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Note

Quantitative separation of bile salts from mixtures by gradient-elution high-pressure liquid chromatography

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It is being increasingly recognised that bile acids play a major role in the aetiology and course of many human diseases [1, 2]. The progress of investigation in this field, however, has been considerably hampered by the lack of a simple method for fractionating the complex bile salt mixtures found in biological materials. In earlier work liquid chromatography was used, but more recently the two main approaches have been to employ gas—liquid chromatography or thin-layer chromatography (TLC). The former necessitates elaborate processes for extraction and purification in addition to the preparation of volatile derivatives, whilst TLC requires the use of time-consuming elution procedures before further analysis can be undertaken. High-pressure liquid chromatography (HPLC) has two inherent advantages over the above techniques; it not only provides comparable resolution in a relatively short time, but it also provides the resolved fractions as eluates free from silicic acid or other chromatographic medium.

The first report of the application of HPLC to the separation of conjugated bile salts appeared as recently as 1976 [3]. Although encouraging, the results showed that human bile contains substances other than bile salts having mobilities in HPLC similar to those of the bile salts themselves. It was therefore essential that the quantitation steps following HPLC should be such as to ensure the specificity of the entire procedure. This publication was succeeded in 1977 by a paper from Parris [4] who demonstrated that reversed-phase HPLC with aqueous methanol as solvent could separate isomers of bile acids and of their conjugates. The same author showed the potential for detection of absorptiometers operating at very short wavelengths of 210 nm or less. However, the loads treated were very small, precluding recovery for confirmatory analysis, while application to clinical samples appears still to be remote. Such difficulties have been obviated by the application of gradient-

elution techniques to HPLC with silicic acid in order to resolve biological bile salt mixtures into fractions suitable for analysis by specific enzyme methods.

EXPERIMENTAL

Apparatus and materials

Stainless-steel 50 × 0.2 cm I.D. columns were packed with small-sized spherical particles coated with chemically bonded silicic acid (Vydac, 30—44 μm; Anachem, Luton, Great Britain) or with a similar pellicular material (Perisorb A, 30—40 μm; Merck, Darmstadt, G.F.R.). Samples were introduced onto the column by means of a 20-μl injection loop attached to a high-pressure change-over valve (Anachem; Fig. 1). The developing solvent was introduced by means of a Milroy pump (Milton Roy, St. Petersburg, Fla. U.S.A.) rated at 3000 p.s.i., equipped with a relief valve (V in Fig. 1; constructed in the laboratory and set to discharge at 2500 p.s.i.). A second Milroy pump was used to form the liquid gradient.

All solvents used were of Analytical Reagent Grade and obtained from BHD (Poole, Great Britain). No attempt was made to remove from the chloroform any ethanol added for stabilisation. Non-radioactive bile salts were obtained

