Short Communications

Unsaturated Monohydroxy Bile Acids in Cholestatic Liver Disease

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In a study of the different bile acid classes in the serum of patients with cholestatic liver disease it was found that 12 out of 15 patients had conjugated monohydroxy bile acids in amounts up to 35% of serum total bile acids (Murphy *et al.*, 1972). These compounds, which were detected after t.l.c. on silica gel (Gregg, 1966), had R_F values corresponding to those of the reference standards tauroolithocholic acid and glycolithocholic acid.

The present paper describes the procedures used to examine the nature of the bile acid (obtained from the pooled sera of five patients with primary biliary cirrhosis) that moved chromatographically like tauroolithocholic acid. Mass-spectral analysis, g.l.c. and g.l.c.–mass-spectral analysis indicated that the compound under examination was a conjugate not of lithocholic acid but of an unsaturated monohydroxy steroid carboxylic acid. The latter contained two double bonds, one of which was located in the side chain.

The same identification procedure was applied to the taurine-conjugated monohydroxy bile acid fraction isolated from the urine of a child with biliary atresia. In accord with the results obtained by Makino *et al.* (1971), this fraction was found to consist of conjugated lithocholic acid and conjugated 3 β -hydroxychole-5-enoic acid; allolithocholic acid was not, however, detected. In specimens of bile and urine obtained from patients with primary biliary cirrhosis lithocholic acid was the main monohydroxy bile acid detected.

Materials and methods

Clostridial cholyglycine hydrolase. A solution (10mg/ml) of a crude extract of *Clostridium welchii* acetone-dried powder [Sigma (London) Chemical Co., London S.W.6, U.K.] in 50mM-sodium phosphate buffer, pH 5.8, was prepared.

Standard bile acid derivatives. Methyl esters of lithocholic acid, deoxycholic acid and cholic acid were obtained, together with cholestane, from Field Instruments Co., Richmond, Surrey, U.K.

G.l.c. The trimethylsilyl ether derivatives were

separated at 230°C on a Gas-Chrom Q (100–120 mesh) column coated with 1% Hi-Eff 8BP obtained from Applied Science Laboratories Inc., State College, Pa., U.S.A. The g.l.c. analysis was kindly performed by Mrs. P. Kyd (Department of Medicine, Royal Free Hospital, London, U.K.).

Mass-spectral analysis. Mass-spectral examinations (A.E.I. mass spectrometer model 902; temperature 250°C; 70eV) were performed by Mr. D. Carter, Mass Spectrometry Department, School of Pharmacy, University of London, London, U.K.

G.l.c.–mass spectrometry. G.l.c.–mass-spectral analysis of the trimethylsilyl ethers of the unknown compounds was provided by Dr. Jan Sjövall (Reimendal & Sjövall, 1972).

Experimental

Isolation of conjugated monohydroxy bile acids.

Bile acids were extracted from serum and bile as previously described (Murphy *et al.*, 1970) and from a 24 h urine specimen with 10 vol. of ethanol. They were then subjected to preparative t.l.c. on silica gel with 1,2-dichloroethane–acetic acid–water (1:1:0.1, by vol.) (Gregg, 1966) as solvent. The fractions with R_F value corresponding to that of tauroolithocholic acid were eluted with chloroform–methanol (2:1, v/v) and assayed quantitatively with hydroxy steroid dehydrogenase. Four preparations were examined in detail.

(i) Serum compound (fraction A): 200 μ g was isolated from 20 ml of pooled serum (mean total bile acid concentration 204 μ M) from five patients with primary biliary cirrhosis.

(ii) Urine compounds (fractions B and C): 150 μ g was obtained from a patient with biliary atresia (fraction B) and 100 μ g from a patient with primary biliary cirrhosis (fraction C).

(iii) Bile compound (fraction D): 30 μ g was provided by Dr. F. Portella of this Department. It had been isolated from the duodenal aspirate of a patient with primary biliary cirrhosis.

Identification procedure. (1) Enzymic hydrolysis (with a crude preparation of the enzyme) was carried out as described by Roovers *et al.* (1968). The resulting free bile acids were subjected to t.l.c. on silica gel

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Fig. 1. Mass spectrum of the methyl ester derivatives of monohydroxy bile acid fraction A from pooled serum

(a) Mass spectrum of methyl esters prepared from fraction A; (b) mass spectrum of standard methyl tauroolithocholate. For experimental details see the text.

with iso-octane – di-isopropyl ether – acetic acid – propan-2-ol (2:1:1:1, by vol.) (Gregg, 1966) as solvent. Only one spot was obtained in the mono-hydroxy bile acid region.

(2) Methylation was achieved with 0.7M-HCl in methanol at 50°C overnight. The resulting esters were separated by t.l.c. on silica gel with acetone–benzene (3:7, v/v) (Hofmann, 1964) as solvent.

(3) Acetylation was performed as described by Anderson & Haslewood (1969). The acetylated methyl esters were isolated by t.l.c. on silica gel with methanol–chloroform (1:3, v/v) as solvent.

The methyl esters derived by this procedure from compounds A, B and D and the acetylated methyl ester prepared from fraction A were subjected to mass-spectral analysis.

The trimethylsilyl derivatives of the methyl esters prepared from fractions A, B and C were prepared and subjected to g.l.c. and also to g.l.c.–mass-spectral analysis.

