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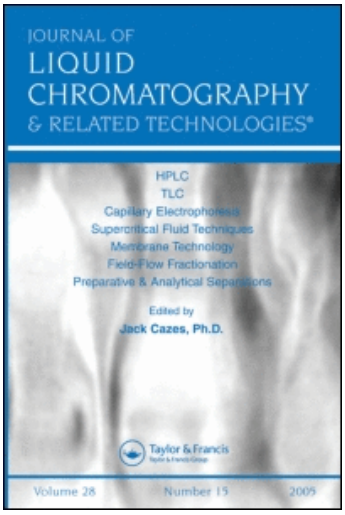

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Evaluation of Mobile and Stationary Phases in Reversed-Phase High-Performance Liquid Chromatography of Conjugated Bile Acids

S. Scalia[a]
[a] Department of Pharmaceutical Sciences, University of Ferrara Via Scandiana, Ferrara, Italy

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EVALUATION OF MOBILE AND STATIONARY PHASES IN REVERSED- PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF CONJUGATED BILE ACIDS

S. Scalia

Department of Pharmaceutical Sciences
University of Ferrara
Via Scandiana 21
44100 Ferrara, Italy

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ABSTRACT

The reversed-phase high-performance liquid chromatographic separation of the ten major conjugated bile acids of man using isocratic conditions is described. Each component of the mobile and stationary phases was examined for its ability to influence the separation selectivity. Manipulation of pH, buffer species, organic modifier and different types of packings showed that optimal resolution was obtained with a mobile phase of methanol-0.02M sodium acetate (60:30) adjusted to pH 4.2 with phosphoric acid, on a Supelcosil LC-18-DB column. Advantages of the optimized phase system are the complete baseline separation of compounds within a short period of time, improved peak symmetry and a high rate of reproducibility. This new chromatographic method, coupled with UV detection at 205 nm, is suitable for the simultaneous determination of bile acid conjugates in routine clinical analysis.

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INTRODUCTION

The commonly occurring bile acids in human fluids [1-4] are cholic acid, deoxycholic acid, chenodeoxycholic acid, ursodeoxycholic acid and lithocholic acid, all present primarily as glycine and taurine conjugates. The individual separation and quantification of these compounds are very important from a clinical point of view since the presence of anomalous amounts of one or more bile acids indicates metabolic disorders and diseases [5-7].

At present reversed-phase high-performance liquid chromatographic (RP-HPLC) methods [1-4, 8-16] are the most widely used for the assay of the bile acid conjugates in biological samples. Consideration of the various chromatographic systems mentioned, indicates that the investigators have used octadecyl packings with a wide range of eluents. Aqueous mobile phases with methanol [1,3,4,9,10], acetonitrile [11-14] or propanol [16,17] have been buffered at both low [1,2,15] and high [12,16,17] pH or modified with ion-pairing agents [11,13,14], although there was little evidence that the eluent has been specifically optimized for the separation, in the majority of instances.

While complete resolution of the ten major conjugated bile acids found in man has been described [1-3,15], in the course of this study it was found, in accordance with other researchers [11], that some of these separations were not as reproducible as the

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literature might suggest leading to a failure to resolve some of the bile acids for proper quantification. These problems prompted a systematic study of the mobile phase optimisation for the isocratic chromatography of these compounds. Various parameters were evaluated, including the pH, the chemical nature and concentration of added buffer species and the type of organic solvent modifier, to find conditions where reproducible and baseline separations of all bile acids investigated could be obtained.

This work also reports on the performance of a series of chemically-bonded RP-supports with different selectivity (i.e. octadecyl-, octyl-, phenyl- and cyano-silica column) in the chromatographic separation of the foregoing bile acids.

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EXPERIMENTAL

Materials

The sodium salts of glycocholic acid (GC), glycodeoxycholic acid (GDC), glycochenodeoxycholic acid (GCDC), glycolithocholic acid (GLC), taurocholic acid (TC), taurodeoxycholic acid (TDC), taurochenodeoxycholic acid (TCDC), taurolithocholic acid (TLC) were obtained from Sigma (St.Louis, MO, U.S.A.); the sodium salts of tauroursodeoxycholic acid

(TUDC) and glycoursodeoxycholic acid (GUDC) were purchased from Calbiochem-Behring (San Diego, CA, U.S.A.). Their purity was checked by HPLC prior to use.

HPLC-grade methanol, acetonitrile, isopropanol and water were supplied by Farmitalia Carlo Erba (Milano, Italy). HPLC-grade sodium acetate was from Baker (Phillipsburg, NJ, U.S.A.). All other chemicals were of analytical grade (Farmitalia). Bile acid standards were used as solutions in HPLC-grade methanol.

Sep-Pak C_{18} cartridges were obtained from Water Assoc. (Milford, MA, U.S.A.).