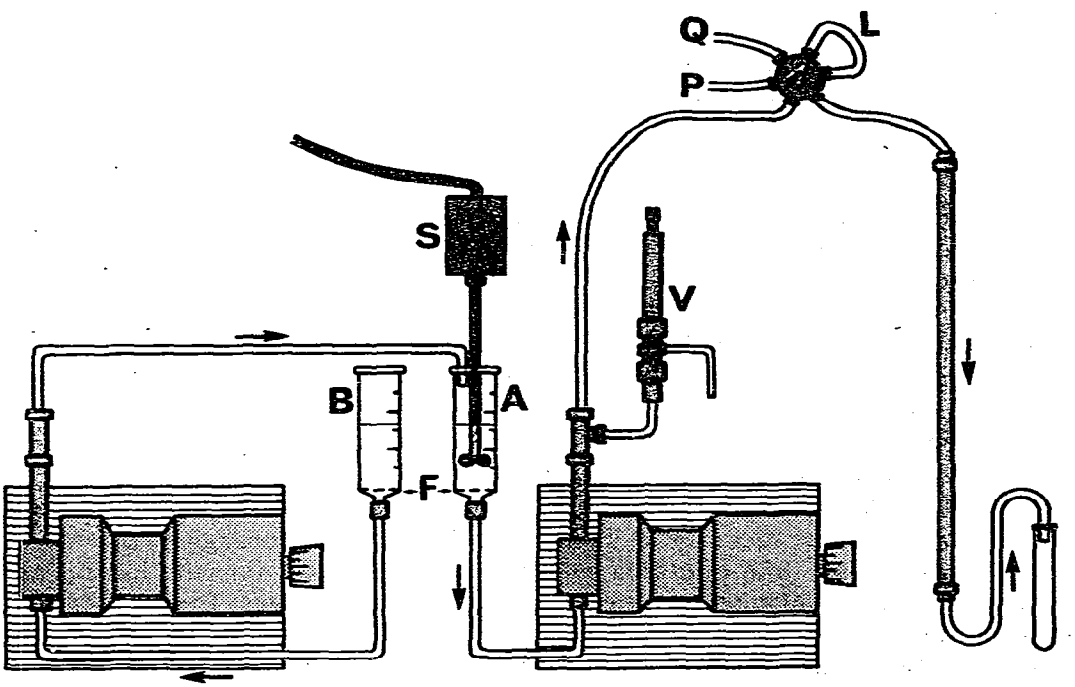

Fig. 1. System for HPLC with polarity gradient. Reservoirs A (in which the gradient forms) and B are glass 20-ml syringe barrels connected to the pumps by PTFE tubing (O.D. 3.2 mm). S is a stirrer. Reservoirs are fitted with paper filters, F. Other tubing is stainless steel of bore 0.25 mm; joints are Swagelok fittings. Loop L is loaded at P from a 100-μl syringe, excess solution being visible in the transparant tube Q while the eluent passes directly to the column. When L is placed in circuit, excess load solution can be withdrawn at P. V, relief valve.

from Maybridge, Tintagel, Great Britain, and from Steraloids (Pawling, N.Y., U.S.A.), except for glycochenodeoxycholic acid, glycocholic acid and chenodeoxycholic acid, which were kindly donated by Weddel Pharmaceuticals. The purity of each substance was assessed by TLC on silica gel G using ethylene dichloride—acetic acid—water (1:1:0.1) [5] as developing agent. Qualitative assessment was made after using sulphuric acid—ultraviolet light for detection [6]. Quantitative results were obtained by using 3α- and 7α-hydroxysteroid dehydrogenases [7, 8] following non-destructive location with iodine vapour. A stock solution (2 m*M* in methanol) of glycochenodeoxycholic acid was prepared directly from a bile acid sample which had been found to be not less than 95% pure. The other bile salts were first purified by TLC and methanolic eluates of the corresponding areas were calibrated using the enzyme method with the glycochenodeoxycholic acid solution as standard.

Radioactive bile acid preparations, [carboxyl-^{14}C] chenodeoxycholic acid, [1-^{14}C]glycocholic acid, and tauro[carbonyl-^{14}C] cholic acid were obtained from The Radiochemical Centre (Amersham, Great Britain).

Methods

All columns were equilibrated with 15 ml chloroform before each run and the flow-rate was assessed by timing the passage of 10 ml of this solvent. The output rate of the second pump was adjusted to the same value.

Bile acids were extracted from bile-rich duodenal aspirates using Amberlite XAD-2 resin on a batch procedure as described by Barnes and Chitranukroh [9]. Some 200—600 μg bile salts (20 μl methanolic solution) were applied to the column and chloroform passed for 0.5 min. The gradient elution was then commenced and half-minute fractions were collected.

The gradient was formed as follows: solution A (chloroform) of volume V, was placed in the reservoir A (Fig. 1) and solution B (ethyl acetate—ethanol, 40:15) added at the same rate (r; approx. 1 ml/min) as that at which the reservoir contents were transferred to the column. The proportion of solvent B in the reservoir A at time t (min) is given by $1\text{-}e^{-(r/V)t}$ where V is the volume (ml) at which the reservoir contents are maintained. In the present context suitable values for r were found to be in the range 0.5—1.1 ml/min, with V ranging from 5 to 15 ml; all components of the mixtures examined then eluted within 30 min. It may be shown that $(V/r)\ln 2 = \lambda$, where λ is the half-life characterising the gradient, and is the time (min) required to produce in the reservoir a mixture comprising equal volumes of A and B. Since the value for r was fixed on chromatographic criteria, V was calculated so as to obtain a half-life of 4—15 min, this range having been determined as optimal in prior experiments with standard mixtures.

