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Identification of Bile Acids and Neutral Sterols in Guinea Pig Bile

Bile Acids and Steroids 163

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A gas chromatographic peak shift technique was utilized for the identification of bile acids in guinea pig bile. Chenodeoxycholic acid and 7-ketolithocholic acid were confirmed as the predominant bile acids, and ursodeoxycholic acid, lithocholic acid, and cholic acid were confirmed as quantitatively minor bile acids. In addition 3-keto-7α-hydroxycholanoic acid and 3,7-diketocholanoic acid were newly identified. The neutral sterols identified in the bile by combined gas-liquid chromatography and mass spectrometry were cholesterol, β-sitosterol, and coprostanol while cholestanol was tentatively identified. Also, campesterol and stigmasterol were tentatively identified by gas-liquid chromatography.

The transformation of ^{14}C-labeled cholesterol to bile acids in three strains of guinea pigs has been reported by Danielsson and coworkers.[1,2] Chenodeoxycholic acid, formed in the liver, and 7-ketolithocholic acid, formed by microbial oxidation of chenodeoxycholic acid in the intestinal tract, were the major bile acids in the bile. Lithocholic and ursodeoxycholic acids, the latter tentatively identified, were present as minor components while trihydroxycholanoic acids were not found. Peric-Golia and Jones isolated chenodeoxycholic and 7-ketolithocholic acids from immature and mature guinea pigs whereas cholic acid was found only in the bile of adult animals.[3,4] Danielsson and Einarsson [2] showed the presence of cholesterol and small amounts of two unidentified polar neutral steroids but additional information regarding the sterol composition of guinea pig bile is not available.

During a study of factors related to the experimental production of gallstones,[5] it was necessary to obtain a more complete evalution of the bile acids and sterols present in guinea pig bile. We therefore employed a gas

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chromatographic peak shift technique for the identification of bile acids. In addition, the major neutral sterols in the bile have been identified by combined gas chromatography-mass spectrometry.

METHODS

Procedure. Guinea pigs (obtained from H. Axell, Färentuna, Sweden) of both sexes ranging in weight from 250 to 700 g were fed a commercial diet (H. Forss, Stockholm) which, according to the manufacturer contained protein 15 %, fat 3.6 %, soluble carbohydrates 42 %, plant fibers 12 %, yeast 2 %, and added salts and fat soluble vitamins. This diet was made into pellets containing 3 % Modocoll (Mo and Domsjö AB Sweden).[5] The animals were fasted for 6—8 h and then killed by striking the head. Bile was obtained directly from the gallbladder immediately after sacrifice and the biles of several animals of the same sex and approximate weight were pooled for the analyses.

The bile was lyophilized and then extracted with 20 volumes of chloroform/methanol, 1:1, (v:v). One aliquot was taken for analysis of bile acids and another for neutral sterols.

Bile acids. Bile acid standards were as in previous investigations of this series. The extracts were submitted to thin layer chromatography (TLC) as described by Eneroth [6] using a solvent system (propionic acid, isoamyl acetate, water, propanol, 15:20:5:10) [7] suitable for the gross estimation of the relative proportions of glycine and taurine conjugates of bile acids.

The extracts were hydrolyzed with 15 % NaOH in 50 % aqueous ethanol for 10 h at 115—120° in stainless steel bombs. After dilution with water the hydrolyzed bile acids were then extracted from acidified solution with ethyl acetate and methylated with diazomethane as previously described.[8]

Preparative TLC of the hydrolyzed bile acids was done on pre-extracted silica gel G using the S1 (benzene, dioxane, acetic acid, 75:20:2) or S7 (trimethylpentane, ethylacetate, acetic acid, 10:10:2) solvent systems of Eneroth.[6] The separated bile acid zones were located with iodine vapors, and after sublimation of the iodine, the zones were extracted with acetone.

