High levels of (24S)-24-hydroxycholesterol 3-sulfate, 24-glucuronide in the serum and urine of children with severe cholestatic liver disease

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Abstract Extracts of urine and serum from children with cholestatic liver disease were analyzed by fast atom bombardment (FAB) mass spectrometry. About half of all spectra showed a peak at *m/z* 657, compatible with the presence of a glucuronidated cholestenediol sulfate. Separation by ion exchange chromatography before and after solvolysis and treatment with β-glucuronidase, combined with analyses by gas chromatography–mass spectrometry and FAB mass spectrometry with collision-induced dissociation, showed that the major compound responsible for the peak at *m/z* 657 was (24S)-24-hydroxycholesterol 3-sulfate, 24-glucuronide. The double conjugate of 27-hydroxycholesterol was also identified and double conjugates of cholestene- and cholestanetriols were also present. Semiquantitative analyses of the double conjugate of 24-hydroxycholesterol in patients whose FAB spectra showed a peak at *m/z* 657 indicated serum levels of 2–18 μM and a daily urinary excretion of 0.1–2.7 μmol/24 h. Eleven of 13 studied patients with a prominent peak at *m/z* 657 in the FAB spectra of their serum or urine either underwent liver transplantation or died. It is concluded that double conjugation of hydroxysterols with sulfuric and glucuronic acids can be an important metabolic pathway, particularly for (24S)-24-hydroxycholesterol. It is speculated that serious cholestatic liver disease may induce an increased formation and release of (24S)-24-hydroxycholesterol from brain (Lütjohann et al. 1996. *Proc. Natl. Acad. Sci. USA.* **93:** 9799–9804) with subsequent extracerebral conjugation with sulfuric and glucuronic acids.—**Meng, L. J., W. J. Griffiths, H. Nazer, Y. Yang, and J. Sjövall.** 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.* 1997. **38:** 926–934.

Supplementary key words 24-hydroxycholesterol • glucuronide • sulfate • infants • cholestasis • fast atom bombardment • mass spectrometry

Many oxysterols, including 24-hydroxycholesterol, have potential 3-hydroxy-3-methylglutaryl-coenzyme A reductase repressor activity (1, 2). 24-Hydroxycholesterol occurs in brain (3), adrenal glands (4), and plasma (5,6) in both free and esterified form, and as the sulfate in human meconium and infant feces (7,8). It can be formed from cholesterol by rat brain microsomes (9) and pig (10) and rat (11) liver mitochondria. However, the biological function and metabolic fates of 24-hydroxycholesterol are not clear. A recent study has indicated that formation of 24-hydroxycholesterol is of importance for cholesterol homeostasis in the brain, and that the major part of 24-hydroxycholesterol in plasma originates from this organ (12). The level of this oxysterol in the circulation was also found to be markedly age-dependent (12). Thus, studies of the occurrence and metabolism of 24-hydroxycholesterol are of importance to increase our understanding of the biological role of this sterol under normal and pathological conditions. In the present communication, we report the identification of a sulfated and glucuronidated form of 24-hydroxycholesterol that is present in high concentrations in the serum and urine of children with severe cholestatic liver disease.

MATERIALS AND METHODS

Patients and samples

Fast atom bombardment mass spectrometry (FABMS) was used in a screening program to detect

Abbreviations: CID, collision-induced dissociation; ES, electrospray; ESMS, electrospray mass spectrometry; FABMS, fast atom bombardment mass spectrometry; FAB, fast atom bombardment; GLC, gas–liquid chromatography; GC/MS, gas chromatography/mass spectrometry; OATOF, orthogonal acceleration time-of-flight; RI, retention indices; TMS, trimethylsilyl.

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TABLE 1. Clinical data and relative intensity of m/z 657 in the FAB spectra of urine and serum samples from patients with cholestatic liver disease

Patient	Relative Intensity[a]		Age	Sex	Diagnosis	Liver Transplantation	Outcome
	Urine	Serum	yr				
1	80	—[b]	0.6	M	Large duct obstruction, cirrhosis	no	died at 1 yr
2	25	25	4	F	Biliary atresia, marked portal fibrosis	yes	died at 5 yrs
3	25	—	4	M	Neonatal hepatitis syndrome, cirrhosis	—[b]	no follow-up
4	20	10	0.2	M	Familial cholestasis, progressive liver disease	no	alive at 4 yrs
5	30	—	0.7	F	Biliary atresia, progressive cirrhosis	yes	alive at 6 yrs
6	35	15	3.5	M	Paucity of bile ducts, cholestasis	—	no follow-up
7	20	15	0.5	M	Neonatal hepatitis syndrome	no	alive at 4.5 yrs
8	35	30	5.5	M	Progressive liver disease	no	died at 8.5 yrs
9	20	15	0.5	F	Chronic active hepatitis	planned	alive at 4.5 yrs
10	20	30	10	F	Progressive cholestatic liver disease	yes	alive at 14 yrs
11	3	30	2	F	Biliary atresia, progressive cirrhosis	yes	alive at 7 yrs
12	10	15	0.6	M	Biliary atresia, progressive cirrhosis	yes	alive at 7 yrs
13	10	20	3	F	Intrahepatic biliary atresia	—	no follow-up
14	15	—	2.5	M	Primary biliary cirrhosis	yes	alive at 8.5 yrs
15	15	4	1.6	M	—	no	died at 2 yrs
16	2	30	0.3	M	Neonatal hepatitis syndrome	—	no follow-up
17	4	15	1	M	Bile plug syndrome, neonatal hepatitis	no	died at 6 yrs
18	30	12	2.7	M	Neonatal hepatitis and portal fibrosis	—	no follow-up

