Analysis of metabolic profiles of bile acids in urine using a lipophilic anion exchanger and computerized gas–liquid chromatography–mass spectrometry

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Abstract A method is described for quantitative analysis of bile acids in urine. Urine is acidified and bile acids are extracted on an Amberlite XAD-2 column. Bile salts are converted to acids on an Amberlyst A-15 column and are separated into groups of unconjugated, glycine, taurine, monosulfated, and polysulfated conjugates using the lipophilic anion exchanger diethylaminohydroxypropyl Sephadex LH-20 (DEAP-LH-20). After solvolysis and hydrolysis, the deconjugated bile acids are purified on DEAP-LH-20, and are converted to methyl ester trimethylsilyl ether derivatives. Identification and quantitation of the individual bile acids is accomplished by computerized gas–liquid chromatography–mass spectrometry.

The daily excretion of bile acids in urine from healthy subjects was 6.4–11 μmoles. The mixture of bile acids was quite complex and differed from that in bile. About 30 bile acids were identified or partially characterized. Three of these were monosubstituted: lithocholic, allolithocholic, and 3β-hydroxy-5-cholenoic acids. Fourteen disubstituted bile acids included epimers of deoxycholic, allodeoxycholic, chenodeoxycholic, allochenodeoxycholic, and hyodeoxycholic acids. 3α-Hydroxy-12-keto-5β-cholanoic acid was the major ketonic bile acid and 3β,12α-dihydroxy-5-cholenoic acid was the major unsaturated bile acid in this group. Nine trihydroxy bile acids included cholic and allocholic acids, epimers of these compounds, hyocholic acid, and a 1-hydroxylated bile acid tentatively characterized as 1,3,12-trihydroxycholanoic acid. Cholestatic subjects excreted tetrahydroxycholanoates carrying hydroxyl groups in positions 1, 3, 6, 7, 12, or 23.

All monohydroxy and the predominant part of dihydroxy bile acids were present in the monosulfate fraction. Exceptions were 3α,12β-dihydroxy- and 3α-hydroxy-12-keto-5β-cholanoic acids, which were found mainly in the glycine conjugate fraction. Most of the trihydroxy bile acids were nonsulfated, and cholic and norcholic acids were the major unconjugated bile acids. The tetrahydroxy bile acids and hyocholic acid were present mainly in the taurine conjugate fraction, while 1,3,12-trihydroxycholanoic acid was predominantly found in the glycine conjugate fraction. Sulfation of trihydroxy bile acids was increased in patients with marked cholestasis. All bile acids in the monosulfate fraction were conjugated and carried the sulfate ester group at C-3. Significant amounts of di- and trisulfates were not found.

The results indicate selective mechanisms for sulfation, hydroxylation, and renal elimination of bile acid conjugates. Analysis of metabolic profiles of bile acids in urine may be a useful method in studies of the function of organs involved in bile acid metabolism.

Supplementary key words ion exchange chromatography · lipophilic Sephadex · Lipidex

The identification of bile acid sulfates (1, 2) and the finding that bile acids in urine may exist predominantly in a sulfated form have resulted in a renewed interest in the analysis of urinary bile acids under different conditions in man (3–15). Studies in this laboratory are concerned with defects in steroid and bile acid metabolism in pregnant women with intrahepatic cholestasis (16–18) and a method was needed for analysis of metabolic profiles of bile acids in urine from these patients and from healthy pregnant women. Existing methods do not include separation of conjugated bile acids and only in some

Abbreviations and trivial names: Lithocholic, 3α-hydroxy-5β-cholanoic; hyodeoxycholic, 3α,6α-dihydroxy-5β-cholanoic; chenodeoxycholic, 3α,7α-dihydroxy-5β-cholanoic; ursodeoxycholic, 3α,7β-dihydroxy-5β-cholanoic; deoxycholic, 3α,12α-dihydroxy-5β-cholanoic; hyocholic, 3α,6α,7α-trihydroxy-5β-cholanoic; cholic, 3α,7α,12α-trihydroxy-5β-cholanoic; and norcholic, 3α,7α,12α-trihydroxy-24-nor-5β-cholan-23-oic acid. The term allo is used for substituted 5α-cholan-24-oic acids. The prefixes glyco, G, and tauro, T, are used for bile acids having glycine or taurine in amide linkage at C-24. U, MoS, DiS, and TriS are abbreviations for unconjugated, mono-, di-, and trisulfated bile acids, respectively. TMS, trimethylsilyl; TIC, total ion current produced by all ions from *m/e* 34 to the molecular weight; FIC, fragment ion current produced by ions of a single *m/e* value; t_R, retention time relative to that of methylcholate TMS ether. GLC, gas–liquid chromatography; MS, mass spectrometry; TLC, thin-layer chromatography.

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studies have sulfated bile acids been separated from nonsulfated ones. Furthermore, these methods do not yield sufficiently clean samples to permit analysis of bile acid profiles in urine from healthy subjects. Attempts were initially made to improve isolation procedures using Sephadex LH-20 (6, 9, 13, 19–21), but difficulties including overloading, memory effects, and separation artifacts were encountered.

The present paper describes a method in which the isolation of bile acid conjugates and sulfates is achieved on a lipophilic anion exchanger (diethylaminohydroxypropyl Sephadex LH-20) (22, 23). The bile acids are then analyzed by computerized gas–liquid chromatography–mass spectrometry (24, 25). Preliminary results of this work have been reported (26, 27). During the completion of this paper the identification of 6α-hydroxylated bile acids in urine from patients with liver disease was reported by Summerfield, Billing, and Shackleton (28).

MATERIALS AND METHODS

Solvents and reagents were of analytical grade and were redistilled before use. Dimethyl formamide was purified on a column of neutral aluminum oxide (Woelm, Eschwege, Germany, activity grade I) immediately before use. Water was redistilled twice in an all-glass apparatus. Ammonium bicarbonate was recrystallized from 80% aqueous ethanol. Absence of contaminating compounds giving peaks with retention times similar to those of bile acid derivatives was ascertained by gas–liquid chromatographic analysis of the methylated and trimethylsilylated residue from about 2 ml of the solvents used.

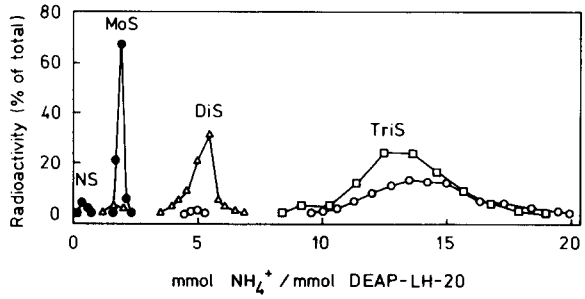

Fig. 1. Separation of nonsulfated (NS), mono- (MoS), di- (DiS), and trisulfated (TriS) products obtained in the synthesis of bile acid sulfates. Products from labeled lithocholic (●), chenodeoxycholic (△), cholic (○), and taurocholic (□) acids are indicated. A column of DEAP-LH-20 in the acetate form (about 0.6 g, 1.0 mmol ion exchanging capacity, 250 × 4.4 mm) was used at a flow rate of about 1.3 ml × min^{-1} × cm^{-2}. The reaction mixture was applied to the column in about 10–15 ml 72% ethanol, and bile acid sulfates were eluted with NH_4HCO_3 or $(NH_4)_2CO_3$ in 72% ethanol (usually stepwise increase of concentration from 0.1–0.2 M to 0.5 M).

Reference compounds. Authentic bile acids were those used in previous studies in this laboratory. Bile acids of the 5α-series were kindly supplied by Drs. W. H. Elliott and A. Kallner. Labeled compounds (Radiochemical Centre, Amersham, England and NEN Chemicals GmbH, Frankfurt/Main, W. Germany) were [24-^{14}C]lithocholic acid (sp act 50 mCi/mmol), [24-^{14}C]chenodeoxycholic acid (sp act 60 mCi/mmol), [24-^{14}C]cholic acid (sp act 53 or 60 mCi/mmol), cholyl[1-^{14}C]glycine (sp act 17.6 mCi/mmol), and [24-^{14}C]taurocholic acid (sp act 58 mCi/mmol). The purity of the bile acids was checked by anion exchange chromatography on DEAP-LH-20 or by thin-layer chromatography (see below).

Bile acid sulfates were prepared essentially as described by Mumma (29). Bile acids (1 mmol) and dicyclohexyl carbodiimide (5 mmol) were dissolved in dimethyl formamide (5 ml) and the solution was cooled on an ice bath. Sulfuric acid (1 mmol per mmol of hydroxyl groups in the bile acid) was mixed with dimethyl formamide (1 ml), cooled to 0°C and slowly (3–4 drops/min) added to the bile acid solution with constant shaking on an ice bath. After 1 hr at 0°C the mixture was diluted with ice-cold 50% aqueous ethanol (6 ml). The precipitate was filtered off in the cold on a small sintered-glass Büchner funnel and was washed with 30 ml of ice-cold 72% (v/v) aqueous ethanol. Reaction products were separated on a DEAP-LH-20 column using a volatile buffer system (**Fig. 1**). Recently, crystalline disodium salts of the 3-sulfates of cholic, chenodeoxycholic, and deoxycholic acids have been prepared by Professor G.A.D. Haslewood[2] who kindly donated samples of these compounds for comparative purposes.

Amberlite XAD-2 (Rohm and Haas, Philadelphia, PA) was washed with 5 volumes of each of the following solvents: 4 M HCl in 72% ethanol, 72% ethanol, 2 M NaOH in 72% ethanol, 72% ethanol, acetone, and water. The resin was stored in water. Columns (8–12 mm ID) were prepared with 10 g of resin suspended in water. When the column bed had settled under gravity flow, it was washed with 5 volumes of ethanol followed by 5 volumes of water. When used for extraction of urine, the columns were eluted at a flow rate not exceeding 0.2 ml × min^{-1} × cm^{-2}.

Amberlyst A-15 (Rohm and Haas) was washed with 5–10 volumes of each of the following solvents: 2 M HCl in 72% ethanol, 72% ethanol, 2 M NaOH

[2] Department of Biochemistry and Chemistry, Guy's Hospital Medical School, London SE1, 9RT, England. A detailed description of the synthesis of these sulfates appeared in *Biochem. J.* **155:** 401–404 (1976).

in 72% ethanol, and 72% ethanol. The resin was stored dry in the sodium form. Columns (10 mm ID) were prepared with 2 g of the resin suspended in 72% ethanol. When the column bed had settled by gravity flow, it was washed with 2 M NaOH in 72% ethanol and 72% ethanol, and was converted to the H^+ form with 2 M HCl in 72% ethanol followed by 72% ethanol until the eluate was neutral. Samples were eluted at a rate of 0.4 ml × min^{-1} × cm^{-2}.

Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden) was sieved before use. The 100–140 mesh fraction was washed with 5 volumes of each of the following solvents: 5% acetic acid in water, 5% acetic acid in ethanol, water, ethanol, chloroform, and methanol. The gel was dried at 60°C and stored at room temperature. For chromatography, columns (180–200 × 10 mm) containing 4 g were packed in chloroform–methanol 3:2 (v/v), saturated with sodium chloride (19). Bile acid mixtures were applied to the columns dissolved in about 1 ml of the same solvent. The columns were eluted with a flow rate of about 0.2 ml × min^{-1} × cm^{-2}.

Diethylaminohydroxypropyl Sephadex LH-20 (DEAP-LH-20). Chlorohydroxypropyl Sephadex LH-20 having a chlorohydroxypropyl group content of 22.4% (w/w) (2.4 mmol × g^{-1}) was synthesized from Sephadex LH-20, 140–170 mesh (22). The product, 194.5 g, was soaked for 30 min in a mixture of methanol (700 ml) and diethylamine (800 ml). Potassium hydroxide in methanol (0.26 M, 1750 ml) was added and the reaction proceeded for 3 hr with continuous stirring and use of a reflux condenser (23). The final product had a diethylamine substitution of 14.0% (w/w), and a titrated amine content of 1.7 mmol/g dry gel. The capacity to take up cholic acid was about 0.3 mmol/g dry gel. The gel was stored dry in its base form at −18°C.

Column beds were prepared in 72% ethanol using 0.6–0.7 g of gel, corresponding to a total anion exchanging capacity of about 1 mmol. Glass columns (300 × 4.4 mm) were used equipped with a 70 ml solvent reservoir and a ground glass joint at the top, and a Teflon fitting covered with a fine-meshed (75 μm) Teflon net at the bottom. The column was connected to a nitrogen pressure line, and a gel bed of 2.8–3.5 ml was packed at a pressure of about 1 kg × cm^{-2}. This bed was washed with potassium acetate–KOH (20 ml, 0.5 M, pH 9.8–10.0, in 72% ethanol) and 72% ethanol until neutral. The gel was then suspended in 20 ml of 0.1 M acetic acid in 72% ethanol, packed in 10 ml of 72% ethanol and washed to neutrality with 20 ml of 72% ethanol. The pressure was released and the column could be left with a stoppered outlet until used. After application of the sample, the columns were eluted at a flow rate of 25–30 ml × hr^{-1}, usually requiring a pressure of 0.2–0.5 kg × cm^{-2}. Columns were used repeatedly after regeneration as described above.

Lipidex-5000 (Packard Instruments Co., Downers Grove, IL) was used to separate bile acid methyl esters (30). Columns, containing 4 g, were prepared in hexane–chloroform 8:2 (v/v). The sample was applied in this solvent and the column was eluted with 5 × 5 ml. Elution was then continued with 7:3, 6:4, and 5:5 (v/v) mixtures of hexane–chloroform, 5 × 5 ml fractions being collected with each solvent. The column was finally eluted with methanol.

