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Identification of Bile Acids in the Serum and Urine in Cholestasis

EVIDENCE FOR 6α-HYDROXYLATION OF BILE ACIDS IN MAN

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In this qualitative study of the pattern of bile acid excretion in cholestasis, methods are described for the isolation of bile acids from large volumes of urine and plasma. The bile acids were subjected to a group separation and identified by combined gas chromatography–mass spectrometry. The techniques were developed to allow identification of the minor components of the bile acid mixture. Four bile acids that have not previously been described in human urine and plasma were detected, namely 3β,7α-dihydroxy-5β-cholan-24-oic acid, 3α,6α-dihydroxy-5β-cholan-24-oic acid (hyodeoxycholic acid), 3α,6α,7α-trihydroxy-5β-cholan-24-oic acid (hyocholic acid) and 3α,7β,12α-trihydroxy-5β-cholan-24-oic acid. In addition three C_{27} steroids were found; 26-hydroxycholesterol and a trihydroxy cholestane, probably 5β-cholestane-3α,7α,26-triol were found in the sulphate fraction of plasma and urine. In the plasma sample, a sulphate conjugate of 24-hydroxycholesterol was found. The presence of these compounds probably reflects the existence of further pathways for bile acid metabolism. It is not yet known whether this is a consequence of the cholestasis or whether they are also present in normal man, at much lower concentrations.

Patients with cholestatic liver disease have an interrupted enterohepatic circulation of bile acids. These compounds accumulate in the systemic circulation and are excreted in the urine. The normal biliary bile acids, cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid), chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid), deoxycholic acid (3α,12α-dihydroxy-5β-cholan-24-oic acid) and lithocholic acid (3α-hydroxy-5β-cholan-24-oic acid) have have been found in the urine and plasma (Carey & Williams, 1965; Sandberg *et al.*, 1965; Gregg, 1967; Norman & Strandvik, 1971; Makino *et al.*, 1971). In addition, ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholan-24-oic acid) and 3β-hydroxychol-5-en-24-oic acid have been identified in both urine and plasma (Makino *et al.*, 1971; Back *et al.*, 1972; Back, 1973), epilithocholic acid (3α-hydroxy-5α-cholan-24-oic acid) in urine (Makino *et al.*, 1971) and another unsaturated monohydroxy bile acid in plasma (Murphy *et al.*, 1972). Van Berge Henegouwen (1974) has identified in urine, by using g.l.c. with different stationary phases, peaks with the retention times of hyocholic acid (3α,6α,7α-trihydroxy-5β-cholan-24-oic acid) and a dihydroxy-3-oxo bile acid.

Preliminary g.l.c. studies in eight patients have confirmed that the pattern of bile acid excretion in urine, plasma and bile in cholestasis is extremely complex. Combined g.l.c.–mass spectrometry (g.l.c.–m.s.) showed that many of the peaks contained more than one compound. This paper reports experiments performed to examine the bile acid pattern in greater detail. Bile acids were extracted from large volumes of urine and plasma from two patients with primary biliary cirrhosis, subjected to a group separation and identified by g.l.c.–m.s. Four bile acids that have not been described in human urine and plasma were detected, 3β,7α-dihydroxy-5β-cholan-24-oic acid, 3α,6α-dihydroxy-5β-cholan-24-oic acid (hyodeoxycholic acid), 3α,6α,7α-trihydroxy-5β-cholan-24-oic acid (hyocholic acid) and 3α,7β,12α-trihydroxy-5β-cholan-24-oic acid. In addition, 26-hydroxycholesterol and a trihydroxycholestane, probably 5β-cholestane-3α,7α,26-triol, were found in the sulphate fraction of plasma and urine. In the plasma sample, a sulphate conjugate of 24-hydroxycholesterol was found. These results support the hypothesis that there is an alternate pathway for bile acid metabolism in man, similar to that described in rats (Mitropoulos & Myant, 1967; Anderson *et al.*, 1972).

Materials and Methods

Source of material

Samples were obtained from two female patients with primary biliary cirrhosis and a severe cholestasis.

A 24h urine collection from one patient was frozen at −20°C until analysed. Plasma was collected in oxalate from the second patient [total bile acids in serum of 350 μmol/litre as measured by the method of Murphy *et al.* (1970)] undergoing plasmaphoresis for a xanthomatous neuropathy, and stored at −20°C.

Reagents

All reagents, except cyclohexane and thioglycollic acid, were of analytical grade. Pyridine was refluxed and redistilled over solid KOH and stored over solid KOH. Deionized distilled water was used throughout. Cholic acid, deoxycholic acid, chenodeoxycholic acid, lithocholic acid, sodium taurolithocholate, sodium glycolithocholate and 3β-hydroxychol-5-en-24-oic acid were purchased from Weddell Pharmaceuticals, London Wall, London EC2M 5XD, U.K. Hyodeoxycholic acid was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Hyocholic acid, isolithocholic acid, 3β,7α-dihydroxy-5β-cholan-24-oic acid, 3α,7β,-12α-trihydroxy-5β-cholan-24-oic acid and allocholic acid were gifts from Professor G. A. D. Haslewood and Dr. I. G. Anderson (Guy's Hospital Medical School, London SE1 9RT, U.K.) and Professor W. Klyne (Westfield College, Kidderpore Avenue, London N.W.3, U.K.). The ursodeoxycholic acid was a gift from Dr. P. Back (Medizinische Klinik der Universität Freiburg/Brsg., West Germany). The sample of 26-hydroxycholesterol was a gift from Dr. K. A. Mitropoulos (M.R.C. Lipid Metabolism Unit, Hammersmith Hospital, Ducane Road, London W.12, U.K.). The purity of the standards was checked by g.l.c. and g.l.c.–m.s. Sulphate salts of lithocholic acid, glycolithocholic acid and taurolithocholic acid were prepared by the method of Palmer & Bolt (1971) and purified by repeated crystallization and preparative t.l.c. Clostridial cholylglycine hydrolase solution was prepared by grinding 200mg of *Clostridium welchii* acetone-treated powder (strain A.T.C.C. 19574, type III, Sigma Chemical Co.) with 20ml of phosphate buffer (66mM) (pH 7.0) containing thioglycollic acid (5mM) at 4°C and centrifuging at 30000*g* for 40min. The enzyme solution was prepared freshly for each experiment.

