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A METHOD FOR THE ESTIMATION OF BILE ACID CONJUGATES AND BILE ACIDS IN BIOLOGICAL FLUIDS

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SUMMARY

A method is described for the determination of bile acids and bile acid conjugates in biological fluids. Bile acids separated on thin-layer chromatography are delineated by a pyrene spray. The pyrene is removed and the bile acids estimated by a modified Pettenkofer reaction.

INTRODUCTION

Several methods have been described for the separation and estimation of bile acids either in plasma or in duodenal contents[1]. The methods vary from the rapid and non-specific to highly specific, but ponderous procedures.

The method described in this paper utilises thin layer chromatography (TLC). It is a sensitive method and can be used for the estimation of bile acids, free or conjugated in duodenal contents or in plasma.

EXPERIMENTAL

Material

Silica gel (Hopkin and Williams, Silica Gel M. F. C. without binder). It must be cleaned from certain contaminants especially iron which can inhibit the Pettenkofer reaction and which lowers the sensitivity of the method. Other substances which are soluble in ethanol also inhibit the Pettenkofer reaction. The silica gel is prepared by washing 500 g with 50 % hydrochloric acid 3–4 times over a period of 24 h. The silica gel is then washed to neutrality using redistilled water, the system being allowed to settle and the supernatant decanted after 2–3 h. In this way undesirable fines are removed. The silica gel is then further washed with boiling ethanol, after filtration it is dried in an oven overnight at 100° and stored until used. The iron content after this treatment is less than 5 μg per g of silica gel.

The glass plates are kept in chloroform between use to ensure removal of all lipid material which may adhere to the plate.

The silica gel is prepared for application to the plates by sieving through a 120-mesh sieve with 20 % by weight of anhydrous calcium sulphate. The anhydrous calcium sulphate is previously boiled in ethanol followed by drying in an oven at 150°. To

the sieved silica gel–calcium sulphate 2 volumes of distilled water are added and the mixture is stirred until a smooth paste is obtained. This paste is spread on the glass plates using a Shandon spreader giving a thickness of 600 μ. The plates are left in the air to dry for 2–3 h and stored in a desiccator until used. If the plates are allowed to dry slowly in the air, the background reading is small. High background readings occur if the plate is heated before adequate drying is achieved. The prepared plates are heated in an oven at 100° for 30 min before use.

Methods

Serum extraction. The serum (1–2 ml) is extracted with 5 volumes of isopropanol at 65° for 30 min under reflux and the extract centrifuged at 3000 r.p.m. for 10 min. The supernatant is decanted and the precipitated protein extracted twice with 1–2 volumes of isopropanol. The pooled isopropanol extracts are evaporated to dryness under nitrogen and the residue completely redissolved in 2.5 *M* sodium hydroxide (10 ml) with gentle warming. The non-polar lipids are removed from this solution by extraction with 3 volumes of petroleum ether (b.p. 60°–80°).

The bile acid conjugates which remain in solution are hydrolyzed in nickel crucibles in an autoclave for 3 h at 110° under a pressure of 15 lbs./in.^{2} (1 kg/cm^2). The hydrolysate is then acidified to pH 4 using 1 *N* hydrochloric acid and extracted with petreoleum ether(b.p. 60–80°) to remove other lipids.

Bile acids are removed by extracting three times with equal volumes of freshly distilled peroxide-free diethyl ether. The ether extract is washed to neutrality with distilled water, which in turn is back-washed with ether. The pooled ether extracts are taken to dryness under nitrogen and redissolved in a small volume of ethanol. The ethanolic solution is applied as a narrow band to a thin-layer plate coated with silica gel, 600μ thick.

Duodenal contents extraction. The duodenal contents (1–2 ml) are extracted at room temperature with 9 volumes of chloroform–methanol (9:1), and the protein filtered from the organic phase. An aliquot is taken from this chloroform–methanol extract and applied directly to the thin-layer plate as described above.

Chromatography. When samples are applied, the plate must not be heated as some of the bile acids are adsorbed to the silica gel with consequent loss in estimation as these adsorbed bile acids do not move in the chromatographic system. The plate is first developed in a chloroform–methanol (9:1) system to separate contaminants from the bile acids and conjugated bile acids which remain at the origin. The plate is then dried under cold air. Bile acids are separated with an isooctane–ethyl acetate–acetic acid system (10:10:2) (ref. 2). Bile acid conjugates are separated using the system amyl acetate–*n*-propanol–propionic acid–water (20:10:15:8).