Thin-layer chromatography was carried out using plates precoated with Kiesel-gel 60 (Merck, Darmstadt). The solvent system trimethylpentane–ethyl acetate–acetic acid 5:5:1 (by vol), was used for unconjugated bile acids (31), and ethyl acetate–ethanol–25% aqueous ammonium hydroxide 5:5:1 or 2:7:1 (by vol) (32), or ethylene chloride–acetic acid–water 10:10:1 (by vol) (33), for conjugated and sulfated bile acids. The zones were visualized by spraying with an anisaldehyde reagent (34) or with 10% phosphomolybdic acid in ethanol.

Gas–liquid chromatography (GLC) and gas–liquid chromatography–mass spectrometry (GLC–MS). The bile acids were analyzed as methyl ester trimethylsilyl (TMS) ether derivatives (35). Samples were dissolved in methanol–diethyl ether 1:9 (v/v) and methylated with diazomethane. TMS ethers were prepared in pyridine–hexamethyldisilazane–trimethylchlorosilane 3:2:1 (by vol) for 30–60 min at 45°C or overnight at room temperature. Samples were taken to dryness under a stream of nitrogen and were immediately dissolved in hexane.

Two types of column packings were used: 1.5% Hi-Eff 8BP or 1.5% SE-30 on Gas-Chrom Q 80–100 mesh. The columns were 2.5 m × 3.4 mm and were used at temperatures of about 210–240°C. Analyses on Hi-Eff 8 BP columns were carried out at the lowest possible temperature, usually about 220°C.

GLC–MS was carried out using a modified LKB 9000 instrument (LKB Instruments, Rockville, MD) (24). The energy of the bombarding electrons was 22.5 eV. The temperatures of the molecule separator and ion source were 250°C and 290°C, respectively. Spectra were taken by repetitive magnetic scanning of the *m/e* range 0–750, and data were recorded on magnetic tape. Evaluation of data was performed on an IBM 1800 computer (25, 36).

Radioactivity determinations were made in a flow counter (Frieseke and Hoepfner) or by liquid scintillation counting (Tri-Carb scintillation spectrometer 4322, Packard Instruments Co.) using Instagel as

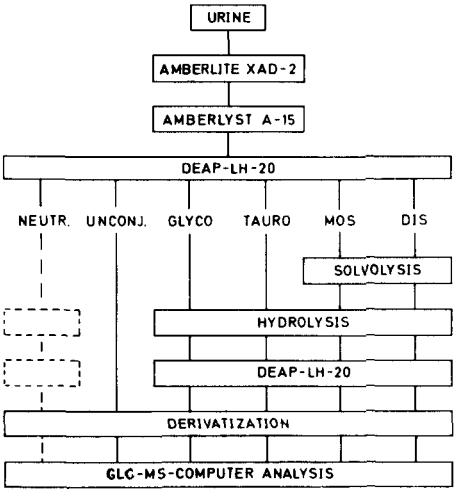

Fig. 2. Outline of procedure for analysis of bile acid profiles in urine.

scintillation liquid. Quenching corrections were made using [4-^{14}C]cholesterol as an internal standard. Radioactive compounds on thin-layer chromatograms were detected by scanning (Berthold Dünnschicht-scanner II) or by scraping and eluting the zone for counting in the liquid scintillation spectrometer.

ANALYTICAL PROCEDURE

An outline of the procedure is given in **Fig. 2.**

Urine was collected in polyethylene bottles which were kept refrigerated during the 24 hr collection period. Aliquots were then stored at −20°C until analyzed.

Twenty ml of urine was filtered and the filter paper was washed with an equal volume of water. The sample was acidified with 4 N HCl to pH 4.0 and passed through the Amberlite XAD-2 column. After washing with 50 ml of water the bile acids were eluted with 50 ml of ethanol containing 0.5 ml of 25% aqueous ammonium hydroxide (v/v). The eluate was taken to dryness, and the residue was transferred in 85% ethanol (v/v) (3 × 3 ml) to a centrifuge tube. After centrifugation, the insoluble material was washed with 85% ethanol (3 × 2 ml). The pooled supernatants were diluted with water to make a 72% (v/v) ethanolic solution, which was passed through the Amberlyst A-15 column. The effluent was allowed to flow directly through the column of DEAP-LH-20, and the two columns were rinsed with 20 ml of 72% ethanol (v/v). The effluent from the DEAP-LH-20 column and an additional 15 ml of 72% ethanol was collected to give a fraction of neutral compounds (**Table 1**). Group separation of bile acids was then achieved using the solvents and buffers given in Table 1. All fractions were collected in 100 ml round-bottomed flasks, taken to near dryness and lyophilized overnight to remove ammonium acetate.

TABLE 1. Buffer systems for group separation of bile acids and their conjugates on DEAP-LH-20

Acetate Concentration (M)[a]	Apparent pH[b]	Volume Used (ml)[c]	Compounds Eluted
0		15[d]	Neutral compounds
0.10[e]	(3.8)	7.4	Unconjugated bile acids
0.30	5.0	16.5	Glycine conjugates
0.15	6.6	11.0	Taurine conjugates
0.34	7.6	11.0	Monosulfates
0.30	9.6	13.0	Di- and trisulfates

[a] The buffers were made from 72% (v/v) aqueous ethanol to which acetic acid was added to give the appropriate molarity.
[b] Measured with a glass electrode. The pH was adjusted by addition of conc. aqueous ammonium hydroxide to the solution of acetic acid in 72% ethanol.
[c] Ml per meq DEAP-LH-20.
[d] Not including volume used for application of sample.
[e] Acetic acid.

The *unconjugated* bile acids were methylated. *Glycine and taurine conjugated* bile acids were hydrolyzed in 15% (w/v) NaOH in 50% aqueous ethanol (6 ml) for 10 hr at 110–120°C (37). The hydrolysate was transferred to a separatory funnel with water (5 × 2 ml), acidified with 4 N HCl to pH 1 and extracted with ethyl acetate (3 × 20 ml). The pooled ethyl acetate portions were washed with water (5 × 20 ml) which was reextracted with 20 ml of ethyl acetate. The ethyl acetate was evaporated and the residue was transferred with 72% ethanol (3 × 3 ml) to a DEAP-LH-20 column. Neutral compounds were eluted and unconjugated bile acids were recovered as described in Table 1.

The fractions containing *sulfated* bile acids were dissolved in 3 ml of ethanol. Ethyl acetate (27 ml) equilibrated with 2 M aqueous sulfuric acid was added and the sample was incubated at 39°C for 16 hr (38). The solution was then neutralized with 0.4 ml 2 M sodium hydroxide and taken to dryness. Hydrolysis and purification on DEAP-LH-20 was performed as described above.

Before GLC–MS analysis, a known amount of coprostanol was added to all samples as an internal standard to correct for variations in injection volume (25). Methyl ester TMS derivatives were then prepared, the samples were dissolved in hexane (50–4000 μl), and 4-μl aliquots were analyzed using Hi-Eff 8 BP and SE-30 as stationary phases. Preliminary identification of bile acids was based on retention times on the two columns and on partial mass spectra generated from fragment ion current (FIC) chromatograms (24, 25, 36). Final identification required complete mass spectra and close resemblance

to mass spectra of reference compounds analyzed under similar conditions.

Bile acids were quantitated either from gas chromatograms obtained with the flame ionization detector or from selected FIC chromatograms obtained in the GLC–MS analyses. Areas of peaks given by the urinary bile acid derivatives were compared with those given by known amounts of reference compounds (external standards). Usually lithocholic, chenodeoxycholic (and deoxycholic) and cholic acids were used as external standards for mono-, di- and tri-(tetra)-hydroxycholanoates, respectively. In the GLC–MS analyses, peak areas in FIC chromatograms were converted to total ion current (TIC) peak areas (25). Differences in TIC response between the bile acid derivatives from urine and the respective external standards were not corrected for. All values are given without correction for losses during the isolation procedure (about 5–30% depending on the fraction analyzed, see Results).

Determination of the position of the sulfate moiety. The fraction containing monosulfated bile acids was dissolved in 1 ml of acetone and 10 μl of chromic acid oxidation mixture was added (39). The reaction proceeded for 10 min at 0°C with constant shaking and was stopped by the addition of 0.3 ml of methanol. Fifty μl of 4 M HCl was added, and after solvolysis for 72 hr at room temperature (2) the reaction was neutralized with sodium hydroxide and evaporated. Keto groups were converted into *O*-methyloximes by reaction with 70 mg methoxyamine hydrochloride in 1 ml of pyridine for 3 hr at 80°C (40). Hydrolysis, extraction, and analysis by GLC–MS was then performed as described above.

RESULTS

Chromatographic methods

Extraction with Amberlite XAD-2. Purification using Amberlite XAD-2 (41) was essential since inorganic and organic compounds in urine interfered with the anion exchange chromatography and acted as eluting buffers. Recoveries of di- and trisulfates were pH-dependent. All bile acids tested were quantitatively adsorbed when the urine was acidified with HCl to pH 4 (**Fig. 3**). The necessary washing volume was 5 ml/g of dry resin. The flow rate was critical; when exceeding 0.25 ml $\times$ min^{-1} $\times$ cm^{-2}, unconjugated bile acids, mainly lithocholic acid, were partly eluted by the water. Bile acids were desorbed with ethanol containing a small amount of ammonium hydroxide (Fig. 3). Recovery was 98–100% as determined for each conjugate class separately in multiple runs. No hydrolysis occurred as judged by thin-layer chromatographic analysis of labeled bile acid sulfates.

Further purification of the material eluted from the XAD-2 column was achieved by extraction of the residue with 85% ethanol. Bile acids were dissolved, and insoluble residue was discarded. When labeled bile acids were added to urine, thin-layer chromatographic analysis showed that only taurocholic acid trisulfate was present in the undissolved material (less than 0.5% of the added amount).

Cation exchange with Amberlyst A-15. Cations that interfered with the separation on DEAP-LH-20 were removed by passing the extract through a column of Amberlyst A-15 in H^+ form. The recoveries of labeled unconjugated, glycine or taurine conjugated, monosulfated, and trisulfated bile acids added to extracts of urine were better than 97% when the column was washed with 20 ml of 72% ethanol after application of the sample.

Anion exchange chromatography on DEAP-LH-20. Several batches of DEAP-LH-20 were synthesized that differed in the degree of substitution with diethylamine. In the range 0–1.5 mmol of diethylamine/g gel there was a good correlation between the content of amine and the capacity of the anion exchanger to bind bile acids. Above this range the binding capacity increased less than expected, possibly due to exclusion effects in the gel matrix. Gels containing about 1.5 mmol/g were chosen for bile acid separations.

Chromatographic conditions were evaluated using extracts of urine to which labeled bile acids were added. For practical reasons, stepwise elution was preferred to gradient elution. Also, in order to obtain a relatively rapid resolution when large amounts of material were present, the following variables were investigated: solvent and buffer composition, pH,

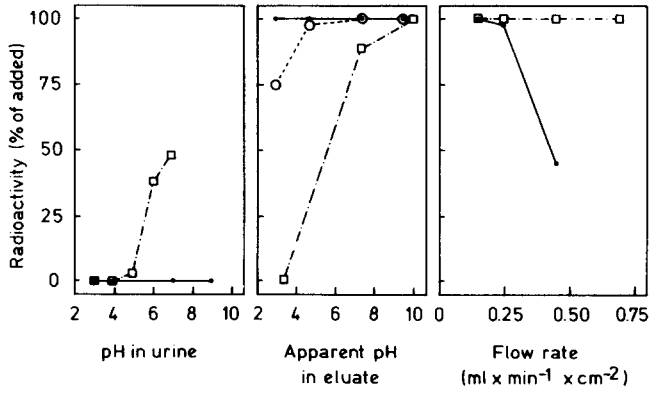

Fig. 3. Effects of pH and flow rate on the extraction by Amberlite XAD-2 of labeled bile acids added to urine. *Left panel*: radioactivity in the water phase; *middle and right panels*: radioactivity in the ethanol phase. Symbols: see Fig. 1.

TABLE 2. Recoveries in the chromatography on DEAP-LH-20 of radioactive bile acids added to extracts of urine

Bile Acid Added[a]	No. of Experiments	Amount Added		Recovery, % in Fraction from DEAP-LH-20					
		cpm × 10^{-2}	ng	N	U	G	T	MoS	DiS + TriS
Cholic	3	73	35	0.1	94.7	0.7	0	0	0
Glycocholic	2	250	390	0	1.8	92.3	3.8	0	0
Taurocholic	2	378	265	0	0	0.7	92.1	1.2	0
Lithocholic-MoS	1	122	80	0	0	0	0	95.2	0
Chenodeoxycholic-DiS	2	495	425	0	0	0	0	0.9	101.9
Cholic-TriS	2	340	450	0	0	0	0	1.8	89.7

[a] MoS, DiS and TriS, mono-, di-, and trisulfated bile acids, respectively.

ionic strength, flow rate, temperature, and amount of ion exchanging groups in the column.

Ethanol–water mixtures were employed since they are good solvents for bile acids and their conjugates and sulfates. Optimal swelling of the gel was obtained with 72% ethanol. In this system, liquid–gel partition or adsorption effects were not seen (as tested with cholesterol and cortisol, which were not separated). Peak broadening occurred with increasing proportions of ethanol or water, possibly due to restricted diffusion. However, the elution order of bile acid conjugates remained unchanged.

