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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF BILE ACIDS WITH A REVERSED-PHASE RADIAL COMPRESSION COLUMN

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SUMMARY

A reversed-phase radial compression column (Waters Assoc. Radial-Pak C_{18}, 10 μm, 8 mm I.D.) has been evaluated for the separation of bile acid standards by high-performance liquid chromatography. With a mobile phase of methanol–water (75:25, v/v) containing 2.5% (v/v) acetic acid and adjusted to pH 5.25 with 10 *M* sodium hydroxide, a mixture of fifteen bile acids could be resolved, and the ten major conjugated bile acids of human bile were well separated in under 20 min at a flow-rate of 2.0 ml min^{-1}. More rapid separations could be achieved at higher flow-rates. Refractive index detection permitted quantitation of 5 nmoles or less.

INTRODUCTION

Quantitation of individual bile acids in biological samples continues to present an analytical problem. The most satisfactory established technique for the analysis of bile acids is gas–liquid chromatography. While this method is sensitive and resolves bile acid isomers[1], it entails a derivatization step which renders it unsuitable for differentiating free and conjugated bile acids without prior group separation[2]. Radioimmunoassays are available for only some of the commonly occurring bile acids[3]. Probably the most widely used technique for analysing native bile acids in biological material has been thin-layer chromatography. Although the conjugated dihydroxy isomers can be thus separated by rather tedious multiple solvent development methods[4], quantitation of these bile acids in biological samples is not very reliable, particularly when the isomers are present in different proportions, such as in human bile (personal observation).

In view of these difficulties interest has focussed in recent years on the application of high-performance liquid chromatography (HPLC). With this technique detection of separated bile acids present in low concentration requires collection and assay of column eluates[5], precolumn derivatization[6], UV absorbance in the 200-nm region[7] or the use of UV-absorbing ion-pairing agents[8]. However, a number of biological materials of interest, such as bile and duodenal fluid, contain bile acids in sufficient quantities to permit detection by refractive index[9]. Good resolution of conjugated bile acids has been obtained on reversed-phase C_{18}-packed stainless-steel columns with methanol–water as the mobile phase[7,9].

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Interest in this laboratory in the changes in the bile acid composition of human bile in patients with large duct obstruction[10] has lead to attempts to establish a suitable HPLC method. The technique of Bloch and Watkins[9] was found to be satisfactory, but improved results were obtained with the C_{18} reversed-phase radial compression column introduced by Waters Associates. In this system the conventional stainless-steel column is replaced by a short, flexible-walled cartridge containing a reversed-phase packing material which is subjected to uniform radial compression (2500 p.s.i.) in a special module. This process is claimed to produce a highly efficient homogeneous bed free of voids and channels, which yields highly reproducible results. Furthermore, the cartridge dimensions enable the use of high flow-rates at low back-pressures so producing more rapid equilibration and separations.

In this report we describe the use of the radial compression system for the separation of mixtures of bile acid standards with particular emphasis on those bile acids found in human bile.

EXPERIMENTAL

Chemicals

The sodium salts of taurocholic acid* (TC), taurodeoxycholic acid (TDC), tauroursodeoxycholic acid (TUDC), taurolithocholic acid (TLiC), glycocholic acid (GC), glycochenodeoxycholic acid (GCDC), glycodeoxycholic acid (GDC), glycoursodeoxycholic acid (GUDC), glycolithocholic acid (GLiC), cholic acid (C), chenodeoxycholic acid (CDC), deoxycholic acid (DC), lithocholic acid (LiC) and the free acid of ursodeoxycholic acid (UDC) were obtained from Calbiochem (Bishops Stortford, Great Britain); the sodium salts of taurohyodeoxycholic acid (THDC) and glycohyodeoxycholic acid (GHDC) and the free acids of hyodeoxycholic acid (HDC), hyocholic acid (HC) and β-muricholic acid (βMC) from Steraloids (Croydon, Great Britain), and sodium taurochenodeoxycholic acid (TCDC) from Sigma London (Poole, Great Britain). HPLC-grade methanol was obtained from Rathburn Chemicals (Walkerburn, Great Britain). All other chemicals were AnalaR grade from BDH (Poole, Great Britain).

