Identification of 27-nor-5β-cholestane-3α,7α,12α,24ξ,25ξ,26-hexol and partial characterization of the bile alcohol profile in urine

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Abstract The nature of the bile alcohols present in urine of an infant with neonatal cholestasis has been investigated. Urine was extracted with Sep-Pak C_{18} cartridges and a glucuronide fraction was isolated by ion exchange chromatography on Lipidex-DEAP. Following enzymatic hydrolysis and purification on Lipidex-DEAP, the bile alcohols were isolated by high performance liquid chromatography. Fourteen compounds were studied by a combination of microchemical reactions and capillary column gas–liquid chromatography–mass spectrometry. Both C_{26} and C_{27} bile alcohols were present. Among the former, three additional isomers of the previously identified 27-nor-5β-cholestane-3α,7α,12α,24ξ,25ξ-pentol were detected. A new C_{26} bile alcohol, 27-nor-5β-cholestane-3α,7α,12α,24ξ,25ξ,26-hexol, was identified, and a 27-norcholestane-pentolone with hydroxyl groups at C-24 and C-25 and a keto group in the ring system was partially characterized. The C_{27} bile alcohols consisted of cholestanepentols, -tetrolones, and -pentolones. 5β-Cholestane-3α,7α,12α,25,26-pentol (5β-bufol), one of its isomers and an isomer of cholestane-3,7,12,24,26-pentol were present. Two cholestanetetrolones and two cholestanepentolones having the keto group in the ring system were partially characterized. The hydroxyl groups in the side chain of the tetrolones were at C-24,26 and C-25,26, respectively, whereas the pentolones had hydroxyl groups at C-24,25 and C-25,26, respectively. The excretion of glucuronidated bile alcohols in urine is suggested to reflect an alternative metabolism of intermediates in the normal biosynthesis of bile acids.—**Karlaganis, G., V. Karlaganis, and J. Sjövall.** Identification of 27-nor-5β-cholestane-3α,7α,12α,-24ξ,25ξ,26-hexol and partial characterization of the bile alcohol profile in urine. *J. Lipid Res.* 1984. **25:** 693–702.

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A variety of bile alcohols are present in sulfated form in bile of lower vertebrates (1). Some of them have recently been found in the bile of a patient with cholestasis and gallstones (2). Patients with cerebrotendinous xanthomatosis excrete large amounts of 23-, 24-, and 25-hydroxylated bile alcohols in feces (3–8) and bile (9), most probably due to a genetic defect in the 26-hydroxylase required for normal bile acid biosynthesis (see 10). Bile alcohols are also excreted as glucuronides in urine of healthy subjects and in elevated amounts in patients with liver disease (11–14). The major compound has been identified as 27-nor-5β-cholestane-3α,7α,12α,24ξ,25ξ-pentol (11, 13, 14), but several similar compounds are excreted (11–13) constituting a complex metabolic profile of bile alcohols in urine. The aim of the present study was to further characterize the components of this profile. Preliminary results of this work were presented at the Seventh International Bile Acid Meeting in Basel 1982 (15).

MATERIALS AND METHODS

Reagents

All solvents were of reagent grade and were redistilled before use. Hexamethyldisilazane and trimethylchlorosilane (Applied Science Labs., State College, PA) were redistilled, and trimethylsilylimidazole (Supelco, Bellefonte, PA) was used as supplied. Methoxyamine hydrochloride (Eastman Organic Chemicals, Rochester, NY) was recrystallized from methanol. Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA) were washed with 5 ml of methanol and 5 ml of water before use. SP-Sephadex C25 (Pharmacia Fine Chemicals, Uppsala, Sweden) was converted to H^+ form with 0.1 M hydrochloric acid and washed with water and 72% methanol. Lipidex-DEAP (diethylaminohydroxypropyl Sephadex LH-20) was from Packard Instrument Co. (Downers Grove, IL) and was

Abbreviations: GLC, gas–liquid chromatography; HPLC, high performance liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; TMS, trimethylsilyl; MO-TMS, O-methyloxime-trimethylsilyl; *m/z*, mass/charge ratio; RI, retention time (Kovats index); CTX, cerebrotendinous xanthomatosis.

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washed by stirring from the top at 70°C for 1 hr in 20% aqueous ethanol and then in ethanol. It was stored at −20°C. Before use, an appropriate amount was washed with 0.5 M potassium acetate/hydroxide solution, pH 10, in 72% ethanol, 0.1 M acetic acid in 72% ethanol and 72% ethanol (16). *Helix pomatia* intestinal juice (Pharmindustry, Clichy, France), 3 ml (300.000 Fishman units), was dissolved in 100 ml of 0.5 M sodium acetate buffer, pH 4.5, and filtered through washed Amberlite XAD-2 (bed size 15 × 1 cm; Rohm and Haas, Philadelphia, PA) in order to remove interfering compounds (17).

