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RETENTION BEHAVIOUR OF BILE ACIDS IN ION SUPPRESSION AND ION-PAIR CHROMATOGRAPHY ON BONDED PHASES

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SUMMARY

The retention behaviour of some free bile acids and succinyl derivatives in acidic form at low pH (3) and as ion pairs at higher pH (7.5) in the presence of quaternary alkylammonium has been studied in a reversed-phase chromatographic system using C_{18} or C_8 bonded stationary phases and mixtures of acetonitrile or methanol and aqueous phosphate buffer as the mobile phase. The influence of pH, organic solvent content, quaternary alkylammonium concentration, stationary phase and temperature on retention has been investigated. The more efficient separations for bile acids either by ion suppression or by ion-pair chromatography are presented. The use of UV detection and determination of response for the bile acids, operating at a wavelength in the range 200–220 nm, indicate that high responses and detection limits of 0.02–1 nmole are obtained below 205 nm.

INTRODUCTION

In recent years a number of chromatographic methods for the separation of bile acids have been reported. Güveli and Barry[1] used column and thin-layer chromatography to separate bile acids and their salts. Gas–liquid chromatography (GLC) and gas chromatography–mass spectrometry coupling (GC–MS) have been applied to the separation and determination of isomeric bile acids with many successful applications[2,3]. A review has recently appeared covering this subject in detail[4]. However, the necessary derivatization of bile acids prior to GLC has inherent disadvantages that hamper its application.

In recent years, a great deal of attention has been paid to studying the analysis of free bile acids and their tauro and glyco conjugates by high-performance liquid chromatography (HPLC). A variety of HPLC systems, including adsorption, liquid–

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liquid partition and reversed-phase systems have been used and HPLC of bile acids is at a stage of rapid development. Consideration of the various chromatographic systems mentioned indicates that most workers used reversed-phase (RP) HPLC with a very wide range of mobile phases, each author having proposed a system for the separation of the bile acids with which he had to deal without any clear explanation about his choice. Aqueous mobile phases with methanol or propanol have been buffered at both low and high pH. In the latter case, if we consider that the separation is applied to acidic solutes, the eluting system does not seem well adapted. Also, a separation referred to as "ion-pairing partition" has been used[5] but the use of quaternary ammonium as the counter ion together with a mobile phase at pH 2.85 makes an ion-pair process doubtful, at least for acids with a pK above 3, which is the case for bile acids. Elliott and Shaw[6] have extensively reviewed advances in this field. Shaw *et al.*[7] investigated the relationship between the retention and structure of bile acids[7]. Parris[8] reported the ion suppression chromatographic (ISC) technique for the separation of bile acids. However, ion-pair chromatography (IPC) of bile acids has received much less attention, and little attention has been paid to the separation of the free bile acids.

It is common knowledge that reversed-phase ion-pair chromatography is very suitable for the separation of ionic compounds. It offers many parameters such as organic–aqueous composition, pH, nature of the counter ion and type of bonded phase for the control of separation selectivity. Another advantage of ion-pair chromatography is that an increase in the photometric detection response can be obtained by a proper choice of the counter ion[9,10]. The retention behaviour of organic acids in IPC has been widely investigated[11–13] and three hypotheses to describe the mechanism of the ion-pair phenomenon have been proposed, although the exact mechanism is still uncertain[13,14]. Bile acids are structurally related to other organic acids and steroids by the presence of a carboxyl group and with a lack of unsaturation or aromaticity in their structure. The ion-pair chromatographic technique offers a possibility for their separation.

In this work, we investigated the retention behaviour of the more common free bile acids and some of their oxo (keto) and succinyl derivatives which differ in the extent of hydroxyl, keto or acidic group substitution, in acidic forms (non-ionized) and as ion pairs in RP-HPLC. We intended to rationalize the problem in order to optimize their separation by studying the influence of the various factors that affect the separation, on the one hand according to the ion suppression technique and on the other hand by ion pairing in a mobile phase at a particular pH. Thus, the influence of pH, mobile phase composition and nature, counter ion and its concentration, stationary phase and temperature on retentions has been studied.

