4-cholenoic acid, 0.66.

(26:8, vol/vol) (21). An aliquot of the urine extract ($\equiv$ 0.5 ml urine) was dissolved in 200 μl of the mobile phase and injected into a 400-μl sample loop. The flow rate was 1 ml/min and 20 1-ml fractions were collected. The acetonitrile was evaporated under a stream of N_2 and the remainder lyophilized. The residue was dissolved in 200 μl of 50% methanol and 10 μl of each fraction analyzed by FAB-MS.

Full identification of urine bile acids using chromatography, microchemical reactions, and mass spectrometry: reference compounds. Methyl 3β,7α-dihydroxy-5-cholenoate and a mixture of the 7α and 7β epimers of methyl 3β-hydroxy-7-methoxy-5-cholenoate were kindly supplied by Dr. K. Uchida, Shionogi Research Laboratories, Osaka, Japan. Methyl 7α-hydroxy-3-oxo-4-cholenoate was synthesized from methyl 7α-hydroxy-3-oxo-5β-cholanoate (Steraloids, Inc., Wilton, NH) using SeO_2 in 96% ethanol and the same method as described for the free acid (22) except that chromatography on neutral alumina was employed for purification. Methyl 3β,7α-dihydroxy-4-cholenoate was prepared from the 3-oxo compound by reduction with $NaBH_4$ (16) and purified by chromatography on alumina. A mixture of the 3α and 3β epimers of methyl 7α-hydroxy-3-methoxy-4-cholenoate was prepared by refluxing methyl 3β,7α-dihydroxy-4-cholenoate with methanol/glacial acetic acid (10:1, vol/vol) (23). Starting from methyl 7α,12α-dihydroxy-3-oxo-5β-cholanoate (Steraloids, Inc.) the same reactions were used to produce methyl 7α,12α-dihydroxy-3-oxo-4-cholenoate, methyl 3β,7α,12α-trihydroxy-4-cholenoate, and methyl 7α,12α-dihydroxy-3-methoxy-4-cholenoate (3α and 3β epimers). Reference compounds (200 μg) were tested with Lifschütz reagent (200 μl).

Microchemical reactions. Conversion of urinary bile acids to methyl ester TMS ethers required solvolysis, deconjugation, methylation of the carboxyl group without methylation of an allylic hydroxyl group, and trimethylsilylation. The reactions used to achieve these modifications could be monitored by FAB-MS of the crude urine extract, FAB-MS of separated urinary bile acids, or their effect on reference compounds, principally methyl 3β,7α-dihydroxy-5-cholenoate. ~ 10 μg of the reference compound was used and the products were analyzed as TMS ethers after addition of 10 μg of triacontane as internal standard. For studies of solvolysis, dehydroepiandrosterone (3β-hydroxy-5-androsten-17-one) sulfate (Sigma Chemical Co., St. Louis, MO) was used.

(*a*) Trimethylsilylation. TMS ethers of substituted methyl cholenoates were prepared using pyridine/hexamethyldisilazane/trimethylchlorosilane (3:2:1, by vol) at 20°C overnight or at 60°C for 30 min. Peak areas relative to the internal standard were compared with those produced by the TMS ethers of substituted methyl cholanoates.

(*b*) Methylation of carboxyl groups was achieved using freshly distilled diazomethane in diethyl ether/methanol (9:1, vol/vol). The effect of these conditions on reference methyl 3β,7α-dihydroxy-5-cholenoate was studied.

(*c*) Solvolysis. The reference compound was incubated with 1 ml of freshly distilled THF/0.1 M H_2SO_4 (200:1, vol/vol) at 20°C for 15 min (24). The reaction was terminated by adding $NaHCO_3$. The products were extracted into ethyl acetate, converted to TMS ethers, and analyzed by GC-MS.

The same solvolysis procedure was used on the sulfated trihydroxycholenoic acid (spot 1) and the sulfated dihydroxycholenoic acid (spot 2) isolated from the urine of MU2 by TLC. In this case, however, the reaction was stopped with 50 μl 7 M NH_4OH. The products were analyzed by TLC and by FAB-MS of the Lifschütz-positive products.

