Journal of Chromatography, 76 (1973) 425–431

CHROM. 6485

THE SEPARATION OF BILE ACIDS FROM NEUTRAL LIPIDS BY THIN-LAYER CHROMATOGRAPHY

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(Received October 10th, 1972)

SUMMARY

A method has been developed to separate bile acids, both in their free and conjugated forms, from neutral lipids by thin-layer chromatography. The average recovery rate, measured by liquid scintillation counting and by enzymatic determinations, is 95%. This method has been applied to the determination of the amount of bile acids in human blood serum and in artificial mixtures.

INTRODUCTION

The separation of bile acids from other substances is a prerequisite for the quantitative analysis of a mixture of bile acids in biological material. Of all the substances that make the isolation of bile acids difficult, neutral lipids are probably the most important. The separation techniques that are currently available have been summarized in recent reviews[1,2]. The techniques most frequently used include liquid–liquid chromatography, adsorption chromatography and ion-exchange chromatography[3–13]. In spite of the time-consuming and difficult procedures, the quantitative accuracy of the techniques is not always entirely satisfactory because some bile acids tend to be separated with the neutral lipids during the separation procedures. The present study was undertaken in order to develop a method of rapidly separating the main bile acids, both free and conjugated, from neutral lipids in biological material.

EXPERIMENTAL

The method of separating bile acids from lipids is based on thin-layer chromatography. Prepared chromatoplates (20 × 20 cm and 20 × 5 cm) coated with Silica

Gel G (Catalogue No. 5721/0025, Merck, Darmstadt, G.F.R.) are washed with p.a. methanol (Merck) and then dried and stored in a desiccator. A 90:54:11 mixture of p.a. chloroform (Merck), p.a. methanol (Merck) and 7.7 *N* ammonia (U.C.B., Brussels, Belgium) is used as the mobile phase. This system has been used for separating phospholipid mixtures[14]. The chromatoplates were run for 2½ h at a constant temperature of 25°.

Qualitative aspects of the separation technique

The R_F values of the following bile acids (Calbiochem, Los Angeles, U.S.A.) were determined: cholic acid (C), deoxycholic acid (DC), chenodeoxycholic acid (CDC), lithocholic acid (LC), taurocholic acid (TC), taurochenodeoxycholic acid (TCDC), taurodeoxycholic acid (TDC), taurolithocholic acid (TLC), glycocholic acid (GC), glycodeoxycholic acid (GDC), glycolithocholic acid (GLC) and hyodeoxycholic acid (HDC). These bile acids were dissolved in methanol (final concentration 1.0 mg/ml), and 10, 30, 50 and 100-μl volumes of the solution were spotted on to chromatoplates. The R_F values of mixtures of bile acids and fat were then determined. After evaporation of 50 μl of each bile acid solution, fat (250 mg of butter in 2:1 chloroform–methanol) was added in amounts sufficient to give final fat:bile acid ratios of 20:1, 50:1 and 100:1. A 30-μl volume of each bile acid–fat mixture was spotted on to the chromatoplates, which were developed with 10% phosphomolybdic acid in ethanol or in iodine vapour in order to make the components visible. Mixtures of different bile acids and fat were also run for separation.

Quantitative aspects of the separation technique

Mixtures of lipids and radioactively labelled bile acids were prepared in the same way as described above. The labelled bile acids included [^{14}C]cholic acid (Philips-Duphar, The Netherlands), [^{14}C]lithocholic acid (New England Nuclear, U.S.A.), sodium-[^{3}H]taurocholate (New England Nuclear, U.S.A.) and [^{14}C]chenodeoxycholic acid (Philips-Duphar, The Netherlands). After the run, the silica gel was scraped off over a length of 2 cm at the starting line, at the zone corresponding to the R_F values of the bile acids and at the front where the lipids are located. The remaining zones were also removed and examined for radioactivity. The bile acids were washed out of the silica gel with five 7-ml volumes of methanol. After evaporation of the methanol in a rotating evaporator, the bile acids were dissolved in 1 ml of methanol; 0.5 ml of this solution was added to 5 ml of Insta Gel (Packard Instrument Co., U.S.A.) and counted in a liquid scintillator, Series 314 A (Packard Instrument Co., U.S.A.). Quench correction was carried out by the internal standard method using [^{3}H]toluene and [^{14}C]toluene (Packard Instrument Co., U.S.A.).