EXPERIMENTAL

Apparatus

The HPLC equipment used was a Chromatem 800 solvent delivery system with a Model 420 microprocessor controller/programmer (Touzart et Matignon, France) and a high-pressure sampling valve (Type 71-25, Rheodyne, U.S.A.) with a 20-μl sample loop. For gradient elution, a Waters M 740 liquid chromatograph with a Model U6K injector was used. The detector used was an SPD-2A ultraviolet spectro-

photometric detector (Shimadzu, Japan) with variable wavelength between 195 and 350 nm.

The columns used were a μBondapak C_{18} column (30 cm × 4 mm I.D.) obtained from Waters Assoc. (U.S.A.) and a 150 × 4.6 mm I.D. stainless-steel column with a polished inner surface and packed by a slurry technique using carbon tetrachloride as the slurry liquid and isooctane as eluent. Nucleosil C_{18} (5 μm) and Nucleosil C_8 (5 μm) (Macherey, Nagel & Co., F.R.G.) were used as packings.

The pH measurements were performed with a TS 4N pH meter (Tacussel) equipped with a glass electrode and a saturated calomel electrode.

Samples

The free bile acids studied, generously supplied by a private laboratory, and their derivatives are listed in Table I. All the succinyl derivatives were obtained by the usual treatment. The samples were used without further purification. All the abbreviations for bile acids in the text will refer to this table.

Solvents and reagents

Acetonitrile, HPLC grade, far UV, was from Fisons Scientific (U.K.). Methanol, HPLC grade, far UV, was from Prolabo (France). Water was de-ionized, distilled in a quartz still and filtered through 0.45 μm cellulose acetate filters (Millipore, U.S.A.). Tetrabutylammonium (TBA) hydrogen sulphate was from Sigma (U.S.A.). 1-Hexanesulphonic acid, sodium salt, was from Eastman Kodak (U.S.A.). All other reagents were of analytical or equivalent grade.

TABLE I

BILE ACIDS STUDIED

No.	*Common name*	*IUPAC name*	*Abbreviation in the text*
	Free bile acids		
1	Cholic	3α,7α,12α-Trihydroxy-5β-cholanic	C
2	Deoxycholic	3α,12α-Dihydroxy-5β-cholanic	DC
3	Chenodeoxycholic	3α,7α-Dihydroxy-5β-cholanic	CDC
4	Ursodeoxycholic	3α,7β-Dihydroxy-5β-cholanic	UDC
5	Hyodeoxycholic	3α,6α-Dihydroxy-5β-cholanic	HDC
6	Lithocholic	3α-Hydroxy-5β-cholanic	LC
	Oxo derivatives		
7	7-Oxocholic	7-Oxo-3α,12α-dihydroxy-5β-cholanic	7-OC
8	12-Oxocholic	12-Oxo-3α,7α-dihydroxy-5β-cholanic	12-OC
9	3-Oxocholanic	3-Oxo-5β-cholanic	3-OCA
10	7,12-Dioxocholic	7,12-Dioxo-3α-hydroxy-5β-cholanic	7,12-OC
11	3,12-Dioxocholanic	3,12-Dioxo-5β-cholanic	3,12-OCA
12	Trioxo cholanic	3,7,12-Trioxo-5β-cholanic	TOCA
	Succinyl derivatives		
13	Succinyldeoxycholic	3α-Succinyl-12α-hydroxy-5β-cholanic	SDC
14	Succinyllithocholic	3α-Succinyl-5β-cholanic	SLC
15	Succinyloxocholic	3α-Succinyl-7-oxo-12α-hydroxy-5β-cholanic	SOC

Procedure

The mobile phase was prepared by mixing known volumes of acetonitrile or methanol with aqueous phosphate buffers or, in the case of ion-pair chromatography, with aqueous phosphate buffers containing the counter ion. The concentration of phosphate in the mobile phase was $2 \cdot 10^{-3}$ *M* in ion suppression and $5 \cdot 10^{-3}$ *M* in ion-pair chromatography.

The pH was adjusted to the desired value by the addition of either phosphoric acid or sodium hydroxide. As the pH measurements were performed directly in the mobile phase, the pHs obtained are only apparent values[15]. The mobile phases were then filtered through a 0.45 μm Fluoropore filter (Millipore) and degassed in an ultrasonic bath before use. For gradient elution, mixed solvents of acetonitrile and aqueous phosphate buffer were used. In all the chromatographic separations the flow-rate of the mobile phase was set at 1 ml/min. The samples were dissolved in the mobile phase or in the mobile phase with methanol depending on their solubility. The separation was performed at ambient temperature (22–23°C) except when evaluating the influence of the column temperature on the retentions. In these experiments, the column was thermostated by a water-jacket connected to a constant-temperature circulating bath and in addition the mobile phases were also thermostated before entering the column. To protect the columns, prolong their lifetime and obtain reproducible capacity factors, at the end of each working day the columns were washed first with water and then with acetonitrile or methanol.