A number of different buffers were tested. The best results were obtained with mixtures of acetic acid and ammonium hydroxide, which had the additional advantage of being volatile.

The pH and ionic strength were selected to elute each group of bile acids within 3–5 column volumes. All bile acids tested were retained on the column in 72% ethanol. Unconjugated bile acids were eluted with 0.1 M acetic acid. By gradually increasing the apparent pH from 3.8 to 9.6, bile acid conjugates were eluted in the order: glycine conjugates, taurine conjugates, monosulfates, disulfates, and trisulfates. Increase of the ionic strength improved peak shape, and an optimum concentration was selected that did not result in overlap between the conjugate groups. With the conditions chosen, no separation was observed between individual bile acids within each group, and separations between unconjugated, glycine and taurine conjugated bile acids within the sulfate fractions were minimal.

Since bile acid sulfates are acid-labile, the stability of lithocholic acid sulfate in 0.5 M acetic acid and 0.5 M formic acid in 72% ethanol was studied. The rate of hydrolysis was about 0.3% per hr in both solutions at room temperature.

Flow rate changes between 0.23 and 3.3 ml × min^{-1} × cm^{-2} had no effect on the chromatographic behavior of the bile acids. Peak broadening of neutral compounds occurred already at the lower flow rates.

A temperature change from 0°C to 45°C decreased peak elution volumes by about 25% without changing elution order or peak resolution.

The retention volume of bile acids was linearly correlated to the total amount of ion exchanging groups in the column. The use of about 1 meq of ion exchanger has been found sufficient for all samples studied so far.

In order to determine recoveries and overlaps between adjacent fractions, labeled bile acids were added to extracts of urine and subjected to chromatography on DEAP-LH-20. The results are summarized in **Table 2.** Recoveries in the appropriate fraction were 90–100% and the overlap was less than 5%. An example of the separation of labeled bile acids added to an extract of urine is shown in **Fig. 4.**

Separation of glycine and taurine conjugated monosulfated bile acids on Sephadex LH-20. At the beginning of this study, various solvent systems were tested for separation of bile acids on Sephadex LH-20 (19).

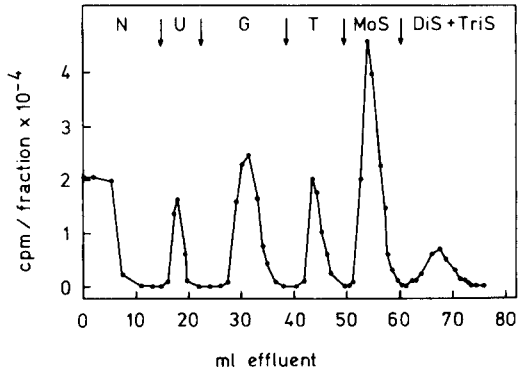

Fig. 4. Separation of bile acids on DEAP-LH-20. Labeled bile acids were added to normal urine, taken through the analytical procedure and separated on a column of DEAP-LH-20 (0.6 g, acetate form). The solvents and buffers listed in Table 1 were used to elute neutral compounds (*N*, cholesterol), unconjugated (*U*, cholic and lithocholic acids), glycine conjugated (*G*, glycocholic acid), taurine conjugated (*T*, taurocholic acid), monosulfated (*MoS*, lithocholic acid sulfate), disulfated (*DiS*, chenodeoxycholic acid disulfate), and trisulfated (*TriS*, cholic acid trisulfate) bile acids. Arrows indicate changes of eluent as given in Table 1. Effluent collected during application of the sample (about 45 ml, see Procedure) not included.

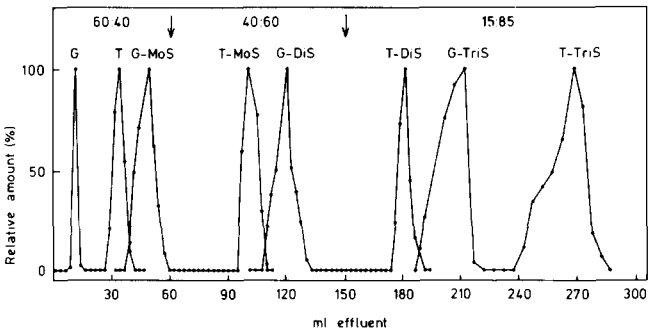

Fig. 5. Separation of a synthetic mixture of sodium salts of glycine (G) and taurine (T) conjugated mono-(MoS), di-(DiS) and trisulfated (TriS) bile acids. Column: 4 g of Sephadex LH-20; solvents: mixtures (v/v) of chloroform and 0.02 M NaCl in methanol: 60:40 (0–60 ml), 40:60 (60–150 ml), and 15:85 (150–300 ml). The approximate amount of bile salt in each fraction of 2.5–5.0 ml was determined by liquid scintillation counting or by scanning of stained thin-layer chromatograms, and is expressed relative to the amount at the peak maximum. Between 2–200 nmol of each compound was applied to the column.

Although these systems were then found to be less satisfactory for crude extracts of urine, they offered the possibility of separating purified sodium salts of glycine and taurine conjugated monosulfates, which were not separated in the DEAP-LH-20 system. This is exemplified in **Fig. 5.** Groups of bile acids were eluted by stepwise decrease of the chloroform content of the solvent mixture chloroform–0.02 M NaCl in methanol.

Individual bile acids within the conjugate groups were not separated, neither were unconjugated bile acids and their sulfates separated from the corresponding glycine conjugates (**Table 3**). Table 3 and Fig. 5 also show that the effect of a taurine moiety on the mobility is similar to that of a sulfate group. The separations are quite different from those obtained on DEAP-LH-20 and the method may therefore serve as a complement to the ion exchange procedure. However, it should be stressed that the compounds travel as sodium salts, and if ammonium or other cations are present they must be exchanged for sodium, e.g., by a cation exchanger or by titration followed by Amberlite XAD-2 extraction (not using NH_4OH in the eluting ethanol). The separations on Sephadex LH-20 are also sensitive to overloading with crude extracts.

Solvolysis and hydrolysis. Two methods of solvolysis were employed: acetone–ethanol–HCl for 72 hr at room temperature (2) and ethyl acetate–ethanol–H_2SO_4 for 16 hr at 39°C similar to the method described by Kornel (38). The more time-consuming acetone solvolysis had been used in several previous investigations (2, 4, 9, 11, 12, 21) and was used initially. However, with this procedure, acetonides may be formed from *cis* glycol structures. Acetone solvolysis also resulted in the formation of substances interfering with the gas–liquid chromatographic analyses of the bile acids. Therefore, solvolysis in ethyl acetate was studied. Under the conditions chosen, labeled lithocholic acid sulfate added to a monosulfate fraction from urine was completely solvolyzed as determined by TLC and radioactivity scanning. Recoveries of synthetic lithocholic acid sulfate and 3β-hydroxy-5-cholenoic acid sulfate were 80% as determined by GLC analysis. When the 3-monosulfates of cholic, chenodeoxycholic, and deoxycholic acids were solvolyzed, no transformation of the bile acid structure was noted except for an extensive acetylation and some ethylation. However, this does not interfere with the analysis since alkaline hydrolysis of conjugated bile acids is always performed. When a monosulfate fraction from urine was solvolyzed by the two methods, the yield of bile acids was the same or better after solvolysis in ethyl acetate, which also gave fewer interfering peaks in the gas–liquid chromatographic analysis.

The major loss of bile acid occurred during hydrolysis. When reference compounds were hydrolyzed, the recovery was about 80%. Significant formation of isomerized, oxidized, or unsaturated derivatives of bile acids added to urine was not observed. After alkaline hydrolysis and extraction with ethyl acetate, it was found necessary to wash the ethyl acetate phases (60 ml) with large volumes of water to remove material that interfered in the subsequent DEAP-LH-20 chromatography. Using 5 × 20 ml of water, the recovery of added labeled cholic acid exceeded 99%.

Purification of hydrolyzed bile acids on DEAP-LH-20. Several compounds (e.g., steroid glucuronides) contaminated the sample after ethyl acetate extraction and seriously interfered in the gas–liquid chromatographic analysis. Rechromatography on DEAP-LH-20 was rapid and resulted in a highly purified fraction of unconjugated bile acids.

Gas–liquid chromatography–mass spectrometry. Because of their excellent gas–liquid chromatographic and mass spectrometric properties, methyl ester TMS

TABLE 3. Separation of sodium salts of lithocholic acid derivatives using a 4 g column of Sephadex LH-20 in the solvent system chloroform–0.02 M NaCl in methanol 30:70 (v/v)

Bile Salt	ml Effluent
Lithocholate	10–15
Glycolithocholate	10–15
Taurolithocholate	15–25
Lithocholate sulfate	20–35
Glycolithocholate sulfate	20–35
Taurolithocholate sulfate	40–50

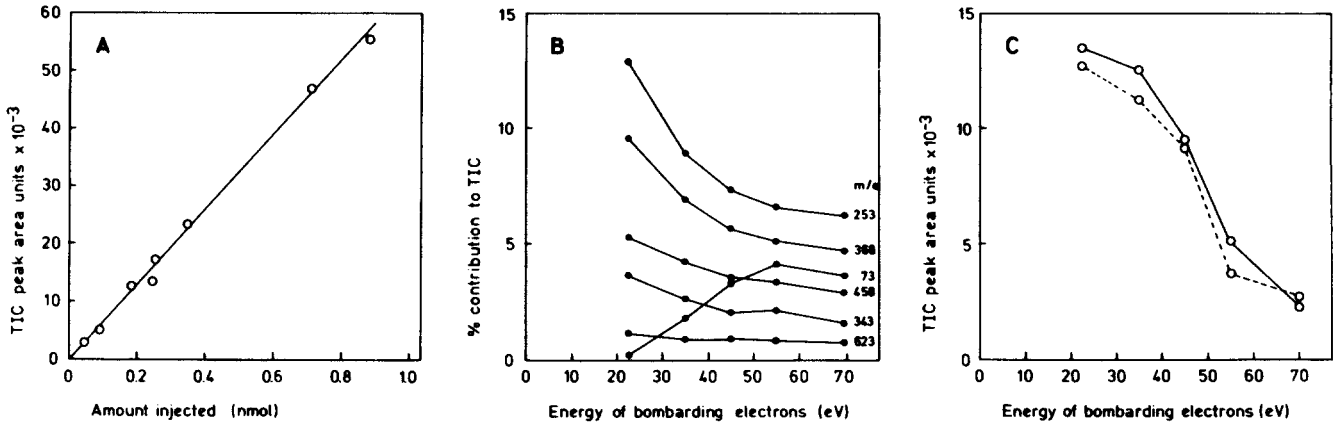

Fig. 6. (*A*) Response (TIC peak area) to different amounts of the TMS derivative of methyl cholate at 22.5 eV. (*B*) Relative ion abundance at varying energy of the bombarding electrons. (*C*) Response (TIC peak area) to 0.25 nmol of the TMS derivatives of methyl cholate (O —— O) and methyl lithocholate (O - - - O), at varying energy of the bombarding electrons.

ether derivatives were used. However it was noted in several cases that the stability of the TMS ethers was limited, resulting in erroneous quantitations unless the samples were analyzed within a day of the TMS ether preparation. This was not solely due to hydrolysis since resilylation did not improve quantitation.

In order to achieve optimal conditions for the quantitative GLC–MS analysis, a study was undertaken to determine the influence of the energy of the bombarding electrons on the total ionization and the fragmentation pattern of the TMS ethers of methyl cholate and lithocholate. At 22.5 eV the TIC peak area was linearly related to the injected amount of bile acid derivatives (**Fig. 6**). The response for the TMS ether of methyl lithocholate was 75–90% of that for the cholic acid derivative. This relation was the same for all energies between 22.5 and 70 eV.

The reproducibility of the mass spectra at 22.5 eV, expressed as the individual variation of the relative abundance of major ions, was within 10% when 0.25 nmol of the derivatives were repeatedly injected. As expected, the relative abundance of diagnostically important ions decreased with increasing energy of the bombarding electrons (Fig. 6). In addition, the total yield of ions decreased (Fig. 6), resulting in a marked progressive decrease of sensitivity with increasing energy of the bombarding electrons.

Miyazaki et al. (42) reported that the fragmentation of TMS ethers of methyl cholate and chenodeoxycholate was affected by the simultaneous ionization of cholesterol TMS ether. This phenomenon (not observed at 60 eV) resulted in erroneous quantitations when 20 eV was used. In order to study whether a similar effect was obtained under the conditions used in our study, the TMS ethers of methyl cholate (0.9 nmol) and methyl chenodeoxycholate (0.05 nmol) were analyzed on SE-30 columns at a temperature where the two compounds were eluted together. The mass spectra revealed no interference between the compounds, and the quantitation of the chenodeoxycholic acid derivative was not affected by the presence of the cholic acid derivative. The reason for the difference between our results and those of Miyazaki et al. (42) is not clear. It may be noted that the amounts of bile acid derivatives are much lower in our study and that the stationary phases are different. It should also be pointed out that ionization of column bleed may be affected when a compound zone passes through the ion source (43).

In view of these results, 22.5 eV was used both for qualitative and quantitative work. Depending on mass spectrum and retention time (usually between 8 and 40 min), the lower limit for quantitation was about 0.01–0.05 nmol of injected bile acid derivative.