Isolation of bile alcohols

Urine, 1020 ml, was collected during a 4-day period from a 13-month-old infant with neonatal cholestasis (sample kindly supplied by Dr. B. Strandvik, Department of Pediatrics, Huddinge Hospital, Sweden). The filtered urine was passed in 100-ml portions through Sep-Pak C_{18} cartridges. Following a wash with 10 ml of water the adsorbed steroids were eluted with 20 ml of methanol. The cartridges were then washed with 10 ml of water and used again for extraction of a second portion of urine. The combined methanolic eluates were diluted with water to 72% methanol. This solution was passed through a column of SP-Sephadex C25 in H form in 72% methanol (bed size 15 × 2 cm, flow rate one drop per sec) followed by a wash with 250 ml of 72% methanol. The combined eluates were taken to dryness. The residue was dissolved in 20 ml of water and neutralized to pH 7 with 1 M aqueous sodium hydroxide. Water and ethanol were added to give 100 ml of 72% ethanol. This solution was passed through a column of Lipidex-DEAP in acetate form (bed size 26 × 1 cm, flow rate two drops per sec, 100 kPa nitrogen pressure) followed by 200 ml of 72% ethanol to remove neutral compounds. A glucuronide fraction was then obtained by elution with 200 ml of 0.25 M formic acid in 72% ethanol (18). Solvents were removed in vacuo and by lyophilization. The enzyme solution (100 ml) was added, and after incubation at 37°C for 24 hr the solution was passed through a glass filter and three Sep-Pak C_{18} cartridges (one-third on each cartridge). The cartridges were washed with 20 ml of water and eluted with 20 ml of methanol. The combined methanolic eluates were taken to dryness, redissolved in 100 ml of 72% ethanol, and passed through Lipidex-DEAP as above. The effluent and an additional wash with 200 ml of 72% ethanol were taken to dryness. One percent of this neutral fraction was used for capillary gas–liquid chromatography (GLC), the rest for preparative fractionation by high performance liquid chromatography (HPLC).

Microchemical reactions

Trimethylsilyl (TMS) ethers were prepared by heating at 60°C for 15 min in pyridine–hexamethyldisilazane–trimethylchlorosilane 3:2:1 (by vol). O-Methyloxime (MO)-TMS ether derivatives were prepared with methoxyamine hydrochloride and trimethylsilylimidazole and purified by filtration through a column of Lipidex-5000 in hexane containing pyridine and hexamethyldisilazane (19). Acetonides (isopropylidene derivatives) were prepared in 2,2-dimethoxypropane and 0.1 M hydrochloric acid and filtered through a column of DEAE-Sephadex A25 (20). Methyl boronates were prepared with 30 μl of a solution of 0.01 M methyl boronic acid (Applied Science Labs.) in ethyl acetate (21). After 15 min at room temperature, the sample was taken to dryness under a stream of nitrogen, and TMS ethers were prepared at room temperature. Periodate oxidations were performed with sodium metaperiodate in water (11). Reductions were made by addition of solid lithium aluminum hydride and diethyl ether to the dry sample (11).

High performance liquid chromatography

Bile alcohols were isolated using a chromatographic system similar to that described by Tint et al. (22). A semipreparative column (25 × 1 cm) filled with LiChrosorb RP18 5 μm (Merck AG, Darmstadt, West Germany) was employed together with a Waters model 401 refractive index detector. The solvent was methanol–water–chloroform 70:27:3 (by vol) and the flow rate was 1.5 ml × min^{-1}. Fifty percent of the sample was dissolved in 1 ml of mobile phase for injection. The effluent was collected manually as each peak appeared on the recorder tracing, and the resulting fractions were rechromatographed in the same system and subjected to analysis by GLC and GLC–MS after different derivatization and microchemical reactions. HPLC elution volumes of the bile alcohols and GLC retention indices of their TMS ethers are listed in **Table 1.**

Capillary column gas–liquid chromatography

GLC was carried out using a Carlo Erba gas chromatograph model 4160 equipped with a flame ionization detector, a Grob-type on-column injector and a 15 m × 0.3 mm i.d. persilanized glass capillary column (23) coated with OV-73 (0.2%, static coating). Hydrogen was used as carrier gas at a flow rate of 4.8 ml/min (100 kPa) and 250°C column temperature. Retention indices were calculated from the retention times of even-numbered C_{28}–C_{38} n-alkanes.

Gas–liquid chromatography–mass spectrometry

Computerized electron impact GLC–MS was carried out using a modified LKB 9000 instrument (19) and a persilanized OV-73 glass capillary column. Operating conditions were as follows: column temperature 250°C, separator temperature 250°C, temperature of ion source 310°C, energy of bombarding electrons 22.5 eV, ionizing

TABLE 1. Chromatographic characteristics and molecular weights of derivatized bile alcohols from urine