Solutions and aqueous solvents were prepared with ultrapure water, obtained from a Milli-Q System [Millipore (U.K.), London, Great Britain], fed from a reservoir of doubly distilled water (Fistream; Fisons Scientific Apparatus, Loughborough, Great Britain). Solvents were filtered through a 0.5-μm fluoropore filter [Millipore (U.K.)] and degassed in a sonic bath. Bile acid standards were prepared in HPLC-grade methanol. Accurate adjustment of the pH of solvents was made with a Model 3500 digital pH meter (Beckman Instruments, Glenrothes, Great Britain), and a glass combination electrode (Russell pH, Auchtermuchty, Great Britain).

HPLC

The HPLC system was composed of modular units, supplied by Waters Assoc. (Instruments) (Northwich, Great Britain), and consisted of a M6000A solvent de-

* All the bile acids studied were 5β-cholan-24-oic acid derivatives with hydroxyl groups in the following positions: C, 3α,7α,12α; CDC, 3α,7α; DC, 3α,12α; LiC, 3α; HC, 3α,6α,7α; HDC, 3α,6α; UDC, 3α,7β; βMC, 3α,6β,7β.

livery system, a Model U6K universal injector, a RCM 100 radial compression module with a Radial-Pak C_{18}, 10 μm (8 mm I.D.) reversed-phase cartridge (column) and a Model R401 differential refractometer with attenuation set at x4. The 100-mV output from the latter was connected to a chart recorder [Rikadenki Model KB; Mitsui Machinery Sales (U.K.), Chessington, Great Britain] with input set at 100 mV. Peak areas were determined with an on-line computer and chromatography software (Triton 3 data collection and analysis system; Trivector Scientific, Sandy, Great Britain).

Of the several mobile phases evaluated, the most satisfactory for many of the bile acid mixtures studied, including the bile acids found in human bile, was methanol–water (75:25, v/v) containing 2.5% (v/v) acetic acid and adjusted to pH 5.25 with 10 *M* sodium hydroxide (solvent system A).

RESULTS

A mixture of fifteen bile acids consisting of C, CDC, DC, UDC and LiC and their taurine and glycine conjugates was resolved into fifteen peaks in 50 min with the Radial-Pak C_{18} cartridge when solvent system A was used with a flow programme of 1 ml min^{-1} for 30 min, 2 ml min^{-1} for 14 min and 3.0 ml min^{-1} to elute the LiC peak (Fig. 1). Complete baseline resolution was not observed for the triplet TDC, UDC and GDC or for the pair CDC and DC. With a constant flow-rate of 2.0 ml min^{-1} more rapid separation of these fifteen bile acids was observed although the degree of resolution was not quite as good (Fig. 2). The ten conjugated bile acids, TUDC, GUDC, TC, GC, TCDC, TDC, GCDC, GDC, TLiC and GLiC, were well separated in under 20 min at a flow-rate of 2.0 ml min^{-1} (Fig. 3).