Preliminary experiments were performed by using Amberlite XAD-2 [Rohm and Haas (U.K.) Ltd., Croydon CR9 3NB, Surrey, U.K.] by the methods of Bradlow (1968), Shackleton *et al.* (1970) and Makino & Sjövall (1972). Despite careful cleaning of this styrene divinylbenzene polymer, 'blank' experiments revealed considerable contamination of the baseline on g.l.c. when 1% Hi Eff 8BP on Gas Chrom Q (Applied Science Laboratories, State College, Pa., U.S.A.) was used. Amberlite XAD-7 [Rohm and Haas (U.K.) Ltd.], after being washed with water then methanol then water again, showed virtually no contamination on g.l.c. with this phase and was therefore used in all subsequent experiments.

Methods

Large-scale separation of bile acids from the urine and plasma samples were performed to decrease the relative contribution of background contamination and to facilitate the identification of minor components by g.l.c.–m.s.

Liquid/solid extraction. A slurry of 70g of Amberlite XAD-7 was poured into an open vertical glass column (25cm × 1cm) with a Teflon tap and 'back-washed' to classify the beads by size. The column was then left to settle. In contrast with Amberlite XAD-2, optimum binding of bile acids only occurred above pH 10. The bile acids were eluted with methanol. Recovery experiments with this resin, on aqueous solutions of bile acids, gave results for free bile acids of 94%, for glycine-conjugated bile acids of 98%, for taurine-conjugated bile acids of 87% and for free and conjugated lithocholate sulphates of 87%. Similar recovery was obtained for bile acids added to urine (92%) and plasma (82%).

Samples (300ml) of plasma were each diluted to 1250ml with water and adjusted to pH 10 with 1M-NaOH. The urine sample (1650ml) was adjusted to pH 10 with 1M-NaOH, and the flocculent precipitate removed by filtration. The samples were percolated through the columns of Amberlite XAD-7 (7.5ml/min) and washed with water at pH 10. The resin was eluted with 100ml of methanol which was taken to dryness under reduced pressure at 60°C.

Solvolysis (Burstein & Lieberman, 1958; Palmer & Bolt, 1971). The gummy brown residue from the methanol eluate of the resin was dissolved in 5ml of ethanol and 2.5ml of 2M-HCl with agitation in an ultrasonic bath, and 45ml of acetone was added. The mixture was heated for 2 days at 37°C and then taken to dryness under reduced pressure. This residue was dissolved in 15ml of methanolic KOH (5g/100ml) and refluced for 1.5h to cleave any ethyl esters formed during solvolysis. The solution was desalted on a column (30ml) of the cation-exchange resin AG 50 W (X8; 50–100 mesh; H^+ form) (Bio-Rad Laboratories, Richmond, Calif., U.S.A.), and taken to dryness under reduced pressure at 60°C. Since the samples were often acidic at this stage, a few drops of NH_3 solution (sp.gr. 0.88) were added and the excess of NH_3 was blown off under a stream of N_2. Solvolysis appeared to be complete and free from artifact formation when standards of sulphated free and conjugated lithocholate were used, since after deconjugation, methylation and silylation, one peak with the retention time of authentic methyl lithocholate trimethylsilyl ether was obtained on g.l.c. Further, as judged by g.l.c., there was no evidence of the formation of artifacts when conjugates of

cholic acid, chenodeoxycholic acid, deoxycholic acid and lithocholic acid were submitted to solvolysis and deconjugation.

Deconjugation (*Nair*, 1969). The products of solvolysis were dissolved in 2ml of methanol, 40ml of 66mM-phosphate buffer, pH 5.6, and 7ml of clostridial cholylglycine hydrolase solution and incubated at 37°C for 1h. The reaction was terminated by the addition of 2ml of 6M-HCl and the free bile acids were extracted with 3×10ml portions of ethyl acetate, dried over anhydrous Na_2SO_4 and taken to dryness under a stream of N_2.

*Group separation on Sephadex LH*20. Preliminary g.l.c.–m.s. analysis of the whole samples revealed a complex bile acid mixture. A group separation was undertaken, to simplify the non-homogeneous peaks and to purify the sample further. By separating the major components, cholate and chenodeoxycholate, larger amounts of the minor components could be injected into the g.l.c.–m.s. instrument.