Compound[a]	Retention Index (TMS)	HPLC-Fraction[b] (ml)	Partial Structure	Molecular Weights TMS	Acetonide TMS	MO-TMS
1	3447	58.2	C_{26}-3,7,12,24,25-pentol	798	694	
2	3469	55.5	C_{26}-3,7,12,24,25-pentol	798	694	
3	3493	55.5	C_{26}-3α,7α,12α,24,25-pentol	798	694	
4	3505	55.5	C_{26}-3,7,12,24,25-pentol	798	694	
5	3537	27.3	C_{26}-pentolone (24,25-ol)	812	708	841
6	3568	55.5	C_{27}-3,7,12,24,26-pentol	812	708	
7	3586	70.8	C_{27}-3,7,12,25,26-pentol	812	708	
8	3613	70.8	C_{27}-3,7,12,25,26-pentol	812	708	
9	3635	30.0	C_{26}-pentolone (24,25-ol)	812	708	841
10	3659	36.9	C_{26}-3α,7α,12α,24,25,26-hexol	886	782	
11	3716	53.4	C_{27}-tetrolone (24,26-ol)	738	634	767
12	3732	31.0	C_{27}-pentolone (24,25-ol)	826	722	855
13	3745	65.1	C_{27}-tetrolone (25,26-ol)	738	634	767
14	3781	35.1	C_{27}-pentolone (25,26-ol)	826	722	855

[a] Numbers refer to those in Fig. 1 and the text.
[b] Retention volume at the middle of the fraction collected.

current 60 μA, accelerating voltage 3.5 kV. Magnetic scanning over the range 0–800 daltons was initiated every 6–8 seconds to give about four to eight spectra over an average GLC peak. Ammonia chemical ionization mass spectra were recorded by direct inlet of derivatized HPLC fractions on a Kratos MS-50 mass spectrometer. The latter analyses were kindly performed by Dr. David Jones through the courtesy of Professor Curt Enzell at the research laboratories of the Swedish Tobacco Company, Stockholm.

RESULTS

The bile alcohols were isolated from urine of a 13-month-old boy with neonatal cholestasis. The relative amount of components with long retention times was higher in this patient than in a series of children with less advanced cholestasis (12). Qualitatively, however, the bile alcohol profiles were similar, and compounds with the same retention times were present in all samples from these patients. The TMS ether derivatives had retention indices between 3400 and 3800, and at least fourteen bile alcohols could be distinguished (**Fig. 1,** Table 1). The mixture was separated by HPLC into fractions which contained only one or a few components. The structures of these were investigated by GLC–MS analysis of different derivatives and after microchemical reactions. Table 1 and **Table 2** give the HPLC mobilities, GLC retention indices, and mass spectrometric characteristics of the parent compounds, while retention indices of products formed after microchemical reactions are listed in **Table 3.**

Compounds 1–4. The retention index and mass spectrum of the TMS ether showed that compound 3 was 27-nor-5β-cholestane-3α,7α,12α,24ξ,25ξ-pentol identified in a previous study (11). The TMS ethers of compounds 1–4 gave very similar mass spectra indicating that they were stereoisomers. They all formed an acetonide, the TMS ether of which gave an intense nuclear fragment ion (**Table 4**).

Compound 5. The mass spectral data for the TMS ether (Table 2), and a quasimolecular ion at m/z 830 (M + 18) in the chemical ionization spectrum were indicative of a 27-norcholestanepentolone with three hydroxyl groups and one keto group in the ring system and hydroxyl groups at C-24 and C-25. The mass spectra of the TMS

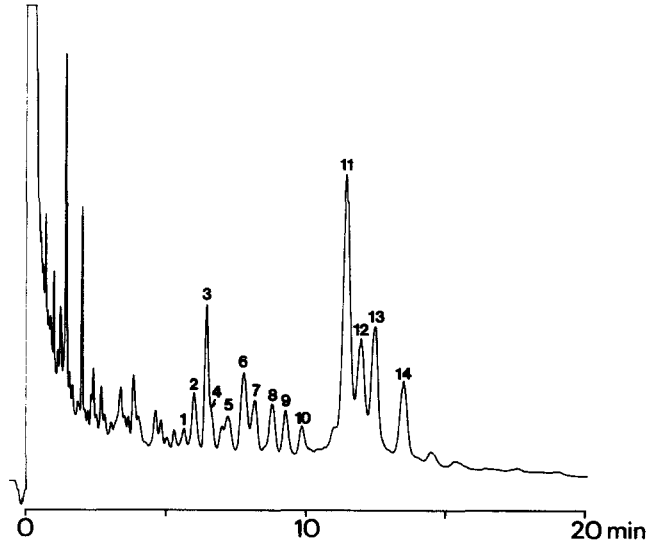

Fig. 1. GLC analysis of the TMS ethers of bile alcohols from the glucuronide fraction of urine from an infant with neonatal cholestasis. The peaks are numbered as the compounds listed in Table 1. A persilanized glass capillary column, 15 m × 0.3 mm, coated with 0.2% OV 73 was used at 250°C with hydrogen at 100 kPa as carrier gas.