RESULTS AND DISCUSSION

The retention behaviour of solutes in the chromatographic system will be described by the capacity factor k' or the relative capacity factor rk', and the resolution by the separation factor α. For calculation of k' the dead time t_0 corresponds to the baseline disturbance on injection of the mobile phase.

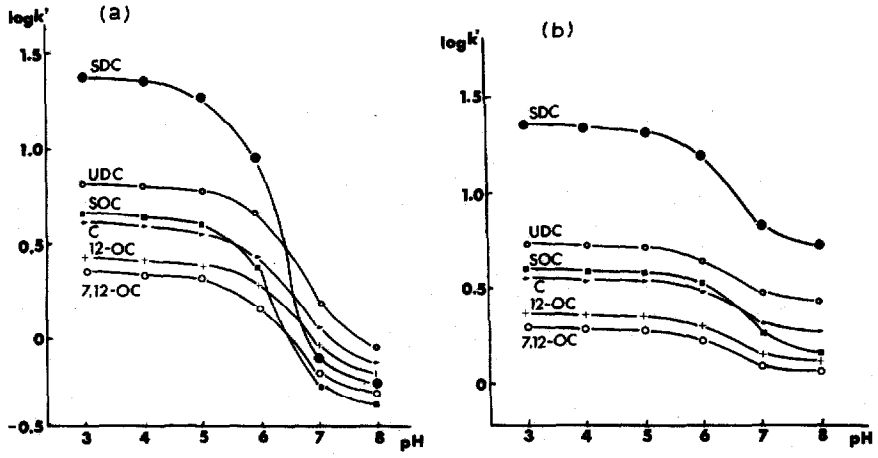

Fig. 1. (a) Change of retention with pH of the mobile phase in ISC. Column: Nucleosil C_{18}. Mobile phase: acetonitrile–phosphate buffer (45:55). (b) Change of retention with pH of the mobile phase in IPC. Column: Nucleosil C_{18}. Mobile phase: acetonitrile–phosphate buffer (45:55) containing 10^{-3} *M* TBA.

Ion suppression chromatography

Influence of pH. The control of pH is a parameter of utmost importance for excellent chromatographic separations of carboxylic acids, as it will dictate the degree of ionization of the species that will exist as neutral undissociated compounds or as anions, and thus it will decide the separation mechanism and retention behaviour. The influence of pH in the case of a mobile phase containing acetonitrile as organic component on the retention of the bile acids is demonstrated in Fig. 1a. Most of the bile acids have apparent pK_a of about 5–6, and above pH 6–7 the drastic decrease in retention is obviously due to the ionization of the bile acids. Fig. 1a shows that the retention of anionic forms is very low, as is general for the organic acids[16]. The anions are hydrophilic and more soluble in the aqueous eluent, undissociated acids are more hydrophobic and more retained on the stationary phase. Moreover, at pH values above 6–7 a greater decrease in retention for succinyloxocholic and succinyldeoxycholic acids, which contain one more carboxylic group than the other bile acids, is observed. This confirms the fact that the dissociation of the carboxylic group is the primary factor for the decrease in retention of the bile acids at high pH.

Influence of the mobile phase composition. Fig. 2a shows the capacity factors for eight bile acids on a Nucleosil C_{18} column as a function of acetonitrile content in the mobile phase buffered at a sufficiently low pH to maintain all the bile acids undissociated. The drastic decrease in the capacity factor for most bile acids with increasing content of acetonitrile in the mobile phase is observed as the usual reversed-phase effect of the solvent strength on the retention of the sample. Fig. 2b shows that similar chromatographic behaviour is obtained with a methanol–phosphate buffer solvent system. Comparing the variations of k' in Fig. 2a and b, it can be seen that for

