Journal of Chromatography, 282 (1983) 603–608
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 138

MEASUREMENT OF CONJUGATED BILE ACIDS BY ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

H. J. WILDGRUBE*, U. FÜSSEL, H. LAUER and H. STOCKHAUSEN
Zentrum der Inneren Medizin der Johann Wolfgang Goethe-Universität Franfurt/Main, Abteilung für Gastroenterologie, Theodor-Stern-Kai 7, D-6000 Frankfurt/Main 70 (F.R.G.)

SUMMARY

The quantitatively most important conjugated bile acids in man were separated by reversed-phase ion-pair chromatography without prior derivatization. As non-polar matrix an Ultrasphere I.P. column (C_{18}) was used, and an ionic alkyl compound, tetrabutylammonium phosphate, was added to the mobile phase, which was a mixture of acetonitrile and water. Under these conditions, the glycine and taurine conjugates of cholic acid, chenodeoxycholic acid and deoxycholic acid were separated within 15 min. At 214 nm, the minimum measurable concentration was 1.3–2.0 nmol/ml. The average recovery from bile was 94%. In ten patients with biliary drainage an average of 79.5% of the bile acids were glycine conjugates.

INTRODUCTION

Bile acids have been determined by thin-layer chromatography (TLC), gas–liquid chromatography and radioimmunological tests with variable success. After the first reports on bile acid analyses by high-performance liquid chromatography (HPLC) in 1977, several researchers attempted an optimization of this method[1–10], especially with regard to separation efficiency, sensitivity and analysis time. Most authors have applied reversed-phase columns. (RP-18) and acidic alcoholic eluents for the separation of underivatized metabolites. However, some studies have shown that these methods do not meet the requirements of routine analysis. We have applied ion-pair chromatography on a reversed-phase column on the basis of the physicochemical behaviour of bile acids.

EXPERIMENTAL

Instrumentation

The apparatus used was a Beckman high-performance liquid chromatograph (Model 110 A pump, Model 210 injection valve with 20-μl sample loop and Model 165 detector at 214 nm, Beckman Instruments, Berkeley, CA, U.S.A.), equipped with a Model C-R1A integrator (Shimadzu, Kyoto, Japan).

0021-9673/83/$03.00

Materials

Sodium salts of conjugated bile acids were from commercial sources: glycocholic acid, taurocholic acid, glycochenodeoxycholic acid, taurochenodeoxycholic acid, glycolithocholic acid and taurolithocholic acid (Calbiochem-Behring, Giessen, F.R.G.), taurodeoxycholic acid (P-L. Biochemicals, Milwaukee, WI, U.S.A.) and glycodeoxycholic acid (Supelco, Bellefonte, A, U.S.A.). Their purity was checked by TLC prior to use. Tetrabutylammonium phosphate (0.5 l/l) was supplied by Beckmann Instruments. Acetonitrile and methanol (LiChrosolv) were from E. Merck (Darmstadt, F.R.G.) For filtration a Millex filter, 0.22 μm (Millipore, Molsheim, France) was used.

Chromatography

An Ultrasphere I.P. column (250 × 4.6 mm) (Beckmann Instruments) was used under ambient conditions. The eluent was prepared by mixing 45 ml double-distilled water, 50 ml acetonitrile and 1 ml 0.5 *M* tetrabutylammonium phosphate. The mobile phase was filtered through a Millex filter and degassed. Conditions: flow-rate, 1.0–1.3 ml/min; pressure, 1500–2300 p.s.i.; temperature, 40°C, isocratic flow. The UV monitor was set to range 0.1 (a.u.f.s.). Peaks were quantified by triangulation.

Sample preparation

Pure bile salts were diluted in methanol. Bile (1 ml) was diluted in methanol (2 ml), centrifuged and filtered through a Millex filter.