Aliquots of the urine extract and of HPLC fractions 5 and 8 were analyzed by GC-MS following solvolysis in acidified THF, methylation with diazomethane, and trimethylsilylation.

(*d*) Deconjugation. Cholylglycine hydrolase (Sigma Chemical Co.) was employed for the hydrolysis of glycine conjugated bile acids as described by Karlaganis et al. (25), except that 12 U of enzyme were used and the products were extracted with a C_{18} cartridge. Experiments were performed on the crude urine extract and HPLC fractions 6 and 10, with FAB-MS monitoring the reaction. Cholylglycine hydrolase was also used for the deconjugation of compounds separated by TLC. In this case 90 U of enzyme and a 3-h incubation were used (26). Experiments were performed on TLC spots 1 and 2 (following solvolysis and repeat TLC) and on spots 3 and 4. The products were extracted (C_{18}), methylated with diazomethane, and analyzed by GC-MS of TMS ethers. In the case of TLC spot 1 an aliquot of the methylated extract was subjected to hydrogenation.

(*e*) Hydrogenation of methyl dihydroxy- and trihydroxycholenoates. The trihydroxycholenoic acid in MU2's urine was identified by treating the methyl ester with H_2/PtO_2 (to saturate the double bond) and comparing the products with reference substituted methyl cholanoates. The bile acid methyl ester from TLC spot 1 was dissolved in 200 μl of glacial acetic acid and reduced with H_2 and 1 mg of PtO_2 at 20°C for 30 min. The reaction mixture was diluted with 4 ml of distilled water, and the bile acid methyl esters were extracted (C_{18}) and dried under a stream of N_2. TMS derivatives were analyzed by GC-MS. The same experiment was performed with reference methyl 3β,7α-dihydroxy-5-cholenoate and methyl 3β,7α,12α-trihydroxy-4-cholenoate.

(*f*) Methylation of allylic hydroxyl groups. The dihydroxycholenoic acid in MU2's urine had one allylic hydroxyl group that could be methylated in acidic methanol. It was also sulfated on one hydroxyl group. If the allylic hydroxyl group was sulfated, methylation before solvolysis would be impossible. Conditions for methylation of the 7α-hydroxy group in reference methyl 3β,7α-dihydroxy-5-cholenoate were studied (27). The sample was dissolved in 1 ml of methanol/concentrated HCl mixture (100:1, vol/vol). After 5–10 min at 20°C, solid $NaHCO_3$ was added, methanol was removed under a stream of N_2, and the products were extracted twice with ethyl acetate. The products were analyzed by GC-MS of the TMS ethers. The crude urine extract and HPLC fractions 5, 6, 8, and 10 were subjected to identical conditions. The products were analyzed by FAB-MS either by direct insertion on the probe or following neutralization, evaporation of methanol, and extraction (C_{18}).

PLASMA

Blood was taken 2 h after a feed. Nonsulfated plasma bile acids were analyzed using a simple packed-column GLC method (28). The results were compared with those obtained from normal infants and infants with cholestasis (26). Plasma from MU2 was also analyzed by a capillary GLC method that included a solvolysis step and was thus capable of detecting sulfated cholic and chenodeoxycholic acids (29).

Results

Routine investigations

The investigations listed in Table I indicated conjugated hyperbilirubinemia with raised transaminases and alkaline phosphatase (isoenzymes not determined). These results are consistent with a giant cell hepatitis and cholestasis (as seen in the sibling's biopsy). There was some evidence of fat-soluble vitamin malabsorption, and the fecal fat excretion was 7 g/24 h (normal < 4.5 g). The investigations in Table II gave no indication of a cause of cholestasis.