The free bile acid extract was methylated with either freshly prepared ethereal diazomethane for 15min or overnight in methanol/acetyl chloride (19:1, v/v). It was then dissolved in 1.5ml of cyclohexane/ethanol (19:1, v/v), with agitation in an ultrasonic bath, and applied to an 8g column of Sephadex LH20, equilibrated with this solvent, in open vertical glass columns (25cm×1cm internal diam.) with Teflon taps. The column was eluted with cyclohexane/ethanol (19:1, v/v) and fractions were collected as follows: monohydroxy bile acid fraction +cholesterol (0–25ml), dihydroxy bile acid fraction (36–65ml), trihydroxy bile acid fraction (76–175ml). Most of the pigments remained at the origin. The fractions were taken to dryness under a stream of N_2 and the separations were checked by t.l.c. in a solvent system of chloroform/acetone/methanol (70:25:5, by vol.) (S VII from Hofmann, 1964). Some trihydroxy and dihydroxy bile acids appeared in the wrong fractions so that the group separation was not complete (See Fig. 1.) The fractions were submitted to g.l.c. and g.l.c.–m.s.

G.l.c. The *O*-trimethylsilyl ethers were prepared by the method of Makita & Wells (1963). Chromatography was performed on glass columns (2.7m×2mm internal diam.) packed with 1% Hi Eff 8BP on Gas Chrom Q (100–120 mesh) in a Pye 104 Series, model 64 gas chromatograph fitted with a heated flame-ionization detector. The conditions were: N_2 carrier flow rate, 20ml/min; H_2 flow rate, 20ml/min; column temperature, 235°C; detector oven and injector oven temperature, 250°C. The retention times of the bile acids, relative to methyl cholate trimethylsilyl ether were calculated from these analyses.

G.l.c.–m.s. was carried out on a Varian-Mat 731 instrument. The chromatographic conditions were similar to those used for gas chromatography. The *m/e* scales of the illustrated spectra differ owing to different paper speeds of the XY plotter (Fig. 3). Impurities eluted from the Sephadex LH20 and silica gel 60 may account for some of the minor peaks in the mass spectra.

Procedure for identification of C_{27} steroids. Preliminary experience with g.l.c.–m.s. revealed that the samples contained C_{27} steroids as well as bile acids. Preparative t.l.c. was used to isolate sufficient amounts of these C_{27} steroids for further analysis. Silica-gel plates (0.25mm DC-Fertigplatten Kieselgel 60, Merck, Darmstadt, W. Germany) were first cleaned by continuous downward elution with methanol for 18h, dried and stored in a desiccator. The dihydroxy and trihydroxy bile acid fractions from three plasma extracts subjected to the group separation were applied to the plates and run in a solvent system of chloroform/acetone/methanol (14:5:1, by vol.) (S VII from Hofmann, 1964). The unknown compounds (A and B) in the dihydroxy fraction had R_{MeC} values (relative to methyl cholate) of 5.30 and 4.75 respectively. The unknown compound (C) from the trihydroxy fraction had an R_{MeC} value of 1.44. These compounds were detected by spraying the plates with a methanolic solution of I_2 (3.5g/100ml), which was then allowed to sublime. The silica gel containing the compounds was scraped off the plates and eluted three times with methanol and ultrasonic agitation. About 1mg of each compound was obtained. N.m.r. spectrometry was kindly performed by Dr. D. N. Kirk and Dr. R. E. Morgan (Westfield College, Kidderpore Avenue, London N.W.3., U.K.).

Results

Fig. 1 shows the g.l.c. analysis of the serum sample after the group separation and demonstrates the ability of this technique to simplify the pattern.

The bile acids identified by g.l.c.–m.s. in these patients are shown in Table 1 with their retention times relative to methyl cholate trimethylsilyl ether (RRT_c). The predominant compounds were cholate and chenodeoxycholate; in addition, deoxycholate, ursodeoxycholate, lithocholate and 3β-hydroxychol-5-en-24-oate were found. A peak with the retention time of allo- or iso-lithocholate and a mass spectrum of a monohydroxy bile acid was present but was not further identified. Apart from lithocholate and epilithocholate, which were only found in the urine specimen, the remainder of the bile acids were present in both urine and plasma.

Four more bile acids were identified in these samples. Spectra were obtained for several other bile acids but these were not fully identified mainly because of the lack of appropriate reference compounds.

3β,7α-Dihydroxy-5β-cholan-24-oate

This compound was only found in the urine specimen and was eluted in the 'trihydroxy' fraction

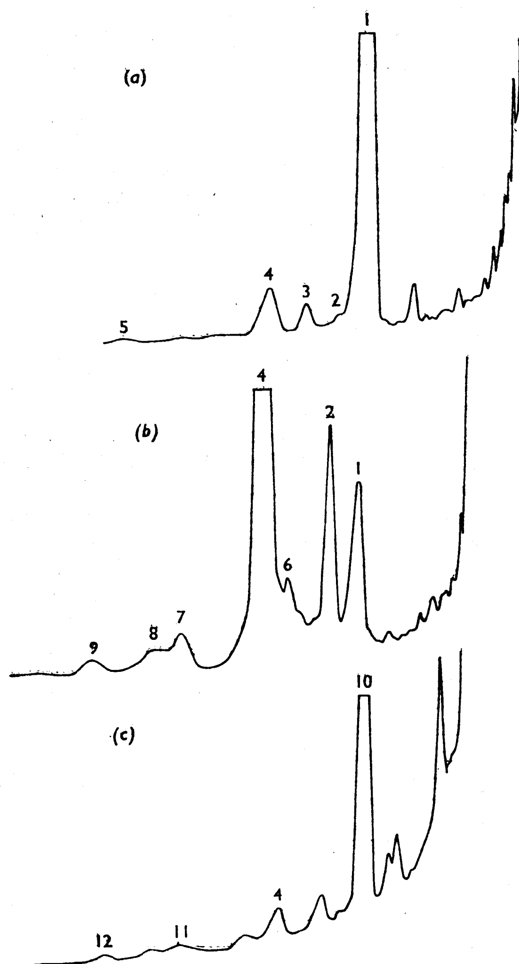

Fig. 1. *Gas-chromatographic separation of plasma bile acid methyl esters and C_{27} steroids as trimethylsilyl ether derivatives after Sephadex LH20 fractionation yielding monohydroxy* (c), *dihydroxy* (b) *and trihydroxy* (a) *fractions*