RESULTS

The glycine and taurine conjugates of the four bile acids that are quantitatively important in humans were separated within 15 min (Fig. 1 and 2). Conjugated bile acids were eluted according to the number of hydroxyl groups in the cholanic acid. Glycine- and taurine-conjugated cholic acid (trihydroxycholanic acid) were followed by the dihydroxy- and monohydroxycholanic acids. Glycine and taurine conjugates of chenodeoxycholic acid and deoxycholic acid were well separated, as can be seen from the capacity factors (Table I). For each of the eight bile salts the sample volume applied was plotted against the area of the chromatogram (detector sensitivity 0.1 a.u.f.s.). With concentrations up to 12 pmol/ml of each bile acid a linear relation (regression coefficient 0.99) was found (Fig. 3). By diluting a mixture of eight pure bile acids the lowest measurable concentration was determined. With a detector sensitivity of 0.01 (a.u.f.s.) at 214 nm, concentrations above 1.3 nmol/ml could be measured from di- and trihydroxylated bile salts at a signal-to-noise ratio of 2:1. Under these conditions the minimum measurable concentration for lithocholic acid conjugates was 2.0 nmol/ml.

The average recovery, measured by addition of pure bile salts to bile, was 94% (S.D. 3%). Furthermore, the reproducibility was determined by repeated analyses of the synthetic mixture of eight bile salts. The intrassay coefficient of variation, calculated from ten chromatograms in sequence, was 0.8% for the capacity factor and 14.2% for the area. The interassay coefficient of variation ($n = 10$) was 6.9% for the capacity factor and 14.2% for the area. One column was still usable after nearly 500 applications.

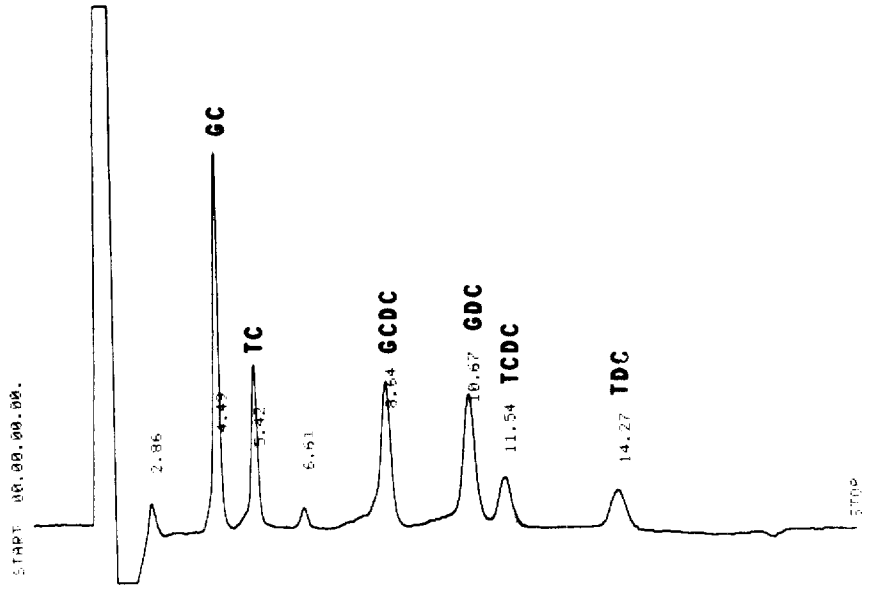

Fig. 1. Separation of a synthetic mixture of conjugated bile acids. Conditions as in Experimental. For abbreviations see Table I.