TABLE 2. Masses and relative intensities of ions in mass spectra of TMS ethers of bile alcohols in urine

Compound[a]	M[b]	Origin of Ions: M-n·TMS	M-Terminal Side Chain-n·TMS	Ring System	Side Chain
1. *m/z*	798	618, 528, 439, 349	681, 591, 501, 411, 321	343, 253	156, 129, 117
%	—	4 2 2 2	7 9 46 52 46	26 20	27 48 22
2. *m/z*	798	618, 528, 439, 349	681, 591, 501, 411, 321	343, 253	156, 129, 117
%[c]	—	5 4 3 3	10 12 56 76 78	32 25	40 69 24
3. *m/z*	798	618, 528, 439, 349	681, 591, 501, 411, 321	343, 253	156, 129, 117
%[c]	—	4 4 2 3	7 7 35 55 77	18 37	28 52 24
4. *m/z*	798	618, 528, 439,	681, 591, 501, 411, 321	343, 253	156, 129, 117
%[c]	—	1 0.2 0.2	2 2 12 21 19	17 19	10 16 3
5. *m/z*	812	—	605, 515, 425, 335	267	156, 129, 117
%[c]	—		3 10 14 21	15	4 13 2
6. *m/z*	812	722, 632, 542, 452, 363	681, 591, 501, 411, 321	343, 253	233, 129, 103
%	—	1 17 16 9 5	8 8 37 54 71	23 44	100 39 24
7. *m/z*	812	722, 632, 542, 452, 363	709, 619, 529, 439, 349	343, 253	219
%[c]	—	3 17 21 6 3	38 13 50 17 13	53 26	42
8. *m/z*	812	722, 632, 542, 452, 363	709, 619, 529, 439, 349	343, 253	219
%[c]	—	1 10 15 11 4	44 10 36 37 24	23 43	47
9. *m/z*	812	542, 452, 363	605, 515, 425, 335	267	156, 129, 117
%	—	4 4 3	14 41 62 100	23	17 52 25
10. *m/z*	886	706, 616, 527,	681, 591, 501, 411, 321	343, 253	217, 204, 156, 129
%	—	2 2 1	13 12 54 81 100	6 12	10 22 57 57
11. *m/z*	738	558, 468, 378	517, 427, 337	269, 267	233, 103
%[c]	—	2 3 1	4 47 3	5 7	18 23
12. *m/z*	826	721[d], 556, 466	605, 515, 425, 335	267	233, 131
%	—	0.4 0.2 0.6	0.2 0.8 1 2	3	0.6 100
13. *m/z*	738	633[d], 468	545, 455, 365	269, 267	219
%	—	3 6	57 100 3	6 6	22
14. *m/z*	826	721[d], 556, 466, 376	633, 543, 453, 363	267	219
%[c]	—	3 3 23 3	45 28 20 8	62	21

[a] Numbers refer to those in Fig. 1 and Table 1.
[b] M^+ was usually absent, M − 15 was sometimes present in low intensity and the molecular weight was confirmed by chemical ionization mass spectra (see text).
[c] Base peak at *m/z* 75.
[d] M − 90 − 15.

ethers of compounds 5 and 9 were similar and these compounds may be isomers. Microchemical reactions were performed on compound 9 and the structure of this compound is discussed below.

Compound 6 was identified as a cholestane-3,7,12,24,26-pentol by comparison with the spectrum of the TMS ether of 5β-cholestane-3α,7α,12α,24,26-pentol published by Tint et al. (24). The configuration of the hydroxyl groups cannot be established by this comparison and remains unknown.

TABLE 3. Retention indices of derivatives and reaction products of bile alcohols

Compound[a]	Partial Structure	Retention Index: TMS	Acetonide TMS	MO-TMS	Reduction TMS	Periodate + Reduction TMS
7	C_{27}-3,7,12,25,26-pentol	3586				3321
8	C_{27}-3,7,12,25,26-pentol	3613				3348
9	C_{26}-pentolone (24,25-ol)	3635	3550[b]	3577[b]	3473[b]	
			3611	3604	3496	
10	C_{26}-3α,7α,12α,24,25,26-hexol	3659	3642			3221
11	C_{27}-tetrolone (24,26-ol)	3716[b]	3687	3653	3577	
		3724		3675[b]	3592[b]	
12	C_{27}-pentolone (24,25-ol)	3732	3588	3669	3570	
				3690[b]		
13	C_{27}-tetrolone (25,26-ol)	3745	3649	3707	3609	
				3724[b]	3628[b]	
14	C_{27}-pentolone (25,26-ol)	3781	3707	3696	3622	
				3718[b]		

[a] Numbers refer to those in Fig. 1 and Table 1.
[b] Major isomer.

TABLE 4. Masses and relative intensities of ions in mass spectra of acetonide-TMS ether derivatives of bile alcohols in urine

Compound[a]		M[b]	M-n · TMS	M-n · TMS-58	M-n · TMS-101	Ring System
3.	*m/z*	694	679[c], 604, 514, 424	636, 546, 456, 366		343, 253
	%	1	8 1 41 38	1 5 22 34		43 100
9.	*m/z*	708	513[c], 438	380		267
	%	—	5 6	16		100
10.	*m/z*	782	602, 512	544, 454	411, 321	343, 253
	%[d]	—	1 1	1 2	2 10	10 45
11.	*m/z*	634	454, 439[c]	396	355, 354	269, 267
	%	—	30 48	100	25 24	56 34
12.	*m/z*	722	527[c]	394		267
	%	—	5	29		100
13.	*m/z*	634	454, 439[c], 364	396		269
	%	—	1 13 3	100		55
14.	*m/z*	722	452	394		267
	%	—	2	15		100

[a] Numbers refer to those in Fig. 1 and Table 1.
[b] Not seen in most of the spectra.
[c] Loss of a methyl group.
[d] Base peak at *m/z* 75.