Results

Mass-spectral analysis. Serum compound A. The mass spectrum of the corresponding methyl ester (Fig. 1a) was significantly different from that prepared from the standard tauroolithocholic acid (Fig. 1b) and published spectra of methyl lithocholate.

In Fig. 1(b) the molecular ion M occurs at m/e 390, $M-H_2O$ at m/e 372, $M-(H_2O+side\ chain)$ at m/e 257 and $M-(H_2O+side\ chain+ring\ D)$ at m/e 215. The corresponding ions in Fig. 1(a) are at m/e 386, 368, 255 and 213. The unsaturated nature of the ring nucleus of compound A was further suggested by the absence from Fig. 1(a) of a fragment corresponding to $M-(H_2O+ring\ A)$, which occurs with methyl lithocholate at m/e 318 and which is present in Fig. 1(b).

The presence of one hydroxyl group in compound A was confirmed by the spectrum of its acetate derivative. The molecular ion, M , of the acetylated

derivative was at m/e 428, i.e. 4 mass units less than that of the acetylated methyl lithocholate.

From these results it appears that compound A is not tauroolithocholic acid but an unsaturated mono-hydroxy steroid carboxylic acid with one double bond in the side chain and another in the nucleus, e.g. 3 β -hydroxychola-5,22-dienoic acid. However, there is evidence that the mass spectrum is not due to a single compound, and therefore the trimethylsilyl derivative was prepared and subjected to g.l.c.–mass-spectral analysis.

Urine compound (fraction B). The mass spectrum of the methyl ester derived from fraction B suggested a molecular ion M at m/e 388, one hydroxyl group, i.e. $M-H_2O$ at m/e 370, and a fully saturated side chain of 115 units, i.e. $M-(H_2O+115)$ at m/e 255. Peaks were also apparent at m/e 129 and $M-129$. This spectrum suggested that the methyl ester derived from fraction B was that of 3 β -hydroxychol-5-enoic acid.

However, other ionic fragments were present, notably at m/e 215, 230, 319 and 368.

Urine compound (fraction C) and bile compound (fraction D). The mass spectra of the methyl ester derivatives prepared from fractions C and D indicated that each fraction contained tauroolithocholic acid; both the ion of the saturated nucleus at m/e 215 and that of $M-H_2O$ at m/e 372 were very prominent.

G.l.c. analysis. The results of g.l.c. analysis of trimethylsilyl derivatives prepared from fractions A, B and C are given in Table 1.

The values in Table 1 indicated that only fraction C contains lithocholic acid as the major bile acid. Only small amounts of lithocholic acid were present in fraction B (<30%), the major component being 3 β -hydroxychol-5-enoic acid. The major peak observed for the methyl derivative of fraction A accounted for more than 90% of the total peak area of the chromatogram and had $R_{cholestone}$ 5.2, which was significantly different from those of the methyl lithocholate and methyl 3 β -hydroxychol-5-enoate.

Table 1. $R_{cholestone}$ values of major and minor peaks of trimethylsilyl derivatives of the methyl esters of compounds A, B and C

For experimental details see the text.

Trimethylsilyl derivative of	$R_{cholestone}$	
	Major peak	Minor peak
Methyl esters prepared from fraction A	5.2	1.5
Methyl esters prepared from fraction B	5.8	3.9
Methyl esters prepared from fraction C	3.9	1.3, 0.8
Methyl cholate	1.7	—
Methyl deoxycholate	2.8	—
Methyl lithocholate	3.9	—
Methyl 3 β -hydroxychol-5-enoate	5.8	—

G.l.c.-mass-spectral analysis. The results of g.l.c.-mass-spectral analysis indicated that fraction B contained conjugated lithocholic acid, 3 β -hydroxychole-5-enoic acid and an unidentified compound, and that fraction C contained conjugated lithocholic acid as the only bile acid.

The analysis of the trimethylsilyl derivative of the methyl ester derived from fraction A did not correspond to that of lithocholic acid or 3 β -hydroxychole-5-enoic acid, but supported the suggestion that compound A is an unsaturated monohydroxy bile acid with two double bonds, one of which is in the side chain.

Discussion

This report extends the findings by Makino *et al.* (1971) that a variety of monohydroxy bile acids are formed in liver disease. The results of g.l.c.-mass-spectral analysis have confirmed the result (indicated by the mass spectrum of the fraction isolated by t.l.c.) that compound A is an unsaturated monohydroxy bile acid containing two double bonds, one of which is in the side chain. Confirmation of the suggestion that this compound is 3 β -hydroxychole-5,22-dienoic acid must, however, await the synthesis of this compound and a detailed examination of its mass spectrum and those of suitable derivatives. The possibility that the unexpected double bond in the side chain could be an artifact and might have arisen during methylation has been considered. Use of the very sensitive technique of g.l.c.-mass-spectral analysis failed, however, to detect this compound in the deri-

vatives prepared from fractions B and C by using the same procedure.

The presence of unsaturated monohydroxy bile acids in serum from patients with cholestatic liver disease has not been previously reported, and extensive studies are needed to establish the relationship between the unsaturated monohydroxy bile acid found in serum and the different monohydroxy bile acids detected in urine and bile.

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