[a]Intensity of m/z 657 relative to that of the most intense peak above m/z 400.
[b]Samples or data not available.

inherited defects in bile acids biosynthesis. Samples of urine and serum from 77 children with cholestatic liver disease of unknown etiology who were treated at King Faisal Specialist Hospital and Research Centre, Saudi Arabia, were analyzed. Urine samples (25 ml) were passed through a washed Sep-Pak C_{18} cartridge (Millipore-Waters, Milford MA) (13) followed by a wash with water; serum samples (2 ml) were diluted with 8 ml 0.1 M sodium hydroxide heated to 64°C and then extracted by the cartridge (13). The cartridges were then mailed to Stockholm, eluted with 7 ml methanol, and diluted to 10 ml with water. This solution was analyzed by FABMS. In addition to the peaks of deprotonated molecules of known bile acids and bile alcohol conjugates, a significant peak at m/z 657 was seen in about half of the spectra. Clinical data for patients showing m/z 657 with a relative intensity greater than 10% in spectra of serum extracts or 15% in spectra of urine extracts are given in **Table 1.** Samples from these patients were selected for study of the structures of compounds producing ions at m/z 657.

Isolation, solvolysis, and hydrolysis of steroid conjugates

The extract corresponding to 10 ml of urine or 1 ml of serum was applied in 70% aqueous methanol on a column (7.5 × 0.4 cm) of the lipophilic anion exchanger Lipidex-DEAP in acetate form (Packard Instrument Co., Downers Grove, IL). After elution of glucuronides with 10 ml of 0.25 M formic acid in 70% ethanol, sulfated steroids and bile acids were eluted with 6.5 ml of 0.3 M acetic acid/ammonium hydroxide, pH 9.6, in 70% ethanol (14).

After removal of ethanol and dilution with water, steroid conjugates in the latter fraction were extracted using a bed of Preparative C_{18} (Millipore-Waters). Aliquots of this extract were taken for FABMS analyses. The remainder was taken to dryness and solvolyzed (15). The desulfated steroids were extracted with Preparative C_{18} and applied in 70% methanol to a column of Lipidex-DEAP. After a wash with 5 ml of 0.1 M acetic acid in 70% ethanol, steroid glucuronides were eluted with formic acid as above. Aliquots of this fraction were taken for FABMS analyses. The remainder was taken to dryness and glucuronides were hydrolyzed with a preparation of *Helix pomatia* intestinal juice (Reactifs IBF, France) (16). The hydrolysate was extracted with Preparative C_{18} and passed through a column of Lipidex-DEAP to obtain the neutral steroids (14).

Gas–liquid chromatography (GLC) and gas chromatography–mass spectrometry (GC/MS)

The neutral steroids were analyzed by GLC and GC/MS after conversion into trimethylsilyl (TMS) ethers (17). For quantitative analyses, a suitable amount of internal standard, *n*-hexatriacontane, was added to aliquots of the neutral steroid fraction. GLC was carried out using a Carlo Erba GC 6000 gas chromatograph connected to a Spectra-Physics SP4270 integrator. An on-column injection system and a fused silica column

(25 m × 0.32 mm coated with a 0.25-μm layer of cross-linked methyl silicone (Quadrex Corp., New Haven, CT)) were used with a flame ionization detector. The samples were injected onto the column in 0.5–1.5 μl of hexane at 60°C. The temperature was programmed to rise from 60°C to 210°C at a rate of 30° min^{-1}, remain at 210°C for 1.5 min, then rise to 320°C at a rate of 2.5°C min^{-1}, and remain at this temperature for 3 min. Retention indices (RI) (18) were calculated from the retention times as compared with those of a series of *n*-hydrocarbons, and the amount of steroids was calculated from the peak area as compared with that of the internal standard.

GC/MS was carried out on a VG 7070E double-focusing mass spectrometer with an electron-impact ion source, a DANI 3800 gas chromatograph, and a VG 11-250 data system (formerly VG Analytical now Micromass Ltd., Manchester, UK). The capillary column (the same as for the GLC) was directly connected and extended into the ion source. An all-glass falling-needle system was used for the injection of the sample at 210°C. After 4 min the temperature was taken to 315°C at a rate of 2.5°C min^{-1} and maintained at this temperature for 5 min. The electron-impact energy was 70 eV and the trap current was 200 mA. Spectra were recorded in the mass range of *m/z* 70–800 at a scan rate of 2 s per decade and a resolution of 1000 (5% valley).

