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STUDIES ON STEROIDS

CXXVIII. SEPARATION AND DETERMINATION OF BILE ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method is described for the simultaneous determination of major bile acids by high-performance liquid chromatography without prior hydrolysis. A mixture of bile acids is divided into the free, glyco- and tauro-conjugate groups by thin-layer chromatography. Separation of each group into cholate, ursodeoxycholate, chenodeoxycholate, deoxycholate and lithocholate is attained in two stages on a μBondapak C_{18} column; first, 0.3% ammonium carbonate–acetonitrile (9:4) is used as a mobile phase for the separation of the last three compounds. Subsequently cholate and ursodeoxycholate are resolved by chromatography in 0.3% ammonium carbonate–acetonitrile (11:4).

INTRODUCTION

Bile acids in biological fluids are usually present in the form of glyco- and tauro-conjugates. The methods commonly used for the quantitation of these conjugates involve prior hydrolysis with alkali under drastic conditions followed by chromatographic separation of the free bile acids. Such procedures have the inevitable disadvantages of the lack of reliability of the analytical results owing to incomplete hydrolysis and formation of artifacts, and of the loss of information about the conjugate form of bile acids[1]. Therefore, it is desirable to be able to analyse directly the intact conjugates in biological materials. In addition, chenodeoxycholic acid and ursodeoxycholic acid are currently under intensive investigation as therapeutic agents for the treatment of gallstones[2,3]. A variety of separation methods, including high-performance liquid chromatography (HPLC)[4,**], have previously been used with limited success for the analysis of bile acids. This paper describes a method for the simultaneous deter-

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** After preparation of this paper another report on the HPLC of bile acids by Parris[5] was published.

mination of the free and glycine- and taurine-conjugated bile acids by HPLC combined with thin-layer chromatography (TLC).

EXPERIMENTAL

Instruments

The apparatus used was a Waters Model ALC/GPC 202 high-performance liquid chromatograph equipped with a Model R 401 differential refractometer and a sample loop injector with an effective volume of 2 ml. μBondapak C_{18} (1 ft. × $\frac{1}{4}$ in. I.D.) and μBondapak-NH_2 (1 ft. × $\frac{1}{4}$ in. I.D.) columns (Waters Assoc., Milford, Mass., U.S.A.) were used under ambient conditions.

Materials

Cholic acid, ursodeoxycholic acid, chenodeoxycholic acid, deoxycholic acid and lithocholic acid were purchased from Sigma (St. Louis, Mo., U.S.A.) and their purity was checked by thin-layer chromatography prior to use. The glyco- and tauro-conjugates were synthesized by the *p*-nitrophenyl ester method in this laboratory. Digitoxigenin was supplied by E. Merck (Darmstadt, G.F.R.). Digitoxigenin 3-suberoylglycine ester and 3-suberoylarginine ester were prepared as previously reported[6,7]. All of the reagents employed were of analytical grade. Solvents were purified by distillation prior to use.

Group separation by TLC

A mixture of bile acids was dissolved in methanol and applied to a thin-layer plate (10 × 20 cm) of silica gel H (Merck). Development was carried out with triple runs employing isooctane–ethyl acetate–acetic acid (5:5:1)[8], isooctane–diisopropyl ether–acetic acid–*n*-butanol–isopropanol–water (10:5:5:3:6:1)[9] and chloroform–methanol–water (70:25:2) as successive solvent systems. Elution of the adsorbent corresponding to R_F 0.05–0.25 (zone 1), R_F 0.25–0.65 (zone 2) and R_F 0.65–0.90 (zone 3) with hot methanol (five 5-ml volumes) followed by evaporation of the eluent gave the tauro-conjugate, glyco-conjugate and free fractions, respectively. The eluate was dissolved in chloroform–methanol (2:1) and filtered, and the filtrate was evaporated to dryness *in vacuo*. HPLC was applied to the residue.