As shown in the next section, the derivatives of

TABLE 4. Fragment ions and equations used for calculation of the amounts of individual components in a mixture of the TMS ethers of three methyl tetrahydroxycholanoates having relative retention times of about 0.80 on the Hi-Eff 8 BP column

Bile Acid	Tentative Structure[a]	Fractional Contribution to TIC[b]		
		m/e 217	*m/e* 367	*m/e* 546
A	B-1,3,7,12-ol	0.12	0.015	0.01
B	B-3,6,7,12-ol	0.02	0.08	0.12
C	Unidentified	0.01	0.005	0.16

[a] See Table 5. B, cholanoic acid.

[b] $AREA_{217} = 0.12TIC_A + 0.02TIC_B + 0.01\ TIC_C$
$AREA_{367} = 0.015TIC_A + 0.08TIC_B + 0.005TIC_C$
$AREA_{546} = 0.01TIC_A + 0.12TIC_B + 0.16TIC_C$
where $AREA_{217,367,546}$ are the peak areas in FIC chromatograms for *m/e* 217, 367, and 546, respectively, and $TIC_{A,B,C}$ are the contributions of bile acids A, B, and C, respectively, to the peak area in the TIC chromatogram. $TIC_{A,B,C}$ are solved and converted to amounts of bile acids.

several bile acids have closely similar retention times. In most cases the individual bile acids in these mixtures could be quantitated from peak areas in specific FIC chromatograms. However, the derivatives of three tetrahydroxycholanoates were eluted together, with a relative retention time of about 0.80 on the Hi-Eff 8 BP column, and no fragment ion was sufficiently specific to permit quantitation of any individual compound in this mixture. This could be achieved by taking advantage of the differences in relative abundance of characteristic ions in the spectra of the individual bile acid derivatives. The ions selected for this purpose, their fractional contribution to the total ion current given by the individual compounds, and the equations used in the calculation are given in **Table 4**. It should be noted that the derivative of allocholic acid may also occur in the same peak as the tetrahydroxycholanoates. However, this compound makes only negligible contributions to the peak areas for the ions used in the equations.

Qualitative composition of bile acids

No bile acids were found in the neutral fraction from the DEAP-LH-20 column; small amounts were unconjugated (see below, Tables 6–8). Thin-layer chromatography of material eluted in the glycine and taurine conjugate fractions gave spots with the mobility of such conjugates. Solvolysis of the material in the monosulfate fraction followed by Amberlite XAD-2 extraction and rechromatography on DEAP-LH-20 yielded bile acids in the glycine and taurine conjugate fractions. Unconjugated bile acid monosulfates did not appear in significant amounts. Only in a few samples were small amounts of bile acids found

TABLE 5. Completely and partially identified bile acids found in urine from healthy subjects and patients with hepatobiliary diseases

Bile Acid[a]	Relative Retention Time[b]		Fraction[c]	Ions Used for Quantitative GLC–MS Analysis, *m/e*[d]
	Hi-Eff 8 BP	SE-30		
23-nor-5β B-3α,7α,12α-ol	0.74	0.70	U,G,T	609, 444, 354, 343, 253
B-tetrol[e]	0.79	1.40	U,G,T	546
B-3,6,7,12-ol[f]	0.82	1.31	U,G,T	546, 367
5α B-3α,7α,12α-ol	0.82	0.95	G,T,MoS	623, 458, 368, 343, 261, 253
B-1,3,7,12-ol[f]	0.84	1.29	G,T	217
5β B-3α,7α,12α,23-ol	0.94	1.44	G,T	546, 456, 343, 253, 143
5β B-3α,7α,12α-ol	1.00	1.00	U,G,T,MoS	623, 458, 368, 343, 253
5α B-3α,7β,12α-ol	1.13	1.09	U,G,T	458, 343, 253
5β B-3α,6β,12α-ol[f]	1.16	1.09	U,G	458, 368, 343, 253
5α B-3α,12α-ol	1.25	0.84	G,MoS	535, 345, 255
5α B-3β,7β,12α-ol[f]	1.32	1.09	U,G,T,MoS	458, 368, 343, 253
5β B-3β,7α-ol	1.34	0.86	MoS	460, 370, 262, 255, 249, 243
5β B-1,3,12-ol[f]	1.38	1.21	U,G,T,MoS	548, 217
5α B-3α,7α-ol	1.40	0.92	MoS	460, 370, 262, 255, 249, 243
5β B-3β,12α-ol	1.43	0.88	MoS	535, 345, 255
5β B-3α,6α,7α-ol	1.45	1.27	U,G,T,MoS	458, 369, 195, 159
5β B-3β,7β,12α-ol	1.48	1.13	U,G	623, 458, 433, 343, 253
5β B-3α,7β,12α-ol	1.59	1.15	U,G,T	623, 458, 433, 368, 343, 253
5β B-3α,12α-ol	1.61	0.92	U,G,T,MoS	535, 370, 345, 255
5β B-3α,12β-ol	1.69	0.85	U,G,T,MoS	535, 370, 345, 255, 208
5β B-3α,7α-ol	1.78	0.95	U,G,T,MoS	370, 262, 255, 249, 243
5β B-3α,6β-ol	1.87	0.97	G,MoS	460, 445, 405, 370, 323, 255
5α B-3α-ol	1.95	0.76	MoS	462, 372, 357, 230, 215
B^{Δ}-diol[e]	1.98	1.06	MoS	458, 368, 353, 327, 253, 158
5β B-3α,6α-ol	2.11	1.02	T,MoS	535, 460, 405, 370, 323, 255
B^{5}-3β,12α-ol	2.21	1.05	G,MoS	458, 368, 343, 329, 253, 129
5α B-3β,12α-ol	2.24	1.09	T,MoS	535, 460, 345, 255
5β B-3α-ol	2.50	0.74	MoS	372, 357, 257, 230, 215
5β B-3α,7β-ol	2.76	1.06	MoS	535, 460, 370, 364, 255, 249, 243
B^{5}-3β-ol	3.10	0.91	MoS	460, 404, 370, 331, 276, 255, 249, 129
5β B-3β-ol-12-one	6.00	—	G,MoS	476, 386, 271, 231, 229, 121
5β B-3α-ol-12-one	7.50	1.25	G,MoS	476, 386, 361, 321, 231, 229, 121

[a] B = cholanoic acid. Configuration at C-5 and of hydroxyl groups are indicated by Greek letters. Superscript denotes position of double bond.
[b] As methyl ester TMS ether, relative to the derivative of cholic acid.
[c] Fractions collected from the DEAP-LH-20 column. For abbreviations see legend to Table 2.
[d] The isotope peaks of the major fragment ions were sometimes used.
[e] Positions of substituents unknown.
[f] Positions of hydroxyl groups and stereochemistry are tentative.

in the di- and trisulfate fraction. Further studies of some of these samples indicated that this was due to contamination with monosulfates.

Position of sulfate groups. Three samples from patients with primary biliary cirrhosis, congenital intrahepatic cholestasis, and liver disease due to contraceptive steroids were studied (see below, Table 8). The monosulfate fractions contained lithocholic, allolithocholic, 3β-hydroxy-5-cholenoic, deoxycholic, 3β,12α-dihydroxy-5β-cholanoic, 3β,12α-dihydroxy-5α-cholanoic, 3α-hydroxy-12-keto-5β-cholanoic, chenodeoxycholic, ursodeoxycholic, cholic, allocholic, hyocholic, and some unidentified trihydroxycholanoic acids in varying proportions. After the reactions described in Materials and Methods the methyl ester MO-TMS derivatives of the following bile acids could be identified: 3α-hydroxy-12-keto-5β-, 3β-hydroxy-12-keto-5β, 3β-hydroxy-12-keto-5α-, 3α-hydroxy-7-keto-5β-, and 3α-hydroxy-7,12-diketo-5β-cholanoic acids. The saturated monohydroxy bile acids remained unchanged. Derivatives of bile acids with hydroxyl groups at C-7 or C-12 were not found, showing that, in the monosulfate fraction, sulfation occurred only at C-3.

Individual bile acids. A list of completely and partially characterized bile acids and of unidentified bile acids that seem to be of particular interest for the evaluation of urinary bile acid excretion is given in **Table 5.** In addition to the bile acids listed, a number of unidentified bile acids were observed, mainly trihydroxycholanoic acids. Since comprehensive reviews of mass spectra of bile acid derivatives have been published (44, 45), only mass spectra of particular interest will be discussed.

Two monohydroxycholanoic acids, lithocholic and 3β-hydroxy-5-cholenoic acids, were found in all urine samples. Allolithocholic acid was a minor component in many samples. One sample from a patient with intrahepatic cholestasis of pregnancy contained small amounts of a compound, the methyl ester TMS ether of which gave a mass spectrum similar to that of the derivative of 3β-hydroxy-5-cholenoate. The retention time (t_R 2.1–2.2 on Hi-Eff 8 BP) was approximately that expected for a 3α epimer of this compound.

A large number of isomeric 3,7- and 3,12-dihydroxycholanoates were found. The identification of 3α,12β-dihydroxy-5β-cholanoic acid is of particular importance for GLC and GLC–MS analyses of bile acids in urine, since this acid is easily confused with deoxycholic acid because of the similarities in retention times (Table 5) and mass spectra. It was frequently the predominant 3,12-dihydroxy bile acid in the glycine conjugate fraction.

Both 3α,6α- and 3α,6β-dihydroxy-5β-cholanoic acids were identified. The former compound was found both in healthy and cholestatic subjects whereas the latter was present only in urine from three patients. The mass spectrum of the TMS ether of methyl 3α,6β-dihydroxy-5β-cholanoate gives a characteristic ion at *m/e* 405, corresponding to loss of the A-ring (40). This peak is much smaller in the spectrum of the 6α epimer.

Some unsaturated dihydroxy bile acids were present. The mass spectrum of the methyl ester TMS ether of the predominant one is shown in **Fig. 7.** The peaks at *m/e* 492 (M − 56), 329 (M − 129) and 129 are all indicative of a 3-trimethylsiloxy-Δ^5 structure (46, 47). As is the case in spectra of all 3,12-*bis*-trimethylsiloxycholanoates, the ABCD-ring fragment ion is the base peak. The retention times on SE-30 and Hi-Eff 8 BP are very similar to those of the TMS ether of methyl 3β,12α-dihydroxy-5α-cholanoate. In previous studies of a number of 3β-Δ^5 and 3β,5α steroid TMS ethers, the 3β-Δ^5 steroid has been found to be eluted slightly ahead of the saturated steroid on SE-30 columns whereas the retention times of the two types of compounds are essentially the same on Hi-Eff 8 BP columns. These retention time relationships are exactly those found for the derivatives of the two bile acids from urine. Therefore, the unsaturated bile acid is identi-

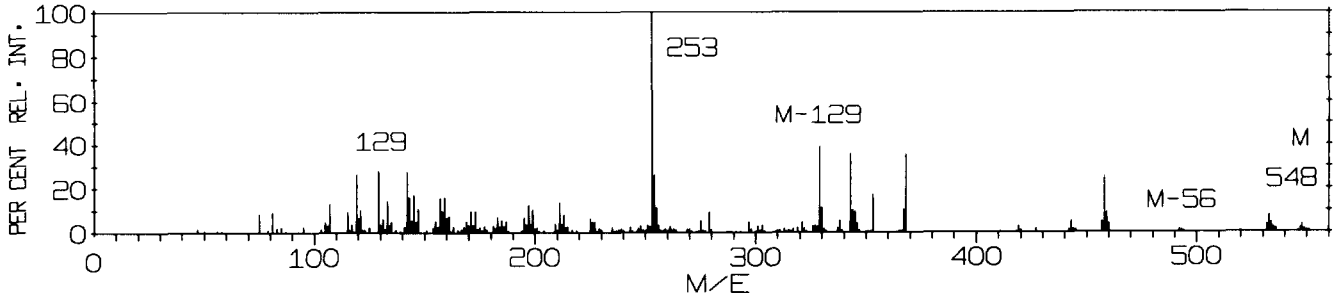

Fig. 7. Mass spectrum of 3β,12α-dihydroxy-5-cholenoic acid methyl ester TMS ether obtained in the analysis of monosulfated bile acids in urine of a patient with intrahepatic cholestasis.

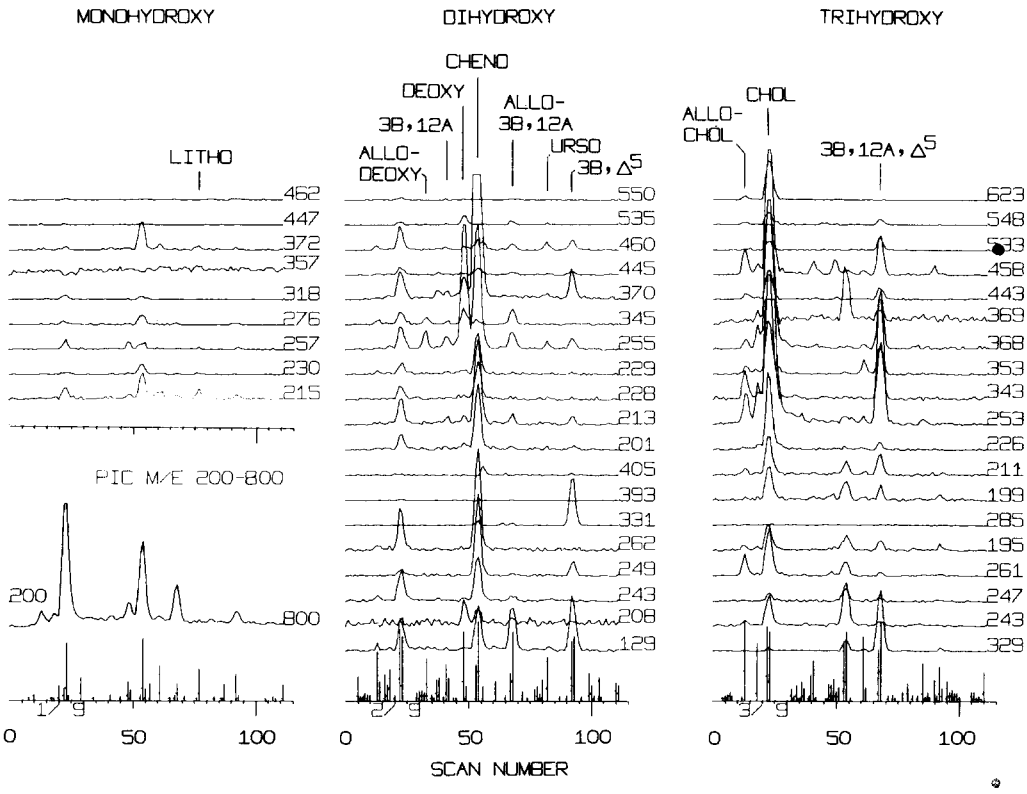

Fig. 8. GLC–MS analysis of a monosulfate fraction from urine of a patient with intrahepatic cholestasis. The lower left chromatogram is a partial ion current (PIC) chromatogram representing current of all ions from *m/e* 200 to 800.

fied as 3β,12α-dihydroxy-5-cholenoic acid. **Fig. 8** shows the GLC–MS analysis of a monosulfate fraction where this was one of the predominant bile acids.