After removal of the solvents from the thin-layer plate, it is sprayed with 0.05% pyrene in petroleum ether (b.p. 60–80°) and the bile acids and bile acid conjugates are visualised under UV (350 mμ) (ref. 3). The outline of the areas occupied by bile acid conjugates are then lightly marked out as dots with a pin. The pyrene is removed to the top of the plate using the chromatographic system chloroform–petroleum ether (b.p. 60–80°) (9:1). The bile acids and bile acid conjugates do not move in this system. Standard mixtures of bile acids and bile acid conjugates are run on each thin-layer plate to facilitate identification and to enable standard graphs to be drawn.

The bile acid areas and bile acid conjugate areas are scraped from the plate into

centrifuge tubes and other areas are removed for comparison as blanks. The quantities of bile acids and bile acid conjugates are estimated using the Pettenkofer reaction described below.

The Pettenkofer chromogen. This is essentially the method developed by REID which is described in some detail by BOYD *et al.*[4]. 5 ml of 70 % sulphuric acid is added to the centrifuge tubes containing the silica gel and left after mixing at 42 ± 2° for 10 min. 1.0 ml of a 0.25 % solution of furfural is then added, the contents are thoroughly mixed and allowed to stand for 1 h. The tubes are then centrifuged for 25 min at 3500 r.p.m., the supernatant removed with a Pasteur pipette and the colour read at 510 mμ, 90 min after addition of the furfural. The amount of free bile acid or bile acid conjugate is estimated by comparison with a calibration graph obtained from TLC of known amounts of the appropriate bile acids. (Fig. 1).

RESULTS

The presence of iron has been shown to inhibit the Pettenkofer reaction. Table I shows the effect of different amounts of ferric chloride on the optical density produced by the Pettenkofer reaction on cholic acid, chenodeoxycholic acid and deoxycholic acid.

The presence of the silica gel in the reaction tube was shown not to affect the Pettenkofer reaction (Table II).

Recovery experiments were carried out using taurocholic acid, glycochenodeoxycholic acid and taurodeoxycholic acid and the results are shown in Table III.

Table IV shows the results of serum bile acids, measured by this technique, in five patients in whom there was no clinical or biochemical reason to suspect liver disease.

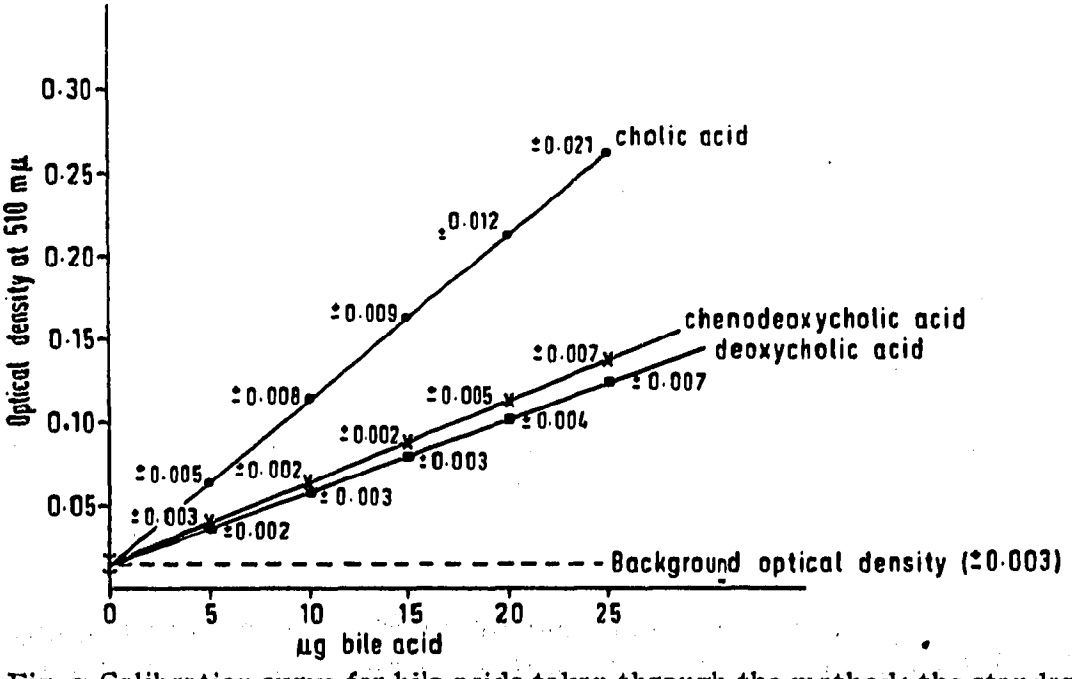

Fig. 1. Calibration curve for bile acids taken through the method; the standard error of the results (n = 10); and the mean background optical density.