Application of the method to the analysis of the bile acids in human blood serum

To 5 ml of blood serum of thirteen normal subjects, 50 ml of 3:1 ethanol–diethyl ether was added, the mixture was vigorously shaken, centrifuged at 4000 r.p.m. for 10 min (MSE Super Minor centrifuge, universal angle rotor, $g = 2{,}600$), and the upper phase was decanted off. A 50-ml volume of 3:1 ethanol–diethyl ether was added to the sediment and the mixture was centrifuged. The upper phases were combined and evaporated. From the residue obtained, the bile acids were extracted in two steps: firstly with two 0.5-ml volumes of 3:1 ethanol–diethyl ether and secondly

TABLE I

R_F VALUES OF SINGLE BILE ACIDS (TWO DETERMINATIONS)

Bile acid	*Free acid*	*Glyco-*	*Tauro-*
LC	0.56–0.53	0.51–0.47	0.56–0.52
DC	0.46–0.44	—	0.51–0.47
CDC	0.46–0.45	0.47–0.45	0.50–0.46
C	0.43–0.42	0.46–0.42	0.44–0.41
HDC	0.47–0.45	—	—

with 0.5 ml of ethanol. The extracts were combined and 1 ml of this solution was spotted in a short band. The remaining 0.5 ml of this solution was used to make the separation visible on another chromatoplate, which was run in parallel. The appropriate zones of the silica gel were removed, the bile acids were extracted with five 7-ml volumes of methanol, the solution was evaporated and the bile acids were analyzed enzymatically[15].

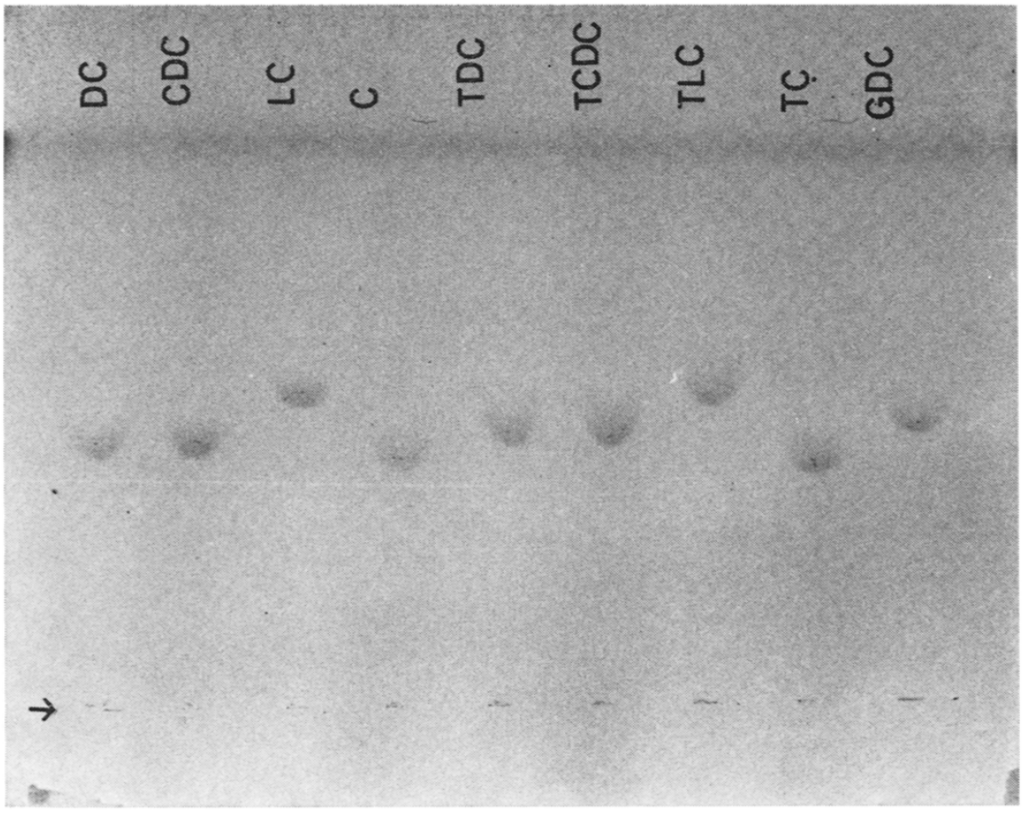

Fig. 1. Migration pattern of bile acids on Silica Gel G. Chloroform–methanol–ammonia (90:54:11) was used as the solvent. The following bile acids were spotted: DC, CDC, LC, C, TDC, TCDC, TLC, TC and GDC. The arrow indicates the starting point, the straight line corresponds to the front.