Preparation of bile acid derivatives for the peak shift study was carried out as previously described.[9] Partial trimethylsilyl (TMSi) ethers of bile acids with hydroxyl groups at C3 and equatorial hydroxyl groups at C6, C7, and C12 were formed with hexamethyldisilazane in dimethylformamide; the retention time on GLC indicating the number of reactive hydroxyl groups. Trifluoracetates (TFA) were prepared by dissolving TMSi derivatives in trifluoracetic anhydride. Methyl esters of bile acids were oxidized as described by Djerassi and coworkers,[10] the retention time on GLC indicating whether a mono-, di-, or tri-ketocholanoate was formed and whether the cholanoate was of the 5α or 5β series. 1,1-Dimethylhydrazones (DMH) of 3-ketocholanoates were prepared by reacting the bile acid methyl esters or the oxidation products of the bile acids with 1,1-dimethylhydrazine [11] (Fluka AG, Buchs, Switzerland).

Gas chromatography (GLC) of the bile acid derivatives was carried out using glass columns packed with 3 % QF-1 coated on acid washed, silanized Gas-Chrom P (100—120 mesh, Applied Science Laboratories Inc., State College, Pa.). An argon ionization detector was used. The column was operated at about 230° and the argon inlet pressure was kept at 1.6—2.0 kg/cm^2. The columns under these conditions had 2500—3500 theoretical plates for methyl deoxycholate with a retention time of about 20 min.

Neutral sterols. An aliquot of the chloroform/methanol extract was hydrolyzed with 2 N KOH in 50 % aqueous ethanol for 3 h at 60°C. The hydrolysate was then extracted with petroleum ether as described previously.[12] The neutral sterols were run on columns of 3 % QF-1 (at about 220°) and 2.2 % SE-30 (at about 240°), before and after preparation of TMSi ethers. The TMSi derivatives of the 3-hydroxysterols were prepared with hexamethyldisilazane and trimethylchlorosilane in pyridine.[13] Mass spectrometry of GLC column effluents (GC-MS) was done using the LKB model 9000 instrument.[12]

Analysis of diet for neutral sterols. 20 g of the diet in $CHCl_3/CH_3OH$, 1:1, (v/v) with added 4-^{14}C-cholesterol was homogenized with an Ultra-Turrax homogenizer (Janke and Kunkel KG, Staufen i. Br., W. Germany). The extract was filtered and then hydrolyzed

and extracted with petroleum ether in the same manner as for the bile sterols. The petroleum ether extract was further purified on a silicic acid column.[12] Corrections were made for losses determined by the recovery of the added labeled cholesterol.

RESULTS

Bile acids. By inspection of TLC of conjugated bile acids, it was estimated that there were somewhat more glycine than taurine conjugates in the bile.

Fig. 1 is a gas chromatogram of the methyl esters of the bile acids. The final identifications of the bile acids responsible for the peaks on this chromatogram are indicated.

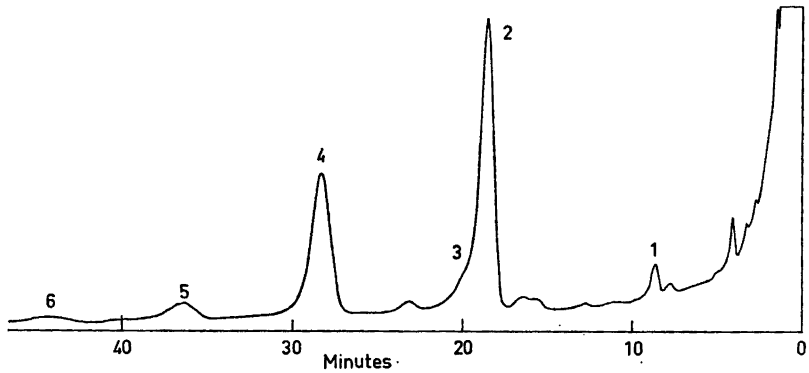

Fig. 1. Gas chromatogram of bile acid methyl esters from guinea pig gallbladder bile. The acids were identified as 1) lithocholic, 2) chenodeoxycholic, 3) ursodeoxycholic, 4) 7-ketolithocholic, 5) cholic and 3-keto-7α-hydroxycholanoic acid, and 6) 3,7-diketocholanoic acids. Conditions: 3 % QF-1 on acid washed, silanized, 100—120 mesh Gas Chrom P, column temperature 230°. Argon inlet pressure 1.8 kg/cm².