FABMS with collision-induced dissociation (CID)

The extracts of urine and serum were analyzed by negative-ion FABMS on a VG 70–250 double-focusing mass spectrometer (Micromass Ltd). Ten μl of the extract was dissolved in a drop of glycerol applied to the FAB probe tip. Excess solvent was removed under a stream of N_2. Xenon atoms were used to bombard the sample, the ion gun conditions being typically 8 keV accelerating potential and 1 mA discharge current. The spectrometer accelerating potential was 6 kV.

Further FAB and electrospray (ES) analyses were carried out on an AutoSpec-TOFFPD double-focusing mass spectrometer (Micromass Ltd). For the recording of negative-ion FAB spectra (more correctly in this instance termed liquid secondary-ion mass spectra), the instrument was fitted with a static FAB source. Samples were dissolved in glycerol which was bombarded with Cs ions from a cesium ion gun operated at an anode potential of 25 kV. The spectrometer accelerating potential was 8 kV. Certain samples were also analyzed by negative-ion ESMS. Samples were introduced into the ES ion source by continuous infusion in an aqueous methanol solvent at a flow rate of 5 μl/min. For the recording of ES mass spectra, the instrument accelerating potential was 4 kV. FAB/CID spectra were generated using He as the collision gas in the first field free region gas cell at a pressure sufficient to reduce the parent $[M-H]^-$ ion beam by 50%. Daughter ion spectra were recorded as linked scans (the ratio magnetic field/electric sector voltage is kept constant) on the $[M-H]^-$ ions. ES/CID spectra were also recorded. The double-focusing sectors of the instrument were used for parent ion selection, xenon was the collision gas in the fourth field free region gas cell, and daughter ions were mass measured on the orthogonal acceleration time-of-flight (OATOF) analyzer (19).

RESULTS

FABMS analysis of conjugates

The high mass region of the negative-ion FAB mass spectrum of the extract of urine from a child with liver disease (patient 1, Table 1) is shown in **Fig. 1.** The peaks at *m/z* 448 and 498 represent glycine- and taurine-conjugated dihydroxycholanoates, respectively, and peaks at *m/z* 528 and 578 represent their sulfates. Glycine-conjugated trihydroxycholanoates give a peak at *m/z* 464, and their sulfates the peak at *m/z* 544. Taurine-conjugated trihydroxycholanoates give a peak at *m/z* 514. The peak at 480 is compatible with taurine-conjugated monohydroxycholenoate and/or glycine-conjugated tetrahydroxycholanoate. Peaks at *m/z* 444, and 460 indicate the presence of glycine conjugates of mono- and dihydroxyoxocholenoates, respectively, and those at *m/z* 494 and 510 of the corresponding taurine conjugates. The peaks at *m/z* 607, 613, 623, and 627 are produced by glucuronidated cholestenetriolone, norcholestanepentol, cholestenetetrolone, and cholestanepentol, respectively.

The peak at *m/z* 657 is compatible with deprotonated molecules of a cholestenediol conjugated with both sulfuric acid and glucuronic acid. When the urine or serum extracts were group-fractionated by ion-exchange chromatography, *m/z* 657 appeared in the FAB spectra of the fraction containing sulfated bile acids and steroids. After solvolysis, this peak disappeared and was replaced by *m/z* 577 which, upon rechromatography, appeared in the spectrum of the fraction containing steroid glucuronides. Hydrolysis of this fraction with β-glucuronidase eliminated the peak at *m/z* 577, and rechromatography yielded a fraction of neutral steroids among which a cholestenediol was detected by GC/MS analysis.

GC/MS analysis of deconjugated steroids

The steroids present as double conjugates with sulfuric and glucuronic acids were analyzed by GLC and