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Instrumentation

The HPLC apparatus used was a Jasco liquid chromatographic system (Model BIP-I pump, Model GP-A40 solvent programmer and Model UVIDEC-100-V variable-wavelength UV-detector, Jasco, Tokyo, Japan) linked to an injection valve with a 20-µl sample loop (Rheodyne, Cotati, U.S.A.) and a chromatographic data processor (Chromatopac C-R3A, Shimadzu, Kyoto, Japan). The detector was set to 205 nm and 0.04 a.u.f.s. Sample injections were made with an Hamilton Model 802 RN syringe (10 µl; Hamilton Bonaduz AG, Bonaduz, Switzerland).

The pH measurements were performed with a Model PHM82 digital pH meter (Radiometer, Copenhagen, Denmark) equipped with a combination glass electrode (Radiometer).

Chromatography

Separations were carried out on stainless-steel columns pre-packed with reversed-phase supports. The columns used were: μBondapak C_{18} (d_p 10-μm, 300x3.9 mm I.D.; Waters Assoc.), Supelcosil LC-18-DB (d_p 5-μm, 150x4.6 mm I.D.; Supelco, Bellefonte, PA, U.S.A.) and LiChrospher CH-8 (d_p 5-μm, 250x4.0 mm I.D.; Merck, Darmstadt, F.R.G.). To study the selectivity of different bonded phases, four IBM HPLC columns (d_p 5-μm, 250x4.5 mm I.D.; IBM Instruments, Danbury, CT, U.S.A.) were used: Octadecyl, Octyl, Phenyl RP, and Cyano RP. Chromatography was performed at ambient temperature, under isocratic conditions.

Mobile phases were prepared by mixing known volumes of methanol, acetonitrile or isopropanol with aqueous phosphate or acetate buffers. The molarity of the buffer and the pH refer to the water phase. The pH was adjusted to the desired value by the addition of either phosphoric acid or sodium hydroxide. The eluents were filtered through a 0.45-μm filter (type HVLP; Millipore S.A., Molsheim, France) and degassed with helium. Details of the chromatographic conditions applied in the HPLC systems are given in Table I.

The capacity factors (K') and the separation factors (α) were determined as defined by Snyder and Kirkland [18]. The injection of sodium nitrate (0.5 mg/ml in eluent) was used to determine the column void volume (Vo).

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TABLE I

Capacity Factors and Separation Factors of Conjugated Bile Acids with Different Chromatographic Systems

System 1: column, μBondapak C_{18}; flow-rate, 1.2 ml/min; eluent, methanol-0.02M phosphate buffer, pH 4.2 (60:30)

System 2: column, μBondapak C_{18}; flow-rate, 1.4 ml/min: eluent, methanol-0.1M phosphate buffer, pH 4.5 (60:40)

System 3: column, μBondapak C_{18}; flow-rate, 1.2 ml/min; eluent, methanol-0.02M sodium acetate (60:30) adjusted to pH 4.2 with phosphoric acid (solvent system A)

System 4: column, Supelcosil LC-18-DB; flow-rate, 1.0 ml/min; eluent, solvent system A

System 5: column, LiChrospher CH-8; flow-rate, 0.7 ml/min; eluent, solvent system A

System 6: column, μBondapak C_{18}; flow-rate, 0.6 ml/min; eluent, methanol-acetonitrile-0.03M phosphate buffer, pH 3.4 (60:10:30)

The separation factor (α) relative to the preciding peak is given.

Bile acid	Chromatographic system											
	System 1		System 2		System 3		System 4		System 5		System 6	
	K'	α	K'	α	K'	α	K'	α	K'	α	K'	α

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TUDC	2.42	-	6.40	-	2.31	-	1.40	-	1.42	-	1.25	-
GUDC	3.15	1.30	6.92	1.08	3.06	1.32	2.00	1.43	2.20	1.16	2.02	1.62
TC	3.84	1.22	10.54	1.52	3.71	1.21	2.72	1.94	1.90	1.34	2.02	1.00
GC	5.00	1.30	11.63	1.10	5.00	1.35	3.93	1.44	2.94	1.34	3.21	1.60
TCDC	7.86	1.57	23.59	2.03	7.63	1.53	5.58	1.42	3.97	1.35	3.76	1.17
TDC	8.96	1.14	25.85	1.09	8.69	1.14	6.57	1.18	4.49	1.13	4.25	1.13
GCDC	10.20	1.14	27.27	1.05	10.35	1.19	8.11	1.23	6.23	1.38	6.13	1.44
GDC	11.78	1.15	30.38	1.11	11.94	1.15	9.68	1.19	7.13	1.14	7.07	1.1
TLC	17.68	1.50	*	-	17.92	1.50	12.69	1.31	9.49	1.33	7.82	1.11
GLC	23.18	1.31	*	-	23.50	1.31	18.67	1.47	15.50	1.63	13.04	1.67

* Not measurable due to excessive retention time

RESULTS AND DISCUSSION

Several chromatographic parameters were manipulated in an effort to optimize the separation of the ten major bile acid conjugates found in man. These parameters included various mobile phase components and analytical RP-columns.