Compounds 7 and 8. The mass spectra of the TMS ethers (Table 2) showed that these compounds were isomers. Oxidation with periodate followed by reduction with lithium aluminium hydride produced two isomers of 27-norcholestane-3,7,12,25-tetrol, the TMS ethers of which gave peaks due to loss of trimethylsilanol and the two terminal carbon atoms of the side chain. Comparison with spectra published by Tint et al. (24) confirmed that the isomers were cholestane-3,7,12,25,26-pentols. The retention index and comparison with authentic material (kindly donated by Professor T. Hoshita) indicated that compound 8 was 5β-cholestane-3α,7α,12α,25,26-pentol, i.e., 5β-bufol.

Compound 9. The mass spectrum of the TMS ether (**Fig. 2**) was very similar to those of the TMS ethers of compounds 1–4, except that most peaks in the middle and high mass range were shifted 14 mass units towards higher mass (Table 2). The peak at *m/z* 267 indicated a keto or methyl group in the ring system. The presence of a keto group was established by formation of a methyloxime. The nuclear fragment ion shifted to *m/z* 296 which was accompanied by an ion at *m/z* 266 (296 − 30).

Reduction of compound 9 with lithium aluminium hydride yielded a 27-norcholestanehexol. The nuclear fragment ion appeared at *m/z* 251 indicating the loss of four trimethylsilanols and the side chain from the parent ion.

When compound 9 and the hexol derived from it were reacted with 2,2-dimethoxypropane only the two hydroxyl groups in the side chain formed an acetonide (Table 4). Thus, there were no vicinal *cis*-hydroxyl groups in the rings system, neither in the parent compound nor in the hexol formed by reduction of the keto group. On the basis of these results, compound 9 was characterized as a 27-norcholestanepentolone with one keto and three hydroxyl groups in the ring system and hydroxyl groups at C-24 and C-25.

Compound 10. The mass spectrum of the TMS ether (**Fig. 3,** Table 2) showed many similarities with the spectrum of the TMS ether of 27-nor-5β-cholestane-3α,7α,12α,24,25-pentol (compound 3, cf. ref. 11). The series of peaks due to loss of the two terminal carbon atoms in the latter compound were present (*m/z* 681 etc.). Peaks at *m/z* 706, 616, 527, and 437 indicated a possible molecular weight of 886, corresponding to a C_{26}-

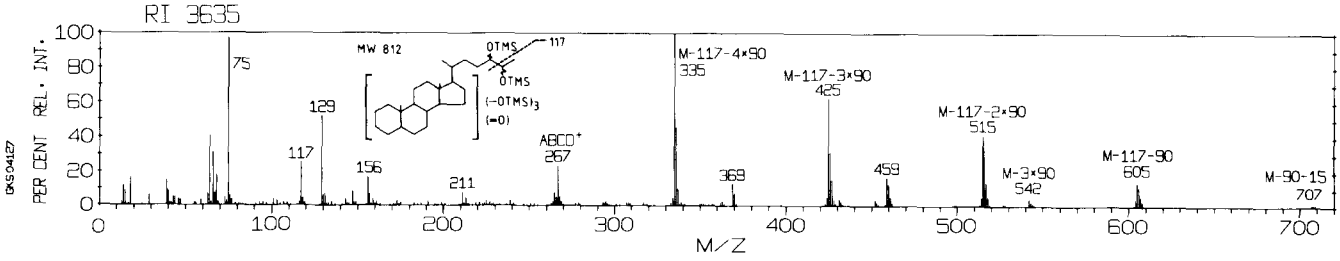

Fig. 2. Mass spectrum of the TMS ether of 27-norcholestanepentolone (compound 9) isolated from urine. The peaks at *m/z* 63–69 are due to a solvent contaminant.

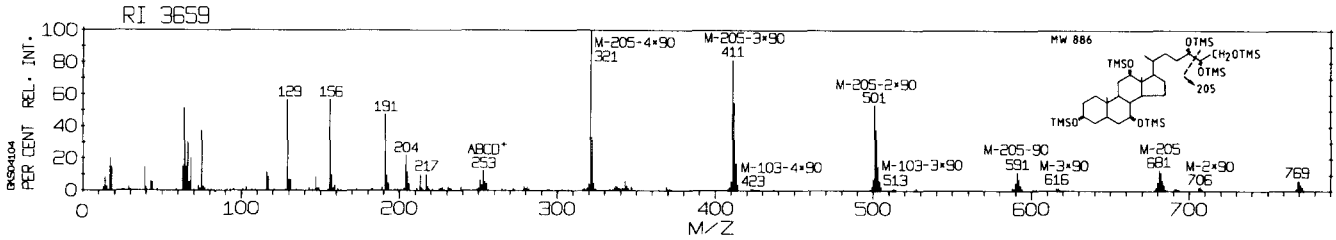

Fig. 3. Mass spectrum of the TMS ether of 27-nor-5β-cholestane-3α,7α,12α,24ξ,25ξ,26-hexol isolated from urine. The peaks at m/z 63–69 are due to a solvent contaminant.

hexol, which was supported by the chemical ionization mass spectrum. The ABCD-ring ion at m/z 253 indicated that three of the hydroxyl groups were in the ring system. In the lower mass end, peaks were present at m/z 129 and 156, whereas the peak at m/z 117 given by the TMS ether of the C_{26}-pentol was small. Instead peaks were found at m/z 191, 204, and 217, typical of TMS ethers of steroids with three neighboring hydroxyl groups (25) and TMS ethers of carbohydrates (26). Compound 10 was therefore considered to be a 27-norcholestane-3,7,12,24,25,26-hexol.