Bile acid analyses

URINE

Analysis by GC-MS. The total peak area indicated a urine bile acid concentration of 23 mg/liter. Almost all bile acids (~ 97%) were found in the sulfate fraction. The GLC analysis of the methyl ester TMS ethers from this fraction showed a complex profile of peaks from retention index (RI) 2966 to RI 3361. The mass spectra indicated the presence of methyl cholenoates with three double bonds (shortest retention time) and a variety of isomeric compounds containing one methoxy group and one or two trimethylsiloxy groups and one or two double bonds. This suggested that the bile acids contained an allylic hydroxyl group that had reacted with methanol on the strong cation exchanger SP-LH-20 (11). Because one of the minor components had the RI of methyl 3β-hydroxy-5-cholenoate (TMS ether) and another peak gave a spectrum that resembled spectra of methyl 3-methoxy-7α-acetoxy-4-cholenoate and 3β-acetoxy-7-methoxy-5-cholenoate (16–19) it was postulated that at least one of the urinary bile acids had a 3,7-dihydroxy-5- or 3,7-dihydroxy-4-cholenoic acid structure.

GLC analyses of the bile alcohol glucuronide and sulfate fractions showed peaks with the retention times of bile alcohol TMS ethers. The total peak area corresponded to a concentration of about 4 mg/l urine, 75% being present in the sulfate fractions.

Analysis by FAB-MS. The urine extract produced a negative ion spectrum with four major peaks (Fig. 1). These corresponded to quasimolecular ions, $[M-1]^-$, of sulfated dihydroxycholenoic (*m/z* 469) and trihydroxycholenoic (*m/z* 485) acids and their respective glycine conjugates (*m/z* 526 and 542). Minor peaks at *m/z* 453 and 510 corresponded to the quasimolecular ions of 3β-hydroxy-5-cholenoic acid sulfate and its glycine conjugate, and the peak at *m/z* 462 to that of a glycine-conjugated trihydroxycholenic acid. Peaks at *m/z* 497, 513, and 529 indicated monosulfated cholestenetriol(s), cholestenetetrol(s), and cholestenepentol(s), respectively. The peaks at 495 and 511 may have been produced by sulfated mono- and dihydroxycholestenoic acids. The FAB spectrum shown in Fig. 1 was unique to MU2 and was not seen with urine from normal infants or infants with other causes of cholestasis (9).

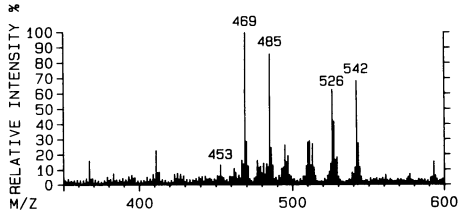

Figure 1. High mass end of the negative ion fast atom bombardment mass spectrum of an extract corresponding to 25 μl of urine from patient MU2.

The Lifschütz reaction. The urine extract from MU2 produced an immediate intense purple color with Lifschütz reagent. This was strong evidence in favour of the presence of one or more cholenoic acids with an allylic hydroxyl group (16–19). No such color reaction was seen with urine extracts from the normal or cholestatic controls. Taken together, the GC-MS data, the FAB-MS data, and the Lifschütz reaction provided accumulating evidence for the presence in MU2's urine of (*a*) a dihydroxycholenoic acid with an allylic hydroxyl group, possibly a 3,7-dihydroxy-Δ^4 acid or a 3,7-dihydroxy-Δ^5 acid and (*b*) a trihydroxycholenoic acid also with an allylic hydroxyl group, perhaps a 3,7,12-trihydroxy-Δ^4 or -Δ^5 acid bearing in mind the normal substitution in human primary bile acids and their precursors. Both of these compounds were present mainly as monosulfates and the glycine conjugates of the monosulfates.

Chromatographic separation of compounds detected by FAB-MS. Table III shows the separation of Lifschütz-positive compounds achieved using TLC with an alkaline solvent system. This TLC system does not separate glycine-conjugated bile salts from the corresponding unconjugated bile salts (20). FAB-MS and the R_f values suggested the identities listed in Table III.

Reversed-phase HPLC using a volatile alkaline buffer separated the four major components identified by FAB-MS (Fig. 2).

Full identification of urine bile acids using chromatography, microchemical reactions and mass spectrometry: reference compounds. The retention indices of the derivatized reference compounds are shown in Table IV. The mass spectra indicated derivatization of all hydroxyl groups and the peak area of the TMS ether of methyl 3β,7α-dihydroxy-5-cholenoate was similar to that given by equal amounts of the derivatives of saturated bile acids, indicating that the trimethylsilylation reaction was complete and did not destroy the 3β,7α-dihydroxy-Δ^5 structure. Only reference compounds with an allylic hydroxyl or methoxyl group produced a purple color with Lifschütz reagent; 7α-hydroxy-3-oxo-Δ^4 compounds did not react.