1) Mobile Phase

The choice of a suitable mobile phase was studied by examining the influence of the pH, buffer species and organic modifier on the chromatographic behaviour of the foregoing bile acids. These optimisation studies were carried out on a μBondapak C_{18} column which was selected as a representative C_{18} packing since it is commonly used in the RP-HPLC analysis of the conjugated bile acids [2,4,14,17].

pH. The influence of the mobile phase pH was studied by examining combination of phosphate buffer solutions, ranging in pH from 2.5 to 6.0, and methanol. The dependence of the K' for the ten conjugates on the pH is illustrated in Fig.1. The bile acid retention increases with decreasing pH owing to ionic suppression which enhances the lipophilic character of the molecules, hence their interaction with the bonded phase. Due to the low pK values of the taurine--conjugates (pK ca. 2) [19] their K' are less influenced by the pH change of the eluent, in the range studied,

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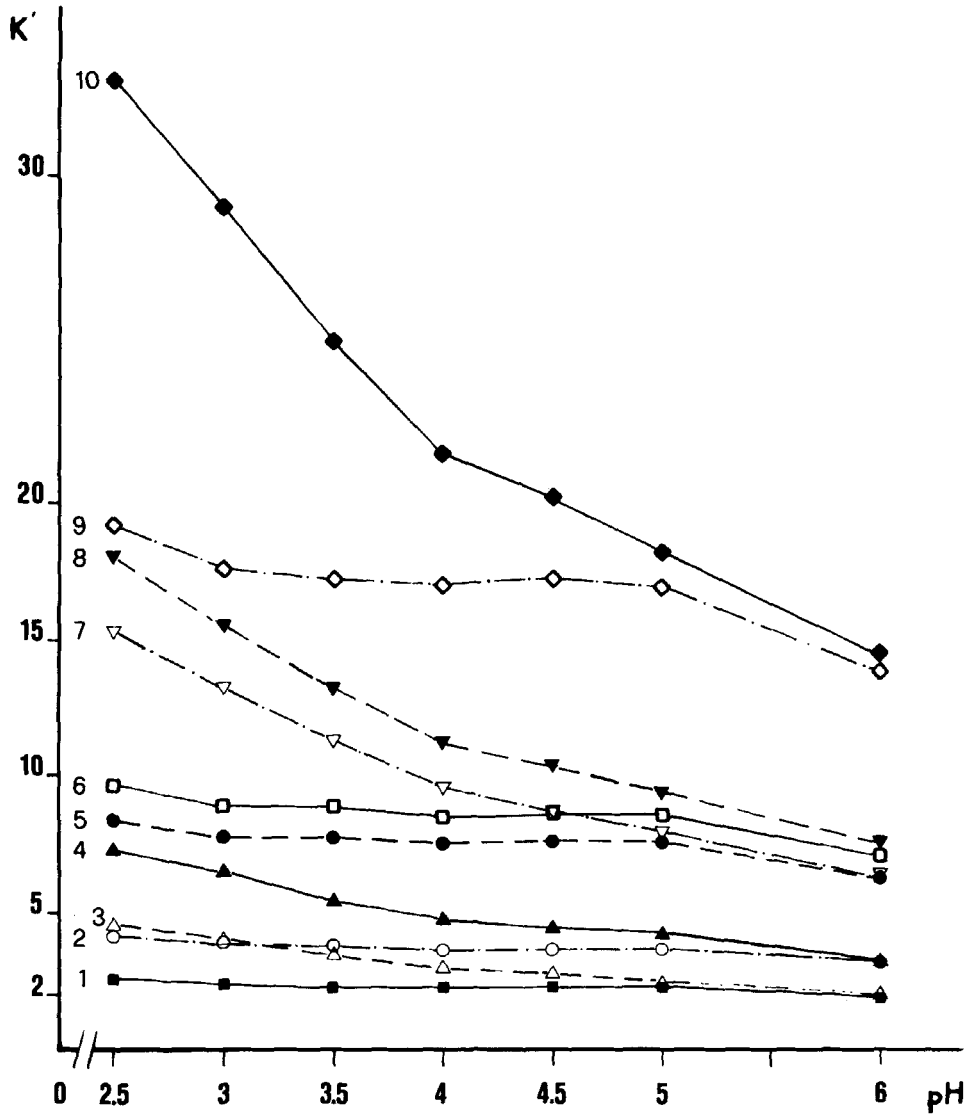

Fig. 1. Influence of the pH of the mobile phase on the K' values of bile acid conjugates. Compounds: 1=TUDC; 2=TC; 3=GUDC; 4=GC; 5=TCDC; 6=TDC; 7= =GCDC; 8=GDC; 9=TLC; 10=GLC. Operating conditions: column, μBondapak C_{18}; mobile phase, methanol-0.02M phosphate buffer (60:30); flow-rate, 1.2 ml/min; UV detection 205 nm.

than those for the glycine-conjugates (pK ca. 4.5) [19]. The reversal in elution order for the pairs GUDC (3), TC (2) and GCDC (7), TDC (6) as the pH decreases (Fig. 1) reflects the greater increase in retention for the glycine-conjugated bile acids.