The peak shift study was done on the bile acids from three zones of the preparative TLC. The zones were selected by comparisons with appropriate reference compounds and contained the seven bile acids seen by GLC done before TLC. Table 1 shows the retention times relative to methyl deoxycholate of the reference compounds and of the bile acid derivatives from each of the three zones. The three compounds (A, B, C) in zone 2 and two of the three compounds in zone 3 gave rise to a single peak on the chromatograms of the oxidation products and on those of the DMH's of the oxidation products. These peaks had retention times characteristic of 3,7-disubstituted compounds. The bile acids identified in the bile were: TLC, zone 1: cholic, zone 2: chenodeoxycholic, ursodeoxycholic and 7-ketolithocholic, zone 3: 3-keto-7α-hydroxycholanoic, 3,7-diketocholanoic and lithocholic acids. There were no peaks compatible with allocholanoates (5α-cholanoates) on the chromatograms of the oxidation products.

Neutral sterols. In Table 2 are listed GLC retention times relative to cholesterol of guinea pig bile sterols chromatographed on 3 % QF-1 and 2.2 %

Table 1. GLC identification of bile acids in guinea pig gallbladder bile by peak shifts of derivatives. Retention times relative to methyl deoxycholate.

TLC zone or reference compound [a]	Methyl ester	TMSi	TFA	Oxidation product	DMH of oxidation product	Direct DMH
1. Cholic (3α,7α,12α)	2.21 2.21	1.25 1.26	1.31 1.35	5.91 5.92	3.20 3.19	2.25 2.23
2. A	1.15	0.63	0.86	2.75	1.44	1.14
2. B	1.24	0.37	0.98			1.23
2. C	1.77	1.08	1.57			1.75
Chenodeoxycholic (3α,7α)	1.12	0.64	0.86	2.79	1.48	1.15
Ursodeoxycholic (3α,7β)	1.24	0.37	0.97	2.77	1.49	1.23
7-Ketolithocholic (3α,7-keto)	1.73	1.08	1.57	2.79	1.48	1.76
3. A	2.27	2.27	1.74	2.74	1.43	1.00
3. B	2.77	2.77	2.78			1.49
3. C	0.53	0.29	0.45	1.00	0.47	0.53
3-Keto-7α-hydroxy-cholanoic	2.24	2.24	1.72	2.76	1.48	1.01
3,7-Diketocholanoic	2.76	2.75	2.79	2.79	1.43	1.48
Lithocholic (3α)	0.51	0.30	0.45	1.01	0.48	0.53

[a] Greek letters denote the orientation of hydroxyl groups at carbons 3, 7, or 12 of cholanoic acid.

TMSi = partial trimethylsilyl ethers, TFA = trifluoroacetates,
DMH = dimethylhydrazones.

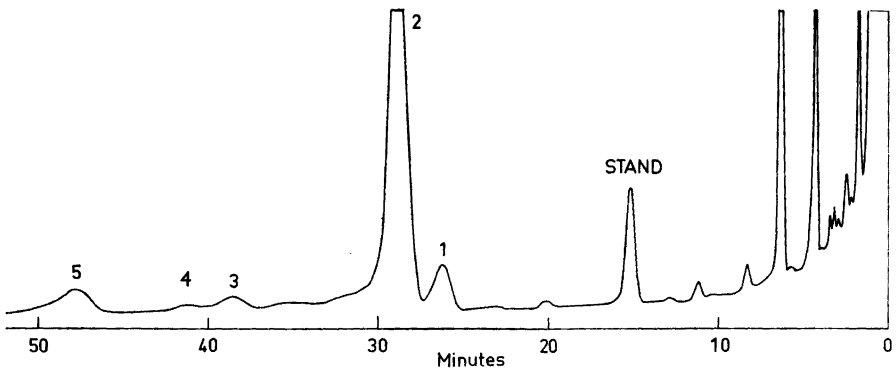

Fig. 2. Gas chromatogram of neutral sterols from guinea pig gallbladder bile. The sterols were identified as 1) coprostanol, 2) cholesterol and cholestanol (tentatively), 3) campesterol (tentatively), 4) stigmasterol (tentatively) and 5) β-sitosterol. Conditions: 2.2 % SE-30, column temperature 240° and argon inlet pressure 1.2 kg/cm².

Table 2. GLC of neutral sterols in guinea pig bile. Retention times relative to cholesterol.

