Studies in the Bile Acids

2. THE NON-KETONIC ACIDS OF HUMAN BILE

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A method of analysis has been described in which a mixture of bile acids from bile can be separated and estimated by a combination of chromatography and infrared spectrometry (Wootton, 1953). This method was applied to a specimen of pooled human bile kindly supplied by the late Dr Konrad Dobriner. To our surprise, we found that the most abundant bile acid in the specimen was chenodeoxycholic acid (3α:7α-dihydroxycholanic acid), although this acid is usually regarded as one of the minor constituents of mammalian bile.

This particular specimen had been collected at autopsy from patients who had suffered from advanced carcinomatosis, and it was considered possible that their clinical condition had influenced the constitution of the bile. It was therefore decided to analyse individual specimens from patients with and without carcinoma. It was also felt that such a study would help to define the natural variability of the proportions of the different bile acids in the human, a matter which is possibly of importance in the aetiology of gall-stones. Furthermore, the use of bile constituents as an 'evolutionary label' (Haslewood & Wootton, 1950) has created interest in the variability within a single species.

In the course of this work, chenodeoxycholic acid was produced from cholic acid (3α:7α:12α-trihydroxycholanic acid) on a preparative scale by a method which has been briefly reported (Anderson, Haslewood, Wiggins & Wootton, 1952). Several esters were made and characterized.

EXPERIMENTAL

Material. Bile specimens were obtained from nine patients at autopsy. Within a few hours of death, the contents of the gall-bladder were mixed with 2 vol. of ethanol. One specimen (case XII) was collected during life from a patient with bile fistula following cholecystectomy; in this case the bile was run directly into a bottle containing ethanol.

Preparation of esters. The bile-ethanol mixture was filtered from precipitated mucin and the filtrate evaporated to dryness on a steam bath and finally under vacuum. The residue, dissolved in water, was mixed with half its weight of charcoal and dilute NaOH solution added until a spot test gave a pink colour with phenolphthalein. After drying at 100°, the powdered solid was extracted with ethanol in a continuous extractor and the ethanol was removed under vacuum.

The ethanol-soluble bile salts (1 g.) were dissolved in 10 ml. 2·5 N-NaOH, heated to 120° overnight in a closed bomb and the reaction mixture diluted and acidified. The precipitated bile acids were collected, washed with water and dried. They were dissolved in methanol containing 1 % (v/v) H_2SO_4 and allowed to stand for 2 days at room temperature, the solution was then diluted with water and extracted with ether. The ether extracts were washed with dilute ammonia solution and water, dried (Na_2SO_4) and evaporated, yielding an amber gum containing the mixed methyl esters of the bile acids.

Separation and identification. The esters were separated on columns of silica gel and the individual fractions were examined and identified by infrared spectrometry as described by Wootton (1953). The instrument used was a single-beam Grubb-Parsons S 3 A with a NaCl prism.

Fractions displaying similar spectra were combined, so that in general each specimen was divided into parts comprising the methyl esters of (*a*) lithocholic acid (3α-hydroxycholanic acid), (*b*) a mixture of chenodeoxycholic acid and deoxycholic acids (3α:7α- and 3α:12α-dihydroxycholanic acids), and (*c*) cholic acid (3α:7α:12α-trihydroxycholanic acid). The amounts of the two esters present in (*b*) were determined in the following way. A number of mixtures were made containing different proportions of the pure synthetic methyl esters of chenodeoxycholic acid and deoxycholic acid. The infrared spectrum of each mixture was obtained and a celluloid transparency prepared from it. The spectrum of the fraction from the column was then compared with the set of transparencies by three independent observers. An analysis of the results indicated that the percentage of each ester could be estimated with a standard deviation of 3·8 %.

Reference compounds

General. Melting points were measured with a hot-stage microscope and are corrected. Optical rotations were determined on solutions in $CHCl_3$ at about 20° using a 0·5 dm. tube and concentrations of 0·8–1·0 %. Microanalyses were done by Weiler and Strauss, Oxford. The light petroleum had b.p. 40–60. 20 % Chromic acid was made as described by Haslewood & Wootton (1950). The infrared spectra were taken in CS_2 solution; concn. about 2 mg./ml., solution thickness 3 mm. These are shown in Fig. 1.