Determination by HPLC

The free and glycine- and taurine-conjugated bile acids were determined by the use of digitoxigenin, its 3-suberoylglycine ester and 3-suberoylarginine ester as internal standards, respectively. To a test sample (50–500 μg) was added a known amount of the internal standard (*ca.* 400 μg) in methanol and the mixture was evaporated to dryness *in vacuo*. The residue was re-dissolved in methanol (100 μl), a 20-μl aliquot of which was injected with a microsyringe into the high-performance liquid chromatograph equipped with a μBondapak C_{18} column. The separation of cholate, ursodeoxycholate, chenodeoxycholate, deoxycholate and lithocholate was carried out in two stages: first, 0.3% ammonium carbonate–acetonitrile (9:4) was used as the mobile phase for the separation of the last three compounds, and subsequently cholate and ursodeoxycholate were resolved by chromatography in 0.3% ammonium carbonate–acetonitrile (11:4).

Recovery test

A synthetic mixture of 100 μg of each free and conjugated cholate, ursodeoxycholate, chenodeoxycholate, deoxycholate and lithocholate was divided into the free and glyco- and tauro-conjugated groups by TLC. After addition of the internal standard to each group, the five bile acids were separated and determined by HPLC as described above.

RESULTS AND DISCUSSION

First, the chromatographic separation of the representative bile acid conjugates was carried out under a variety of conditions. On a μBondapak-NH_2 column the use of aqueous ammonium dihydrogen phosphate solution–methanol or acetonitrile failed to separate the bile acids owing to overlapping with a solvent peak and undesirable leading. For the reversed-phase chromatography on a μBondapak C_{18} column, the choice of a suitable eluent was first studied by examining combinations of the buffer solution and organic solvent. The conjugated bile acids were not separated satisfactorily when aqueous methanol containing ammonium dihydrogen phosphate or ammonium carbonate was employed as the mobile phase. As they were eluted with aqueous acetonitrile containing the phosphate salt, the conjugates were not completely resolved owing to leading and/or tailing. Of four eluents tested, the aqueous ammonium carbonate solution–acetonitrile system appeared to be most promising for efficient separation (Table I).

TABLE I

CAPACITY RATIOS OF CONJUGATED BILE ACIDS

Conditions: μBondapak C_{18} column (1 ft. × $\frac{1}{4}$ in. I.D.); detector, Model R 401 differential refractometer. Mobile phase: (A) 0.02 *M* $NH_4H_2PO_4$–methanol (1:2); (B) 0.3% ammonium carbonate–methanol (4:7); (C) 0.03 *M* $NH_4H_2PO_4$–acetonitrile (2:1); (D) 0.3% ammonium carbonate–acetonitrile (5:2).

Compound	*Mobile phase*			
	A (*1.5 ml/min*)	*B* (*1.5 ml/min*)	*C* (*2.5 ml/min*)	*D* (*2 ml/min*)
Glycocholate	2.2	2.0	2.2	2.2
Glycochenodeoxycholate	4.0	3.3	3.4	5.7
Glycodeoxycholate	5.6	4.2	4.9	6.7
Glycolithocholate	9.6	8.0	—	—
Taurocholate	2.5	1.9	1.6	2.7
Taurochenodeoxycholate	3.3	3.3	2.8	10.8
Taurodeoxycholate	4.1	3.7	3.3	12.3
Taurolithocholate	7.8	7.1	—	—
	(1.9 min)*	(1.9 min)	(1.2 min)	(1.5 min)

* The figures in parentheses represent the t_0 values.

The retention value was markedly influenced by the ratio of water to acetonitrile but not by the concentration of the salt. The free bile acids and their glyco- and tauro-conjugates were separated satisfactorily on a μBondapak C_{18} column when 0.3% ammonium carbonate–acetonitrile (either 9:4 or 11:4) was employed. The sepa-

ration of the five bile acids is illustrated in Fig. 1 for the tauro-conjugates. It should be noted that the capacity ratios of unconjugated cholate, ursodeoxycholate, cheno-deoxycholate, deoxycholate and lithocholate were almost identical with those of the corresponding glyco- and tauro-conjugates (Table II). The bile acids were eluted in a definite order irrespective of the conjugate form, suggesting that the capacity ratio would be dependent upon the number, position and configuration of the hydroxyl group on the steroid nucleus rather than the functional group of the side-chain.