A few samples contained a bile acid, the derivative of which (t_R 1.98 on Hi-Eff 8 BP) gave a mass spectrum indicating the presence of two trimethylsiloxy groups and one double bond in the ring skeleton. Characteristic intense peaks were seen at *m/e* 158 and 327. The origin of these ions is not known and the compound remains unidentified.

A large number of trihydroxycholanoates were found. Most of these appeared to be substituted at C-3, C-7, and C-12. Both 3β and 7β epimers and 5α isomers of cholic acid were present. In some cases the configurations given in Table 5 should be regarded as tentative.

Some of the trihydroxy bile acids had a 6-hydroxy group. Hyocholic acid was the predominant one and was found in almost all samples. β-Muricholic (3α, 6β,7β-trihydroxy-5β-cholanoic) acid was found only in one subject with intrahepatic cholestasis of pregnancy and is not listed in Table 5. However, the peaks at *m/e* 285 and 195 typical of a 3,6β,7-*tris*-trimethylsiloxy structure (44) have been observed in some analyses but no attempts have been made to further characterize the minor components giving these peaks.

The derivative of one of the trihydroxy bile acids had retention times identical with those of the TMS ether of methyl 3α,6β,12α-trihydroxy-5β-cholanoate, and the mass spectrum closely resembled that of the reference compound. The characteristic ions due to loss of the A-ring (M − 145 at *m/e* 493 and M − 145 − 90 at *m/e* 403) were clearly seen.

A commonly occurring trihydroxy bile acid gave an intense base peak at *m/e* 217 when analyzed as the methyl ester TMS ether (**Fig. 9**). This ion corresponds to the well-known ion of mass 129 with an additional TMS group, and it is found in C_{19} steroids with a 1,3-, 15,17- or 17,18-*bis*-trimethylsiloxy structure (48, 49). Its occurrence in a spectrum of a bile acid derivative strongly suggests a 1,3-*bis*-trimethylsiloxy structure. The presence of two hydroxyl groups in the A-ring is strongly supported by the peak at *m/e* 316 formed by loss of the A-ring and its substituents (44). Trihydroxy bile acids with only one hydroxyl group in the A-ring give a corresponding peak at *m/e* 314. Further support for the presence of two hydroxy groups in the A-ring are the peaks at *m/e* 142 and 143, which are particularly prominent in steroids with a 2,3-*bis*-trimethylsiloxy structure (50).

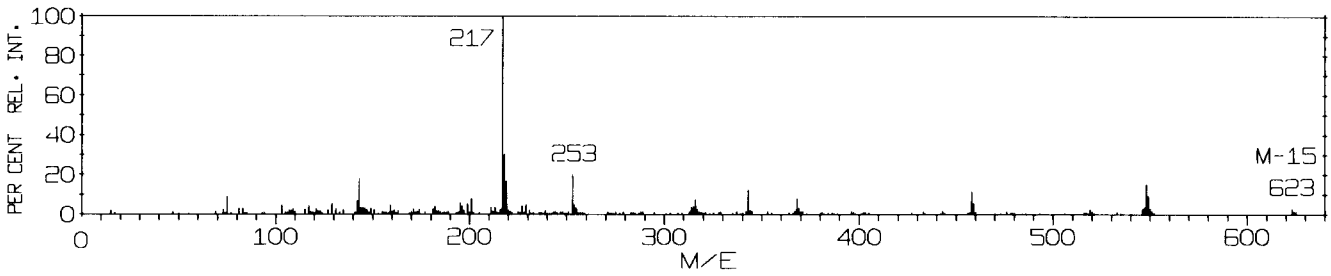

Fig. 9. Mass spectrum of the methyl ester TMS ether derivative of the bile acid tentatively identified as 1,3,12-trihydroxycholanoic acid obtained in the analysis of the taurine conjugated bile acids in urine from a patient with cholestasis of pregnancy.

However, this structure does not give rise to an ion of mass 217. The position of the remaining hydroxyl group is not clear. The ABCD ring fragment at *m/e* 253 shows that it is not in the side chain. Lack of indications for fragmentation through the B-ring suggests that it is in the C-ring. On biological grounds the tentative structure of 1,3,12-trihydroxycholanoic acid is suggested for this new bile acid. **Fig. 10** shows the GLC–MS analysis of glycine conjugated bile acids from the urine of a patient with biliary cirrhosis, where this bile acid was a prominent component.

In addition to the common C_{24} bile acids, most urine samples had norcholic acid as a major component in the unconjugated fraction. The mass spectrum of the methyl ester TMS ether is shown in **Fig. 11.** The presence of the 24-nor side chain is clearly seen from the ions at *m/e* 609 (M − 15), 444 (M − 2 × 90), 354 (M − 3 × 90) and 253 (M − 3 × 90 − side chain).

Tetrahydroxycholanoic acids were excreted in significant amounts in urine from cholestatic subjects. 3α,7α,12α,23-Tetrahydroxy-5β-cholanoic acid was identified on the basis of retention times and mass spectra (Fig. 11) of the methyl ester TMS ether, which were identical with data for the authentic compound.

The mass spectrum of the methyl ester TMS ether of the most commonly occurring tetrahydroxy bile acid is shown in **Fig. 12.** A peak is seen at *m/e* 711 (M − 15) and the base peak is at *m/e* 546 (M − 2 × 90). The ABCD ring fragment at *m/e* 251 and the side chain ion at *m/e* 115 are clearly seen, showing that the four hydroxyl groups of this bile acid are in the ring system. The spectrum is analogous in

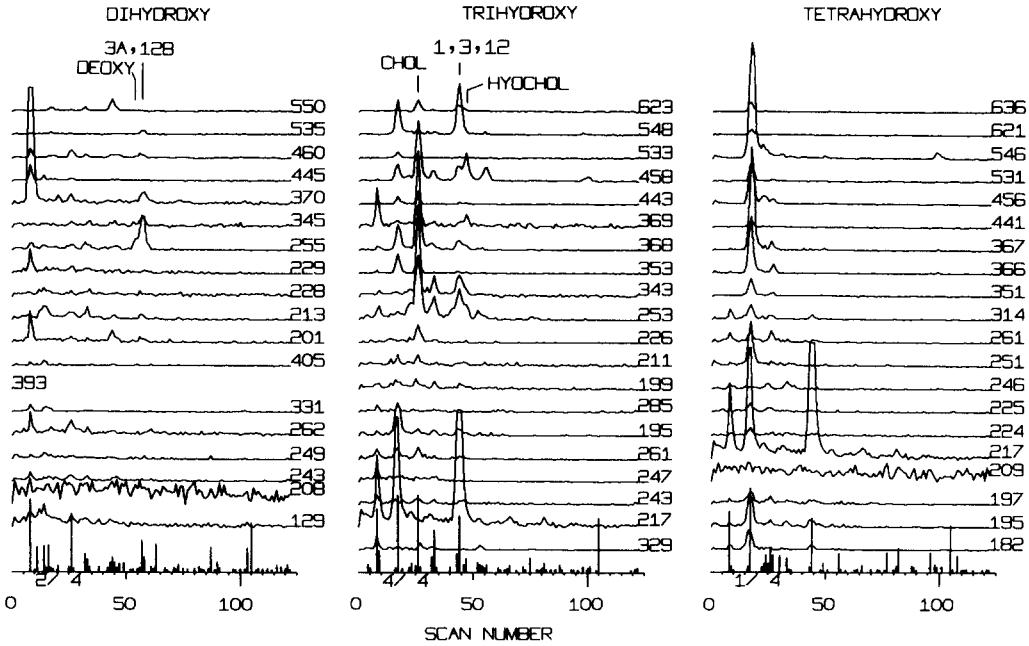

Fig. 10. GLC–MS analysis of glycine conjugated bile acids from urine of a patient with biliary cirrhosis. This sample contained large amounts of the bile acid tentatively identified as 1,3,12-trihydroxycholanoic acid.

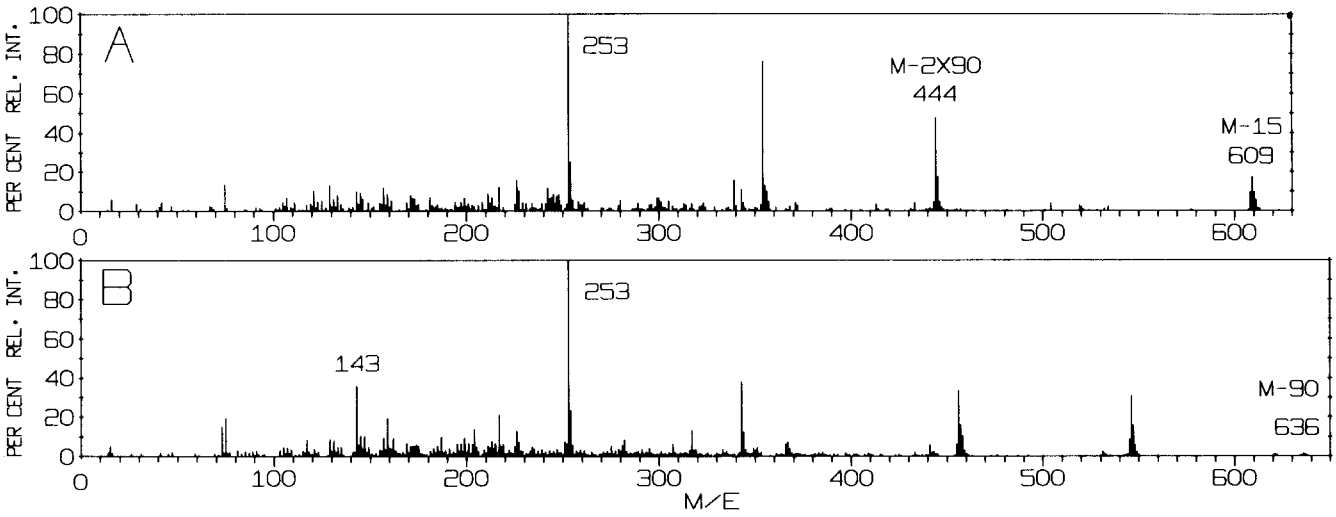

Fig. 11. (*A*) Mass spectrum of the methyl ester TMS ether derivative of norcholic acid obtained in the analysis of unconjugated bile acids in urine from a patient with cholestasis of pregnancy. (*B*) Mass spectrum of the methyl ester TMS derivative of 23-hydroxycholic acid obtained in the analysis of taurine conjugated bile acids in urine from a patient with biliary cirrhosis.

many respects to that of the derivative of hyocholic acid, showing a prominent base peak at *m/e* (M − 2 × 90), the second most intense peak at *m/e* 367 (corresponding to *m/e* 369 in the spectrum of the hyocholic acid derivative), and a peak at *m/e* 195 typical of the 6,7-*bis*-trimethylsiloxy structure. Hyocholic acid and this tetrahydroxycholanoic acid occur predominantly in the taurine conjugate fraction, and the tetrahydroxy acid is most abundant in those samples where hyocholic acid is prominent. On biological grounds it is therefore tentatively identified as 3,6,7,12-tetrahydroxycholanoic acid. **Fig. 13** shows the GLC–MS analysis of a sample where this compound was present in larger amounts than hyocholic acid.