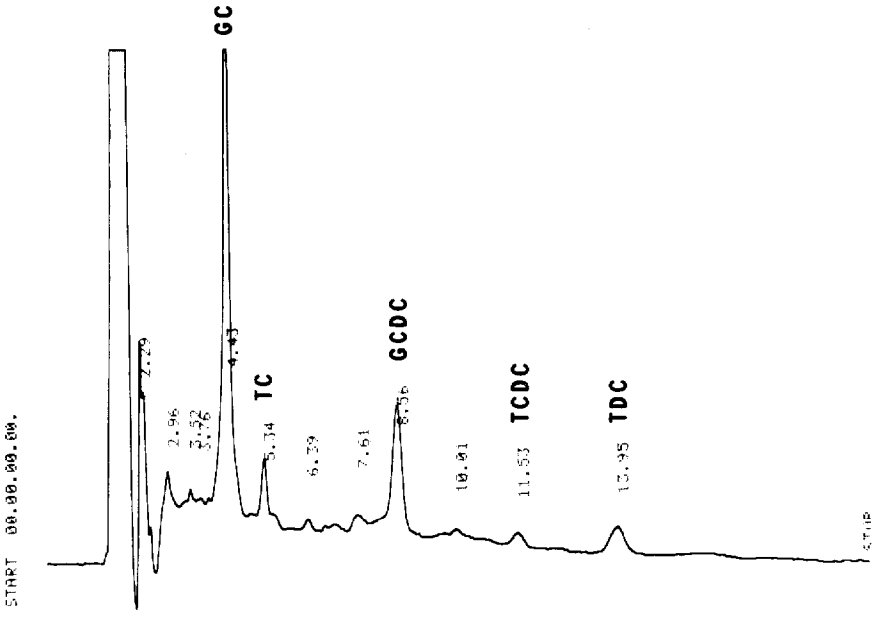

Fig. 2. Separation of conjugated bile acids from human bile. For abbreviations see Table I.

TABLE I

CAPACITY FACTORS, k', FOR EIGHT BILE ACID CONJUGATES

Conditions as in Experimental.

Bile acid	k'
Glycocholic acid (GC)	0.38
Taurocholic acid (TC)	0.53
Glycochenodeoxycholic acid (GCDC)	0.93
Glycodeoxycholic acid (GDC)	1.17
Taurochenodeoxycholic acid (TCDC)	1.30
Taurodeoxycholic acid (TDC)	1.62
Glycolithocholic acid (GLC)	3.18
Taurolithocholic acid (TLC)	4.52

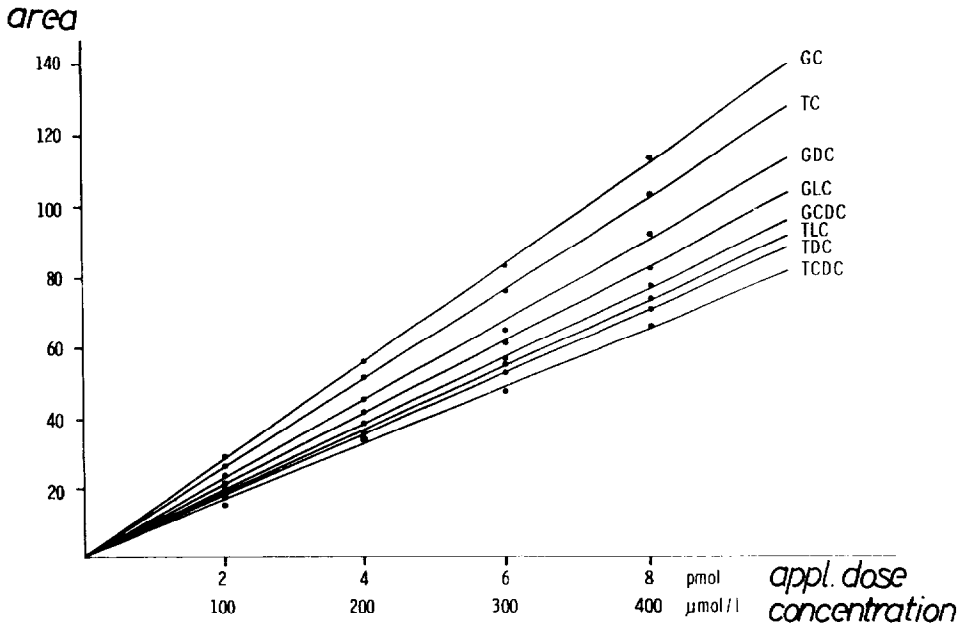

Fig. 3. UV (210 nm) detector response. For abbreviations see Table I.

DISCUSSION

Reversed-phase (RP) liquid chromatography on a chemically bonded organic stationary phase has become a widely used separation mode. It generally gives best results for non-polar, non-ionized metabolites, when the mobile phase is an aqueous organic solvent mixture in which the samples have different affinities for the polar mobile phase and the non-polar stationary phase. However, ionized substances are best separated on RP columns by means of further modifications of the procedure. One method is the so-called ion suppression mode. In this technique, the pH of the mobile phase is adjusted according to the pK of the substances to be analyzed. The undissociated metabolites may then be separated by common eluents. Ion suppres-