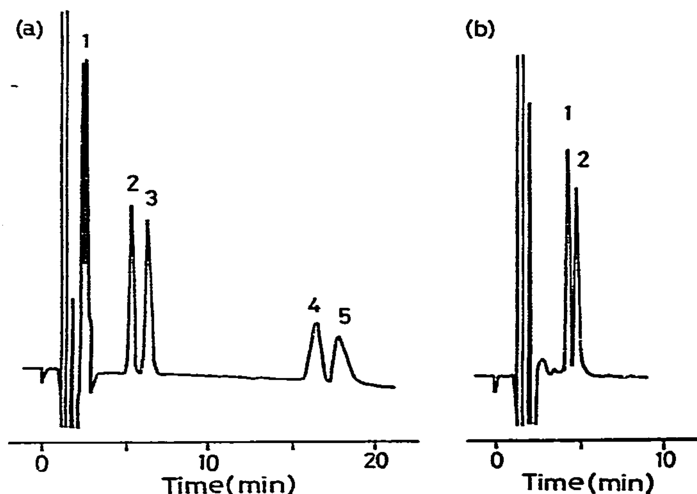

Fig. 1. (a) Separation of a mixture of taurine-conjugated bile acids. 1 = Tauroursodeoxycholate, taurocholate; 2 = taurochenodeoxycholate; 3 = taurodeoxycholate; 4 = digitoxigenin 3-suberoyl-arginine ester (internal standard); 5 = taurolithocholate. Conditions: μBondapak C_{18} column; mobile phase, 0.3% ammonium carbonate–acetonitrile (9:4), 2 ml/min; detection, Waters Model R 401 differential refractometer. (b) Separation of tauroursodeoxycholate (1) and taurocholate (2). Mobile phase: 0.3% ammonium carbonate–acetonitrile (11:4), 2 ml/min; other conditions as in (a).

A calibration graph was constructed by plotting the ratio of the peak area of each bile acid to that of an internal standard, *i.e.*, digitoxigenin for free bile acids, digitoxigenin 3-suberoylglycine ester for glyco-conjugates and digitoxigenin 3-suberoylarginine ester for tauro-conjugates, against the amount of the bile acid. Detection with a differential refractometer showed a linear response to each bile acid in the range of 10–100 μg.

For the simultaneous determination of bile acids in biological fluids on the basis of the above results, fractionation into the free and glyco- and tauro-conjugate groups was required prior to HPLC. A mixture of bile acids could be divided into three groups according to their mobilities by TLC. A typical chromatogram obtained by triple development with three solvent systems is illustrated in Fig. 2. The eluate from each zone was submitted to HPLC. Glycolithocholate was located in the zone of free bile acids, but in the subsequent step it was readily separated from other components.

The recovery test for free and conjugated bile acids applied to TLC was then carried out. A synthetic mixture of free bile acids and their glyco- and tauro-conjugates was submitted to TLC and the eluates from three fractions were deter-

TABLE II

CAPACITY RATIOS OF FREE AND CONJUGATED BILE ACIDS

Conditions: 1 ft. × ¼ in. I.D. column; detector, Model R401 differential refractometer; mobile phase, 0.3% ammonium carbonate–acetonitrile, (A) 9:4, (B) 11:4; 2 ml/min.