At pH 6, the conjugated bile acids, containing the same steroidal nucleus, exhibit similar K' values (Fig. 1). This chromatographic behaviour can be explained by considering that at this pH the conjugating moieties are ionized, hence the observed retention is primarily influenced by the lipophilic portion of the bile acid molecules. Surprisingly a satisfactory separation of these acidic solutes has been described at pH 6 on a flexible-walled column [9] packed with the same phase (i.e. μBondapak C_{18}) and eluted with the same methanol--phosphate buffer system reported in Fig. 1. Lowering the pH of the aqueous portion of the mobile phase confers improved separation selectivity to the chromatographic system (Fig. 1), although the resolution of GUDC (3) and TC (2) is lost below pH 3.5. Fig. 1 illustrates that optimal resolution of the conjugated bile acids in question occurs in the pH range 3.9-4.3. A chromatogram of a typical separation of a standard mixture of compounds 1-10 with a methanol-0.02M phosphate buffer, pH 4.2 (60:30) eluent is shown in Fig. 2 and the K' and α values observed are listed in Table I (chromatographic system 1). The ten bile acid conjugates are baseline separated except for TCDC (5) TDC (6) and GCDC (7).

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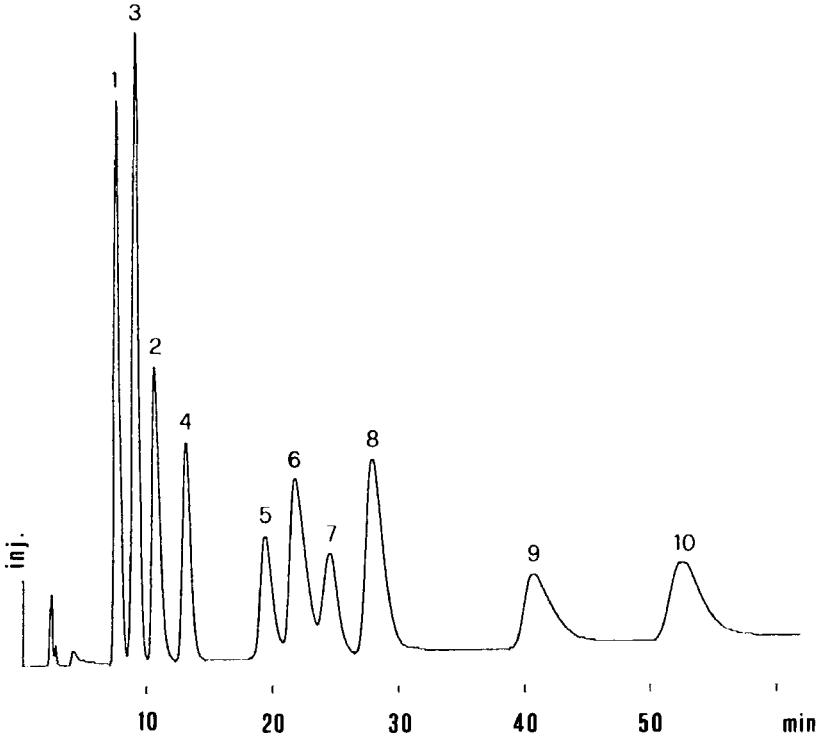

Fig. 2. Separation of a synthetic mixture of conjugated bile acids (ca. 0.5-0.8 μg of each component) on a μBondapak C_{18} column with a methanol-0.02M phosphate buffer, pH 4.2 (60:30) eluent. Other conditions and peak identifications as in Fig. 1.

Buffer. The influence of the concentration and nature of the mobile phase buffer on the chromatography of the foregoing compounds was also investigated. Varying the molarity of the phosphate buffer, in the range 15-50 mM, did not enhance the resolution reported in Fig. 2, although with increasing ionic strength in the mobile phase the K' value for each conjugate rose.

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Moreover even the high salt concentration (0.1M potassium phosphate, pH 4.5) of the chromatographic system used in ref. 4 failed to resolve the ten conjugates for proper quantification (Table I, chromatographic system 2).

Although the majority of investigators [2,4,9,10, 12,15-17] preferred phosphate as a buffer for the RP--HPLC analysis of the conjugated bile acids, in this study it was found that when potassium phosphate was replaced by sodium acetate as buffering ion in the mobile phase, improved separation was observed. Fig. 3 shows the complete baseline resolution of the ten bile acids on the μBondapak C_{18} column using methanol-0.02M sodium acetate (60:30) adjusted to pH 4.2 with phosphoric acid as the mobile phase (solvent system A). The K' and α values measured from the chromatogram in Fig. 3 are listed in Table I (chromatographic system 3). The overall separation was not affected by variation of the eluent pH in the range 4.2-4.3 or by change in the molarity of the buffer from 20 to 30 mM (not shown). By contrast, other published methods [2,3,9] require the exact pH adjustment to three digits of the mobile phase for satisfactory resolution of these conjugated steroids.