Microchemical reactions. (*a*) Methylation of carboxyl groups. No side products or losses of methyl 3β,7α-dihydroxy-5-cholenoate were observed following treatment with diazomethane.

(*b*) Solvolysis of reference dehydroepiandrosterone sulfate in acidified THF was > 75% complete after 15 min at 20°C. At least 80% of the reference methyl 3β,7α-dihydroxy-5-cholenoate was recovered intact. Solvolysis altered the TLC mobility of the Lifschütz-positive material in spot 1 from R_f 0.09 to 0.25. The ions *m/z* 485 and 542 in the FAB spectrum of the starting material changed to *m/z* 405 and 462 in the product. Spot 2 (R_f 0.13) moved to R_f 0.37 and the FAB spectrum changed from

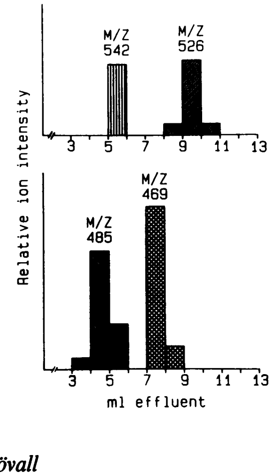

Figure 2. Separation of urinary bile acids from patient MU2 by reversed-phase HPLC. 5% of each fraction (except for fraction 7, which was lost) was analyzed by FAB-MS and the intensities of the negative ions from the bile salts are expressed relative to the intensity of *m/z* 459 from the glycerol matrix.

Table IV. Retention Indices of Methyl Ester Trimethylsilyl Ether Derivatives of Reference Bile Acids

Cholenoic acid structure*	Retention index
Δ^5-3β,7α-diOH	3204
Δ^4-3β,7α-diOH	3200
Δ^5-3β-OH-7α-OMe	3251
Δ^5-3β-OH-7β-OMe	3319
Δ^4-3β,7α,12α-triOH	3214
Δ^4-3-oxo-7α-OH	3298
Δ^4-3-oxo-7α,12α-diOH	3360

* Δ^4 and Δ^5 indicate the position of the double bond; α and β, the orientation of hydroxy (OH) or methoxy (OMe) groups.

m/z 469 and 526 to *m/z* 389 and 446. This indicated that the trihydroxycholenoic acid sulfate and its glycine conjugate (spot 1) and the dihydroxycholenoic acid sulfate and its glycine conjugate (spot 2) had been solvolyzed to produce the nonsulfated compounds. There was no evidence of any methylation or dehydration products. The solvolysis was almost complete as judged by TLC.

The gas chromatogram obtained from the urine of MU2 following solvolysis, methylation, and trimethylsilylation showed two major peaks, A and B. Peak A had an RI of 3203, compatible with the TMS ether of methyl 3β,7α-dihydroxy-5-cholenoate or 3β,7α-dihydroxy-4-cholenoate (Table IV). The mass spectrum (Fig. 3; ionization energy 22.5 eV) when compared with the reference compounds clearly indicated methyl 3β,7α-dihydroxy-5-cholenoate. The intense base peak at *m/z* 458 (M-90) was present in the spectra from both the Δ^4 and the Δ^5 compound and indicates the ease with which the allylic trimethylsiloxy group is lost (cf. TMS ether of 7α-hydroxycholesterol [30]). However, only the Δ^5 compound produced an ion of *m/z* 249 (C- and D-rings with side chain [31] and 135 (also present in the spectra of the TMS ether and acetate [17] of methyl 3β-hydroxy-7-methoxy-5-cholenoate). The Δ^4 compound produced a prominent ion at *m/z* 196, probably formed by cleavage between C-6 and C-7, and allylic cleavage between C-9 and C-10 to produce an ion containing the A-ring and C-6. (An equivalent ion at *m/z* 138 was seen in the 3-methoxy-Δ^4 derivatives.) The Δ^4 compound also showed a prominent ion at *m/z* 209 that is absent in Fig. 3. The characteristic *m/z* 129 of TMS ethers of 3β-hydroxy-Δ^5 compounds was more clearly seen in the mass spectrum of 3β,7α-dihydroxy-5-cholenoate (bis TMS ether) when mass spectra were produced with an ionization energy of 70 eV. GC-MS of HPLC fraction 10 and TLC spot 2 (following solvolysis, methylation, and trimethylsilylation) produced a single peak, again readily identified as the derivative of 3β,7α-dihydroxy-5-cholenoic acid.