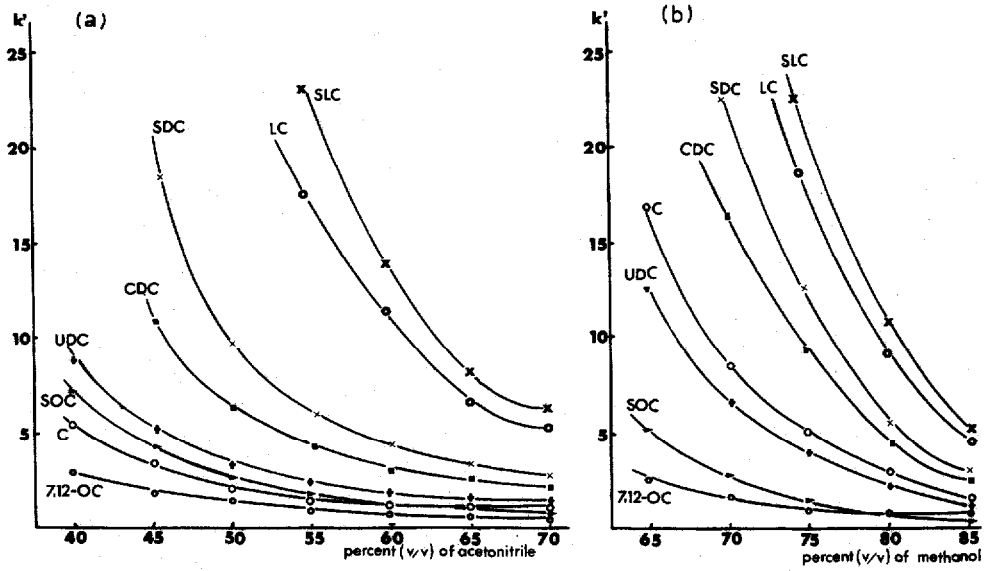

Fig. 2. (a) Dependence of capacity factors (k') in ISC on the percentage of acetonitrile in the eluent. Column: Nucleosil C_{18}. Mobile phase: acetonitrile–phosphate buffer, pH 3. (b) Dependence of capacity factors (k') in ISC on the percentage of methanol in the eluent. Column: Nucleosil C_{18}. Mobile phase: methanol–phosphate buffer, pH 3.

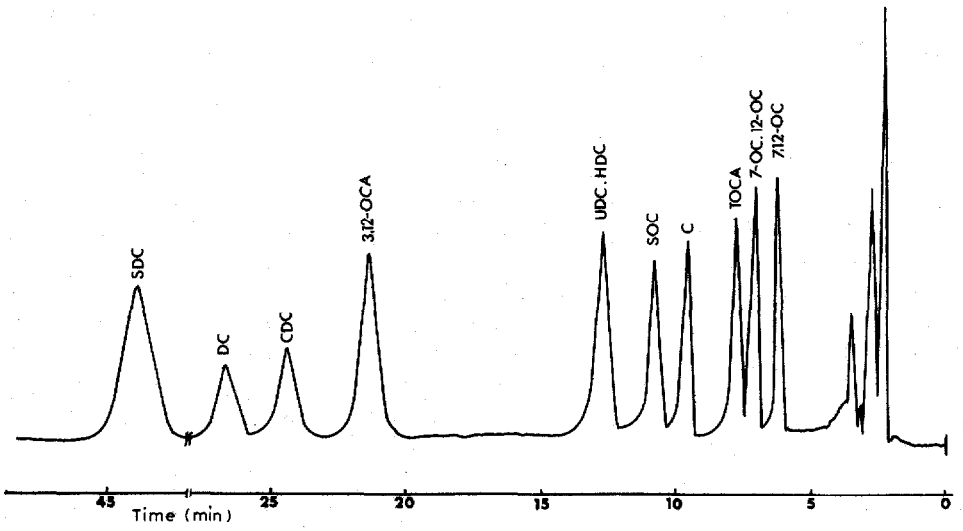

Fig. 3. Separation of bile acids using ISC with acetonitrile as the eluent. Column: μBondapak C_{18}. Mobile phase: acetonitrile–phosphate buffer (45:55), pH 3.

the same organic solvent content, the k' values are much higher for methanol than for acetonitrile. On the other hand, the order of elution for C, UDC and SOC differs according to the organic solvent used. Also, for C and SOC with acetonitrile solvent and for 7,12-OC and SOC with methanol, the order of elution reverses with increasing organic solvent content in the mobile phase.