The methyl ester TMS ether of a third type of tetrahydroxycholanoic acid gave a prominent base peak at *m/e* 217 (**Fig. 14**) indicative of a 1,3-*bis*-trimethylsiloxy structure. A molecular ion or M − 15 were not seen. Thus, the compound might also be an unsaturated trihydroxycholanoate. This possibility was excluded by the chromatographic mobility of the methyl ester on a Lipidex 5000 column in hexane–chloroform mixtures (30) which was that of a tetrahydroxycholanoate. The additional hydroxyl groups are in the BCD-rings as evidenced by the peaks at *m/e* 251 (ABCD-rings), 314 (BCD-rings and side chain) and 115 (side chain) (44). The peaks at *m/e* 182, 195, and 261 indicate fragmentation through the B-ring. Thus, the ion of mass 182 may represent the A-ring minus one TMS group and be formed by cleavage of the 5,6 and the 9,10 bonds, and the ion of mass 195 may arise by cleavage of the 6,7 and the 9,10 bonds. The ion at *m/e* 261 may be the same as that formed from the TMS ethers of methyl cholate and allocholate. These peaks support the presence of a 7-hydroxy group. On biological grounds, the fourth hydroxyl group is placed at C-12 and the tentative structure 1,3,7,12-tetrahydroxycholanoic acid is suggested. The presence of this compound in a sample that also contained the tentative 1,3,12-trihydroxycholanoic acid is shown in Fig. 10, where it appears

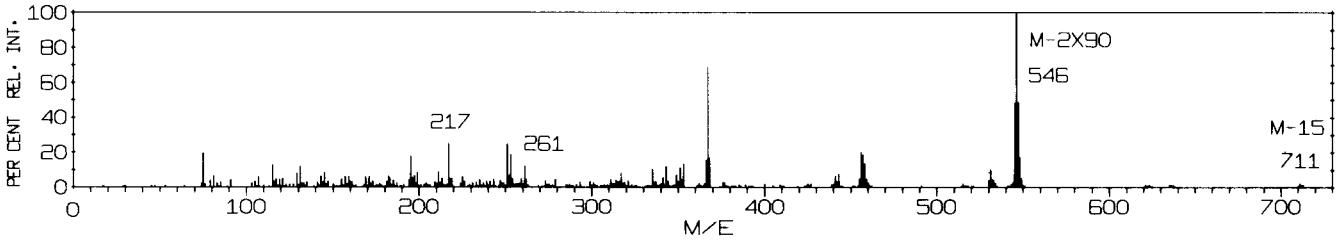

Fig. 12. Mass spectrum of the methyl ester TMS ether derivative of the bile acid tentatively identified as 3,6,7,12-tetrahydroxycholanoic acid obtained in the analysis of glycine conjugated bile acids in urine from a patient with intrahepatic cholestasis. The peaks at *m/e* 253, 261, 343, 353, 368, 458, and 623 are due to the presence at the same retention time of the derivative of allocholic acid. The peak at *m/e* 217 arises from the derivative of the bile acid tentatively identified as 1,3,7,12-tetrahydroxycholanoic acid.

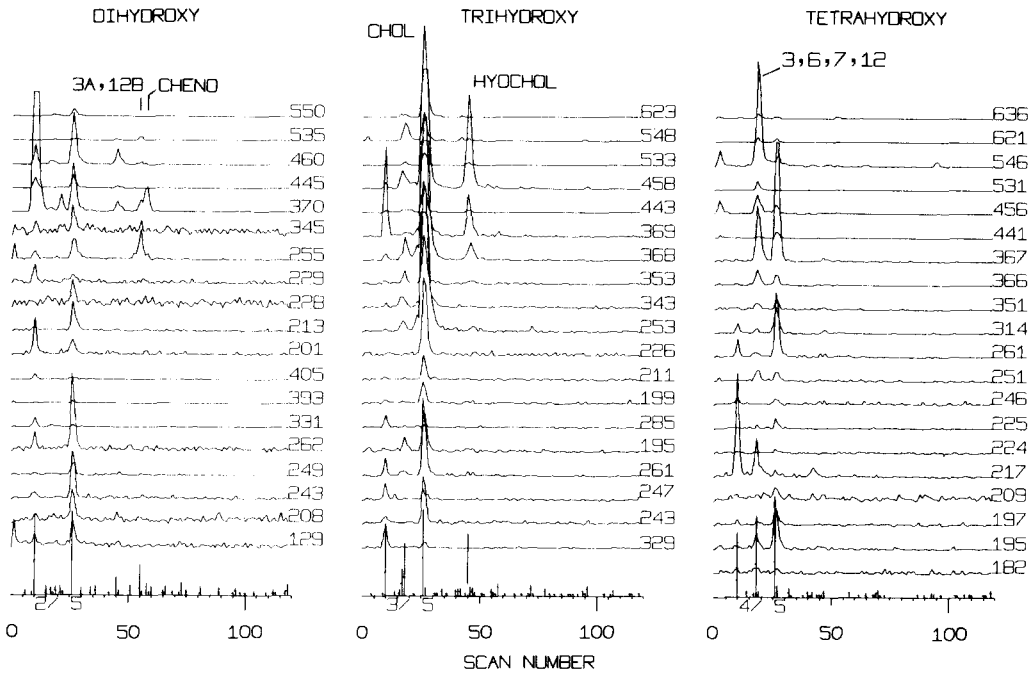

Fig. 13. GLC–MS analysis of the glycine conjugated bile acids in urine from a patient with intrahepatic cholestasis. In this fraction both hyocholic and the tentatively identified 3,6,7,12-tetrahydroxycholanoic acids were major bile acids.

together with the other prominent tetrahydroxycholanoic acid.

The derivative of an infrequently occurring tetrahydroxycholanoic acid gave an intense ion at *m/e* 546 as the most prominent feature. It did not give an ion of mass 217, and a small peak at *m/e* 367 was the isotope peak of that at *m/e* 366. These characteristics served to distinguish this unknown compound from the two tetrahydroxycholanoates appearing at approximately the same retention time.

Only a few ketocholanoic acids were found. By far the predominant one was 3α-hydroxy-12-keto-5β-cholanoic acid. Its 3β epimer was occasionally found. In a few cases a dihydroxy-monoketocholanoate was detected.

Quantitative composition of bile acids

Accuracy and precision of the method. Known amounts of glycocholic, glycodeoxycholic, taurodeoxycholic, and taurolithocholic acids and lithocholic acid sulfate were added to urine samples that were then carried through the entire procedure. In nine experiments where about 100 nmol of bile acid were added to 20 ml of urine from healthy subjects, the recoveries were 67–95%. When losses during hydrolysis were corrected for by comparison with directly hydrolyzed reference compounds, the recoveries were 93–112%.

The precision of the method was tested by analysis of four 20-ml aliquots of a urine sample from a pregnant woman with marked pruritus due to intrahepatic cholestasis. The amounts of individual bile acids in each conjugate fraction varied between 0.03 and 23.08 μmol/24 hr. Mean values and standard deviations for the different compounds are given in **Table 6.**

Bile acid excretion. Results of analyses of 24-hr urine samples from five healthy subjects are summarized in **Table 7**. The daily excretion was very similar in

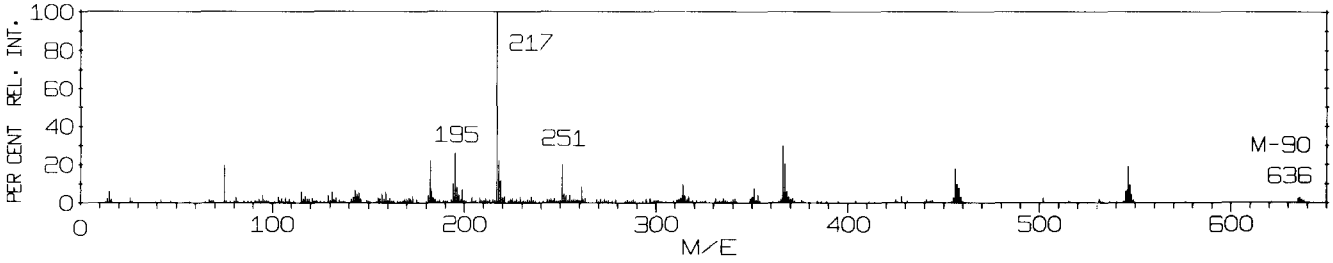

Fig. 14. Mass spectrum of the methyl ester TMS ether of a bile acid tentatively identified as 1,3,7,12-tetrahydroxycholanoic acid obtained in the analysis of the glycine conjugated bile acids in urine from a patient with cholestasis of pregnancy.

four women (mean 7.0 ± 0.8 μmol/24 hr) and the difference between two samples collected with an interval of 11 months from the same woman was small. The bile acid excretion was independent of the 24-hr urine volume, which varied between 0.6 and 2.2 l in the four subjects. The "classical" bile acids, cholic (2–7% of the total), chenodeoxycholic (3–18%), deoxycholic (8–25%), and lithocholic (10–24%) acids constituted 40–80% of the total bile acids.

The major part of the bile acids was found in the sulfate fraction (43–87% of the total), which contained predominantly mono- and disubstituted acids. The glycine conjugate fraction (4–50% of the total) contained mainly bile acids substituted at C-12, notably the 12β-hydroxy and 12-keto analogues of deoxycholic acid. Unconjugated bile acids (3–10% of the total) were predominantly trihydroxylated. The taurine conjugate fraction was usually the smallest one and contained the main part of hyocholic acid in samples where this acid was present.

Results of analyses of 24-hr urine samples from a pregnant woman and from some women with cholestatic liver disease are shown in **Tables 6** and **8**. The excretion of bile acids was increased in the patients with biliary cirrhosis and congenital intrahepatic cholestasis. Lithocholic and deoxycholic acids comprised less than 6% of the total bile acids, 60–70% of which consisted of cholic and chenodeoxycholic acids. The urinary elimination of bile acids was much increased in the patient with marked pruritis of pregnancy whereas the percentages of deoxycholic and chenodeoxycholic acids were the same as in healthy subjects.

In these patients the major part of the bile acids (71–84% of the total) was found in the monosulfate fraction. Bile acids in the taurine conjugate fraction constituted a higher percentage of the total in the cholestatic than in the healthy subjects and were usually the predominant nonsulfated bile acids. About 75% of the tetrahydroxylated bile acids were excreted in this fraction where they constituted 25–40% of the bile acids. Hyocholic and 1,3,12-trihydroxycholanoic acids were also major bile acids in the taurine conjugate fraction. The fraction of unconjugated bile acids was always the smallest one.

Bile acids in plasma. For comparative purposes the

TABLE 6. Results of four parallel determinations of bile acids in a urine sample from a pregnant woman with intrahepatic cholestasis. Values are given as μmol/24 hr ± standard deviation

Position of Substituents[a]	Unconjugated Mean ± SD[b]	Glycine Conjugated Mean ± SD	Taurine Conjugated Mean ± SD	Monosulfated Mean ± SD	Total Mean
3α,7α,12α[c]	3.14 ± 0.95	0.14 ± 0.00	0.27 ± 0.01	0.03 ± 0.00	3.58
3,6,7,12[d]	0.16 ± 0.04	1.25 ± 0.10	2.96 ± 0.10	trace	4.37
3α,7α,12α[e]		0.24 ± 0.01	0.39 ± 0.03	1.13 ± 0.15	1.76
1,3,7,12[d]	0.04 ± 0.01	0.72 ± 0.03	2.46 ± 0.09	trace	3.22
3α,7α,12α	0.26 ± 0.03	3.20 ± 0.10	4.48 ± 0.12	11.92[f] ± 1.81	19.86
3α,7β,12α[d,e]	0.08 ± 0.01	0.73 ± 0.05	0.62 ± 0.16		1.43
3α,12α[e]				0.19 ± 0.03	0.19
3β,7β,12α[d,e]				1.14 ± 0.20	1.14
1,3,12[d]		2.54 ± 0.37	4.63 ± 0.13	1.09 ± 0.18	8.26
3α,7α[e]				0.56 ± 0.08	0.56
3β,12α				1.26 ± 0.26	1.26
3α,6α,7α		0.18 ± 0.01	0.69 ± 0.04	0.68 ± 0.17	1.55
3α,7β,12α	0.03 ± 0.00	0.23 ± 0.02	0.23 ± 0.07		0.49
3α,12α	0.11 ± 0.01	0.44 ± 0.02	0.43 ± 0.03	23.08[f] ± 2.29	24.06
3α,12β		0.45 ± 0.02	0.42 ± 0.03		0.87
3α,7α		0.24 ± 0.03	0.26 ± 0.06	11.25[f] ± 1.63	11.75
3α,6β				0.23 ± 0.11	0.23
3α[e]				0.45 ± 0.11	0.45
3α,6α			0.14 ± 0.02		0.14
3β,12α-Δ[5]			0.10 ± 0.02	1.14 ± 0.12	1.24
3β,12α[e]				0.61 ± 0.08	0.61
3α				2.40 ± 0.44	2.40
3α,7β				0.40 ± 0.04	0.40
3β-Δ[5]				2.26 ± 0.48	2.26
3β-12-one				0.26 ± 0.03	0.26
3α-12-one				4.02 ± 0.70	4.02
Total	3.82 ± 1.02	10.36 ± 0.77	18.08 ± 2.07	64.10 ± 11.70	96.36

[a] In 5β-cholanoic acid unless otherwise noted. Greek letters denote configuration of hydroxyl groups. Δ[5] = 5,6 double bond.
[b] Standard deviation.
[c] In 24-norcholan-23-oic acid.
[d] Tentative, see text.
[e] In 5α-cholanoic acid.
[f] About 0.2% of these amounts were present in the di- and trisulfate fraction.