The reproducibility of the separation afforded by the solvent system A was also investigated by testing different reversed-phase packings. When the acetate and phosphate mobile phases were compared on a Supelcosil LC-18-DB column, improved separation selectivity and peak symmetry were attained by the former system. The

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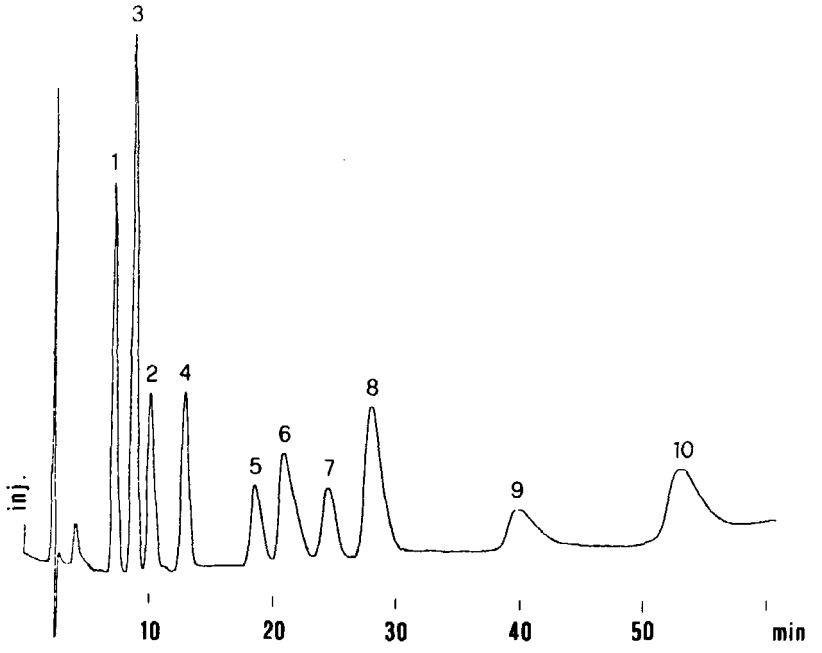

Fig. 3. Separation of a synthetic mixture of conjugated bile acids on a μBondapak C_{18} column with an eluent consisting of methanol-0.02M sodium acetate (60:30) adjusted to pH 4.2 with phosphoric acid. Other conditions and peak identification as in Fig. 1.

baseline resolution of the ten conjugated bile acids achieved on the Supelcosil LC-18-DB column with the solvent system A eluent is illustrated in Fig. 4 and the K' and α are given in Table I (chromatographic system 4). Although there are no significant differences in selectivity between the two octadecyl (C_{18}) bonded phases tested, the use of the Supelcosil LC-18-DB (Fig. 4) rather than the μBondapak C_{18} column

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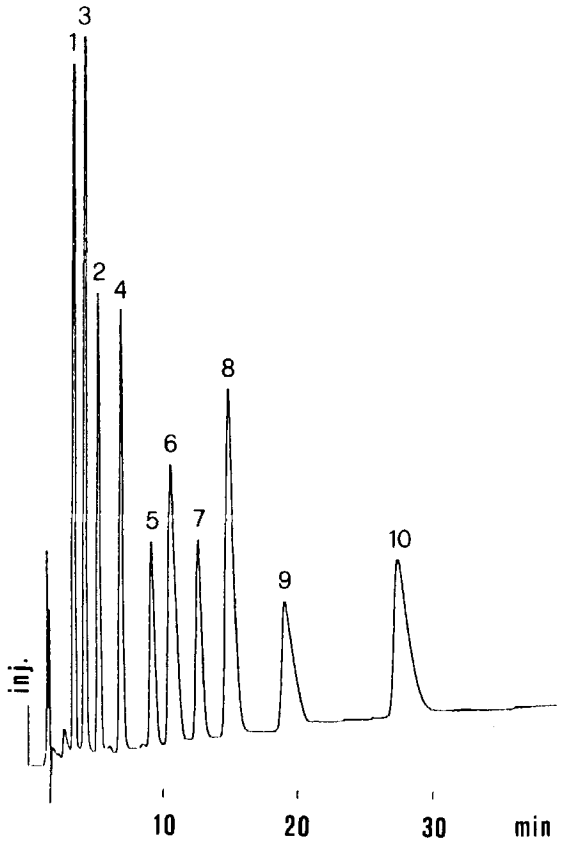

Fig. 4. Chromatogram of the separation of a standard mixture of conjugated bile acids on a Supelcosil LC-18-DB column. Mobile phase as in Fig. 3. Flow-rate, 1.0 ml/min. Other conditions and peak identification as in Fig. 1.

(Fig. 3) provided more efficient resolution of the conjugates within a shorter period of time (28 min). Previously described isocratic HPLC separations of the same compounds on stainless-steel C_{18} columns required longer elution time [1,2,4,7] and only a radial compression system [3] has been reported to produce more rapid, though not as effective, separations. For the latter method, however, the application to the analysis of biological samples was not investigated.