Bile sterol or reference compound [a]	3 % QF-1 (220°)		2.2 % SE-30 (240°)	
	Free	TMSi[b]	Free	TMSi[b]
1. Coprostanol (5β,3β-ol)	0.87 0.88	0.52 0.52	0.91 0.92	0.96 0.98
2. Cholesterol (Δ^5-3β-ol)	1.00 1.00	0.70 0.70	1.00 1.00	1.27 1.27
2 A. Cholestanol (5α,3β-ol)	1.07 1.09	0.70 0.72	1.00 1.00	1.27 1.28
3. Campesterol (24α-Methyl-Δ^5-3β-ol)	1.32 1.34	0.97 0.95	1.33 1.34	1.70 1.71
4. Stigmasterol (24α-Ethyl-$\Delta^{5,22}$-3β-ol)	1.32 1.37	0.97 0.97	1.43 1.44	1.81 1.85
5. β-Sitosterol (24α-Ethyl-Δ^5-3β-ol)	1.58 1.57	1.02 1.08	1.67 1.66	2.09 2.14

[a] Notations refer to structural features of cholestane.
[b] Trimethylsilyl ethers.

SE-30 before and after preparation of TMSi derivatives. The values for references were obtained from standards as used previously or determined by Eneroth *et al.* [12] in this laboratory. Attempts were not made to identify minor components. For example, small poorly defined peaks were noted one of which may have been lathosterol (5α-cholest-7-en-3β-ol). Fig. 2 is a chromatogram of free neutral sterols on 2.2 % SE-30. The final identifications of the compounds responsible for the peaks are indicated. Cholestanol can be seen only as the free compound in the tail of the cholesterol peak on QF-1. Stigmasterol and campesterol were separated only on SE-30 columns and not on QF-1 columns.

The structures attributed to the neutral sterols by GLC were confirmed by combined GC-MS for cholesterol, coprostanol, and β-sitosterol (analyzed as the TMSi ethers on a QF-1 column). Mass spectra taken at the end of the peak of cholesterol TMSi ether showed fragments which were two mass units higher than those due to the cholesterol derivative indicating the presence of cholestanol TMSi ether.

Diet. Each animal ate about 30 g of the diet daily. The sterols found in the diet were cholesterol, campesterol, stigmasterol, and β-sitosterol. Copro-

stanol could not be detected; if it comprised more than 5 % of the sterols it would have been seen. Cholesterol comprised 0.008 % of the diet and it was estimated that there were about 21 mg of total sterols in 30 g of diet.

DISCUSSION

There was a somewhat greater concentration of glycine than taurine conjugates in the gallbladder bile as estimated from inspection of the TLC plates. The relative proportions of these conjugates depend in part upon diet and species.[14] This might explain why Danielsson *et al.*[1,2] reported a predominance of glycine conjugates while Peric-Golia *et al.*[3,4] found more taurine conjugates.

The peak shift technique for the identification of bile acids has been shown to be valid in the analysis of fecal bile acids where the structures were confirmed by mass spectrometry.[15] Chenodeoxycholic acid and 7-ketolithocholic acids were the predominant bile acids found in the bile. Of the other bile acids, lithocholic and ursodeoxycholic acids have been previously reported by Danielsson and coworkers[1,2] while cholic acid was found by Peric-Golia and Jones[3,4] in the bile of mature guinea pigs. 3-Keto-7α-hydroxycholanoic and 3,7-diketocholanoic acids have been found previously in human feces[15] but have not been reported before in bile. They are probably formed by oxidation of chenodeoxycholic acid in the intestine.[14] GLC is a more sensitive technique than paper and column chromatographic procedures and so the detection of trace amounts of additional bile acids is not surprising. However, the relative proportions of bile acids in the bile may be influenced by diet or intestinal flora.[16] Also it has been suggested that plant sterols might be converted to bile acids.[17]

There have not been previous systematic studies of the neutral sterols in guinea pig bile and the effect of diet on these compounds in bile is not known. The presence of coprostanol and plant sterols in bile has not been previously reported. The coprostanol might have been absorbed from the intestine where it is formed from cholesterol. The plant sterols probably represent dietary sterols that have been absorbed and then excreted in the bile.[18] None of the sterols identified corresponded with the two polar steroids reported by Danielsson and Einarsson[2] and specific attempts were not made to identify them.

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