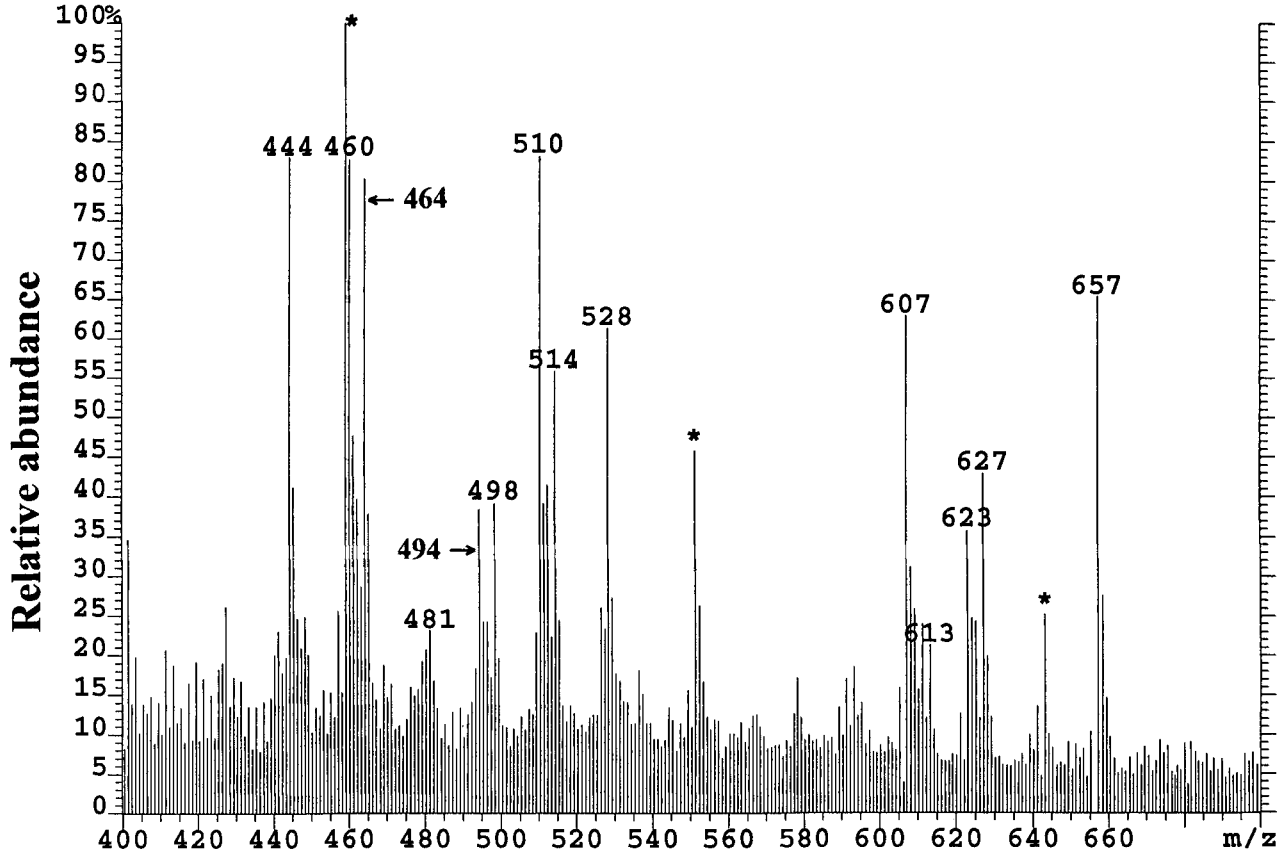

Fig. 1. The high mass region of the negative-ion FAB mass spectrum of a urine extract from patient 1 (Table 1). Peaks marked with an asterisk are from the glycerol matrix.

GC/MS after isolation by ion-exchange chromatography, solvolysis, rechromatography on Lipidex-DEAP, and hydrolysis of the glucuronide fraction. The total ion chromatogram obtained in the GC/MS analysis of the TMS ethers of these steroids in serum from patient 2 (Table 1) is shown in **Fig. 2.** The mass spectrum of the predominant compound (peak 2) showed prominent peaks at *m/z* 145, 159 and 413 [M–43–90] typical of the TMS ether of 24-hydroxycholesterol (8). The retention index was also identical to that of this reference compound. The stereochemistry at C-24 was determined by GC/MS analyses of the TMS ether mixed with the TMS ethers of deuterated (24R)- and (24S)-24-hydroxycholesterol (12). The 24-hydroxycholesterol in serum and urine was found to be the 24S isomer.

Six additional steroids were found to be present as sulfate-glucuronide double conjugates (peaks 1 and 3–7, Fig. 2). Peak 4 gave a mass spectrum identical to that of 27-hydroxycholesterol TMS ether. The spectrum of peak 1 showed a molecular ion at *m/z* 548 and peaks at *m/z* 458 and 368 indicative of the TMS ether of a cholestanediol. Intense peaks at 145 and 159 and loss of a fragment of mass 43 from the molecular ion and with additional loss of trimethylsilanol provide evidence for the presence of a 24-trimethylsiloxy group. Thus, the compound producing peak 1 is partially identified as 5ξ-cholestane-3ξ,24ξ-diol. The spectrum of peak 3 also showed intense fragment ions at *m/z* 145 and 159 and other peaks compatible with a cholestenetriol carrying two hydroxyl groups in the ring system in addition to that at C-24.

Peak 5 gave a spectrum with a very intense fragment ion at *m/z* 131 indicative of the TMS ether of a 25-hydroxysterol (20). The RI was higher than that of 25-hydroxycholesterol TMS ether indicating the presence of additional hydroxyl groups. However, other fragment ions were too weak to permit further characterization of this compound. The spectra recorded at peaks 6 and 7 indicated that these represented a cholestenetriol and a cholestanetriol, respectively. The locations of the hydroxyl groups were not obvious from the spectra.