Optimal resolution of compounds 1-10 was afforded by the solvent system A also on an octyl (C_8) bonded phase packing. As shown in Fig. 5 a separation of ten well defined peaks of the glycine and taurine conjugates was obtained on a LiChrospher CH-8 column using solvent system A as the mobile phase. The K' and α values measured from the chromatogram in Fig. 5 are given in Table I (chromatographic system 5). The use of a C_8 support has not been reported before for the complete separation of the ten conjugated bile acids in question. The retention for these bile acids on μBondapak C_{18} and Supelcosil LC-18-DB columns are higher than that observed with the same mobile phase on the LiChrospher CH-8 column (Table I), where the interactions between the C_8 alkyl groups and the hydrophobic portion of the steroid molecules are lower. Compared with the C_{18} bonded phase, the C_8 packing exhibits a different selectivity leading to the reversal in elution order in the case of GUDC (3) and TC (2).

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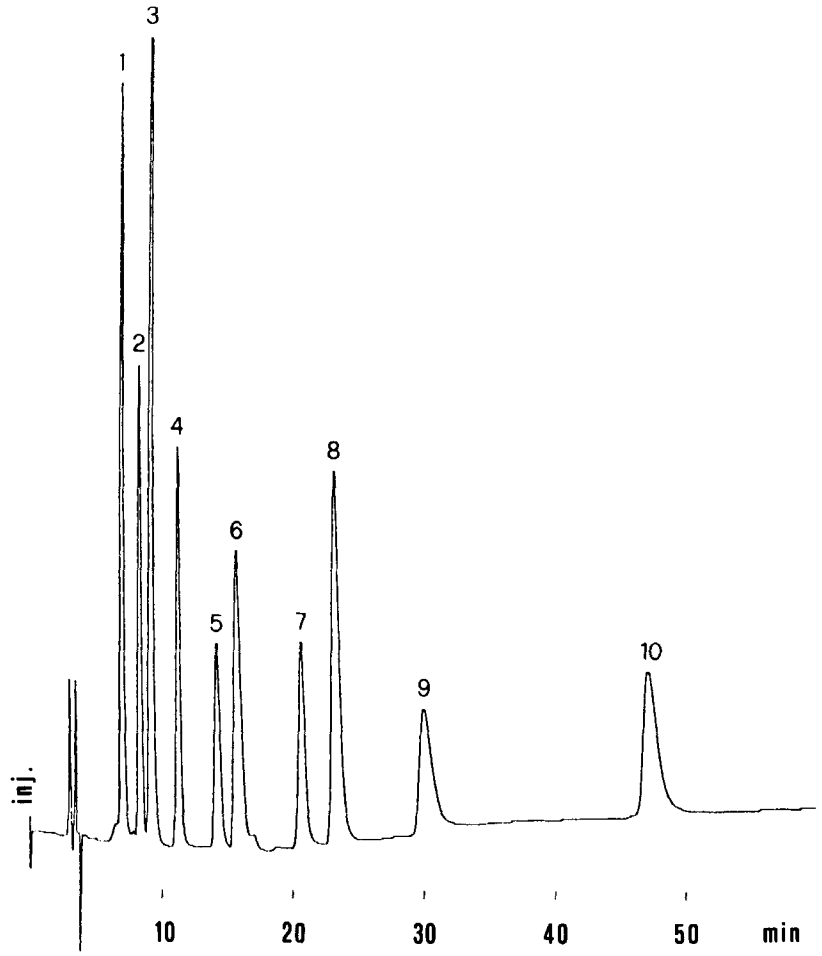

Fig. 5. Chromatogram of the separation of a standard mixture of conjugated bile acids on a LiChrospher CH-8 column. Mobile phase as in Fig. 3. Flow-rate, 0.7 ml/min. Other conditions and peak identification as in Fig. 1.

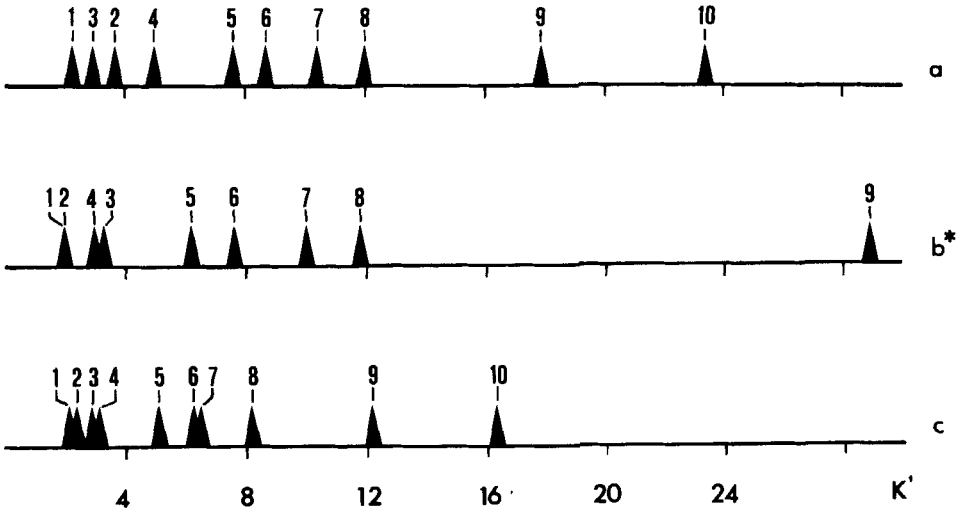

Fig. 6. Diagramatic representation of the separation selectivity of conjugated bile acids with three different organic modifiers using μBondapak C_{18}. a=methanol-0.02M sodium acetate, pH 4.2 (60:30); b=acetonitrile-0.02M sodium acetate, pH 4.2 (30:70); c=isopropanol-0.02M sodium acetate, pH 4.2 (31:69). Compounds 1-10 as in Fig. 1. *GLC was not included because of its prolonged elution time.