sion chromatography is often used for the analysis of bile acids. The majority of investigators have used methanol or propanol in phosphoric acid or phosphate buffer (pH 2–3.4) as eluents with reversed-phase materials[1–10]. Acidification of the mobile phase leads to various undesirable effects, *e.g.*, the functional groups of the column material may be partially covered by these acids, reducing its separation efficiency. Our investigations on the analysis of human bile with methanol and phosphoric acid or phosphate buffer as mobile phase have confirmed these experiences. Far more important, however, were the following observations: (a) in the usual elution period, up to 20% of the applied bile acids may remain on the column; (b) the bile acids retained on the stationary phase were only released after long stripping procedures, combined with the regeneration of the stationary phase; if the stripping procedure was omitted, some of the retained bile salts were eluted together with the next sample or at the end of the day as a complex bile acid mixture; (c) the lifetime of the column was uncommonly short; at pH 2 and high ionic strength, repeated analyses on LiChrosorb RP-18 (Merck) μBondapak C_{18} (Waters Assoc., Milford, MA, U.S.A.) and Ultrasphere ODS (Beckman) showed that, under the conditions given by Maruyama *et al.*[6], the plate number of the columns was reduced by 30–50% after only ten samples.

Hofmann[11] and Fransson and Schill[12] have adequately extraced bile acids as ion pairs with tetrabutylammonium phosphate. We have concentrated on another liquid chromatographic technique, which is known as ion-pair chromatography. This technique is based on the pioneering work of Schill and co-workers[13,14]. There have been many significant practical contributions to the technique[15–20], but the theoretical basis of ion-pair chromatography is still not fully understood[21].

Free and conjugated bile acids are amphipathic molecules. The pK values of these metabolites vary considerably. While the ionization of free bile acids is low (pK *ca* 6), it is increased by conjugation: either by the carboxyl group of glycine (pK *ca*

TABLE II

CONJUGATED BILE ACIDS IN HUMAN BILE (n = 10) OF PATIENTS WITH NASOBILIARY DRAINAGE

In most cases conjugated lithocholic acid was not detected.

	Cholic acid (%)		*Chenodeoxy cholic acid (%)*		*Deoxy cholic acid (%)*		*Remaining (%)*
	GC	*TC*	*GCDC*	*TCDC*	*GDC*	*TDC*	
1	30.1	7.0	29.1	10.4	15.6	3.9	3.9
2	52.4	2.1	22.6	5.4	13.9	0.5	3.1
3	31.6	1.5	44.8	–	18.4		3.7
4	35.5	8.0	29.5	7.3	9.7	0.1	9.9
5	36.1	5.5	41.4	8.6	2.5		5.9
6	36.7	6.8	49.5	5.2			1.3
7	29.9	11.2	30.1	4.8	11.7	7.4	9.1
8	49.7	6.9	15.8	9.7	14.3	3.1	0.5
9	24.6	7.2	44.1	4.6	9.7	0.5	9.3
10	31.2	14.4	24.9	11.7	12.0	2.7	3.1
Mean	35.3	7.1	33.2	6.7	10.8	1.8	4.9
S.D.	9.3	3.8	11.1	3.5	5.7	2.4	3.4

4) or the sulphonate group of taurine (pK ca. 2)[22]. For the strongly acidic metabolites the addition of a pairing reagent (*e.g.*, tetrabutylammonium phosphate) allows the control of retention, because both the ionic nature of the sample and the ionic strength of the eluent are under control. With increasing concentration of the modifier the capacity factor is increased. The retention of each bile acid decreases rapidly between 0.5 and 1.5 tetrabutylammonium phosphate. Above this concentration no dramatic increase in selectivity is observed.

The eight most important conjugates of bile acids in man were completely separated by this technique. In a short time for quantification external standardization with synthetic bile acids is preferred. Although bile acid concentration and peak area are linearly related, there is a significant difference in response to different compounds, *e.g.*, glycocholic acid and taurochenodeoxycholic acid (Fig. 3).

Simultaneous determination of conjugated bile acids in bile was carried out in ten patients suffering from gallstones (Table II). Surprisingly, the ratios of glycine-conjugated to taurine-conjugated bile salts were not necessarily identical among major bile acids. The present method may provide a more precise knowledge of the metabolic profile and turnover of bile acids.

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