Compound	*Free*		*Glycoconjugate*		*Tauroconjugate*	
	A	*B*	*A*	*B*	*A*	*B*
Cholate	1.2	2.7	1.2	2.7	1.2	2.7
Ursodeoxycholate	1.2	3.0	1.2	3.0	1.1	3.0
Chenodeoxycholate	2.9	—	2.9	—	3.3	—
Deoxycholate	3.6	—	3.6	—	4.1	—
Lithocholate	12.3	—	11.4	—	13.6	—
	(1.2 min)*	(1.2 min)				

* The figures in parentheses represents the t_0 values.

mined by HPLC. The recoveries of bile acids were satisfactory, as shown in Table III.

The present procedure is more advantageous than previous methods, as free and conjugated bile acids can be separated, characterized and determined simultaneously under mild conditions without prior hydrolysis. Shaw and Elliott[4] have previously carried out the separation of conjugated bile acids by HPLC with a certain measure of success. Their study, however, did not deal with ursodeoxycholic acid and lithocholic acid. In addition, the 3,7- and 3,12-dihydroxy derivatives were not distinctly separated owing to undesirable tailing even when a recycling mode was employed. The present method, which is capable of determining free and conjugated bile acids with

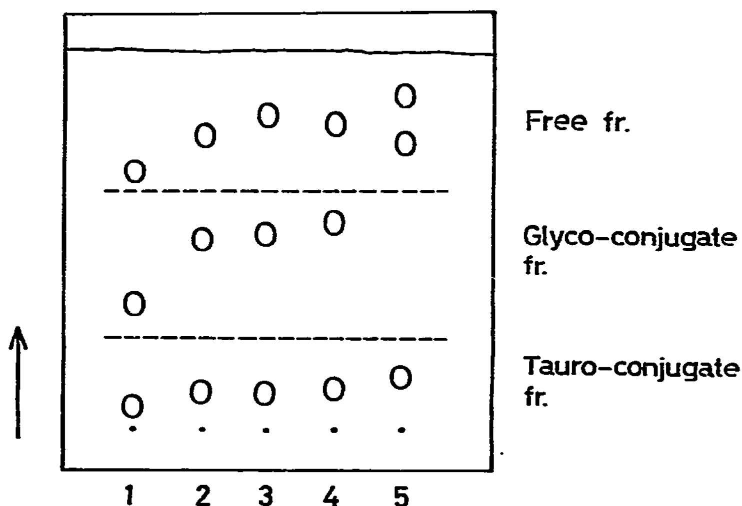

Fig. 2. Thin-layer chromatogram of free and glycine- and taurine-conjugated bile acids. 1 = Cholate; 2 = ursodeoxycholate; 3 = chenodeoxycholate; 4 = deoxycholate; 5 = lithocholate. Adsorbents, silica gel H. Solvent systems: (a) isooctane–ethyl acetate–acetic acid (5:5:1); (b) isooctane–diisopropyl ether–acetic acid–*n*-butanol–isopropanol–water (10:5:5:3:6:1); (c) chloroform–methanol–water (70:25:2).

TABLE III

RECOVERY TEST FOR FREE AND CONJUGATED BILE ACIDS USING TLC

A mixture of 100 μg each of free and conjugated bile acids was applied. The recovery of each bile acid was determined by the method described in the text. The figures represent the mean recovery rate $\pm$ standard deviation (%). Number of determinations (n) = 4.

Compound	*Free*	*Glyco-conjugate*	*Tauro-conjugate*
Cholate	100.9 ± 3.6	93.2 ± 4.3	93.4 ± 1.7
Ursodeoxycholate	99.2 ± 1.9	96.8 ± 2.2	100.0 ± 1.5
Chenodeoxycholate	102.1 ± 3.4	100.4 ± 8.3	108.2 ± 4.2
Deoxycholate	96.4 ± 2.6	100.5 ± 4.6	106.2 ± 5.7
Lithocholate	98.8 ± 5.6	99.6 ± 2.6	102.3 ± 6.3

satisfactory precision and accuracy, appears to be much more advantageous in applicability to biological fluids.

Quantitation of major bile acids in human bile by the proposed method is being conducted in these laboratories, and the details will be reported elsewhere.

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