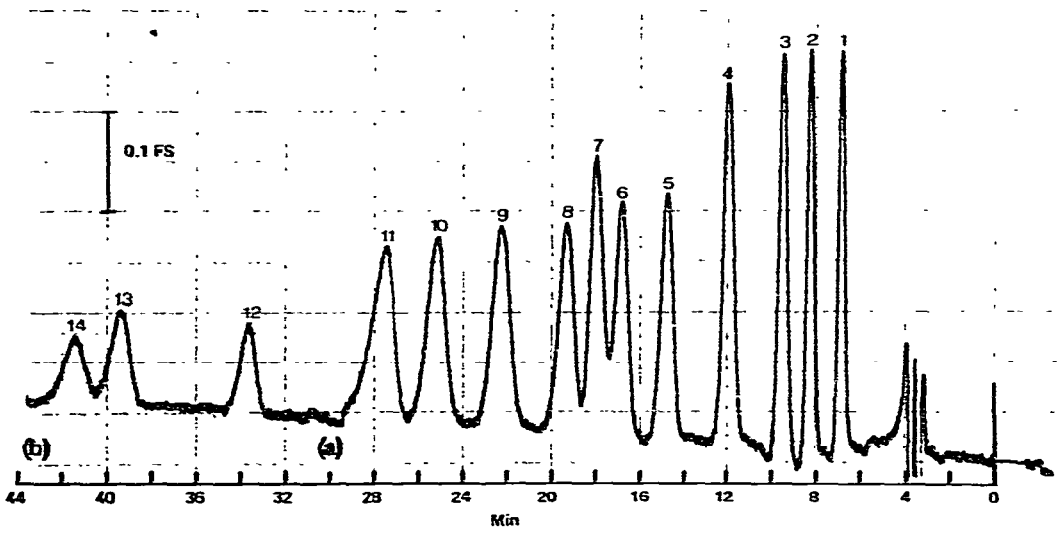

Fig. 1. Separation of fifteen bile acid standards (50 μg each) with a simple flow programme. Solvent system A: flow-rate 1.0 ml min^{-1} to (a), 2.0 ml min^{-1} from (a) to (b) and 3.0 ml min^{-1} from (b) to elute LiC (50 min; not shown). Peaks: 1 = TUDC; 2 = GUDC; 3 = TC; 4 = GC; 5 = TCDC; 6 = TDC; 7 = UDC; 8 = GCDC; 9 = GDC; 10 = C; 11 = TLiC; 12 = GLiC; 13 = CDC; 14 = DC.

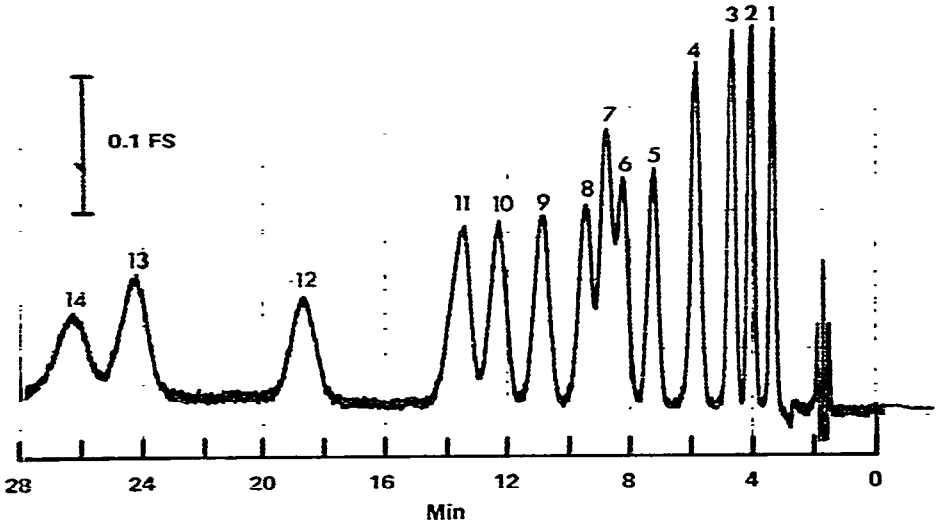

Fig. 2. Separation of fifteen bile acid standards (50 μg each) with constant flow-rate of 2.0 ml min^{-1}. Solvent system and peak identification as in Fig. 1 (LiC peak not shown).