The group separation was not complete. Some dihydroxy and trihydroxy bile acids appeared in the wrong fractions. The following compounds are indicated: 1, cholate; 2, unidentified trihydroxy bile acid; 3, hyocholate; 4, chenodeoxycholate; 5, ursodeoxycholate; 6, 3α,7β,12α-trihydroxy-5β-cholan-24-oate; 7, 26-hydroxycholesterol; 8 and 9, unidentified dihydroxy bile acids; 10, cholesterol; 11, lithocholate; 12, 3β-hydroxychol-5-en-24-oate. This plasma was collected from a patient with primary biliary cirrhosis.

of the group separation. The trimethylsilyl ether derivative had the same RRT_c (1.25) and mass spectrum as the authentic compound. The spectrum (Fig. 2) of this derivative showed the base peak at m/e 370 ($M-2\times90$) and peaks at m/e 460 ($M-90$) and m/e 255 (ABCD ring). The peaks at m/e 243, 249 and 262 are characteristic of a 3,7-bis(trimethylsiloxy) structure in a dihydroxy bile acid (Sjövall *et al.*, 1971).

3α,6α,7α-Trihydroxy-5β-cholan-24-oate (hyocholate)

Hyocholate was present in both the urine and plasma specimens and was eluted in the 'trihydroxy' fraction in the group separation. The trimethylsilyl ether derivatives had the RRT_c (1.40) and mass spectrum of authentic hyocholate. The spectrum of the derivative (Fig. 3) showed a peak at m/e 548 ($M-90$) and the base peak at m/e 458 ($M-2\times90$). The peak at m/e 253 represents the ABCD ring and the prominent peak at m/e 369 the presence of vicinal trimethylsiloxy groups [$M-(2\times90+89)$] (Sjövall *et al.*, 1971).

3α,7β,12α-Trihydroxy-5β-cholan-24-oate

This compound was also present in the urine and plasma specimens, and was found in both the 'dihydroxy' and 'trihydroxy' fractions in the group separation. The trimethylsilyl ether derivative had the RRT_c (1.53) and mass spectrum of the authentic compound. The spectrum (Fig. 4) shows peaks at m/e 623 ($M-15$), m/e 548 ($M-90$), m/e 458 ($M-2\times90$), m/e 368 ($M-3\times90$) with the base peak at m/e 253 (ABCD ring). The peak at m/e 261 is diagnostically important for C_{24} trihydroxy bile acids. The peak at m/e 208 is consistent with the presence of a 12-hydroxy group and that at m/e 243 indicates a 3,7-bis(trimethylsiloxy) structure. The prominent peak at m/e 433 [$M-(90+115)$] suggests a 7β-trimethylsiloxy group (Sjövall *et al.*, 1971).

3α,6α-Dihydroxy-5β-cholan-24-oate (hyodeoxycholate)

This compound was only found in the urine specimen and was eluted in the 'trihydroxy' fraction in the group separation. The trimethylsilyl ether derivative had the RRT_c (2.00) and mass spectrum of authentic hyodeoxycholate. The spectrum (Fig. 5) showed peaks at m/e 460 ($M-90$), the base peak at m/e 370 ($M-2\times90$), m/e 345 [$M-(90+115)$] and m/e 255 (ABCD ring). Although this spectrum shows a peak at m/e 262 the expected peak at m/e 249 is missing.

Unidentified dihydroxy bile acids

The peak on g.l.c. with the retention time of methyl lithocholate trimethylsilyl ether (RRT_c 2.30) in both the urine and plasma was deformed and was

Table 1. *Bile acids and C_{27} steroids found in urine and serum specimens*

Retention times are relative to methyl cholate trimethylsilyl ether. A, B and C refer to the C_{27} steroids obtained by preparative t.l.c. of the plasma extract.

Relative retention time	Urine	Serum	Proper name	Trivial name	Unidentified bile acids
1	+	+	3α,7α,12α-Trihydroxy-5β-cholan-24-oate	Cholate	
1.20	+	+			Trihydroxy
1.25	+	−	3β,7α-Dihydroxy-5β-cholan-24-oate		
1.40	+	+	3α,6α,7α-Trihydroxy-5β-cholan-24-oate	Hyocholate	
1.51	+	+	3α,12α-Dihydroxy-5β-cholan-24-oate	Deoxycholate	
1.53	+	+	3α,7β,12α-Trihydroxy-5β-cholan-24-oate		
1.59		+	24-Hydroxycholesterol	(A)	
1.64	+	+	3α,7α-Dihydroxy-5β-cholan-24-oate	Chenodeoxycholate	
1.77	+	−	3α,Hydroxy-5α-cholan-24-oate or 3β-hydroxy-5β-cholan-24-oate	Epilithocholate or Isolithocholate	
2.00	+	−	3α,6α-Dihydroxy-5β-cholan-24-oate	Hyodeoxycholate	
2.15	+	+		26-Hydroxylated cholestanetriol (C)	
2.19	+	+	26-Hydroxycholesterol	(B)	
2.30	+	−	3α-Hydroxy-5β-cholan-24-oate	Lithocholate	
2.37	+	+			Dihydroxy
2.56	+	+	3α,7β-Dihydroxy-5β-cholan-24-oate	Ursodeoxycholate	
2.73	+	+			Dihydroxy
2.83	+	+	3β-Hydroxychol-5-en-24-oate		
2.86	+	−			Trihydroxy