TABLE I

THE EFFECT OF THE ADDITION OF FERRIC CHLORIDE TO BILE ACIDS (50 μg), AND THEIR CHROMOGEN IN THE PETTENKOFER REACTION

Optical density measured on Unicam SP 600 at 510 mμ.

Ferric chloride added μg (Fe^{3+})	*Cholic acid*	*Chenodeoxycholic acid*	*Deoxycholic acid*
	O.D.	*O.D.*	*O.D.*
210	0.311	0.101	0.166
105	0.420	0.205	0.186
21	0.531	0.306	0.202
10.5	0.560	0.319	0.240
4.2	0.590	0.371	0.290
0	0.630	0.390	0.330

TABLE II

THE EFFECT OF IRON-FREE WASHED SILICA GEL ON THE CHROMOGEN PRODUCED BY THE PETTENKOFER REACTION

Silica gel was added to a solution of 24 μg of bile acid, the gel was dried in an oven. The Pettenkofer chromogen was developed and the chromogen read after centrifugation.

Optical density measured on Unicam SP 600 at 510 mμ

Weight silica gel (g)	*Taurodeoxycholic acid*	*Taurochenodeoxycholic acid*	*Taurocholic acid*
	O.D	*O.D.*	*O.D.*
1.0	0.190	0.258	0.206
1.5	0.193	0.250	0.200
2.0	0.185	0.256	0.225
2.5	0.173	0.238	0.233
3.0	0.162	0.280	0.210

TABLE III

RECOVERY EXPERIMENTS WITH CONJUGATED BILE ACIDS MEASURED AS FREE ACIDS AFTER THE FULL ANALYSIS

Analysis consisting of TLC, Pettenkofer reaction, and chromogen measuring on Unicam SP 600 at 510 mμ. Background absorption (from 35 plates) average 0.018.

Conjugated bile acids	*Recovery (%) ± standard-deviation (n = 6)*	
	20 μg added	*5 μg added*
Taurocholic acid	86 ± 11.6	110 ± 9
Glycochenodeoxycholic acid	88 ± 8.4	87 ± 19
Taurodeoxycholic acid	89 ± 12.0	109 ± 21

DISCUSSION

We have used TLC to separate bile acids and bile acid conjugates principally because it is rapid and does not require the preparation of any bile acid derivatives before the chromatography stage. Furthermore with the technique described it is

TABLE IV

SERUM BILE ACIDS IN INDIVIDUALS FREE FROM LIVER DISEASE, MEASURED BY TLC–PETTENKOFER CHROMOGEN

Ages of the individuals, 23–37 years; 5 ml serum, 1–2 h after breakfast, were estimated.

Individual	*Cholic acid (mg/100 ml)*	*Chenodeoxycholic acid (mg/100 ml)*	*Deoxycholic acid. (mg/100 ml)*
1	0.16	0.18	0.26
2	0.14	0.14	0.29
3	0.12	0.18	0.16
4	0.11	0.18	0.25
5	0.08	0.17	0.23

possible to achieve separation of bile acids and bile acid conjugates from other lipids which may interfere with or enhance the chromogen obtained.

One disadvantage of TLC is the contribution of "fines" to the spectrophotometer readings resulting in high blank values. This has been overcome in this method by the manner in which the silica gel is prepared. Another possible disadvantage of TLC is the presence of iron. This method ensures that the silica gel is free from iron and so allows greater sensitivity.

It is essential also that bile acids are located accurately on the thin-layer plate. Various methods described have utilised a water spray but this is insensitive and tends to blur the spots for compounds with only slightly dissimilar R_F values, *e.g.* chenodeoxycholic acid and deoxycholic acid. The pyrene spray described in this method is sensitive and does not destroy or affect the R_F of the bile acids. It is essential, however, that the pyrene is recrystallized before use, as oxidation products of the pyrene can affect the ease with which pyrene is removed from the areas in which the bile acids are located.

The Pettenkofer reaction is sensitive for cholic acid, chenodeoxycholic acid, deoxycholic acid and hyodeoxycholic acid and their taurine and glycine conjugates, but unfortunately this colorimetric method is of no use in the estimation of the muricholic acids or the monohydroxylated acids. This method is simple, accurate and enables a measurement to be made of the principal bile acids and bile acid conjugates in the peripheral blood or the duodenal contents without the need for internal standards.

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