Peak B had an RI (3235) similar to that of the TMS derivative of methyl 3β,7α,12α-trihydroxy-4-cholenoate (3221). The mass spectrum (Fig. 4) showed the same base peak at *m/z* 548 (M-90) but, in contrast to the reference Δ^4 compound, no clear peak at *m/z* 196, a modest peak at *m/z* 209, and (at 70 eV) a peak at *m/z* 129, all suggesting the Δ^5 analogue. The ion at *m/z* 247 (cf. TMS ether of methyl cholate) supported this structure by indicating the presence of a trimethylsiloxy group in the C- or D-ring. Thus peak B was tentatively identified as methyl 3β,7α,12α-trihydroxy-5-cholenoate tris TMS ether. The same compound was produced by solvolysis and derivatization of HPLC fraction 5 and TLC spot 1.

(*c*) Deconjugation. When the crude urine extract was incubated with cholylglycine hydrolase, FAB-MS indicated a decrease in the intensities of the peaks at *m/z* 526 and 542 relative to those at *m/z* 469 and 485. This supported the conclusion that the peaks at *m/z* 526 and 542 were produced by the glycine conjugates of the bile acid sulfates with quasimolecular ions at *m/z* 469 and 485. However, this experiment also suggested that the sulfated glycine conjugates may be poor substrates for cholylglycine hydrolase. Similar experiments using HPLC fractions 6 and 10 showed that the glycine-conjugated dihydroxycholenoic acid sulfate was hydrolyzed to a greater extent than the glycine-conjugated trihydroxycholenoic acid sulfate. Following solvolysis, deconjugation, methylation, and trimethylsilylation, GC-MS analysis of TLC spot 1 produced a single peak identical with peak B. This showed that even when deconjugation was performed after solvolysis only one trihydroxycholenoic acid was released, suggesting that this compound was present in the urine both as the monosulfate and the glycine conjugate of the monosulfate. Likewise, GC-MS analysis of spot 2 following solvolysis and deconjugation showed only the presence of 3β,7α-dihydroxy-5-cholenoic acid. GC-MS analysis of spots 3 and 4 following deconjugation, methylation, and trimethylsilylation indicated the presence of small amounts of 3β,7α-dihydroxy-5-cholenoic

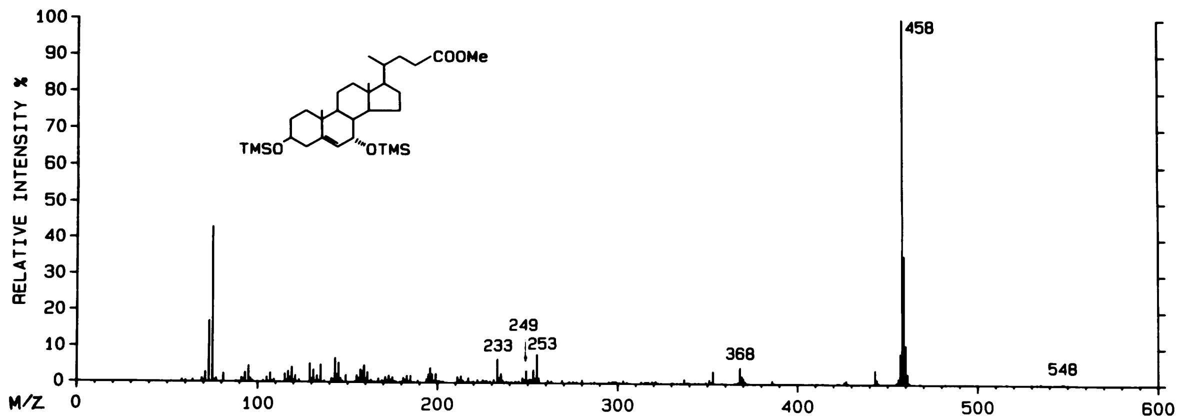

Figure 3. Electron impact mass spectrum of the methyl ester trimethylsilyl ether derivative of the dihydroxycholenoic acid obtained from the urine of patient MU2. (Peak A; ionization energy, 22.5 eV.)