TABLE 7. Bile acid excretion in urine of healthy subjects

Position of Substituents[a]	Daily Excretion of Bile Acids (μmol 24 hr)[b]																					
	A.T. ♀ 33 y (Jan. 1974)				A.T. ♀ 34 y (Dec. 1974)				A.M. ♀ 24 y				A.A. ♀ 27 y				I.F. ♀ 20 y[c]			B.A. ♂ 30 y			
	U	G	T	MoS	U	G	T	MoS	U	G	T	MoS	U	G	T	MoS	G	T	MoS	U	G	T	MoS
3α,7α,12α[d]	0.16				0.07		tr.																
3α,7α,12α[e]		0.11		0.02		0.06																	
3α,7α,12α	0.15	0.15	tr.	0.12	0.11	0.24	0.31	0.05	0.30	0.02	0.06	0.07	0.21	0.14		0.06	0.05		0.07	0.49	0.20		0.04
3α,6β,12α[f]	tr.									0.04			0.20				0.08						
3α,12α[e]				0.05				0.03				0.03							0.03				0.04
3β,7β,12α[e,f]				0.13	0.02		0.02	0.05				0.22			0.06	0.51[g]			0.25				0.32
1,3,12[f]		0.10				0.18			0.07	0.04				0.25	0.07		0.37			0.24	0.49		
3α,7α[e]				0.08				tr.															
3β,12α				0.09						0.01	0.01	0.33				0.28			0.18				0.18
3α,6α,7α		tr.	0.03	0.03			0.10	0.04	0.09	tr.	0.02				0.01			tr.	tr.		tr.		
3β,7β,12α	0.03				0.03	0.18							0.07										
3α,7β,12α		0.03				0.04			tr.														
3α,12α		tr.		0.59	tr.		0.09	0.91	0.06	0.02	0.09	2.96	0.07	0.13	0.14	1.23[g]	0.74	0.03	0.71		0.51	0.05	1.25
3α,12β		0.38		tr.		0.26	tr.	0.03		0.17			0.09	1.15	0.01	0.08	0.49		0.05		1.73		
3α,7α				1.37		0.02	0.08	1.13				0.91				0.18			0.53				0.77
3α[e]				0.28															0.08				
3α,6α																			0.11				
3β,12α-Δ[5]				tr.				0.06															
3β,12α[e]				0.09				0.17				0.08							tr.				0.08
3α				1.77				1.23				1.49				1.05			1.56				1.14
3α,7β				0.18				0.27				0.25							0.07				0.14
3β-Δ[5]				0.46				0.42				0.28				0.10			0.08				0.75
3β-12-one																	0.10						
3α-12-one		0.70		0.35				0.50						0.29			1.02				2.56		
Total	0.34	1.47	0.03	5.61	0.23	0.98	0.60	4.89	0.52	0.30	0.18	6.62	0.64	1.96	0.29	3.49	2.85	0.03	3.72	0.73	5.49	0.05	4.71

[a] In 5β-cholanoic acid unless otherwise noted. Greek letters denote configuration of hydroxyl groups. Δ[5] = 5,6 double bond.
[b] U, G, T, MoS see footnote to first page. tr. = <0.01 μmol 24 hr.
[c] The unconjugated fraction was lost.
[d] In 24-norcholan-23-oic acid.
[e] In 5α-cholanoic acid.
[f] See Table 5 and text.
[g] About 5% of these amounts were present in the di- and trisulfate fraction.

TABLE 8. Urinary bile acid excretion in normal pregnancy and in some cholestatic liver diseases

Position of Substituents[a]	Daily Excretion of Bile Acids (μmol/24 hr)[b]																	
	I.T. 27 y. 32nd Week, Normal Pregnancy				U.Å. 25 y. 38th Week, Slight Cholestasis[c]			S.G. 28 y. Liver Disease due to Contraceptive Steroids[d]				M.H.R. 55 y. Primary Biliary Cirrhosis				S.M.K.N. 21 y. Congenital Intrahepatic Cholestasis			
	U	G	T	MoS	G	T	MoS	U	G	T	MoS	U	G	T	MoS	U	G	T	MoS
3α,7α,12α[e]	0.20							0.12				0.18				0.34			
Tetrol[f]																	0.62		
3,6,7,12[f]					0.02	0.04						tr.	0.23	0.77	tr.	0.07	1.05	1.90	
3α,7α,12α[g]				0.08							0.05		0.08		0.08		0.06	tr.	1.57
1,3,7,12[f]					0.05	0.20							0.07	0.17		0.03	tr.	0.30	
3α,7α,12α,23													0.10	0.56		tr.		0.86	
3α,7α,12α	0.29	tr.		0.30	0.07	0.25	0.96	0.32	0.09	0.08	0.18	0.45	0.91	1.05	2.65	0.80	1.44	3.02	20.98
3α,7β,12α[f,g]		0.14							0.04				0.05						
3α,12α[g]		0.18		0.21							0.21								0.06
3β,7β,12α[f,g]				2.88			0.50				0.12				0.01				
3β,7α															0.13				1.10
1,3,12[f]		0.38	0.45		0.20	0.96	0.70	0.05	0.08	tr.			0.11						
3α,7α[g]				0.13			0.34				0.25								
3β,12α				0.51							1.50				0.14				0.12
3α,6α,7α				0.11		0.13	0.18			0.04			1.91	2.99	0.28	0.02	0.48	2.08	0.05
3α,7β,12α								0.05						0.08			0.04		
3α,12α		0.09	0.11	3.95		0.17	3.21	0.08	0.06	0.05	2.46				0.82				1.89
3α,12β		0.11			0.03	0.08	0.38		0.19	0.04	0.43								
3α,7α		0.23		1.48	0.13	0.10	2.87	0.03			1.05				14.63	0.08	0.10	0.34	28.93
3α,6β											tr.		0.18		0.02				
3α[g]				0.09							0.16				0.15				3.14
Diol-Δ[f]				0.17							0.14				0.14				
3α,6α				tr.											0.44				
3β,12α-Δ[5]				0.26			tr.			tr.	0.11				0.21		0.14		
3β,12α[g]			0.32	0.37			0.43				0.72								0.08
3α				1.94			0.74				1.59				1.96				0.43
3α,7β				0.28							0.18				0.29				0.54
3β-Δ[5]				0.16			0.24				0.45				0.59				5.27
3α-12-one		0.04		0.04			2.01		0.34		1.98								
Total	0.49	1.17	0.88	12.96	0.50	1.93	12.56	0.65	0.80	0.21	11.58	0.63	3.64	5.62	22.54	1.34	3.93	8.50	64.16

[a] In 5β-cholanoic acid unless otherwise noted. Greek letters denote configuration of hydroxyl groups. Δ[5] = 5,6 double bond.
[b] U, G, T, and MoS, see footnote to first page and Table 1. tr. = <0.01 μmol/24 hr.
[c] The unconjugated fraction was lost.
[d] Mild jaundice and pruritus produced by contraceptive steroids. Sample collected after seven days of steroid administration. Serum alkaline phosphatases and alanine aminotransferases were normal and slightly increased, respectively.
[e] In 24-norcholan-23-oic acid.
[f] See Table 5 and text.
[g] In 5α-cholanoic acid.

bile acid profile in blood was analyzed in the pregnant woman with intrahepatic cholestasis, the urine analysis of whom is shown in Table 6. Plasma (2 ml) was diluted with NaCl solution (18 ml, 0.15 M) and carried through the procedure in the same way as urine except that extraction with Amberlite XAD-2 was performed at 64°C, pH 7.0. The result of the analysis is shown in **Table 9.** As expected, the bile acid concentration was higher than normal. Cholic (49% of the total) and chenodeoxycholic (20%) acids were the major bile acids, present mainly in the taurine conjugate fraction. All lithocholic acid (7% of the total) was sulfated. Tetrahydroxy bile acids were not detected. The presence of unconjugated deoxycholic acid is notable.

DISCUSSION

In healthy humans only small amounts of bile acids appear in urine and the nature of these bile acids is largely unknown. Urinary bile acid elimination may become quantitatively important in conditions associated with cholestasis but, in spite of numerous studies, knowledge about the metabolic profiles of bile acids in urine remains fragmentary (9, 11, 13, 15, 28). Evidently the bile acid mixture may be very complex both with regard to the mode of conjugation (13, 51–53) and the nature of individual acids (26, 28), and existing methods are not sufficiently developed to permit detailed studies. The aim of the present work has been to devise procedures that

TABLE 9. Bile acid concentrations, μmol/l, in plasma from a pregnant woman with intrahepatic cholestasis

Position of Substituents[a]	Unconjugated	Glycine Conjugated	Taurine Conjugated	Monosulfated	Total
3α,7α,12α	(—)[b]	1.27	2.76	0.03	4.06
3α,7β,12α[c]		0.06	0.06		0.12
1,3,12[d]			0.38	0.05	0.43
3β,12α				0.06	0.06
3α,6α,7α			tr.[e]		tr.[e]
3α,12α	0.17	0.14	0.31	0.61	1.23
3α,12β			0.07		0.07
3α,7α		0.21	0.84	0.56	1.61
3α,6β				tr.[e]	tr.[e]
3β,12α[c]				0.01	0.01
3α				0.61	0.61
3β-Δ[5]				0.06	0.06
Total	0.17	1.68	4.42	1.99	8.26

[a] In 5β-cholanoic acid unless otherwise noted. Greek letters denote configuration of hydroxyl groups. Δ[5] = 5,6 double bond.
[b] Unconjugated cholic acid could not be quantitated (^{14}C-labeled cholic acid added to the sample for recovery studies).
[c] In 5α-cholanoic acid.
[d] Tentative, see text.
[e] tr = <0.01 μmol/l.

would allow analysis of the small amounts of unconjugated, glycine and taurine conjugated, and sulfated bile acids present in urine from healthy subjects, and be sufficiently flexible to permit analysis of additional types of conjugates without the need for major modifications.

Extraction and group separation of bile acids

Amberlite XAD-2 was chosen for the extraction and preliminary purification of bile acids since it has been successfully used in a variety of applications involving similar organic compounds in urine. Previous studies have indicated satisfactory recoveries of some unconjugated, glycine and taurine conjugated, and monosulfated bile acids (5, 9, 54). The present study has demonstrated the importance of controlling flow rate and pH to obtain maximum recoveries. Unconjugated nonpolar bile acids were partly lost at higher flow rates, and trisulfated bile acids required an acidic pH for extraction and an alkaline pH for elution from Amberlight XAD-2. The latter finding probably explains the recent results of Matsui, Hakozaki and Kinuyama (55) who obtained a poor yield of steroid diconjugates. These authors decreased losses by washing the XAD-2 columns with less water. This procedure is unsatisfactory since it gives extracts containing a high percentage of urea and inorganic salts.

Separation of bile acids into groups according to mode of conjugation has been achieved by countercurrent extraction (56), partition chromatography on cellulose (57), ion exchange (58, 59) and electrophoretic (60) methods. More recently Sephadex LH-20 has been used for the separation of sodium or potassium salts of nonsulfated and sulfated bile acids (6, 9, 13, 19–21). Of these methods, counter-current extraction is unsuitable for routine analyses, and high voltage electrophoresis has insufficient capacity for crude extracts of urine. Sephadex LH-20 chromatography does not permit sufficient separation of different conjugate classes and when used on crude extracts, sometimes gives separation artifacts due to overloading, memory effects, and dependency on counterion composition.

Ion exchange chromatography offers high capacity and can give large separation factors for different conjugates. However, conventional polystyrene resins show undesirable adsorption effects, and large volumes of aqueous solutions of electrolytes and strong acids are required to elute bile acids (59). To diminish these disadvantages in separations of steroid conjugates and acidic lipids, ion exchangers based on polysaccharides have been used, e.g., DEAE-cellulose and DEAE-Sephadex (61, 62). However, the hydrophilic nature of these ion exchangers is a drawback in work with lipid soluble compounds where it is desirable to use organic solvents. For this reason less polar derivatives of such ion exchangers have been synthesized (22, 23).

The ion exchanger used in the present study, diethylaminohydroxypropyl Sephadex LH-20, was selected on the basis of good swelling properties in mixtures of organic solvents and water, high capacity, negligible adsorption effects, chemical inertness, and high rates of ion exchange in organic solvents. Column dimensions and volumes of eluting solvents can be kept small, resulting in rapid separations. The columns are readily regenerated and can be used repeatedly for several months. Since volatile buffers are used, laborious purification of the fractions is not necessary prior to analysis. Preliminary experiments indicate that it may be possible to perform the separations about 10 times faster if the buffer compositions are modified.

A major reason for the extensive purification achieved with the procedure is the two-stage selectivity afforded by the use of DEAP-LH-20, first to separate the conjugates and then to purify the deconjugated bile acids as a group. The second step is very rapid since only one fraction has to be collected. Using aqueous ethanol as solvent there is no separation of bile acids due to differences in the steroid skeleton. It may be a disadvantage that glycine and taurine conjugated monosulfates are not separated from each other. However, this separation may be

achieved by chromatography of the sodium salts on Sephadex LH-20.

A possible source of error in the DEAP-LH-20 chromatography is the elution of bile acids in the neutral fraction. To avoid this loss it is necessary to pass the extract of urine through an Amberlyst A-15 column in the H^+ form prior to the separation on DEAP-LH-20. The cation exchanger probably removes unknown cations forming neutral ion pairs with the bile acids and competing with the cationic groups on the gel.

In this study only conventional bile acid conjugates have been analyzed. Conjugates with arginine and ornithine have been described (52, 53, 63) and would be expected to elute ahead of glycine conjugates (59) or possibly be taken up by Amberlyst A-15. The occurrence of bile acid glucuronides in urine has been proposed (51) and is supported by a recent study (64), and such conjugates might be eluted with monosulfates. Glucuronides of neutral steroids are eluted in the glycine and taurine conjugate fractions (65). If required, it should be possible to modify the present method to include these and other as yet unknown conjugates.

As is the case in many other methods for bile acid analysis, solvolytic and hydrolytic steps are responsible for the main losses in the procedure. Our results indicate that solvolysis in ethyl acetate–ethanol (38) is superior to solvolysis in acetone–ethanol (2) and does not produce artifacts (except for esterifications). Although the alkaline hydrolysis does not yield unsaturated or oxidized products or epimers from the common hydroxycholanoates, it is not known to what extent these vigorous conditions affect, for example, the tetrahydroxycholanoates. However, there is no evidence supporting formation of significant amounts of artifactual bile acids. The selective distribution of many bile acid isomers between the conjugate fractions would be difficult to explain on the basis of artifact formation.