The hydroxyl groups in the side chain reacted with 2,2-dimethoxypropane to form acetonide(s). The mass spectrum of the TMS ether of the product(s) (**Fig. 4,** Table 4) showed peaks due to loss of the elements of acetone, trimethylsilanol, the isopropylidene group, and the side chain. The hydroxyl groups also reacted with methyl boronic acid to form methyl boronate(s). The GLC peaks of the TMS ether derivatives of both the acetonide and the methyl boronate were broad, indicating formation of at least two forms of derivatives as would be expected from a glycerol structure at the end of the side chain.

Oxidation of compound 10 with periodate followed by reduction with lithium aluminium hydride yielded a cholanetetrol. The TMS ethers of this compound and authentic 5β-cholane-3α,7α,12α,24-tetrol (kindly donated by Professor H. Danielsson) gave identical mass spectra and retention times. Based on these findings the new bile alcohol is identified as 27-nor-5β-cholestane-3α,7α,12α,-24ξ,25ξ,26-hexol.

Compound 11 was a major compound constituting 29% of the total bile alcohols in the sample studied. In a series of infants with and without cholestasis (12) the relative amount varied between 6% and 25%. The mass spectrum of the TMS ether derivative (Table 2) showed peaks at m/z 233 and 103 indicating a 24,26-trimethylsiloxy structure in a C_{27} sterol (24). Peaks at m/z 267 and 269 indicated presence of a keto group in the ABCD-rings. These ions and those interpreted to arise from losses of trimethylsilanol and the three terminal carbon atoms led to the assumption that the compound was a cholestanetetrolone. The hydroxyl groups in the side chain formed an acetonide (Table 4). The keto group could be converted into a methyloxime which appeared to decompose partly upon GLC and was lost to a large extent in the GLC–MS analysis.

Reduction of compound 11 with lithium aluminium hydride gave two isomeric cholestanepentols in about equal amounts, which also showed signs of decomposition upon GLC and GLC–MS. The mass spectra of the TMS ethers showed peaks at the same masses as that of the TMS ether of compound 6, but with very different relative intensities. This fact, the spectrum of the TMS ether, the thermal lability mentioned above, and the formation of two isomers in equal amounts upon reduction with lithium aluminum hydride indicate that the keto group is not at any of positions 3, 7, or 12.

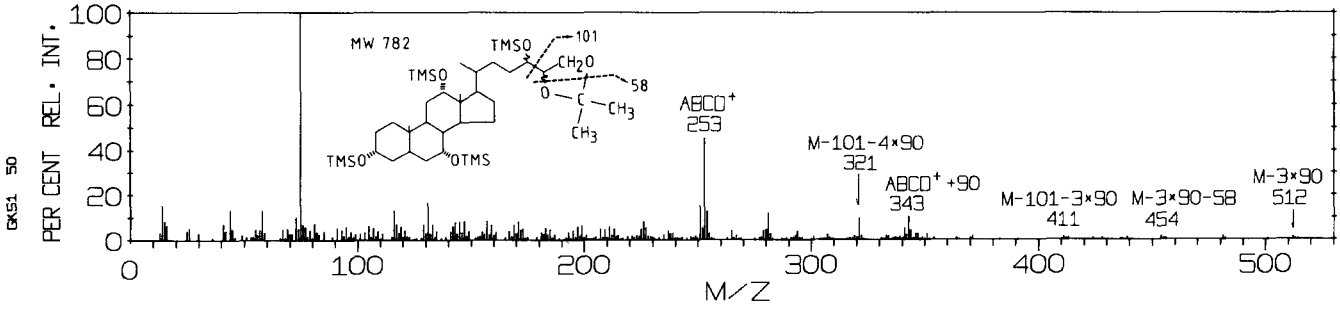

Fig. 4. Mass spectrum of the TMS ether of the acetonide of 27-nor-5β-cholestane-3α,7α,12α,24ξ,25ξ,26-hexol isolated from urine. Only one of the three possible structures of the acetonide is depicted. Formation of several acetonides was indicated by a broad peak in the GLC tracing.

Compound 12. The mass spectrum of the TMS ether derivative (Table 2) showed a pronounced base peak at m/z 131 and a small peak at m/z 233 indicating a 24,25-bis-trimethylsiloxy structure of a C_{27} bile alcohol (24). The peak at m/z 267 indicated the presence of a keto group in the ABCD-rings and the compound was assumed to be a cholestanepentolone. This was supported by the chemical ionization spectrum showing a peak m/z 809 (M + 1 − 18) and peaks due to loss of trimethylsilanols from M + 1. In addition to the ions listed in Table 2, a series of ions at m/z 678 (2% relative intensity), 588 (3%), and 498 (4%) was seen in the GLC–MS analysis. Fragment ion current chromatograms showed that these ions and those in Table 2 gave GLC peaks with the same retention time (± one scan), which makes it likely that all ions were formed from one compound. The presence of a contaminant cannot be completely ruled out and the origin of m/z 678 and its companion ions remains unknown.