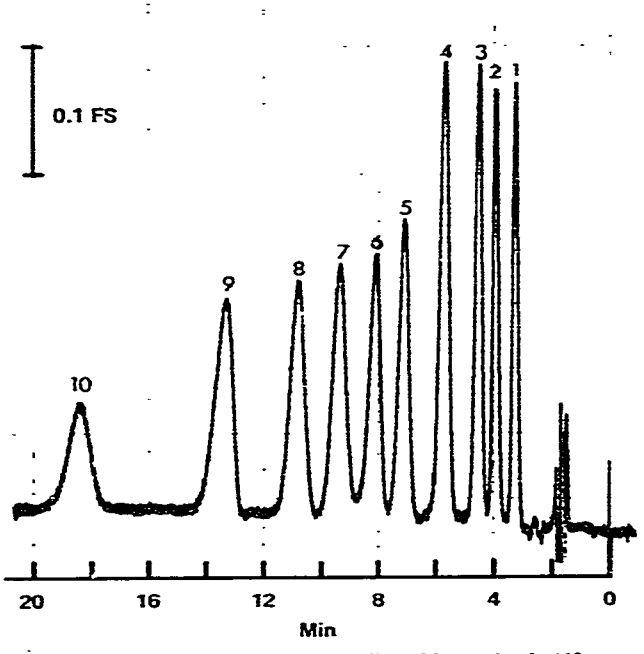

Fig. 3. Separation of ten conjugated bile acid standards (60 μg each). Solvent system A, flow-rate 2.0 ml min^{-1}. Peaks: 1 = TUDC; 2 = GUDC; 3 = TC; 4 = GC; 5 = TCDC; 6 = TDC; 7 = GCDC; 8 = GDC; 9 = TLiC; 10 = GLiC.

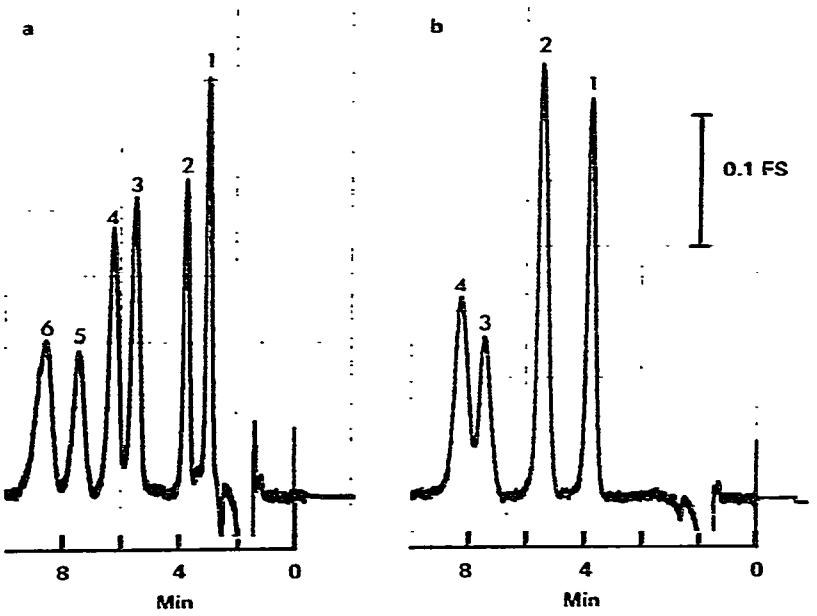

Fig. 4. Separation of conjugated dihydroxy bile acid (a) and the more polar free bile acid (b) standards (50 μg each). Solvent system A; flow-rate 2.0 ml min^{-1}. Peaks: (a), 1 = THDC; 2 = GHDC; 3 = TCDC; 4 = TDC; 5 = GCDC; 6 = GDC; (b), 1 = βMC; 2 = UDC; 3 = HDC; 4 = C.

With solvent system A THDC was partially resolved from TUDC and GUDC, being eluted between them, GHDC did not separate from TC, nor βMC from TCDC and HDC and GDC were only partially resolved. However, the six conjugates of the dihydroxy-isomers HDC, CDC and DC were separated in 10 min (Fig. 4a) and the more polar of the free bile acids (βMC, UDC, HDC and C) were resolved in under 12 min (Fig. 4b) with a flow-rate of 2.0 ml min^{-1}. A mixture of the three trihydroxy isomers (C, HC and βMC) was not resolved with solvent system A.

The pH of the methanol–water solvent system had an important influence on retention and resolution (Table I). As the pH was increased the bile acids were retained longer on the Radial-Pak C_{18} cartridge (k' values) but the earlier bile acids were eluted as sharper peaks (W values) and there was better overall separation and resolution (α and R values). Assuming a peak shape that approximates a Gaussian distribution, values of R = 1.0 and 1.5 indicate an overlap of adjacent peaks of approximately 2.0% and 0.03% respectively[11]. The order of elution of conjugated bile acids was also affected by the nature and ionic strength of the buffering ions. Thus, separation of a mixture of eight conjugated bile acids with methanol–0.05 M potassium dihydrogen orthophosphate (75:25, v/v) adjusted to pH 4.75 with acetic acid resulted in earlier elution of taurine conjugates compared with the separation obtained with solvent system A.