Gas–liquid chromatography–mass spectrometry

Due to the complexity of the bile acid mixture in urine it was usually necessary to analyze the samples on two types of columns. The combined use of SE-30 and Hi-Eff 8 BP columns allowed the identification of compounds eluted as mixtures and of isomeric bile acids. The SE-30 column facilitated detection of ketonic bile acid derivatives that have long retention times on Hi-Eff 8 BP columns. The GLC–MS analyses revealed that peaks with retention times identical to those of bile acid derivatives were sometimes due to other compounds. This was particularly true for samples containing small amounts of bile acids. The GLC–MS analyses also showed that several peaks were due to mixtures of bile acid derivatives.

Since authentic samples of sufficient purity were not available for many of the bile acids found in urine, quantitations had to be made by comparison of calculated TIC peak areas of mono-, 3,7-di-, 3,12-di-, and tri-(tetra-)substituted cholanoates with TIC peak areas given by the derivatives of lithocholic, chenodeoxycholic, deoxycholic, and cholic acids, respectively. Thus, differences in TIC response between bile acids within these groups were not corrected for. However, the error introduced by this procedure had to be accepted and it may be noted that the ratio between TIC responses of the cholic and lithocholic acid derivatives was usually between 1.0 and 1.30, depending mainly on the condition of the column. When samples containing elevated bile acid levels were analyzed by GLC using a flame ionization detector and by GLC–MS, the results were essentially the same for the major compounds.

Bile acids in urine

The daily excretion of bile acids in healthy humans was between 6.4 and 11.0 μmol in the five subjects studied. These values are ten times higher than those found by Makino et al. (10) probably reflecting differences in methodology. The bile acid composition was very different from that reported for bile and blood (for ref. see 10, 66) with respect to both conjugation and steroid structure.

All the monosubstituted and by far the predominant part of the disubstituted bile acids were found in the monosulfate fraction, whereas the trihydroxycholanoates were mainly nonsulfated. The percentage of sulfated bile acids in urine from healthy subjects was much higher than previously reported (13) and similar to that in urine from patients with liver disease (6, 10, 12, 13, 15). However, a greater proportion of cholic acid was sulfated in the samples from patients with marked cholestasis. The reported excretion of disulfates in urine from cholestatic patients (13) could not be confirmed. This might be due to differences between patient groups or analytical procedures. The previous study employed Sephadex LH-20 to separate bile acid sulfates and, since this method is sensitive to the composition of cations in the sample, separation artifacts may be encountered.

The sulfate group was at C-3 in all the major bile acids studied, and chromatographic mobilities indicated that the sulfated bile acids were conjugated with glycine or taurine. No evidence was obtained for sulfation at C-7 as described for cholic acid in the large intestine of mice (67). The extent of sulfation appeared to depend both on the number of hydroxyl

groups in the steroid skeleton (6, 12, 15) and on specific structural features of the bile acid. Thus, deoxycholic acid was almost completely sulfated whereas the 12β epimer was sulfated only to a limited extent. One of the trihydroxycholanoates (tentatively identified as 3β,7β,12α-trihydroxy-5α-cholanoic acid) was found almost exclusively in the sulfate fraction whereas the 1-hydroxylated trihydroxycholanoic acid was essentially nonsulfated. This specificity is less likely explained by differences in renal excretion, but the possibility of renal metabolism of bile acids may be considered. Sulfation may occur in a number of organs and it is possible that the bile acid sulfates in urine are of both hepatic and renal origin. In agreement with previous reports (10, 13, 15) the percentage of sulfated bile acids was much lower in blood than in urine. This has been interpreted as being due to a higher renal clearance of sulfated than of nonsulfated bile acids (10, 13, 15).

The ratio between nonsulfated glycine and taurine conjugated bile acids was higher than reported for bile in most healthy subjects. In the cholestatic patients, however, taurine conjugates were the major nonsulfated bile acids. The renal clearance rates for glyco- and taurocholate are similar in the dog (68), and the ratios between these compounds were about the same in blood and urine of the patient studied. However, a similar relationship was not observed for many other glycine and taurine conjugated bile acids, indicating selectivity in the renal elimination of such conjugates.

A major part of the trihydroxy bile acids in urine from healthy subjects was unconjugated. The presence of unconjugated cholic acid in patients with liver disease (57) could also be confirmed. However, the relative amount of these acids was much lower in the patients than in the healthy subjects. The clearance of cholate from plasma is slower than that of its conjugates (69) and this could be compatible with a relatively high proportion of unconjugated cholate in blood (70) and urine, possibly resulting from hydrolysis of the conjugates in the intestine.

Disregarding the nature of conjugation, the urinary bile acids may be subdivided into groups according to type of substitution of the steroid skeleton or modification of the side chain. One group might arise by oxidoreductions yielding 3β,7β and 12β epimers from the common biliary bile acids. Formation of 3β epimers is a major process in the intestinal tract (39) and 7β epimers (71) are possibly formed by bacterial oxidation of 7α-hydroxycholanoates followed by hepatic reduction of the 7-keto intermediate (see 72, 73). Ursodeoxycholic acid has been found as a sulfate in plasma from pregnant women with intrahepatic cholestasis (21) and its presence in urine is therefore to be expected. This acid has also been identified in meconium, where its origin is unknown (74). Oxidation of deoxycholic acid at C-12 is an important reaction in the intestinal tract but only small amounts of the 12β epimer of deoxycholic acid are present in feces (39). In contrast, the relative amount of this epimer was high in urine. The major part was present in the glycine conjugate fraction where the corresponding 12-keto acid was also found. In the patient studied, the ratio between nonsulfated deoxycholic acid and its 12β epimer was much higher in blood than in urine. Deoxycholic acid occurred in urine mainly in the sulfate fraction where the amount of 12β epimer was negligible. These findings indicate that oxidoreduction at C-12 of glycine conjugated bile acids takes place in the kidney. If this is not the case, sulfation has to be markedly stereospecific for the 12α epimer.

Two bile acids in urine possessed a 3β-hydroxy-Δ^5 structure. The major one, 3β-hydroxy-5-cholenoic acid, was first found in urine from children with biliary atresia (3) and adults with liver disease (11). It has also been found in the sulfate fraction from bile (30, 75) and meconium (74). The present results show that it is one of the major bile acids besides cholic, chenodeoxycholic, deoxycholic, and lithocholic acids in urine from healthy humans. It may be an intermediate in a minor pathway for bile acid biosynthesis starting with degradation of the sterol side chain (76, 77). Neutral C_{19} and C_{21} steroids with a 3β-hydroxy-Δ^5 structure are typically sulfated (see 78), and it may be speculated that under normal conditions any 3β-hydroxy-5-cholenoic acid formed in the liver is sulfated and excreted.

The origin of 3β,12α-hydroxy-5-cholenoic acid is unknown. In rat liver, bile acids and sterols with a planar A/B ring fusion may be 12α-hydroxylated (79) and if this is the case also in the human liver, 3β-hydroxy-5-cholenoic acid might be the precursor.

A third group of bile acids had a 5α configuration. Such bile acids may be either secondary, formed from normal bile acids by intestinal bacteria (80), or primary, formed from cholestanol (81). Since allocholic acid was present in the samples from patients with marked cholestasis and low excretion of lithocholic and deoxycholic acids, this acid may be formed without involvement of bacterial metabolism. However, the presence of allodeoxycholic acid and its 3β epimer in essentially all samples from healthy subjects and their absence or low relative amounts in samples from patients with cholestasis indicate that these acids are of bacterial origin. Bacterial epimerization of the 3α-hydroxy group in 5α-steroids, leading to the more stable equatorial configuration, is also a common reaction (82). Allolithocholic acid

was present in largest amounts in the two patients with highest excretion of 3β-hydroxy-5-cholenoic acid. This correlates well with the previous finding of allolithocholic acid in children with biliary atresia who excrete little or no lithocholic acid (3), and indicates a possible precursor role of 3β-hydroxy-5-cholenoic acid.

With the exception of allocholic acid, the allo bile acids were present almost exclusively in the sulfate fraction. This may be a reflection of the selective sulfation of steroids with a 3α,5α configuration, also observed for neutral steroids in the C_{19} and C_{21} series (see 82).

Only two bile acids with a modified side chain were found. Norcholic acid was a major unconjugated bile acid that correlates well with the early observation that it is a poor substrate for the conjugating enzymes in rat liver (83). Theoretically, norcholic acid could be formed by β-oxidation of a side chain differing from that of cholesterol by one carbon atom (83). However, two of the patient samples contained 23-hydroxycholic acid, which could conceivably represent the precursor. Norcholic acid was not found in blood from the patient with pruritus of pregnancy, in whom it was the major unconjugated bile acid in urine. This may indicate a high renal clearance of this acid.

A fifth group of bile acids may be classified as hydroxylation products of common bile acids. Two positions were hydroxylated: C-1 and C-6. The major 6-hydroxylated bile acid was hyocholic acid which has also been found by van Berge Henegouwen (15) and Summerfield, Billing, and Shackleton (28) in urine from patients with liver disease. Our study has shown that hyocholic acid is excreted by healthy humans although the elimination increases in patients with cholestasis. Only trace amounts of hyocholic acid were found in blood from the pregnant woman with cholestasis, where it was one of the major taurine conjugated bile acids in urine. Generally, in the cholestatic patients, the main part of hyocholic acid was present in the taurine conjugate fraction, and it is possible that the taurine conjugate of chenodeoxycholic acid (particularly predominant in these subjects) served as the preferred substrate for a 6α-hydroxylase. This reaction would be analogous to the 6α-hydroxylation of taurolithocholate demonstrated with human liver microsomes (84). It may also be noted that taurine conjugated bile acids are the substrates in 6β- and 7α-hydroxylations in rat liver (see 85). Hyodeoxycholic acid was present in some urine samples, particularly in those from cholestatic subjects. In analogy with the other dihydroxy bile acids, it was usually found in the sulfate fraction.

Bile acids hydroxylated in the 6β position were also detected. The 6β epimer of hyodeoxycholic acid was found in three samples from cholestatic patients. A bile acid present in healthy subjects was characterized as 3α,6β,12α-trihydroxy-5β-cholanoic acid based on identity of retention times and mass spectrum with those of the reference compound. However, there are at least four biologically important asymmetric carbon atoms and only one isomer was available as reference compound. The identification must therefore be regarded as tentative. The presence of this compound in urine from cholestatic patients could not be established with certainty.

In the rat, 6β-hydroxylated bile acids are predominant metabolites of chenodeoxycholic acid. Such a metabolite, 3α,6β,7β-trihydroxy-5β-cholanoic (β-muricholic) acid, was detected only in one urine sample (from a pregnant woman with intrahepatic cholestasis). Thus, it appears that 6α-hydroxylation of bile acids is a more common reaction in humans than 6β-hydroxylation.

Whereas the excretion of 1β-hydroxylated cortisol metabolites has been known for a long time (86–88), the presence of 1-hydroxylated bile acids has not been reported previously. The 1,3-diol structure is firmly supported by the mass spectra of the TMS derivatives. Furthermore, in ongoing studies aiming at the synthesis of these compounds by microbial 1β-hydroxylation, a compound has been obtained that has a mass spectrum and retention time identical with those of the methyl ester TMS ether of the compound tentatively characterized as 1,3,12-trihydroxycholanoic acid[3]. This bile acid was one of the major trihydroxycholanoates in urine from healthy subjects, occurring almost exclusively in the glycine conjugate fraction. In the pregnant women with intrahepatic cholestasis it was also one of the major trihydroxycholanoates, present mainly in the taurine conjugate fraction. Only small amounts of this bile acid were found in the blood from one of these women. The two patients with low output of deoxycholic acid in urine excreted only minute amounts of 1,3,12-trihydroxycholanoic acid. Taken together, these findings might indicate a renal 1-hydroxylation of bile acids similar to what has been shown for the D vitamins (see 89). However, fetal human liver microsomes (90) and guinea pig liver slices (86) have also been shown to be capable of 1β-hydroxylation of steroids.

In addition to the 1-hydroxylated trihydroxycholanoate, a tetrahydroxy bile acid having a 1,3-diol structure was found in samples from patients

[3] Carlström and Sjövall, unpublished results.

with marked cholestasis. Tetrahydroxy bile acids have not been found previously in man, but the present study indicates that several acids of this type may be formed in patients with liver disease and be excreted in urine mainly as taurine conjugates. None of these acids were detected in blood from the patient with cholestasis of pregnancy.

The physiological or clinical significance of the complexity of bile acids in urine is not clear. The bile acid metabolite profile in urine is the net result of metabolic reactions and transport processes in the liver, intestinal tract, and kidney. Differences between bile acids in rates of chemical transformations, uptake into and release from cells, and plasma protein binding will result in a bile acid mixture in urine different from that in bile and blood. It is possible that the bile acids in urine of healthy subjects reflect formation of minor products in the liver and during the enterohepatic circulation, similar to what is seen for porfyrins and bile pigments. In cholestatic conditions, the pattern of bile acids in urine approaches that in bile (with the exception of sulfation). As expected, secondary bile acids decrease or disappear and monohydroxy bile acids become relatively minor components in these patients. Studies of larger groups of patients are required to determine whether specific changes occur in different diseases and to assess the clinical value of analyses of metabolic profiles of bile acids in urine.

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