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Organic modifier. Methanol, acetonitrile and isopropanol were used as organic modifiers in conjunction with the acetate eluent described above, using the μBondapak C_{18} column (Fig.6). For the same K' to be obtained the percentage of acetonitrile or isopropanol needed in the mobile phase are lower than that of methanol due to the higher eluotropic strength of the former.

Replacing methanol by acetonitrile caused a remarkable difference in selectivity with changes in the retention sequence of the first eluted samples (Fig. 6b). However acetonitrile as organic modifier failed to resolve TUDC (1) and TC (2) and produced poor separation for the pair GUDC (3) and GC (4). Moreover the two conjugates of lithocholate, GLC (9) and TLC (10) exhibited excessively long retention times which reflects their lower solubility in acetonitrile with respect to methanol [4]. By using isopropanol instead of methanol, as the organic eluent in the mobile phase, the elution order between GUDC (3) and TC (2) was reversed (Fig. 6c). Compared with the methanol eluent a loss of baseline resolution was observed for the three pairs of samples: TUDC (1) - TC (2), GUDC (3) - GC (4) and TDC (6) - GCDC (7). These results indicate that methanol is preferable to acetonitrile or isopropanol for optimal separation selectivity of the conjugated bile acids.

The effect of ternary solvent systems on the chromatography of the foregoing compounds was also evaluated using various combinations of methanol, acetonitrile and acetate buffer as the mobile phases. Satisfactory resolution was not attained however (not shown); also the ternary solvent conditions given by Nakayama and Nakagaki [2] failed, on the same μBondapak C_{18} column, to afford a complete separation of the ten conjugated bile acids (Table I, chromatographic system 6).

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2) Stationary Phase

The influence of the packing on the chromatographic separation of bile acid conjugates was studied using octadecyl, octyl, phenyl and cyanopropyl phases bonded to the same silica supports (Fig. 7). These observations have been made using the solvent system A eluent, which gives optimal resolution of all compounds examined.

As shown in Fig. 7, the concentration of organic modifier required to elute the steroids with similar K', is lower for the less hydrophobic supports, cyano and phenyl. The elution sequence of the bile acids was unchanged in chromatography on C_{18}-, C_8-, phenyl or cyano-columns, with the exception of TC (2) which eluted before GUDC (3) on the latter three columns (Fig. 7a-d).

In accordance with the results previously stated, optimal separation selectivity for compounds 1-10 was afforded by the C_{18}- and C_8-stationary phases, whereas the resolution of several component peaks was lost on cyano- and phenyl-columns (Fig. 7d,c). The use of less hydrophobic columns and more polar mobile phases therefore decreases the overall resolution of the bile acids studied. Also in the case of bile acid 3--glucuronides, the C_{18} support has been found to provide more efficient separations than the cyano-phase [20].

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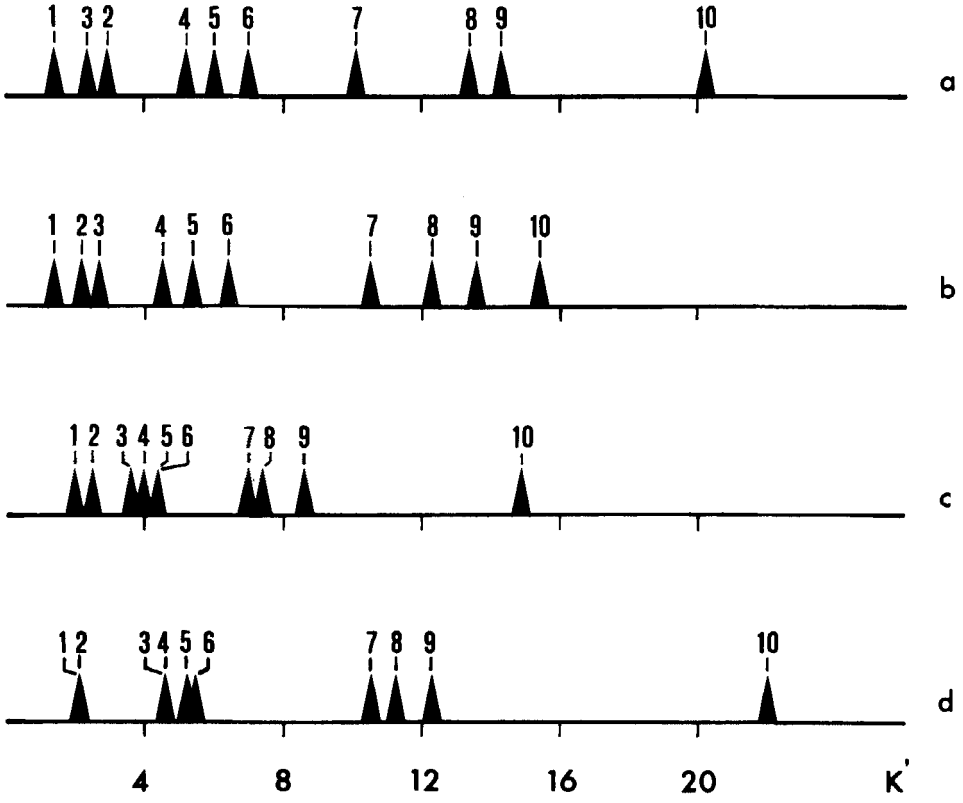