Good separation of conjugated bile acids was also obtained with ethanol–water (60:40, v/v) containing 2.5% (v/v) acetic acid and adjusted to pH 5.15 with 10 M sodium hydroxide. UDC, C, CDC and DC were also well resolved with ethanol–

TABLE I

EFFECT OF MOBILE PHASE pH ON RETENTION AND SEPARATION OF SIX CONJUGATED BILE ACIDS

The mobile phase was methanol–water (75:25, v/v) containing 2.5% (v/v) acetic acid and the pH was adjusted with 10 *M* sodium hydroxide. Flow-rate 1.0 ml min^{-1}. The peak width at base (W), the capacity factor (k'), the selectivity or separation factor relative to the preceding peak (α) and the resolution factor relative to the preceding peak (R), were determined as defined by Parris[11].

Bile acid	*Retention (min)*	*W (min)*	*k'*	*α*	*R*
pH 4.95					
TC	6.4	1.0	0.94	–	–
GC	9.2	1.4	1.79	1.80	2.33
TCDC	9.2	1.4	1.79	1.00	0
TDC	10.6	1.2	2.21	1.23	1.08
GCDC	14.2	1.3	3.30	1.49	2.88
GDC	16.2	1.5	3.91	1.18	1.43
pH 5.15					
TC	6.8	0.9	1.06	–	–
GC	9.3	1.2	1.82	1.72	2.38
TCDC	10.0	1.2	2.03	1.12	0.58
TDC	11.3	1.2	2.42	1.19	1.08
GCDC	14.8	1.4	3.48	1.44	2.69
GDC	17.0	1.6	4.15	1.19	1.47
pH 5.25					
TC	7.3	0.9	1.21	–	–
GC	9.5	1.1	1.88	1.55	2.20
TCDC	10.9	1.1	2.30	1.22	1.27
TDC	12.3	1.3	2.75	1.19	1.17
GCDC	15.2	1.4	3.61	1.32	2.15
GDC	17.4	1.6	4.27	1.18	1.47

water (55:45, v/v), containing 2.5% (v/v) acetic acid and adjusted to pH 5.00 with 10 *M* sodium hydroxide.

The low back pressures obtained with the radial compression system permit the use of high flow-rates and, consequently, rapid separations. At 4.0 ml min^{-1} the taurine and glycine conjugates of C, CDC and DC were separated in under 6 min, although there was some loss of baseline resolution.

The relationship between the amount of bile acid applied to the column and the detector response was determined by applying different amounts of a mixture of taurine and glycine conjugates of C, CDC and DC and recording the computer determined peak areas for each separated bile acid. Linear responses were obtained for each bile acid over the range 2.5–200 μg (Fig. 5). At the attenuation setting of the differential refractometer used in this study the limit of detection was 5 nmoles or less depending on the degree of the retention of the bile acid.