The profiles of doubly conjugated sterols were similar for the patients studied. An infant with inborn deficiency of 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/iso-

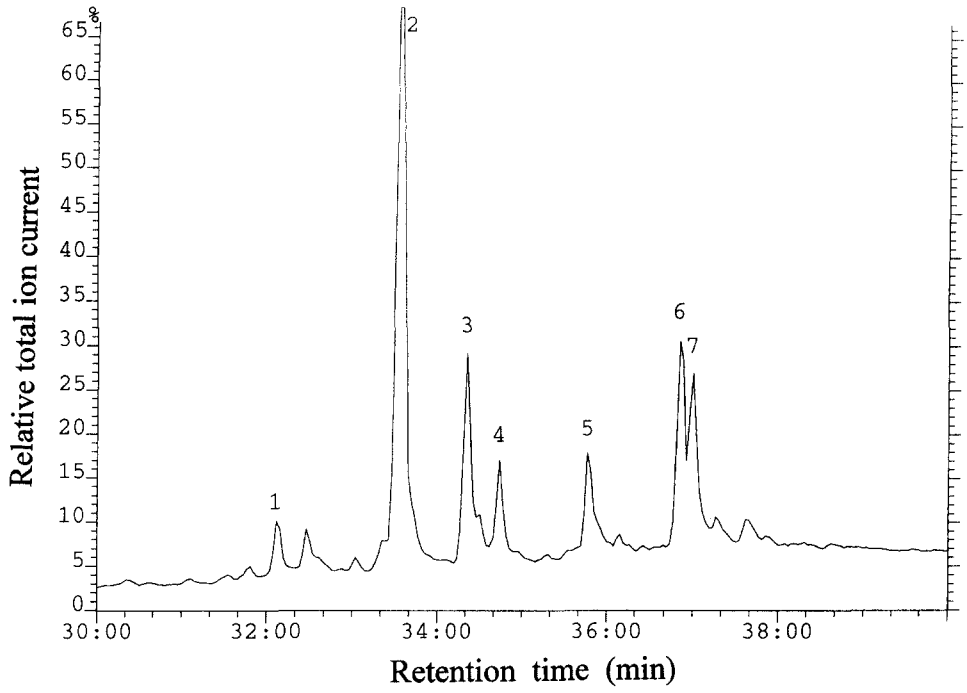

Fig. 2. The total ion current chromatogram obtained in the GC/MS analysis of TMS ethers of sterols released from the fraction of double conjugates from the serum of patient 2 (Table 1). The numbered peaks correspond to (see text): 5ζ-cholestane-3ζ,24ζ-diol (1); (24S)-24-hydroxycholesterol (2); cholestenetriol containing a 24-hydroxy group (3); 27-hydroxycholesterol (4); hydroxysterol containing a 25-hydroxy group (5); cholestenetriol (6); cholestanetriol (7).

merase (not listed in Table 1) constituted an exception. FAB spectra of urine and serum from this patient indicated the presence of sulfate-glucuronate double conjugates of cholestenediol, cholestenetriol and cholestenetetrol (*m/z* 657, 673 and 689, respectively). GC/MS analyses of these compounds after sequential removal of the conjugating groups showed that 24-hydroxycholesterol was only a minor component. Most steroids seemed to have a 5-cholestene-3,7-diol structure with additional hydroxyl groups in the rings or side chain. Two compounds dominated, possibly being isomers of 5-cholestene-3,7,24,25-tetrol. The condition of this patient was greatly improved by treatment with chenodeoxycholic acid (21).

Determination of the positions of conjugation

The positions of conjugation with sulfuric and glucuronic acids were determined by FAB/CID and ES/CID. Shown in **Fig. 3** is the FAB/CID spectrum of the deprotonated molecules of *m/z* 657 recorded as a linked scan. The spectrum shows fragmentation characteristic of steroid sulfates and glucuronides (19, 22, 23). The presence of the sulfate group is immediately identified by the ions at *m/z* 96, 577, and 559 corresponding to $[SO_4]^-$, $[M-H-SO_3]^-$, and $[M-H-H_2SO_4]^-$ ions, respectively. Similarly, fragment ions $[M-H-176]^-$ (*m/z* 481) and $[M-H-194]^-$ (*m/z* 463) are characteristic of the glucuronic acid conjugating group. In the gas phase the HSO_4 group is more acidic than the glucuronic acid group and will primarily be the site for deprotonation. Detailed studies have been carried out on the CID of lipid sulfates and it has been shown that many of the fragmentations of these molecules occur remote from the site of charge, i.e., the SO_4 group (22, 24). These so-called charge-remote fragmentations allow the structures of the parent ions to be determined. The fragment ions at *m/z* 217, 257, and 311 are characteristic of 3-sulfoxy-Δ^5 steroids (22). The fragment ion at *m/z* 379 is also characteristic of a 5-cholesten-3-ol sulfate. The fragment ion at *m/z* 437 is highly indicative of the glucuronic acid group being linked to the 24-hydroxyl group. ES/CID spectra of the deprotonated molecules at *m/z* 657 were also recorded on the OATOF analyzer. The fragmentation patterns were essentially similar to those obtained by FAB/CID; however, more low-mass ions corresponding to fragments of the glucuronide moiety were observed (data not shown). Shown in **Fig. 4** is the ES/CID spectrum of the deprotonated molecules of the glucuronide (*m/z* 577) obtained after solvolysis and rechromatography on Lipidex-DEAP. Low mass fragment ions characteristic of the glucuronide moiety were observed, as were $[M-H-176]^-$ (*m/z* 401)