Fig. 3 shows the separation of a mixture of twelve bile acids having two or three substituents on a μBondapak C_{18} column using acetonitrile–phosphate buffer (45:55) as the mobile phase at pH 3. In this way a chromatogram with good peak symmetry for each bile acid is obtained. The same separation was obtained for a mixture of these bile acids on a Nucleosil C_{18} column using the same mobile phase. The separation of the same compounds on Nucleosil C_{18} with methanol–phosphate (70:30) as the mobile phase is shown in Fig. 4. A similar separation on the μBondapak column using the same methanolic mobile phase was also obtained.

Ion-pair chromatography

Influence of pH. As mentioned above, it is possible to modify the chromatographic retention of the bile acids by introducing a quaternary ammonium ion into the mobile phase. However, the retention depends very much on the pH of the eluent. Fig. 1b shows the changes in the retention of various bile acids with variation of the pH of the mobile phase containing TBA as counter ion. At low pH (up to 5) the k' values remain relatively constant. This is a similar situation as in Fig. 1a. At pH 5–7, the retention of bile acids decreases slowly, but at pH above 7 the decrease in retention is less than that in Fig. 1a. This is due to the counter ion which is present in the chromatographic system and governs the retention of the bile acids according to a common mechanism that we shall call ion pairing for the sake of simplicity.

Comparing Fig. 1a and b, it is obvious that the addition of the counter ion to a reversed-phase chromatographic system will affect the retention of the bile acids only

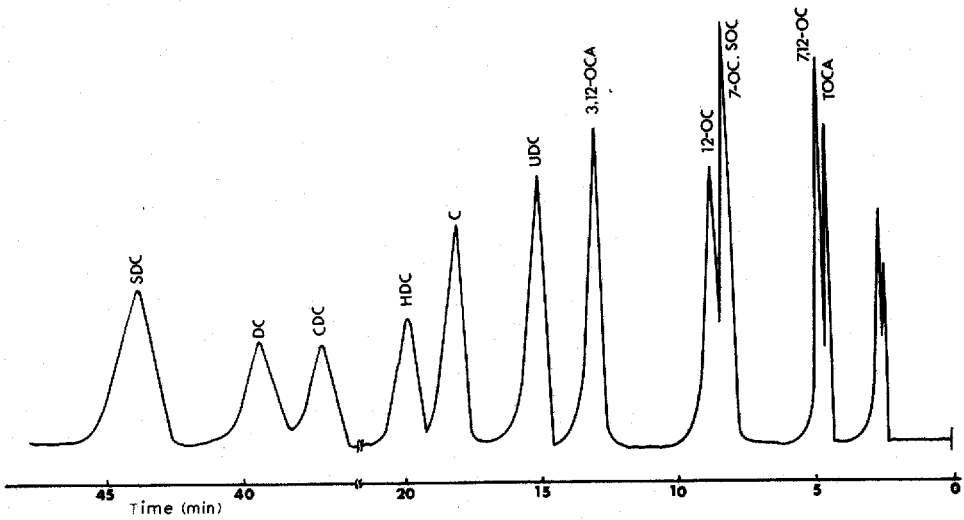

Fig. 4. Separation of bile acids using ISC with methanol as the eluent. Column: Nucleosil C_{18}. Mobile phase: methanol–phosphate buffer (70:30), pH 3.

when they are in the dissociated form. The apparent dissociation constants for free bile acids, pK_a, are 5–6. The inflection points on the bile acids curves are located at pH 5–7. This is in good agreement with the fact that the inflection point, as mentioned by Tilly-Melin *et al.*[16], must be at a pH value near the pK_a value. However, it should be noted that, in any case, the k' values are higher at low pH than at high pH with ion pairing.

Variations in pH influence not only the retention of the bile acids but also their peak shape. At pH below 3 or above 7, whether TBA is present or not in the mobile phase, chromatographic peaks with good symmetry and a narrow band for each bile acid are obtained. At pH 3–7, cholic acid and di- and monosubstituted bile acids give peaks with much greater band broadening and significant tailing. This phenomenon can be explained by the intermediate values of the degree of ionization corresponding to the intermediate pH range. At pH below 3 all the bile acids are non-ionized species, at pH above 7 all are completely dissociated and exist as anions, but at pH 4–6, non-ionized species and anions are simultaneously present in the chromatographic system. The band broadening and tailing can be caused by different chromatographic behaviour of the species which migrate along the column and are concerned with several equilibria: dissociation equilibrium between the acidic and ionic forms and chromatographic equilibrium involving mainly the non-dissociated form. These phenomena result in diffusion and a reduction in the mass-transfer process.