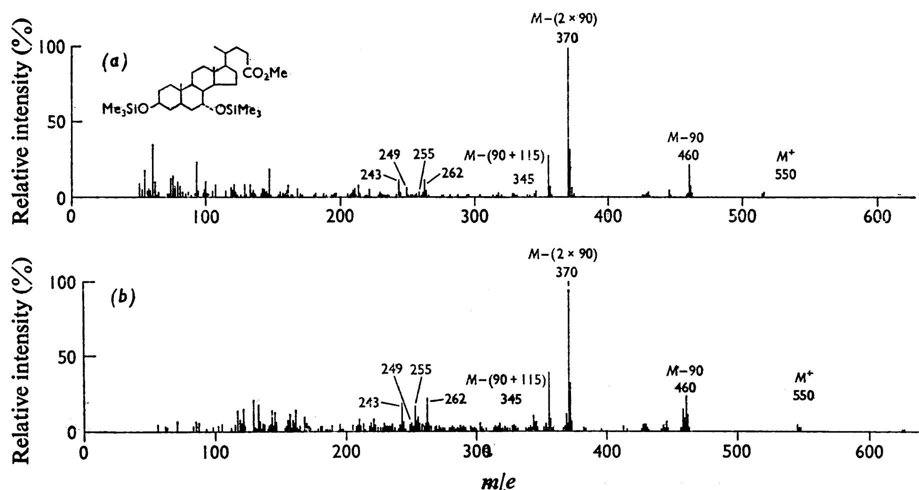

Fig. 2. *Mass spectra of the trimethylsilyl ether derivatives of methyl* 3β,7α-dihydroxy-5β-cholan-24-oate (a) *and the urine compound* (b)

found on g.l.c.–m.s. to contain at least three components in addition to lithocholate. One appeared to be a dihydroxy bile acid (RRT_c 2.37), the other two were C_{27} steroids (see below). In the urine specimen these compounds accounted for about 30% of the lithocholate peak and in the plasma at least 80% of the peak. The compound with RRT_c 2.37 showed a base peak at m/e 255 (ABCD ring) and very prominent peaks at m/e 213 (ABC), m/e 355 [$M-(2\times90+15)$] and m/e 370 ($M-2\times90$). Other peaks present were m/e 345 [$M-(90+115)$] and m/e 460 ($M-90$). These ions indicate the compound is a dihydroxy bile acid.

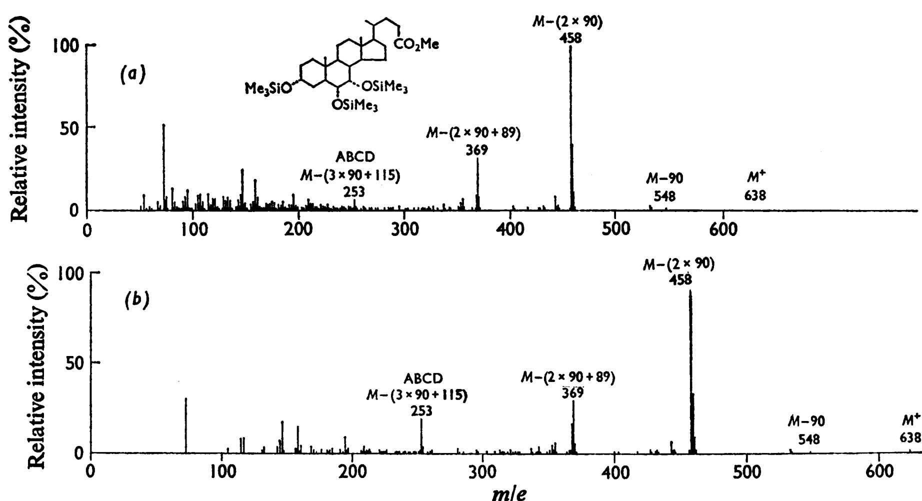

Fig. 3. *Mass spectra of the trimethylsilyl ether derivatives of methyl hyocholate* (*a*) *and the serum compound* (*b*)