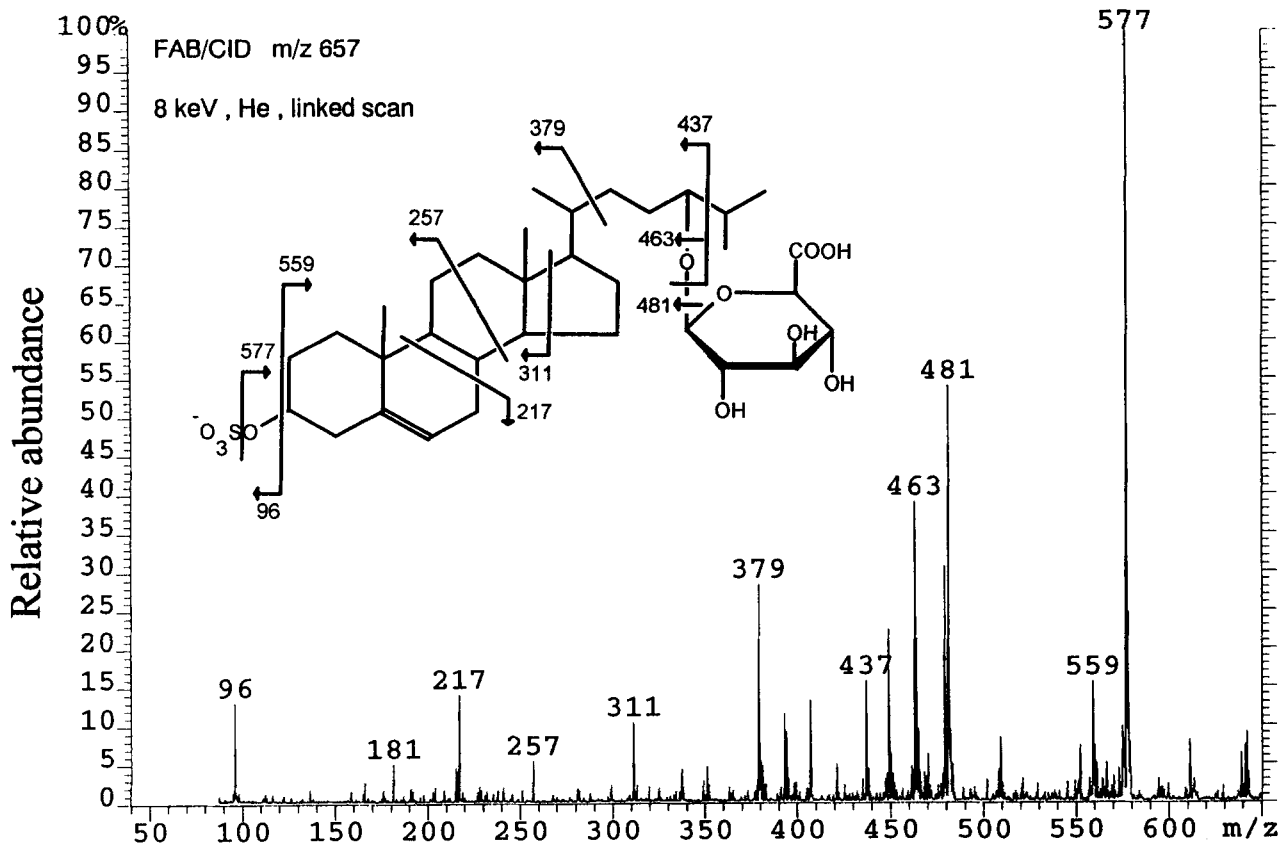

Fig. 3. FAB/CID spectrum of $[M-H]^-$ ions (m/z 657) of the sulfated and glucuronidated double conjugate of 24-hydroxycholesterol. Fragment ions at m/z 559 are formed by loss of H_2SO_4, those at m/z 481 and 463 by loss of $C_6H_8O_6$ and $C_6H_{10}O_7$, respectively.

fragment ions. The fragment ion at m/z 439 corresponds to fragmentation at the B/C ring junction as shown in the inset to Fig. 4. Intense fragmentations at this position are characteristic of 3-hydroxy-Δ^5 structures (22). This CID spectrum of the steroid glucuronide provides further evidence for the ion at m/z 657 corresponding to the deprotonated cholestenediol sulfated at C-3 and glucuronidated at C-24.

Serum concentrations and urinary excretion of doubly conjugated sterols

The concentrations in serum and excretion in urine of 24(S)-24-hydroxycholesterol as double conjugate were estimated by GLC analysis after sequential removal of the sulfate group, isolation of glucuronides, and hydrolysis of the latter. The results are given in **Table 2.** It should be emphasized that the values represent minimum concentrations and excretion, as the recoveries in the initial extraction and in the removal of conjugating groups can only be assumed to be satisfactory based on analogies with known conjugates. It is notable that the levels of the 24(S)-24-hydroxycholesterol double conjugate in serum were in the μM range. The renal clearances of this compound in two subjects were 0.03 and 0.12 L × day^{-1}, respectively.