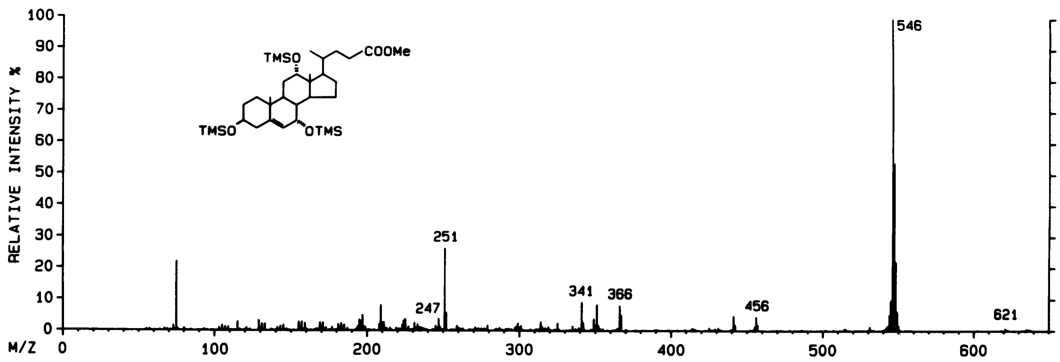

Figure 4. Electron impact mass spectrum of the methyl esther trimethylsilyl ether derivative of the trihydroxycholenoic acid obtained from the urine of patient MU2. (Peak B; ionization energy, 22.5 eV.)

acid, the trihydroxycholenoic acid, and 3β-hydroxy-5-cholenoic acid (trace only). This indicated that these compounds are present in urine in a nonsulfated form to a minor extent.

(*d*) Hydrogenation. Treatment of the methyl trihydroxycholenoate from TLC spot 1 with H_2/PtO_2 produced three saturated bile acid methyl esters identified by GC-MS of their TMS ethers as methyl 3β,7α,12α-trihydroxy-5β-cholanoate (25%), methyl 3β,12α-dihydroxy-5β-cholanoate (60%), and methyl 3β,12α-dihydroxy-5α-cholanoate (15%). The trihydroxycholanoates could have arisen from 3β,7α,12α-trihydroxy-4- or 3β,7α,12α-trihydroxy-5-cholenoate by hydrogenation but the hydrogenolysis products established that the 7α hydroxy group was allylic and therefore the only possible structure of the starting material was 3β,7α,12α-trihydroxy-5-cholenoate. This conclusion was supported by treatment of reference compounds with H_2/PtO_2. [Methyl 3β,7α-dihydroxy-5-cholenoate yielded the 3β,7α-dihydroxy-5β-cholanoate (14%), 3β-hydroxy-5β-cholanoate (55%), and 3β-hydroxy-5α-cholanoate (31%). Methyl 3β,7α,12α-trihydroxy-4-cholenoate yielded the 5β (35%) and 5α (11%) isomers of 3β,7α,12α-trihydroxycholanoate, and the 5β (37%) and 5α (17%) isomers of 7α,12α-dihydroxycholanoate].

Thus the major trihydroxycholenoic acid present in the urine as the monosulfate was identified as 3β,7α,12α-trihydroxy-5-cholenoic acid. This compound was probably also present at high concentration as the glycine conjugate of the monosulfate. It was present in small amounts in nonsulfated form.

(*e*) Methylation of allylic hydroxyl groups. Treatment of methyl 3β,7α-dihydroxy-5-cholenoate with methanol/HCl resulted in methylation of the 7α-hydroxy group followed by isomerization as expected (19, 27).