The hydroxyl groups in the side chain formed an acetonide (Table 4). The keto group formed two methyloximes (Table 3), probably syn/anti isomers. The TMS ethers of these gave a base peak at m/z 131 and peaks at m/z 634, 544, 454, 364, corresponding to loss of 131 mass units and trimethylsilanols, accompanied by peaks due to loss of CH_2O (from the methyloxime group). The ABCD-ring fragment ions were at m/z 296 and 266.

Reduction with lithium aluminium hydride yielded a single compound (Table 3), the TMS ether of which gave mass spectral peaks due to sequential losses of trimethylsilanol from an assumed molecular ion at m/z 900. A series of peaks at m/z 589, 499, 409, and 319 were compatible with combined losses of trimethylsilanols and 131 mass units. The side chain fragment ion, m/z 131, remained a pronounced base peak, and the ABCD-ring ion was shifted to m/z 251, showing that the keto group had been reduced. This C_{27}-hexol reacted with dimethoxypropane and two isomers were separated. The base peak was at m/z 558 (M − 58 − 2 × 90) and the ABCD-ring ion was at m/z 251, showing that hydroxyl groups in the ring system did not form an acetonide. The presence of two isomers might be due to formation of two epimers in the reduction, the TMS ethers of which did not separate on the GLC column. The results indicate that compound 12 is a C_{27}-pentolone with a keto group in the ring system and a 24,25-dihydroxy side chain.

Compound 13. The peaks at m/z 267 and 269, the peak at m/z 219, and intense peaks at m/z 455 and 545 in the mass spectrum of the TMS ether (Table 2) suggested that compound 13 was a cholestanetetrolone with hydroxyl groups at C-25 and C-26 (24). This was supported by the chemical ionization spectrum. The hydroxyl groups of the side chain formed an acetonide (Table 4). The keto group formed two isomers of a methyloxime (Table 3). The spectrum of the MO-TMS derivative retained the side chain ion at m/z 219 and showed series of peaks due to losses of trimethylsilanol together with the methoxy group from the methyloxime or of the 26-methylene with the trimethylsiloxy group (− 103).

When the keto group was reduced with lithium aluminium hydride, two isomers were formed in equal amounts. The spectra of the TMS ether derivatives were compatible with a cholestanepentol structure. Both the results of the microchemical reactions and the spectra of the parent compound and its products indicate that the keto group is not in position 3, 7, or 12.

Compound 14. The peak at m/z 267, the peak at m/z 219, and intense peaks at m/z 633 and 543 in the mass spectrum of the TMS ether (Table 2) indicated a 25,26-bis-trimethylsiloxy structure (see compound 13) in a cholestanepentolone. This was supported by a peak at m/z 809 (M + 1 − 18) in the chemical ionization spectrum.

The hydroxyl group in the side chain formed an acetonide (Table 4). The keto group yielded a methyloxime which gave the expected series of peaks at m/z 734, 644, 554, and 464 arising by loss of trimethylsilanols and the methoxy group from the molecular ion (M = 855). The ABCD ring ions were at m/z 296 and 266. The terminal side chain remained at m/z 219.

Reduction with lithium aluminium hydride yielded a C_{27}-hexol, the TMS ether of which gave two series of peaks due to loss of trimethylsilanols with and without loss of the terminal $CH_2OSi(CH_3)_3$ group. The reduction of a keto group in the ring system was evidenced by appearance of a peak at m/z 251.

Thus, the microchemical reactions support the conclusion that compound 14 is a cholestanepentolone with hydroxyl groups at C-25 and C-26. The position of the keto group is unknown. It is very unlikely to be at C-7 or C-12, but C-3 is a possibility.

DISCUSSION

Fourteen compounds of the bile alcohol profile were studied. The C_{26} steroids consisted of four pentols, two pentolones, and one hexol. The C_{27} steroids consisted of three pentols, two tetrolones, and two pentolones. This is a minimum number since there are many possibilities of stereoisomerism, and single peaks in the bile alcohol profile may have contained more than one compound (cf. Fig. 1).

Among the C_{26} bile alcohols, three additional isomers of the previously identified 27-nor-5β-cholestane-3α,7α,12α,24ξ,25ξ-pentol (11) were detected. It appears possible that these compounds are isomeric at C-24 and C-25. Dayal et al. (27) have isolated one isomer from

feces of patients with CTX (probably identical to our major isomer) and reported the configuration at C-24 to be R. The stereochemistry at C-25 and of the other C_{26} bile alcohols found in this study remains unknown.