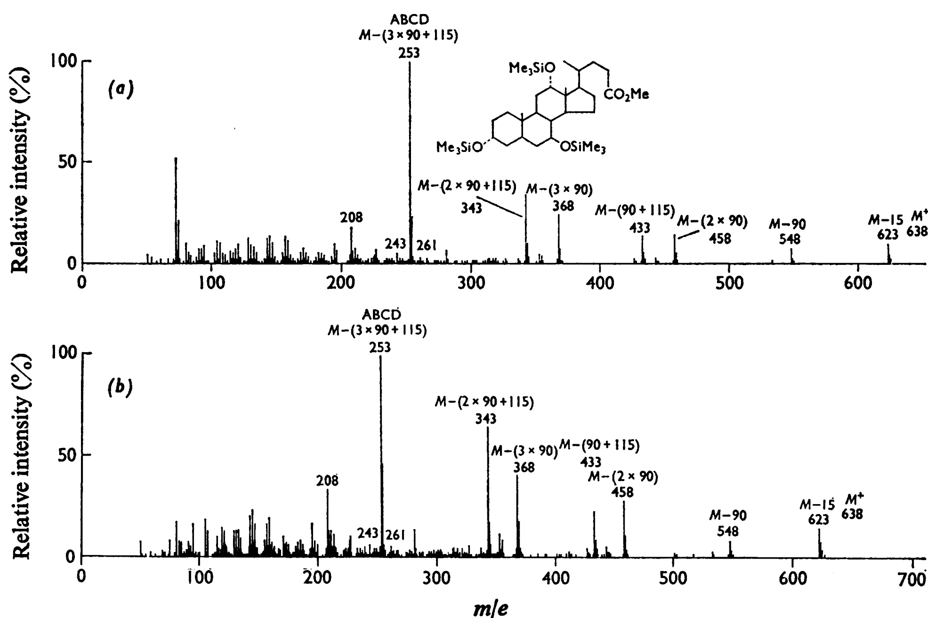

Fig. 4. *Mass spectra of the trimethylsilyl ether derivatives of methyl* $3\alpha,7\beta,12\alpha$*-trihydroxy-5*β*-cholan-24-oate* (*a*) *and the urine compound* (*b*)

A peak at *m/e* 208 suggests this bile acid may have a 12-hydroxyl group (Sjövall *et al.*, 1971). Another dihydroxy bile acid (RRT_c 2.73) was present in trace amounts.

Unidentified trihydroxy bile acids

One compound (RRT_c 1.20) was found running close to cholate in both the urine and plasma specimens. It was largely separated from cholate in the group separation, being eluted in the 'dihydroxy' fraction. The spectrum showed the most intense peaks at *m/e* 226 (ABC − 15) and *m/e* 253 (ABCD ring) either of which formed the base peak in different spectra. The peak at *m/e* 343 [$M-(2\times90+115)$] was more intense and that at *m/e* 368 ($M-3\times90$) less intense than in the spectrum of

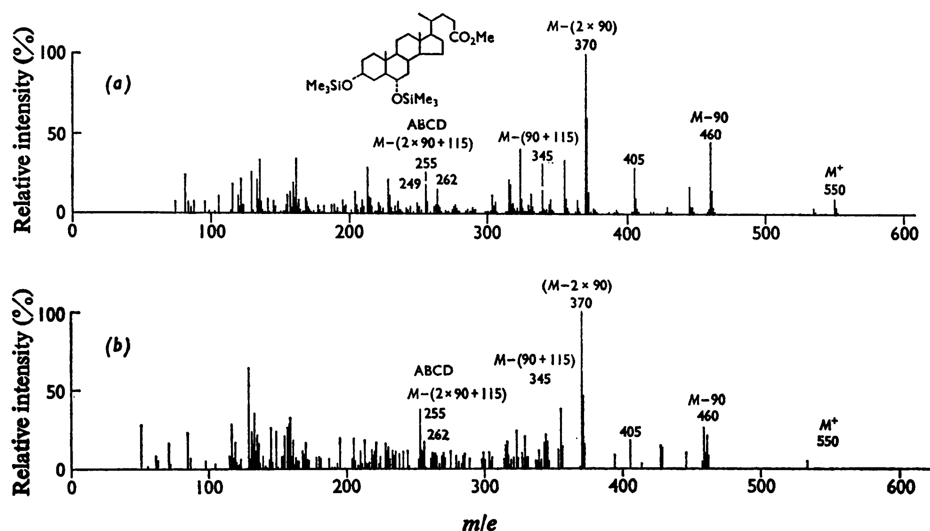

Fig. 5. *Mass spectra of the trimethylsilyl ether derivatives of hyodeoxycholate (a) and the urine compound (b)*

methyl cholate trimethylsilyl ether. Other peaks were present at *m/e* 443 [$M-(2\times90+15)$], *m/e* 458 ($M-2\times90$) and *m/e* 533 [$M-(90+15)$]. No peak was present at *m/e* 208 and those at *m/e* 243 and 261 were only 1% of base peak. The spectrum differed from that of allocholate (3α,7α,12α-trihydroxy-5α-cholan-24-oate), α-muricholate (3α,6β,7α-trihydroxy-5β-cholan-24-oate) or β-muricholate (3α,6β,7β-trihydroxy-5β-cholan-24-oate). A second unidentified trihydroxy bile acid (RRT_c 2.86) appeared to be present in trace amounts.

26-*Hydroxycholesterol*

This compound was found in the urine and plasma specimens and was eluted in the 'dihydroxy' fraction in the group separation (compound B). The trimethylsilyl ether derivative had the RRT_c (2.19) and mass spectrum of 26-hydroxycholesterol. The mass spectrum showed a base peak at *m/e* 129 and prominent peaks at *m/e* 417 ($M-129$), *m/e* 456 ($M-90$) and *m/e* 546 (M^+). Further, the n.m.r. spectrum of the plasma compound was identical with that of authentic 26-hydroxycholesterol.