Treatment of the urine extract (and HPLC fractions 5, 6, 8, and 10) with methanol/HCl resulted in a shift of all four major quasimolecular ions by 14 or (with longer incubation times) 28 D. This data permitted a number of conclusions. Firstly, 3β,7α-dihydroxy-5-cholenoic acid monosulfate could be methylated in two positions, clearly the carboxyl group and the allylic (7α) hydroxyl group. Therefore, the sulfate moiety must be attached to the 3β-hydroxyl group. The 3β,7α,12α-trihydroxy-5-cholenoic acid monosulfate likewise had a nonsulfated 7α-hydroxy group. This, and the ease with which solvolysis was achieved (32) suggested that this compound was also sulfated at C-3. Finally, the fact that the glycine conjugates underwent identical reactions again suggested that they contained the same bile acids.

The approximate percentage composition of the urinary bile acid mixture excreted by MU2 was determined (*a*) from the peak heights produced by FAB-MS analysis (Fig. 1) and (*b*) from the results obtained using the modified solvolysis, deconjugation, and methylation reactions but no fractionation steps. The results were as follows: 3β,7α,12α-trihydroxy-5-cholenoic acid, 45–50%; 3β,7α-dihydroxy-5-cholenoic acid, 45–50%; 3β-hydroxy-5-cholenoic acid, < 8.5%. Regardless of the method of urinary bile acid analysis, chenodeoxycholic acid, and cholic acid could not be detected.

PLASMA

No cholic or chenodeoxycholic acid could be detected in the plasma of MU2 when it was analyzed by packed column GLC of bile acid methyl ester trifluoroacetates (sensitivity, 0.05 μM). The range of values found (for 2-h postprandial samples) in infants of MU2's age with and without cholestasis is shown in Table V. When the plasma sample was subjected to an analysis procedure that included solvolysis of sulfated bile acids, cholic and chenodeoxycholic acids were still undetectable (< 0.01 μM). We did not have sufficient plasma to permit a further analysis using methods that would have allowed us to detect the presence of sulfated allylic bile acids.

Discussion

MU2 suffered from a unique form of giant cell hepatitis that can be diagnosed by testing a urine extract with Lifschütz reagent or by FAB-MS. The diagnosis can be confirmed by GC-MS analysis of the urine bile acids if appropriate methodology is used. The condition appears to be an autosomal recessive one; the parents were healthy but consanguinous and one daughter and two sons were affected. Untreated it led to cirrhosis before the age of 5 yr.

The major bile acids in MU2's urine were 3β,7α-dihydroxy-5-cholenoic acid and 3β,7α,12α-trihydroxy-5-cholenoic acid. Both were excreted mainly in sulfated form. No chenodeoxycholic acid or cholic acid was found in plasma or urine. It seems unlikely that these normal bile acids were excreted in bile.

Table V. Analysis of Plasma Nonsulfated Bile Acids by Packed Column GLC of Methyl Ester Trifluoroacetates: Results Obtained on Plasma from MU2, Normal Infants, and Infants with Cholestasis

	Plasma concentration (μM)		
Bile acid	MU2	Normal infants	Infants with cholestasis
		$n = 16$	$n = 18$
Cholic acid	<0.05	0.15–3.50	7–317
Chenodeoxycholic acid	<0.05	3.23–5.32	25–359

The results suggest a defect in bile acid biosynthesis affecting the conversion of 3β-hydroxy-Δ^5 intermediates to the 3α-hydroxy-5β(H) compounds. In the major pathway from cholesterol, 7α-hydroxycholesterol is oxidized to 7α-hydroxy-4-cholesten-3-one by a microsomal 3β-hydroxy-Δ^5-steroid dehydrogenase/isomerase (2). This reaction apparently did not occur in patient MU2. This may have been due to an absence of active enzyme or protection of the 3β-hydroxy-Δ^5 structure by sulfation. The former explanation appears more likely and would be analagous to the 3β-hydroxy-Δ^5-steroid dehydrogenase deficiency affecting steroid hormone biosynthesis. MU2 showed no signs of defective steroid hormone synthesis. It has been established, both in vivo in man (33) and in vitro in the rat and rabbit (34, 35), that the enzymes required for steroid and bile acid synthesis are different and that the liver contains at least two different enzymes, only one being active on 7α-hydroxycholesterol (35).