Samples of plasma and urine from two cholestatic infants whose FAB spectra showed no peak at m/z 657 were also analyzed by GLC and GC/MS. The double conjugate of 24-hydroxycholesterol was found in plasma in one of these patients at a concentration of 0.4 μM. It was below the detection limit (about 0.05 μM) in the other patient, in whom the cholestenetriol containing a 24-hydroxyl group (peak 3, Fig 2) was present at a concentration of 0.39 μM.

Excretion of the double conjugate of 24-hydroxycholesterol in urine was in most cases accompanied by significant excretion of glycine (m/z 444) and taurine (m/z 494) conjugates of 7α-hydroxy-3-oxo-4-cholenoate (12 of 18 patients) and/or 7α,12α-dihydroxy-3-oxo-4-cholenoate (m/z 460 and 510) (17 of 18 patients) (Fig. 1). Eleven of the 13 patients who could be followed up (Table 1) either underwent liver transplantation or died of their liver disease. The FAB spectra from six of these showed simultaneous presence of the double conjugate of 24-hydroxycholesterol and urinary excretion of conjugates of 7α-hydroxy-3-oxo-4-cholenoate.

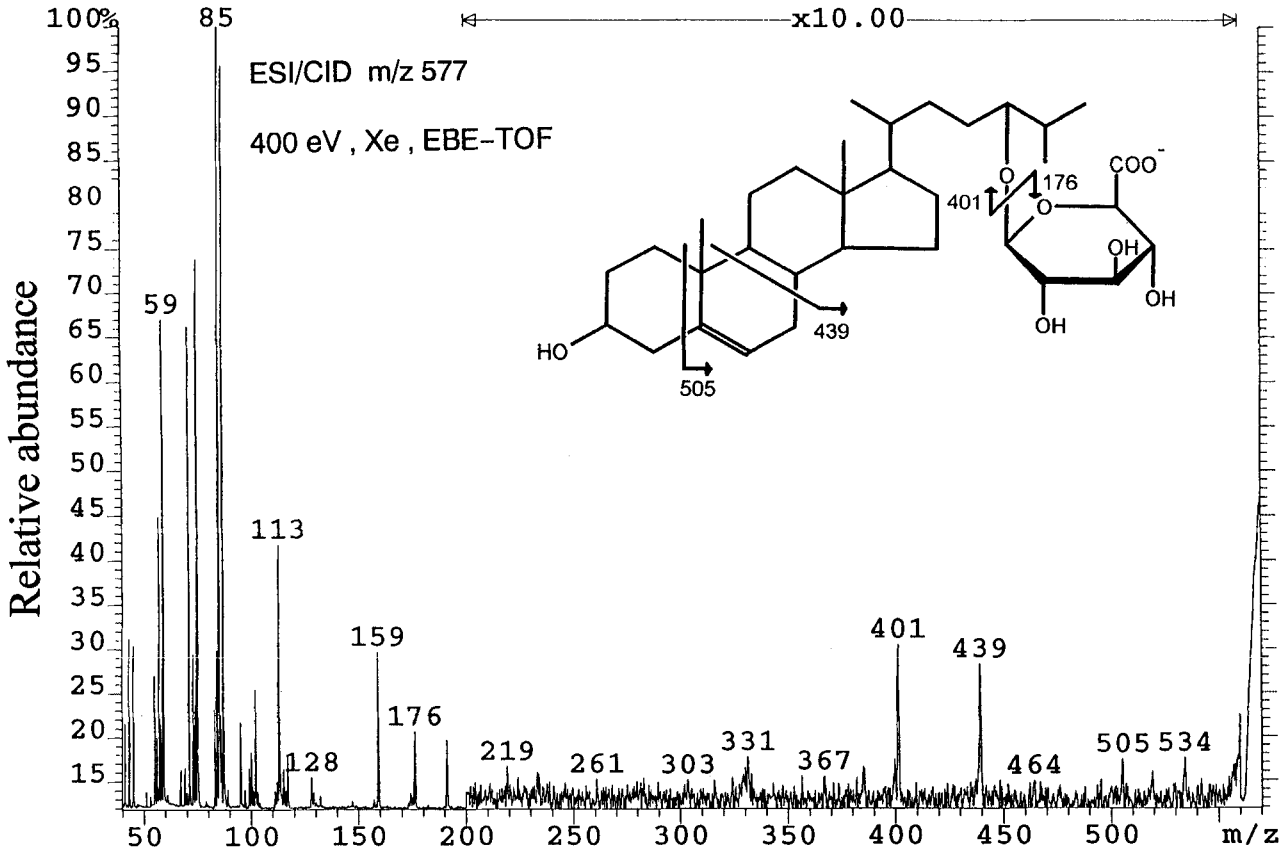

Fig. 4. ES/CID spectrum of $[M-H]^-$ ions (m/z 577) of the glucuronide of 24-hydroxycholesterol obtained after removal of the sulfate group of the double conjugate. Fragment ions at m/z 176 have molecular formula $C_6H_8O_6$, and those at m/z 401 correspond to $[M-H-176]^-$.