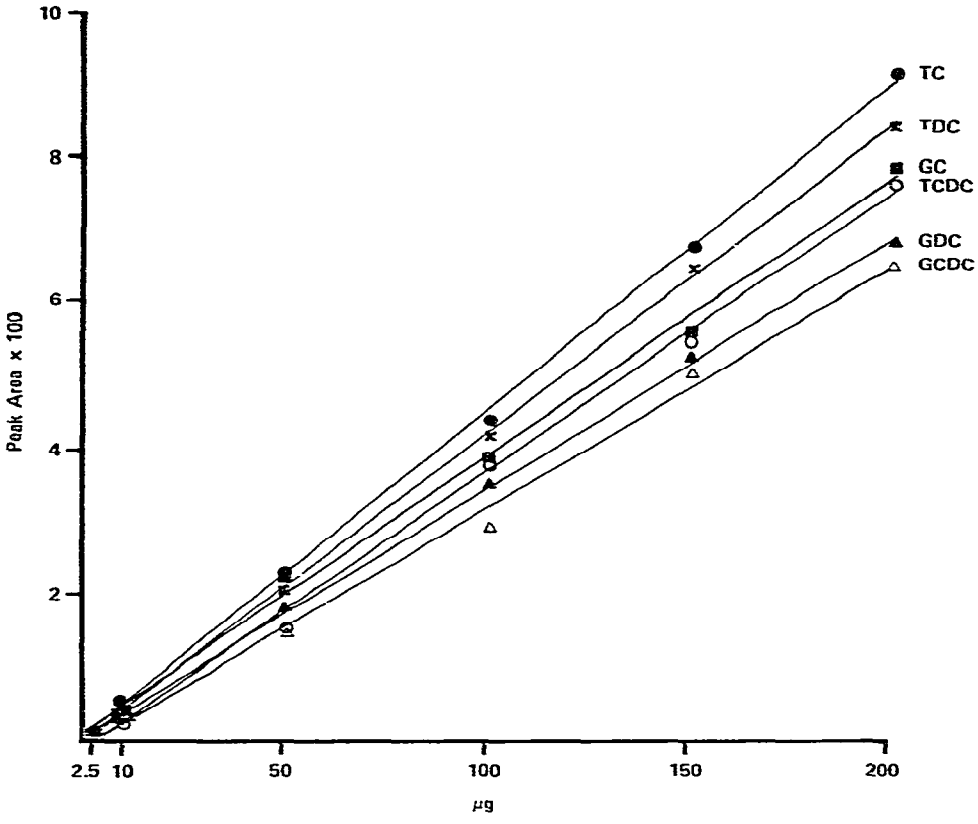

Fig. 5. Relationship between amount of bile acid applied and detector response (peak area). Solvent system A; flow-rate 2.0 ml min^{-1}. Differential refractometer attenuation × 4 (for details see text).

DISCUSSION

The 10-μm C_{18} (8 mm I.D.) radial compression cartridge proved to be very satisfactory for the rapid separation of quite complex mixtures of bile acid standards. Solvent system A was found to be an excellent mobile phase for the separation of the major bile acids of human bile including the conjugates of UDC. Modification of the mobile phase may be required for optimum resolution of the bile acids found in other species. Relative retention times of bile acids were affected by pH, the nature and ionic strength of the buffer and the water content and nature of the organic phase. In an earlier study[12] the Waters radial compression system was used to resolve bile acids previously separated into groups on an ion-exchange column, a procedure that is clearly not necessary.

Although modification of the mobile phase could be used to alter the relative retention times of some bile acids, in common with other studies of reversed-phase HPLC of bile acids[7,9,13], elution was generally in the order of taurine conjugates: glycine conjugates: free acids, and trihydroxyl: dihydroxyl: monohydroxyl bile acids. The earlier elution of bile acids with hydroxyl group(s) in the β position and/or at C-6, observed in the present study, is in agreement with a more detailed study of the effects

of the stereochemical configuration of substituents based on a conventional C_{18} reversed-phase column[14].

The physical characteristics of the Radial-Pak cartridge allow the use of high flow-rates and, consequently, more rapid elution of separated solutes. At a flow-rate of 4.0 ml min^{-1} the six major conjugated bile acids of human bile could be separated, albeit with some loss of resolution, in less than 6 min, a quarter of the elution time at 1.0 ml min^{-1}. The radial compression technique therefore affords a more rapid means of separating bile acids than has been reported for conventional reversed-phase columns[7,9,13]. In addition, the radial compression cartridges are robust, easy to use and relatively inexpensive; they maintain performance for at least as long as conventional HPLC columns.

The refractive index detector used in this stidy enabled the quantitation of 5 nmoles or less of individual bile acids and, particularly when combined with on-line computer analysis, appears suitable for application to the study of the major bile acids in bile and in the serum of patients with liver disease. The use of UV detection in the 200-nm region may provide improved sensitivity, particularly for conjugated bile acids[15].

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