In the major pathway of bile acid biosynthesis, modifications of the ring structure precede side chain cleavage (2). Our studies on MU2 indicate that shortening of the side chain can occur without prior completion of the nuclear modifications. This provides further support for the proposed alternative pathways of bile acid synthesis via 26-hydroxycholesterol (36–38), 3β-hydroxy-5-cholenoic acid (39, 40), and/or 3β,7α-dihydroxy-5-cholenoic acid (19, 41). Yamasaki and coworkers have identified the Lifschütz-positive bile acids in hydrolyzed human bile as 3β,7α-dihydroxy-4- and 3β,7α-dihydroxy-5-cholenoic acids (17). Although the concentrations were probably very low, this shows that side chain cleavage of 7α-hydroxycholesterol can occur in normal individuals. The Δ^4 acid was not found in the urine from our patient, further evidence for the absence of an active 3β-hydroxy-Δ^5 steroid dehydrogenase/isomerase. The excretion of 3β,7α,12α-trihydroxy-5-cholenoic acid in an amount similar to that of the 3β,7α-dihydroxy acid indicates the existence of a 12α hydroxylase active on 3β-hydroxy-Δ^5 intermediate(s).

The bile acids in MU2's urine were only partially conjugated with glycine and no taurine conjugates were detected. This suggests defective conjugation of the unsaturated bile acids, because urinary bile acids in patients with other causes of cholestasis, whether sulfated or nonsulfated, are conjugated for the most part with glycine or taurine (10, 42). The urinary bile acids were mainly sulfated. This is usually the case with mono- and dihydroxy bile acids, whereas trihydroxy bile acids are mostly nonsulfated (10, 42). Sulfation depends both on the number of hydroxy groups and the stereochemistry, and 3β-hydroxy-Δ^5 steroids of medium polarity are largely present as 3-sulfates in man (43, 44). This is particularly true in infants (45, 46). Thus, the 3β-hydroxy group of the di- and trihydroxycholenoic acids may be rapidly sulfurylated in the absence of further oxidation.

The mechanism by which impaired synthesis of chenodeoxycholic and cholic acids led to disease in this family is uncertain. Did the affected individuals have cholestasis (reduced bile flow) and, if so, why? The clinical diagnosis of intrahepatic cholestasis is usually made on the basis of biochemical criteria (eg., elevated plasma concentrations of primary bile acids, conjugated bilirubin and alkaline phosphatase) and biopsy evidence (particularly retention of bile pigment in hepatocytes and canaliculi). Plasma bile acid concentrations, as measured by the use of conventional methods, clearly cannot be used as a criterion of cholestasis in this family; by all the other criteria, cholestasis was present. If bile flow was reduced, can this be explained by reduced synthesis of chenodeoxycholic and cholic acids and increased synthesis of 3β,7α-dihydroxy-, 3β,7α,12α-trihydroxy-, and possibly 3β-hydroxy-5-cholenoic acids? One can only speculate. Chenodeoxycholic and cholic acids stimulate bile acid–dependent bile flow (1), and in their absence 3β-hydroxy-5-cholenoic acid causes marked cholestasis in the rat and hamster (47). The properties of 3β,7α-dihydroxy-5-cholenoic acid are less clear. The nonsulfated bile acid does not produce cholestasis in the hamster (41) but this may be because it is efficiently converted to chenodeoxycholic acid (19). In MU2 this conversion was blocked, and in these circumstances the dihydroxycholenoic acid or its sulfate may have induced cholestasis or at least failed to produce bile acid–dependent bile flow.

Even in the absence of a full explanation of the pathogenesis of the disease, treatment by oral administration of cholic and chenodeoxycholic acids could be attempted. If an adequate pool of these exogenous bile acids was built up, this should lead to (*a*) improvement in the micellar solubilisation of fats and fat soluble vitamins, (*b*) stimulation of bile flow and hence elimination of toxic substances from the liver, and (*c*) inhibition of cholesterol 7α-hydroxylase. This may diminish the production of toxic metabolites from cholesterol.

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