Chenodeoxycholic acid. This preparation was accomplished by the reduction of 3:7-diacetoxy-12-ketocholanic acid by the method of Huang-Minlon (1946). The starting material (4·3 g., m.p. 226–228°) was dissolved in a mixture

of 40% KOH (12·5 ml.) ethylene glycol (150 ml.) and 90% hydrazine hydrate solution (5 ml.). After refluxing for 1·5 hr., the condenser was removed and heating continued until the b.p. rose to 195°. Refluxing was then continued for 4 hr. The reaction mixture was diluted and saturated with NaCl and the crude chenodeoxycholic acid was collected as an amorphous white powder (3·0 g.).

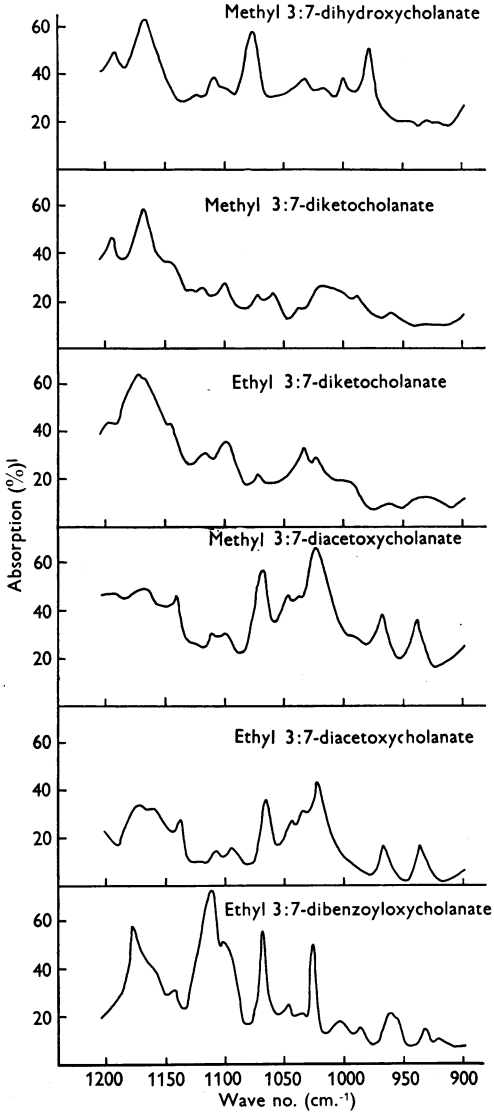

Fig. 1. Infrared-absorption spectra of derivatives of 3:7-diketo- and 3:7-dihydroxy-cholanic acid.

Methyl chenodeoxycholate. The acid (0·5 g.) was dissolved in 50 ml. methanol containing 1% (v/v) H_2SO_4. After 48 hr. at room temperature, the solution was diluted and extracted with ether. After washing, drying and evaporating the ether, a colourless gum remained. After solution in ether:light petroleum *methyl chenodeoxycholate* (0·45 g.) crystallized slowly as sheaves of colourless needles, m.p. 85–87° $[\alpha]_D + 12°$. (Found: C, 72·7; H, 10·2. $C_{25}H_{42}O_4$ requires C, 73·8; H, 10·4%.) Several preparations which were analysed had low C content. On one occasion, sublimation in high vacuum yielded a small amount of crystalline material, m.p. 113–114°, with an unchanged infrared spectrum. It is concluded that the normal form contains solvent of crystallization.

A number of attempts were made to prepare the ethyl ester by a similar method. No crystalline material could be recovered.

Methyl diacetylchenodeoxycholate (*methyl* 3α:7α-*diacetoxycholanate*). Methyl chenodeoxycholate (0·05 g.) was dissolved in a mixture of 4 ml. acetic acid, 1·5 ml. acetic anhydride and 0·05 ml. 60% (w/v) $HClO_4$. After standing for 0·5 hr. at room temperature, the diluted mixture was extracted with ether. The gummy residue remaining after evaporation of the washed and dried ether was dissolved in a mixture of ether:light petroleum (3:7, v/v) and passed through a column of silica gel. The recovered material crystallized from light petroleum as white prisms (0·03 g.), m.p. 127–128°, $[\alpha]_D + 14°$. (Found: C, 71·1; H, 9·1. Calc. for $C_{29}H_{46}O_6$: C, 71·0: H, 9·4%.)

Ethyl diacetylchenodeoxycholate (*ethyl* 3α:7α-*diacetoxycholanate*). This was made in a similar way, starting with the gummy ethyl ester. It crystallized in a similar form, m.p. 106–108°, $[\alpha]_D + 12°$. (Found: C, 71·5; H, 9·2. Calc. for $C_{30}H_{48}O_6$: C, 71·4, H, 9·5%.) The m.p. was unchanged after admixture with the natural ester, m.p. 106–108°, isolated from natural sources and kindly supplied by Prof. G. A. D. Haslewood.