24-*Hydroxycholesterol*

This compound was found in the plasma sample and was eluted in the dihydroxy fraction in the group separation (compound A). The trimethylsilyl ether derivative had an RRT_c of 1.59 and the mass spectrum of 24-hydroxycholesterol (Gustafsson & Sjövall, 1969). Ions formed by the loss of the side chain were prominent with the base peak at *m/e* 145 and a major peak at *m/e* 159. Other ions present were those at *m/e* 323 [$M-(2\times90+43)$], *m/e* 413 [$M-(90+43)$] and *m/e* 546 (M^+). The RRT_c of 24-hydroxycholesterol (1.59) is so close to that of chenodeoxycholate (1.64) on Hi Eff 8 BP that the smaller 24-hydroxycholesterol peak is hidden under the much larger bile acid peak. This probably accounts for the failure to identify this steroid in the urine specimen since it was not subjected to the preparative-thin-layer separation.

26-*Hydroxylated 5β-cholestanetriol*

This compound was found in the urine and plasma samples and was eluted in the trihydroxy fraction (compound C). The trimethylsilyl ether derivative had an RRT_c of 2.15. The mass spectrum showed a molecular ion at *m/e* 636 and ions at *m/e* 546 ($M-90$), *m/e* 456 ($M-2\times90$) and *m/e* 366 ($M-3\times90$). A prominent ion was present at *m/e* 145. This mass spectrum was suggestive of a trihydroxylated cholestane. Evidence from n.m.r. spectrum confirmed the presence of a steroid and provided strong evidence for the presence of a 26-hydroxyl group. Further, the n.m.r. spectrum was consistent with the presence of one of the other hydroxyl groups in the 3 (α or β) position and the last in either the 6α or 7 (α or β) position. The definitive identification of the structure of this compound was limited by the lack of the appropriate reference mass spectrum. To establish the state of conjugation of these three steroids, a portion of the crude serum extract from the XAD-7 extraction was silylated (Makita & Wells, 1963). The

silyl ethers formed were extracted into hexane in a hexane/water (1:1, v/v) partition. Analysis of this extract by g.l.c. and g.l.c.–m.s. revealed the presence of cholesterol only. The aqueous layer from this partition was taken to dryness and solvolysed as described above. The residue was silylated and the silyl ethers were again extracted into hexane. This fraction contained cholesterol, 24-hydroxycholesterol, the 26-hydroxylated cholestanetriol and 26-hydroxycholesterol. These results suggested that C_{27} steroids were present in the serum as solvolysable conjugates.

Discussion

The aim of this qualitative study was to identify as many components as possible of the complex mixture of bile acids and steroids present in the urine and serum in cholestasis. These studies of the pattern of bile acid excretion in cholestasis were not exhaustive. The methods used were inappropriate for the identification of ketonic bile acids and bile acids or cholesterol derivatives with more than three hydroxyl groups in the steroid ring. However, it is noteworthy that these investigations on the urine and serum from two different patients have shown, in general, a qualitative similarity in the bile acid pattern in the two samples. Similar results have been shown in studies on other patients (J. A. Summerfield, J. Cullen, S. Barnes, & B. H. Billing, unpublished work).

Methodology

The identification of minor bile acid components in biological fluids requires procedures that give a high degree of purification without introducing contamination. Amberlite XAD-7 was selected since it produced only trivial background contamination on g.l.c. with 1% Hi Eff 8BP. The enzymic deconjugation method was chosen in preference to strong alkaline hydrolysis since the formation of artifacts is less likely. No artifact formation was observed for the standard conjugated bile acids and lithocholate sulphates submitted to solvolysis and deconjugation, as judged by g.l.c., and recoveries were satisfactory.

The principal limitation to the identification of minor bile acid components is the resolution obtained on g.l.c. Preliminary g.l.c.–m.s. analyses of the whole urine and serum samples had revealed the non-homogeneous nature of many of the peaks seen on g.l.c. It was therefore necessary to introduce a group separation of the unconjugated bile acids before g.l.c.–m.s. analysis. Chromatography on an inert support (Sephadex LH20, Pharmacia, Uppsala, Sweden) was adapted for bile acid separation by methylating the bile acid carboxyl group and eluting the column with a solvent of low polarity (cyclohexane/ethanol, 19:1, v/v). It was noteworthy that the elution volume of the compounds studied appeared to depend on the number of substituent hydroxyl groups and not the size of the molecule. For example, dihydroxy bile acids (C_{24}) were eluted in the same volume as the dihydroxy compounds, 26-hydroxycholesterol (C_{27}) and 24-hydroxycholesterol (C_{27}). This technique simplified the g.l.c.–m.s. analysis considerably, by resolving many of the complex peaks and further minimizing background contamination.

The non-homogeneous nature of the peaks on g.l.c. with 1% Hi Eff 8BP in these columns illustrates the difficulty of making quantitative measurements of bile acids by g.l.c. and the importance of first ensuring the homogeneity of a peak by g.l.c.–m.s. Further, the finding of the 6α-hydroxylated bile acids, hyocholate and hyodeoxycholate, in cholestasis demonstrates that these bile acids are unsuitable for use as internal standards in human bile acid g.l.c. analyses.

Biological significance

The results of this study show the complexity of bile acid metabolism in cholestasis. Although cholate and chenodeoxycholate usually predominated, the unusual components were quantitatively important in a few samples. For example, in the urine of a patient with carcinoma of the bile duct 3α,7β,12α-trihydroxy-5β-cholan-24-oate and chenodeoxycholate were the principal bile acids excreted (J. A. Summerfield, J. Cullen, S. Barnes & B. H. Billing, unpublished work). We have also confirmed the observations of Makino *et al.* (1971), Back *et al.* (1972) and Back (1973) in finding ursodeoxycholate and 3β-hydroxychol-5-en-24-oate in both urine and plasma in cholestasis.