DISCUSSION

This study identifies for the first time the 3-sulfate, 24-glucuronide of (24S)-24-hydroxycholesterol. It was found in the serum and urine of many infants with advanced cholestatic liver disease. Previous studies have demonstrated the occurrence of 24-hydroxycholesterol in the free form and as fatty acid or sulfate esters (3, 4, 7–9, 12). Our results indicate that formation of the double conjugate with sulfate and glucuronate may, under some conditions, be the quantitatively most important pathway of conjugation. The highest serum level of the

TABLE 2. Serum concentrations and urinary excretion of hydroxysterols conjugated with both glucuronic and sulfuric acids

		Quantity of Doubly Conjugated Sterols in Patients[a]							
		Serum					Urine		
Sterol	RI[b]	2	6	9	12	13	1	12	13
		μmol/L					μmol/24 hr		
(24S)-24-hydroxycholesterol	3383	7	18	2	2	1.7	2.7	0.06	0.2
27-hydroxycholesterol	3461	0.7	1.1	0.9	0.6	0.5	0.04	0.03	0.02
5ξ-Cholestane-3ξ,24ξ-diol	3261	0.3	0.4	0.2	0.05	nd	nd	nd	nd
Cholestenetriol[c]	3441	3	0.5	1.1	0.05	0.15	0.03	0.002	0.001
Hydroxysterol[d]	3535	0.7	0.2	0.7	0.06	0.6	0.05	0.01	0.002
Cholestenetriol	3600	0.8	1.2	0.2	nd	0.01	nd	nd	nd
Cholestanetriol	3616	1.3	5.7	0.7	nd	0.4	0.01	nd	nd

[a]Patient numbers correspond to those in Table 1; nd, not detected.
[b]Retention index.
[c]Containing a 24-hydroxy group.
[d]Containing a 25-hydroxy group.

double conjugate in the patients studied was 18 μM and the highest urinary excretion was 2.7 μmol per day. These are minimum values as the recoveries in the analytical method are not known. They may be compared with levels of about 0.15 μM for the sterol (free and as fatty acid ester) in healthy adult subjects (12). The importance of combined sulfate and glucuronate conjugation is further emphasized by the presence of double conjugates of a number of saturated and unsaturated sterols with 2–4 hydroxyl groups. One of these was identified as 27-hydroxycholesterol and several of the others carried a 24-hydroxyl group.

The origin of the double conjugate of (24S)-24-hydroxycholesterol is not known. The stereochemistry at C-24 was the same as that of 24-hydroxycholesterol in brain, liver, adrenals, and plasma from humans (12). The 3-sulfate of 24-hydroxycholesterol is the major hydroxycholesterol sulfate in meconium and infant feces (7, 8), most likely excreted via bile. This process would be impaired in cholestasis and an increased concentration of 24-hydroxycholesterol 3-sulfate in the hepatocytes could conceivably lead to glucuronidation of this substrate. The FAB spectrum of bile from patient 11 showed a minor peak at m/z 657 (data not shown) indicating that the double conjugate may be excreted in bile. However, cholestasis alone does not result in elevated serum levels of the double conjugate, as the FABMS analyses revealed its presence in only half of the cholestatic infants subjected to screening. Thus, high concentrations of the double conjugate in serum are more likely to reflect an increased formation of 24-hydroxycholesterol and/or its 3-sulfate.

The liver (10, 11, 25) or the brain (3, 9, 12) are the most likely sites of an increased formation of 24-hydroxycholesterol. A recent study strongly suggests that a major part of 24-hydroxycholesterol in the circulation originates from the brain (12). As a pure speculation, the liver disease and cholestasis may result in retention of compounds that affect brain metabolism and increase the conversion of cholesterol to 24-hydroxycholesterol. Simultaneous quantitative analyses of the free and all conjugated forms of 24-hydroxycholesterol in arterial and venous blood from different organs will be required to determine the origin of 24-hydroxycholesterol and the sites of conjugation. Our study indicates that the formation of a double conjugate is a selective process as most of the sterols found in this group carried a 24-hydroxyl group.

High levels of the double conjugate of 24-hydroxycholesterol was an indicator of grave liver disease. All but two of the 13 patients who were followed up underwent liver transplantation or died. Most of the patients also excreted bile acids with a 3-oxo-Δ^4 structure in urine. This is another sign of serious liver disease (26, 27) although 7α,12α-dihydroxy-3-oxo-4-cholenoic acid is also a normal urinary constituent in early infancy (28). The presence of a peak at m/z 657 in the FAB spectra of serum or urine may have a prognostic significance and indicate that liver transplantation will be required.

In conclusion, this study has identified (24S)-24-hydroxycholesterol as a 3-sulfate 24-glucuronide in serum and urine and has indicated that double conjugation with sulfuric and glucuronic acids can be an important metabolic pathway for hydroxycholesterols and bile alcohols. This pathway should be considered in future studies of the biological functions and metabolism of 24-hydroxycholesterol and related compounds. The presence of elevated levels of the double conjugate of 24-hydroxycholesterol as revealed by an ion at m/z 657 in FAB spectra of serum or urine may be used as a new parameter of prognostic importance in the clinical evaluation of infants with severe cholestatic liver disease.

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