In qualitative studies the fractions obtained were evaporated to dryness under a steam of nitrogen with the tubes in water at 60°, the residues were dissolved in methanol—chloroform (3:1) and subjected to TLC using ethyl acetate—ethanol—acetic acid (40:20:2) as mobile phase and with phosphomolybdic acid for detection. In quantitative studies the fractions obtained were evaporated to dryness and assessed using, for bile extracts, the enzymatic methods mentioned above.

RESULTS

Qualitative studies with various mixtures of bile salt standards indicated that with a gradient half-life of 4—8 min, satisfactory resolution of the common bile acids could be obtained within 30 min. Bile salts were clearly separated according to whether they were taurine- or glycine-conjugated, and di- or trihydroxy acids (Table I). The maximum amount of bile acid mixture that could be applied to the present column if "tailing" and other overload effects were to be avoided was about 500 μg for roughly equal amounts of six components.

With human duodenal aspirates it was found essential that some initial extraction be made, and in all subsequent work the method of Barnes and Chitranukroh [9] was used. Bile acids were assayed before extraction and in each half-minute fraction obtained from HPLC. In this way it was possible to monitor the recovery. In six duodenal aspirates values for total 3α-hydroxy bile salts obtained by summation of the HPLC fractions were some 92.3±1.7 (S.D.) % of those obtained by analysis of the unchromatographed extracts.

The absence of cross-contamination between eluted peaks was demonstrated by the addition of radioactive bile acids (approx. 0.1 μCi) to each of