Fig. 7. Diagramatic representation of the separation selectivity of conjugated bile acids with four different reversed-phase supports. a=IBM C_{18}, methanol-sodium acetate, pH 4.2 (60:30); b=IBM C_8, methanol-sodium acetate, pH 4.2 (65:35); c=IBM Phenyl, methanol-sodium acetate, pH 4.2 (40:60); d=IBM Cyano, methanol-sodium acetate, pH 4.2 (30:70). Compounds 1-10 as in Fig. 1.

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CONCLUSIONS

In order to increase the reproducibility and enhance the separation of previously published HPLC procedures for the simultaneous analysis of the conjugated bile acids, optimization of the mobile and stationary phases in isocratic RP-HPLC has been examined. Solvent system A was found to ensure high reproducible chromatography and to afford the complete baseline separation of the ten major bile acid conjugates present in humans, suitable for accurate quantitative analysis. The Supelcosil LC-18-DB column achieved the shortest analysis time while giving optimal resolution and efficiency. This separation module (chromatographic system 4) is less laborious than others reported in the literature since preliminary derivatisation [10] or group separation [8] are not required, and presents several advantages over the previously described HPLC procedures. The isocratic feature of the system enhances the simplicity and reproducibility of the method and makes it preferable to gradient elution procedures [12,14], particularly for routine clinical analysis. Good resolution of the foregoing compounds has also been obtained with radial compression systems [3,9,15], nevertheless the use of stainless-steel columns, as described here, makes the method available for laboratories having only a conventional HPLC equipment. Other authors [1,2] have reported complete separations of the ten human conjugated bile acids on stainless-steel C_{18} columns,

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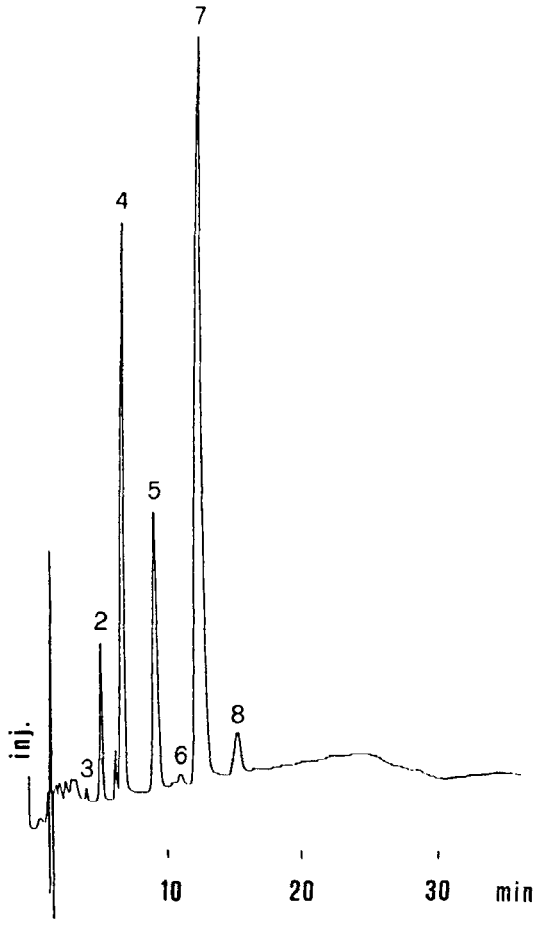

Fig. 8. RP-HPLC separation of conjugated bile acids from a sample of human gastric juice after Sep-Pak C_{18} purification. Conditions as in Fig. 4; peak identification as in Fig. 1.

but the longer analysis time (50 and 60 min) and the lack of good reproducibility [11] are disadvantages.

The rapid and simple isocratic RP-HPLC system (chromatographic system 4) developed here is being applied in this laboratory to the assay of the conjugated bile acids in gastric juice specimens from patients with gastric ulcers, and the results will be reported elsewhere. A representative chromatogram of the conjugated bile acid pattern in human gastric juice, after Sep-Pak C_{18} purification [9], is illustrated in Fig. 8. Using the UV detection at 205 nm, amounts as low as 20-30 ng of individual compound per injection can be measured. Moreover the well separated peaks permit unequivocal identification, accurate and rapid quantification of the conjugated bile acids suitable for routine analysis.

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