As a control, serum extracts of six patients were each divided into three equal parts. The first part was used to determine the bile acid concentration in serum. To the second and third parts, known amounts of sodium taurocholate (NaTC) and lithocholic acid were added. These bile acids were selected because of the differences in their physicochemical characteristics. Bile acids were determined enzymatically after separation according to the procedure described above.

RESULTS

Qualitative analysis

The R_F values of the bile acids cited above are given in Table I. All values lie between the extremes of 0.56 for lithocholic acid and 0.41 for sodium taurocholate. Fig. 1 shows that the free and conjugated bile acids move together in a relatively narrow band. From left to right, Fig. 1 shows the spots of deoxycholic acid, chenodeoxycholic acid, lithocholic acid, cholic acid, taurodeoxycholic acid, taurochenodeoxycholic acid, taurolithocholic acid, taurocholic acid and glycodeoxycholic acid; the arrow indicates the starting line. Fig. 2 illustrates the separation of lipids, litho-

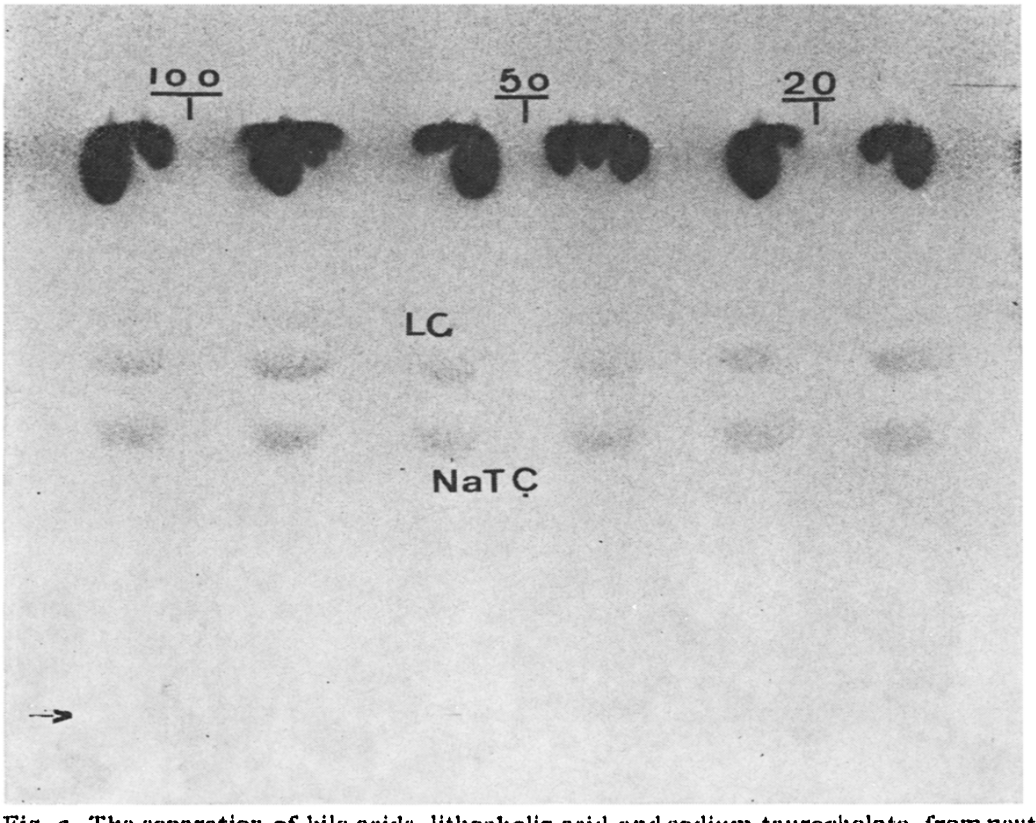

Fig. 2. The separation of bile acids, lithocholic acid and sodium taurocholate, from neutral lipids. From left to right, the fat:bile acid ratios are 100:1, 50:1 and 20:1; 30 µl of each of these fat–bile acid mixtures are spotted in duplicate. The arrow indicates the starting point and the lipids migrate to the front.

cholic acid and sodium taurocholate. From left to right, the six chromatograms correspond to two runs with 30 μl of a 100:1 fat–bile acid solution, two runs with 30 μl of a 50:1 solution and two runs with 30 μl of a 20:1 solution. In all these runs, the bile acids stay close together and are well separated from the neutral lipids.