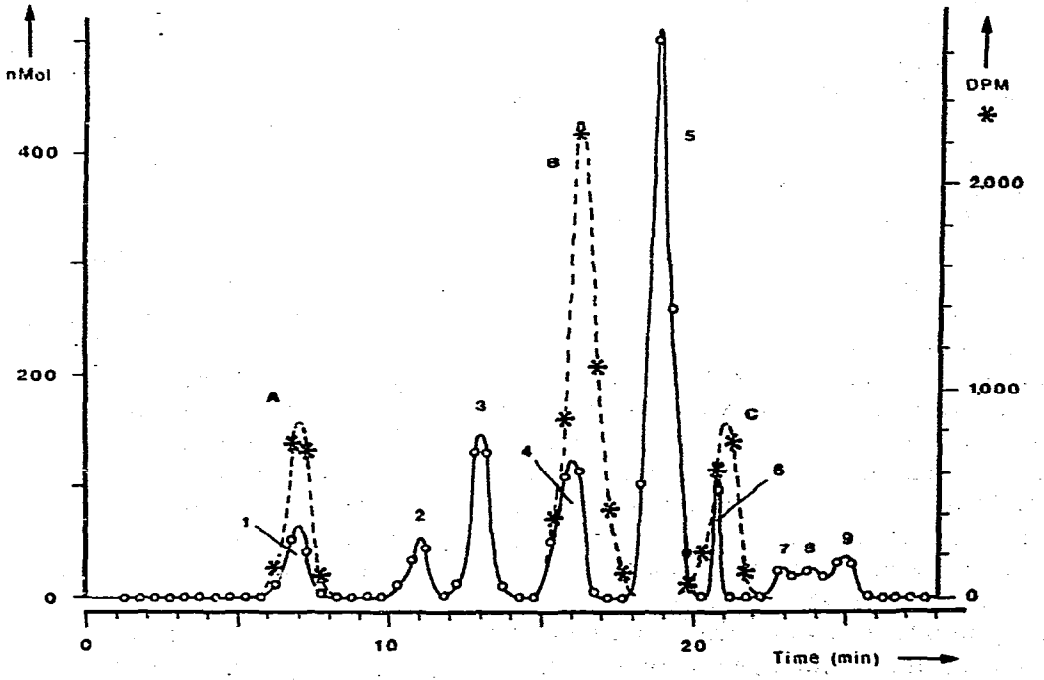

Fig. 2. Separation by gradient HPLC with Perisorb A (30—40 μm) of extract from bile-rich duodenal fluid. Peaks: 1 = dihydroxy bile salts; 2 = cholate; 3 = taurine conjugated dihydroxy bile salts; 4 = taurocholate; 5 = glycochenodeoxycholate; 6 = glycocholate; 7—9, unknown; A = [^{14}C]chenodeoxycholic acid; B = tauro[carbonyl-^{14}C]cholic acid; C = [1-^{14}C]glycocholic acid. Curves drawn through the points conform with those seen on the absorption patterns (280 nm) obtained by chromatography, on the same column and in similar conditions, of aromatic solutes.

TABLE I

SEPARATION OF COMPONENTS OF A 6-PART MIXTURE OF BILE SALTS

Load, 800 μg total. A: Perisorb A; gradient half-life, 3.4 min; flow-rate, 0.79 ml/min. B: Vydac; gradient half-life, 8 min; flow-rate, 0.78 ml/min. V_e = elution volume.

Substance	A		B	
	V_e(ml)	t(min)	V_e(ml)	t(min)
Na deoxycholate	6.2	7.9	3.2	4.1
Cholic acid	8.1	10.3	5.5	7.1
Na taurodeoxycholate	10.7	13.5	8.8	11.3
Na taurocholate	13.1	16.6	11.4	14.6
Na glycodeoxycholate	15.5	19.6	12.9	16.5
Na glycocholate	16.9	21.4	18.1	23.2

three samples. Recovery was 96.8±1.4% and occurred in a single peak. To one sample all three radioactive labels were added, the conjugated markers being eluted with the corresponding bile acids in the sample (Fig. 2).

As part of an otherwise unrelated investigation [10] the columns presently described were used in similar conditions for chromatography of esters of phthalic acid and of cholesterol and its esters. In that work, effluent was passed directly through the cell of a Uvicord II monitor (LKB) before collection, thus providing a continuous strip-chart of absorption at 281 nm. Sharp symmetrical peaks of Gaussian form were seen; this evidence was considered to justify the drawing of the continuous curves in Fig. 2 through the scattered points actually obtained.

DISCUSSION

The success of any method for the quantitative resolution of the bile acid mixture present in biological samples depends on the homogeneity of each fraction and the specificity of the final reaction. With the possible exception of gas chromatography—mass spectrometry (GC—MS) [11] few of the methods described to date fulfil these criteria. GC—MS, however, necessitates the preparation of volatile derivatives and the use of technically exacting and time-consuming techniques [12]. Even then, the possibility of the formation of artefacts during these chemical preparative steps is not easily precluded. All previously established methods for fractionating bile acid mixtures are compromises and clearly that which we now describe is no exception. The level of specificity now attained, however, is comparable with that achieved with TLC in conjunction with 3α-hydroxysteroid dehydrogenase [13]. Its advantages over TLC are those of higher resolution and rapidity, and reside also in the fact that the fractions are obtained directly as eluates in solvents that are free of silicic acid, volatile and which do not react with bile salts.

The resolution obtained is similar to that described by Shaw and Elliot [3] and, very recently, by Parris [4]. Obviously, our use of discrete fractions must confer the effect of broadening the peaks, and thus of impairing resolution. This effect could be reduced by taking fractions at intervals of less

than 0.5 min, or would be avoided entirely if a flow-through detector could be employed. Application of short-wave ultraviolet absorption, the feasibility of which was established by Parris [4], does however impose crippling limitations on the choice of solvents, as that author points out. On balance, however, it might appear that the useful future for HPLC in the analysis of bile salts could be exploited most profitably by reserving maximum flexibility of conditions, meanwhile taking advantage of the highly specific enzymatic or radioimmunological methods already available.

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