Influence of counter ion concentration. The influence of tetrabutylammonium ion on the retention of carboxylic acids is well documented[17]. The relationship between the retention of the bile acids, as expressed by k', and the TBA concentration is demonstrated in Fig. 5. In each instance, the k' value first increases sharply between 0 and $5 \cdot 10^{-3}$ M, increases slowly between 5 and $15 \cdot 10^{-3}$ M and finally remains relatively constant with further increase in concentration. For most k' values a maximum is attained at about $15 \cdot 10^{-3}$ M but for some of them a slight decrease is observed as the counter ion concentration increases up to $20 \cdot 10^{-3}$ M. The same type

of curve was reported by Knox and Hartwick[18]. The k' values of SOC and SDC continue to increase at counter ion concentrations higher than $1 \cdot 10^{-2}$ *M*. The greater charge on the ionized succinyl bile acid (-2) is probably responsible for its greater attraction for oppositely charged counter ions. These observations show that the retention of the bile acids at low TBA concentration can be regulated by the quaternary ammonium ion concentration, but at higher concentrations the control of retention by the counter ion concentration is very limited. Fig. 5 illustrates that the optimum concentration for most bile acids is between $10 \cdot 10^{-3}$ and $15 \cdot 10^{-3}$ *M*. However, in general, the column lifetime will become shorter as the counter ion concentration increases[19], and as we observed that the peak symmetry decreases with increasing concentration of the counter ion; therefore, the concentration of the counter ion that should be used in ion-pair chromatography of bile acids is below $1 \cdot 10^{-2}$ *M*.

Influence of mobile phase composition. The effect of acetonitrile content in the mobile phase on the retention of the bile acids as ion pairs with TBA is shown in Fig. 6a. The k' values increase with decreasing acetonitrile content. However, the extent and rapidity of the change in the k' values vary with the type of bile acids. In particular, the two carbonyl groups of SOC and SDC bring about larger and more rapid variations. Obviously, the control of retention of the bile acids could be improved by decreasing the acetonitrile content in the eluent, but the experiments show that the peak symmetry decreases considerably with decreasing acetonitrile content, and a tendency for tailing to occur is observed, especially for bile acids with high capacity factors: succinyldeoxycholic acid gives a significantly asymmetric peak below an acetonitrile content of 35%. Fig. 6b shows a similar effect of the methanol content. As already observed in Fig. 2, acetonitrile has a greater effect than methanol on the retention of bile acids.

Fig. 7 shows the separation of twelve bile acids with acetonitrile–phosphate

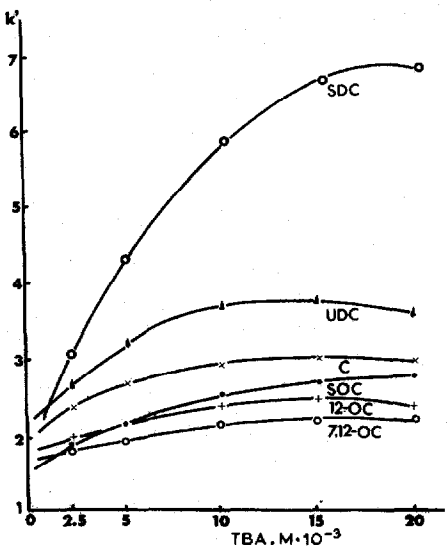

Fig. 5. Effect of counter ion concentration in the eluent on the retention of bile acids with TBA. Column: Nucleosil C_{18}. Mobile phase: acetonitrile–phosphate buffer (45:55), pH 7.5.