Methyl 3:7-*diketocholanate.* Methyl chenodeoxycholate (0·03 g. in 2 ml. acetic acid) was oxidized by the dropwise addition of 20% chromic acid until a permanent brown colour remained. After 20 min., the mixture was diluted with water. Long needles slowly separated, m.p. 160–162°, raised by recrystallization from aqueous ethanol to 162–164° (0·02 g.) $[\alpha]_D - 36°$. (Found: C, 74·2; H, 9·6. Calc. for $C_{25}H_{38}O_4$: C, 74·6; H, 9·5%.)

Ethyl 3:7-*diketocholanate.* This was prepared in the same way from the ethyl ester. The *ethyl* 3:7-*diketocholanate* crystallized as long needles, m.p. 126–129°. $[\alpha]_D - 38°$. (Found: C, 74·8; H, 9·8. $C_{26}H_{40}O_4$ requires C, 75·0; H, 9·7%.)

Ethyl dibenzoylchenodeoxycholate (*ethyl* 3α:7α-*dibenzoyloxycholanate*). The ethyl ester (0·1 g.) in pyridine (1 ml.) and benzoyl chloride (0·2 ml.) was allowed to remain overnight at room temperature. The product was washed by decantation with dilute H_2SO_4 and dissolved in ether. The residue after evaporating the washed ether was taken up in benzene:light petroleum (1:1, v/v) and passed through a column of Al_2O_3. The gummy ester was left in contact with ether:light petroleum and crystallized after several months. Recrystallized from ether:light petroleum, the *ethyl dibenzoylchenodeoxycholate* formed long needles (0·075 g.) m.p. 99–102°, $[\alpha]_D - 31°$. (Found: C, 75·4; H, 8·2. $C_{40}H_{52}O_6$ requires C, 76·5; H, 8·3%.)

RESULTS

The proportions of each of the four non-ketonic acids present in human bile are given in Table 1, which also shows the ages of the patients and the pathological conditions found at autopsy (except on

Table 1. *Proportions of the different bile acids in human bile specimens*

Case no.	Pathological condition	Age (years)	Acids (as % of total) Litho-cholic acid	Deoxy-cholic acid	Chenodeoxy-cholic acid	Cholic acid
I	Coronary thrombosis	83	—	28	44	28
III	Gastric carcinoma and liver metastases	60	—	6	49	45
VI	Intestinal obstruction	46	—	18	40	42
VII	Tuberculous meningitis	65	5	23	34	38
VIII	Subarachnoid haemorrhage	61	—	28	37	35
IX	Sarcoma of the ileum and liver metastases	54	—	3	37	60
X	Carcinoma of lung and liver metastases	65	—	28	42	30
XI	Pulmonary tuberculosis	26	—	17	51	32
XII	Bile fistula after cholecystectomy	46	—	8	32	60
XIII	Gastroenteritis	3	—	12	47	41

case XII which was a post-operative specimen). The amounts found were rather variable, but on inspection there is some semblance of a pattern. Lithocholic acid was only detected in one specimen. Chenodeoxycholic acid was abundant and constituted 30–50 % of the total. The remainder seems to be distributed between deoxycholic acid and cholic acid, and, when the former was scarce, the latter was correspondingly abundant.

DISCUSSION

These results amply confirm our previous impression that chenodeoxycholic acid is a major constituent of human bile. The large quantities present have presumably been overlooked because this substance is exceedingly difficult to isolate by classical techniques, and, moreover, many reported analyses depend on colour reactions of very doubtful specificity. It is, however, noteworthy that Murarami (1952) has found very similar amounts by a colorimetric method which distinguishes only dihydroxy and trihydroxy acids.

There appears to be no reason to suppose that carcinomatosis affects the proportions of the different acids in any way, nor is there any obvious correlation with age. It remains to be determined whether this pattern is in any way characteristic of the human species, or whether other mammalian biles, now thought to contain mainly cholic acid, will be found to be similar. Further work on this point is in progress.

SUMMARY

1. A convenient method of preparing chenodeoxycholic acid (3α:7α-dihydroxycholanic acid) from 3α:7α-diacetoxy-12-ketocholanic acid is described. Several esters of chenodeoxycholic acid are characterized and their infrared spectra given.

2. Analysis of human autopsy bile specimens by chromatography and infrared spectrometry has shown that chenodeoxycholic acid is an abundant constituent, while lithocholic acid is scarce. There is no obvious connexion with age or the presence of carcinomatosis.

3. It is still uncertain whether this finding is specific to the human species or is part of a common mammalian plan.

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