Quantitative analysis

The recovery of radioactive bile acids is nearly complete in the zone corresponding to the R_F values of the bile acids: 95.5% ± 0.5 S.D. ($N = 11$). Not more than 1.1% ± 0.2 S.D. ($N = 11$) of the radioactivity remains at the starting line and

TABLE II

BILE ACIDS IN HUMAN SERUM

Males		*Females*	
Initials	*Amount (μmole/l)*	*Initials*	*Amount (μmole/l)*
Y.G.	3.5	V.V	8.7
G.V.	5.2	V.S.	5.6
W.H.	6.3	M.B.	9.4
H.K.	2.4	G.G	6.7
G.C.	3.2	B.V.	2.8
J.H.	1.5	J.M.	3.7
		T.D.	10.6

2.2% ± 0.4 S.D. ($N = 11$) moves with the lipid fraction. The mean loss of radioactivity at other levels of the chromatoplates is about 1%.

Bile acids in human blood serum

Table II summarizes the bile acid concentration in the blood serum of a series of normal male and female subjects. To check the recovery of the procedure, serum from six patients was analyzed before and after the addition of known amounts of sodium taurocholate and lithocholic acid. The results are given in Table III.

TABLE III

RECOVERIES OF SODIUM TAUROCHOLATE AND LITHOCHOLIC ACID FROM SERUM EXTRACTS AFTER SEPARATION BY THIN-LAYER CHROMATOGRAPHY

Determinations were carried out enzymatically, and the concentrations are expressed in μg of bile acid per 5 ml of serum.

Patient	*Bile acid in serum (μg/5 ml)*	*Amount of bile acid added (μg)*		*Total bile acid determined (μg)*		*Recovery of bile acid (%)*	
		NaTC	*LC*	*NaTC added*	*LC added*	*NaTC*	*LC*
S.V.	9.5	22.5	30.2	32.6	34.7	102.6	83.4
I.S.	9.6	22.5	30.2	30.5	35.5	92.9	85.8
H.V.	12.9	22.5	30.2	33.1	40.8	89.8	92.4
K.M.	22.5	22.5	30.2	44.2	51.9	96.4	97.4
A.D.	19.2	25.0	30.2	42.4	47.0	92.8	92.1
E.B.	0	25.0	—	25.1	—	100.4	—

DISCUSSION

Bile acids in biological material cannot be analyzed quantitatively unless they are separated from other substances in this material. The separation of bile acids from neutral lipids is hampered by the fact that they may be present as free or conjugated acids and as mono-, di- or trihydroxycholanic acids, which have different physicochemical properties.

The present method permits the isolation of the main bile acids from neutral lipids by thin-layer chromatography. It yields one narrow band in which both free and conjugated bile acids are close together and whose R_F values can be reproduced easily. This separation technique permits very good recoveries. Labelled bile acids were retrieved at the rate of 95.5%. The enzymatic tests of known amounts of bile acids added to the serum extracts gave results of the same order of magnitude, averaging 95.8% for sodium taurocholate and 90.2% for lithocholic acid. These bile acids were chosen because of their different physicochemical characteristics. In addition, the values of bile acid concentrations in human serum correspond to those reported in the literature[16]. This method is simple and time-saving, especially in the study of biological fluids in which bile acids are present in fairly high concentrations. Duodenal and jejunal contents can thus be spotted directly on to the chromatoplates in small amounts. All bile acids are separated from the lipids and are present in sufficiently high concentrations to permit further analysis. The method by-passes extractions that might lose the most lipophilic bile acids in the neutral lipid fraction. It also avoids preliminary procedures that are sometimes performed, such as hydrolysis of the bile acids. These results indicate that the present method might be very useful in the quantitative separation of both free and conjugated bile acids from biological material, especially from lipids.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the skillful technical assistance of Miss S. RUTTEN and Miss L. VANDENBULCKE.

This work was supported by Grant No. 20054 of the F.W.G.O., Brussels, Belgium.

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