C_{27} steroids

26-Hydroxycholesterol was found in both urine and plasma probably as a sulphate conjugate. This compound has been found in the urine of patients with biliary atresia (Makino *et al.*, 1971) and in the faeces of infants under 4 months old, but not in older infants (Gustafsson & Sjövall, 1969).

In the rat, an alternative pathway for the synthesis of chenodeoxycholic and 6-α-hydroxylated bile acids, where the formation of 26-hydroxycholesterol is the first committed step, has been demonstrated by Mitropoulos & Myant (1967). Anderson *et al.* (1972) have shown that 26-hydroxycholesterol can be metabolized to bile acids in man. It is therefore possible that the 26-hydroxycholesterol present in specimens from our two patients may be the precursors of bile acids.

The cholestanetriol found in both the urine and serum was also present as a sulphate. Evidence from n.m.r. pointed strongly to this compound having one hydroxyl group in the 26 position. It is possible that the compound was 5β-cholestane-3α,7α,26-triol, an

intermediate in the normal pathway for bile acid synthesis (Danielsson, 1973). However, confirmation of this structure must await appropriate reference mass spectra.

The third C_{27} steroid found in the serum was 24-hydroxycholesterol. Failure to detect it in the urine was probably technical since it would have been obscured by the much larger chenodeoxycholate peak on g.l.c. This compound has been identified in the urine of patients with biliary atresia (Makino *et al.*, 1971). A sulphate ester of 24-hydroxycholesterol has been found in the faeces of infants (Gustafsson & Sjövall, 1969), The metabolic fate of this compound in man is unknown.

6-Hydroxylated bile acids

Hyocholate was identified in both the urine and serum specimens and hyodeoxycholate in the urine specimen: both of these bile acids were present in small amounts. Hyocholate and lithocholate were present in similar amounts, but only traces of hyodeoxycholate were found. To obtain a satisfactory mass spectrum for hyodeoxycholate, it was necessary to extract bile acids from large volumes of urine for purification and subsequent group separation. Apart from the observations of Van Berge Henegouwen (1974) of hyocholate in urine in cholestasis, 6α-hydroxylated bile acids have not been found in man. In this respect, it is noteworthy that Trülzsch *et al.* (1974) found that human liver microsomal preparations were capable of 6α-hydroxylating lithocholate (3α-hydroxy-5β-cholan-24-oate) to hyodeoxycholate (3α,6α-dihydroxy-5β-cholan-24-oate) although Björkhem *et al.* (1973) were unable to confirm these observations.

The presence of 6α-hydroxylated bile acids in cholestasis in addition to 26-hydroxycholesterol, 3α-hydroxychol-5-en-24-oate and lithocholate, lends support to the view that an alternative pathway such as that suggested by Mitropoulos & Myant (1967) may exist in this condition.

3β,7α-Dihydroxy-5β-cholan-24-oate and 3α,7β,12α-trihydroxy-5β-cholan-24-oate

Although there was no clinical evidence of a urinary-tract infection in either patient, the formation of 3β,7α-dihydroxy-5β-cholan-24-oate could have resulted from infection since it was only found in the urine specimen. This bile acid has been found in human faeces (Eneroth *et al.*, 1966). 3α,7β,12α-Trihydroxy-5β-cholan-24-oate was found in both the urine and serum specimen. On g.l.c. this compound (RRT_c 1.53) runs close to deoxycholate (RRT_c 1.51) and may have been confused with deoxycholate in some previous studies, although it has been found in human bile (Sjövall, 1959) and faeces (Hirofugi, 1965). It is unlikely that the formation of 3α,7β,12α-trihydroxy-5β-cholan-24-oate was due to a urinary-tract infection since it was also found in the serum specimen. The possibility that this 7β epimer of cholate was derived from the bowel is made less likely by its occurrence in large amounts in the urine of a patient with carcinoma of the bile duct and a presumably negligible enterohepatic circulation of bile acids (J. A. Summerfield, J. Cullen, S. Barnes & B. H. Billing, unpublished work). Finally, a 7β epimer of chenodeoxycholate (ursodeoxycholate) has been found in the urine of a child with biliary atresia and no significant enterohepatic circulation (Back, 1971). Ursodeoxycholate has also been found in the urine and serum of the patients we have studied. These observations support an hepatic origin for the 7β epimers of cholate and chenodeoxycholate found.

Conclusion

The finding of large numbers of bile acids and C_{27} steroids raises many questions about bile acid metabolism in the cholestatic syndrome. First, whether they are synthesized in the liver at all or are formed as the result of bacterial action in the bowel owing to an incomplete cholestasis, and are then reabsorbed and presented to an incompetent liver so that the plasma concentration rises. Secondly, the unusual bile acids could be the result of an occult urinary-tract infection. Thirdly, it is conceivable that the kidney has a metabolic role and that some of the epimers may be formed in the kidney.

If these numerous bile acids and C_{27} steroids are synthesized *de novo* in the liver, they must reflect further metabolic pathways. The question then arises as to whether their presence is the result of cholestasis or whether they also occur in normal individuals but at concentrations too low for detection by currently available techniques.

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