The mass spectra of TMS ethers of the C_{26}-pentols show a characteristic series of peaks at m/z 681, 591, 501, 411, and 321, due to fragmentation between C-24 and C-25. The intensities of these peaks are high in contrast to those formed by C_{27}-pentols with a 24,25-diol structure where the charge is predominantly localized to the terminal part of the side chain (m/z 131) (24). The same series of peaks was also given by a compound identified as 27-nor-5β-cholestane-3α,7α,12α,24ξ,25ξ,26-hexol (compound 10). This bile alcohol has not previously been found in animals or humans. A similar series of peaks shifted by 14 units towards higher mass was given by two apparently isomeric and previously unknown bile alcohols. These were found to be C_{26}-pentolones with the keto group in the ring system and the same 24,25-diol structure. The position of the additional oxygen substituent remains to be established.

The C_{27} bile alcohols consisted of pentols and ketonic compounds. Cholestane-3,7,12,24,26-pentols occur in lower vertebrates (1) and one isomer with this structure was present. This may be the same compound as that detected in urine of adult patients with liver disease (13). The stereochemistry of the mammalian compound has not been established. The same is true of one of the isomers of cholestane-3,7,12,25,26-pentol found in this study, whereas comparisons with the authentic compound indicated that the other isomer was 5β-cholestane-3α,7α,12α,25,26-pentol, i.e., 5β-bufol previously found in lower vertebrates (1) and rabbit bile (28).

The ketonic C_{27} bile alcohols have not previously been found in animals or humans. However, only the position of hydroxyl groups in the side chain and the number and nature of substituents in the ABCD-rings were identified. The two cholestanetetrolones had hydroxyl groups at C-24,26 (compound 11) and C-25,26 (compound 13), respectively, and the cholestanepentolones were hydroxylated at C-24,25 (compound 12), and C-25,26 (compound 14), respectively.

The quantitative importance of the different bile alcohols can be assessed from the results of our previous study of children with and without liver disease (12). Assuming that peaks with the same retention times represent the same bile alcohols in all subjects, eight of the compounds were quantitated. While their relative proportion varied considerably, five compounds constituted the major part of the bile alcohols both in children with cirrhosis and in healthy children (listed in order of decreasing quantitative importance): 27-nor-5β-cholestane-3α,7α,12α,24,25-pentol (compound 3), cholestane-3,7,12,24,26-pentol (compound 6), cholestanetetrolone (24,26-ol) (compound 11), 5β-cholestane-3α,7α,12α,25,26-pentol (compound 8), and 27-nor-5β-cholestane-3α,7α,12α,24ξ,25ξ,26-hexol (compound 10).

This pattern is quite different from those in bile and feces of a patient with cholestasis (2) and patients with CTX (3, 4, 9). While the major bile alcohols in urine have five or six oxygen substituents, tetrols are predominant in bile. Bile alcohols may be excreted as glucuronides, sulphates, or in unconjugated form. The pattern in each group may differ depending on the specificity of enzymes catalyzing conjugation or hydrolysis. In man, conjugation with glucuronic acid is typical of polar steroid hormone metabolites with a 5β configuration, and this is consistent with the occurrence of bile alcohols in the glucuronide fraction of urine. Biliary C_{25}- and C_{26}-bile alcohols were presumably sulfated in a patient with cholestasis (2), but were conjugated with glucuronic acid when injected into male bile-fistula rats (29). Bile alcohols similar to those occurring as glucuronides in bile of patients with CTX (9) are probably excreted as sulfates in rabbit bile (28). Thus, there are species differences, and both the state of conjugation and the number of polar groups will determine the route of excretion of bile alcohols.

The pathophysiological significance of the bile alcohols in urine is not known. Both the C_{26}- and C_{27}-bile alcohols are formed from cholesterol in man (3, 30). According to current views, cholic acid is formed from cholesterol via 5β-cholestane-3α,7α,12α-triol, 5β-cholestane-3α,7α,12α,26-tetrol, 3α,7α,12α-trihydroxy-5β-cholestanoic acid, and 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid (31). One could speculate that the last-mentioned acid (varanic acid) is a branching point. Decarboxylation of a 24-keto intermediate could lead to 27-nor-5β-cholestane-3α,7α,12α,24-tetrol as suggested by Noma et al. (32) as a possible route for formation of ranol in bullfrogs. Hydroxylation at C-25 would then produce 27-nor-5β-cholestane-3α,7α,12α,24,25-pentol. Further metabolism could conceivably lead to 27-nor-5β-cholestane-3α,7α,12α,24,25,26-hexol or cholic acid. The C_{27} bile alcohols might represent side products from other intermediates in the synthesis of bile acids. The microsomal fraction of human liver can catalyze hydroxylations of 5β-cholestane-3α,7α,12α-triol at carbon atoms 23–26 and the mitochondrial fraction catalyzes 26-hydroxylation of a number of intermediates in bile acid biosynthesis (31, 33). The existence in different compartments of hydroxylating systems that can produce compounds that may or may not be substrates of enzymes catalyzing reactions leading to a bile acid side chain opens the possibility for formation of an array of side products in different states of hydroxylation. Many of these may be substrates of glucuronyl transferases, and hydroxylation and glu-

curonidation may be competing reactions, particularly when the hydroxylations occur in the microsomal fraction. Additional oxygenation of the ring system may be analogous to that observed for bile acids, particularly in patients with liver disease (20). Further studies are needed to establish whether the relative proportion of different bile alcohols may give a selective reflection of disturbances in different compartments of the liver.

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