Short Communication

The Isolation and Identification of 3 α ,7 α -Dihydroxy-5 β -Cholestan-26-oic Acid from Human Bile

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The conversion of cholesterol (cholest-5-en-3 β -ol), a C₂₇ sterol, into C₂₄ bile acids is thought to proceed through C₂₇ bile acid intermediates in man. Evidence for this hypothesis has come from the investigation of a C₂₇ bile acid, 3 α ,7 α ,12 α -trihydroxy-5 β -cholestan-26-oic acid. This trihydroxy compound has been found in both lower-vertebrate (Briggs, Whitehouse & Staple, 1959) and human bile (Carey & Haslewood, 1963), and has been demonstrated to be derived from cholesterol and efficiently converted into cholic acid (3 α ,7 α ,12 α -trihydroxy-5 β -cholanoic acid) in man (Carey, 1964). The presence in human bile of the corresponding dihydroxy C₂₇ bile acid, 3 α ,7 α -dihydroxy-5 β -cholestan-26-oic acid, is described in the present communication.

Materials. Bile was obtained from a 47-year-old man who had a T-tube placed in his common duct after cholecystectomy for cholelithiasis. At the time of the bile collection, 14 days after surgery, his liver-function tests were normal. Approx. 1500 ml of bile was collected over a 2-day period. The faecal urobilinogen was less than 1mg/100g of stool, indicating a complete bile fistula.

The following chemicals were used: chenodeoxycholic acid (3 α ,7 α -dihydroxy-5 β -cholanoic acid) was from Sigma Chemical Co., St Louis, Mo., U.S.A.; methylsuccinic anhydride was from Eastman Organic Chemicals, Rochester, N.Y., U.S.A.; light petroleum was distilled at 60–70°C; alumina (aluminium oxide for chromatography, Woelm neutral-activity grade I) was purchased from Woelm, Eschwege, Germany. The half-methyl esters of methylsuccinic acid were prepared by refluxing methylsuccinic anhydride with anhydrous methanol for 30 min and were used without further purification.

Authentic DL-25-methyl 3 α ,7 α -dihydroxy-5 β -cholestan-26-oate was synthesized by electrolytic coupling of chenodeoxycholic acid and the half-methyl ester of methylsuccinic acid by the method of Bridgewater (1956). The product was isolated from the reaction mixture by using alumina and Celite chromatography. Crystallization of the methyl ester from acetone–water gave crystals with m.p. 51–56°C. Mild hydrolysis of the ester with ethanolic potassium hydroxide gave the free acid, which was crystallized from hot ethyl acetate–light

petroleum. This acid had m.p. 157–160°C [Fischmeister (1960) reported m.p. 157–159°C]. The mass spectrum of the methyl ester of this acid was identical with that reported by Dean & Alpin (1966). Additional samples were obtained from Dr P. D. G. Dean (Department of Biochemistry, University of Oxford, Oxford, U.K.) and Professor G. A. D. Haslewood (Department of Biochemistry, Guy's Hospital Medical School, London, U.K.). One of these samples was obtained from alligator bile (Dean & Alpin, 1966) and the other synthesized as described above. All samples of this compound were identical as determined by t.l.c. and mass spectroscopy.

Methods. Methylation was done by using diazomethane. Mass-spectroscopic analysis was carried out with a Hitachi–Perkin–Elmer mass spectrograph. I.r. spectroscopy was done with the potassium bromide micro-pellet technique in a Perkin–Elmer instrument. All weights were taken after drying for 24 h in an evacuated desiccator over phosphorus pentoxide. Melting points are uncorrected.

Hydrolysis and chromatographic separation of bile. To prevent the production of bacterial metabolites, all bile was collected in ethanol. Each fraction was adjusted to contain 50% (v/v) ethanol and stored at 5°C. Initial separation of the bile acids in this bile was as follows. Portions (300 ml) of the mixture were boiled for 5 min, filtered and evaporated on a steam bath under N₂. The residue was hydrolysed in 4.5 M-sodium hydroxide for 5 h at 120°C at 15 lb/in². The hydrolysate was diluted with an equal volume of water, the pH adjusted to 1 with 12 M-hydrochloric acid, and the mixture extracted with chloroform. The chloroform extracts were evaporated. The residue from each of the evaporated chloroform extracts was placed on a Celite column (3.6 cm × 30 cm) with aq. 70% (v/v) acetic acid as the stationary phase, and was eluted with increasing concentrations of benzene (0, 20, 40 and 60%, v/v) in light petroleum.

Since it was suspected that this compound should be less polar than chenodeoxycholic acid, which is eluted in the 40%-benzene fraction, the material in the last quarter of the 0%-benzene fraction and the first half of the 20%-benzene fraction was taken for further analysis. This pigmented material

Fig. 1. Mass spectra obtained from synthetic DL-25-methyl $3\alpha,7\alpha$ -dihydroxy- 5β -cholestan-26-oate (a) and the methyl ester of the compound isolated from human bile (b). Peaks of relative intensity less than 5% of the base peak at $412 m/e$ are not shown.

(33.4mg) was methylated with diazomethane, placed on a 10g Celite column (1cm $\times$ 30cm) with aq. 71% (v/v) ethanol as the stationary phase, and eluted with light petroleum. The product was eluted free of pigment as a single peak. However, t.l.c. indicated an impurity and the material was further purified by preparative t.l.c. on silica gel G with benzene-acetone (7:3, v/v) (Eneroth, 1963). The silica gel from the area corresponding to that of the synthetic DL-25-methyl $3\alpha,7\alpha$ -dihydroxy- 5β -cholestan-26-oate control was scraped off and washed in hot methanol three times. The combined methanol washes were centrifuged, filtered and evaporated. The residue weighed 2.75mg.

Identification of methyl $3\alpha,7\alpha$ -dihydroxy- 5β -cholestan-26-oate. This compound isolated from human bile was identical with that isolated from alligator bile and that synthesized as described above. T.l.c. with methanol-acetone-chloroform (1:5:14, by vol.) (Eneroth, 1963) demonstrated that the material was a single spot having the same mobility as the synthetic compound and that isolated from the alligator. In this system the R_f for methyl chenodeoxycholate is 0.51 and that for methyl $3\alpha,7\alpha$ -dihydroxy- 5β -cholestan-26-oate is 0.56. The i.r. spectrum of the methyl ester of the synthetic material in a potassium bromide micro-pellet was identical with that of the methyl ester of the material isolated from human bile. The mass spectrum of the compound isolated from human bile was also identical with that of the synthetic material in the region greater than $200 m/e$, as shown in Fig. 1.

Discussion. The presence in human bile of $3\alpha,7\alpha$ -dihydroxy- 5β -cholestan-26-oic acid has not been

previously reported. Previous studies have demonstrated the presence of this dihydroxylated C_{27} bile acid in the bile of *Alligator mississippiensis* (Dean & Alpin, 1966). Earlier investigations conducted with the alligator indicated that labelled cholesterol was converted into a compound with the mobility of a dihydroxy bile acid, probably $3\alpha,7\alpha$ -dihydroxy- 5β -cholestan-26-oic acid (Briggs *et al.* 1959).

The conversion of $3\alpha,7\alpha,12\alpha$ -trihydroxy- 5β -cholestan-26-oic acid into cholic acid has been demonstrated to involve β -oxidation and removal of the terminal C_3 fragment of the side chain as propionic acid (Suld, Staple & Gurin, 1962). It is reasonable to suggest that $3\alpha,7\alpha$ -dihydroxy- 5β -cholestan-26-oic acid may be metabolized in a similar fashion to chenodeoxycholic acid in man.

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