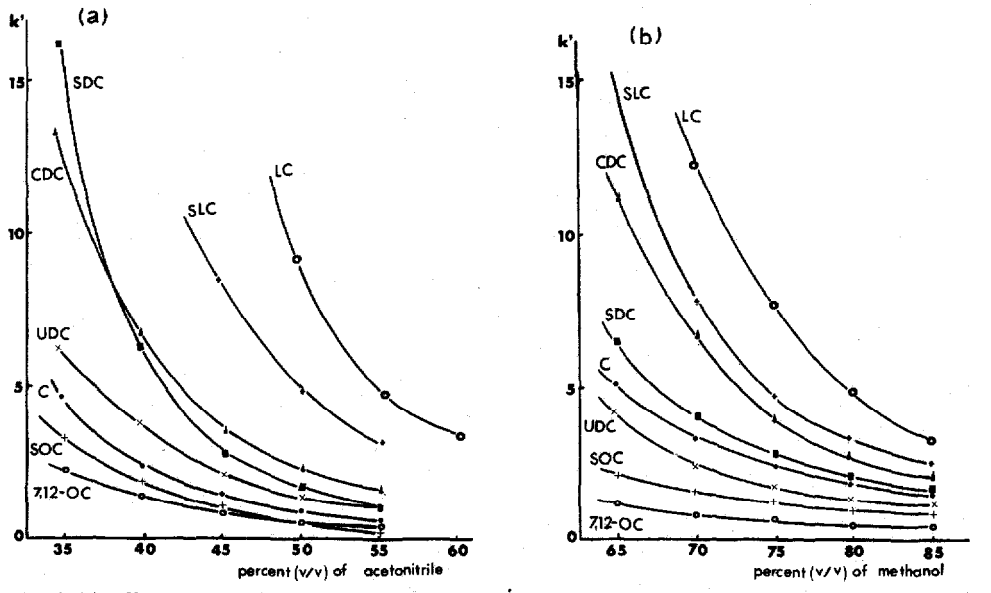

Fig. 6. (a) Effect of acetonitrile content in the eluent on the retention of bile acids using IPC. Column: Nucleosil C_{18}. Mobile phase: acetonitrile–phosphate buffer, pH 7.5, with $5 \cdot 10^{-3}$ *M* TBA. (b) Effect of the methanol content in the eluent on the retention of the bile acids in IPC. Column: Nucleosil C_{18}. Mobile phase: methanol–phosphate buffer, pH 7.5, with $5 \cdot 10^{-3}$ *M* TBA.

buffer containing TBA as the mobile phase on a μBondapak C_{18} column. All the bile acids, except 7-OC and 12-OC, are well separated with reasonable separation factors. Similar separations of the same compounds can be obtained on Nucleosil C_{18} using the same solvent system, but the separation factors are slightly lower than those on μBondapak C_{18}. Fig. 8 shows the separation of these bile acids on Nucleosil C_{18} with

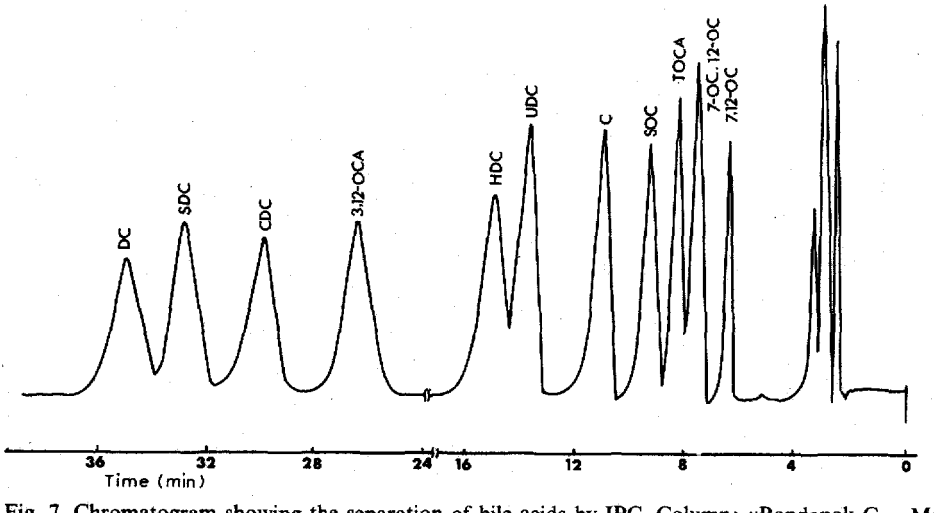

Fig. 7. Chromatogram showing the separation of bile acids by IPC. Column: μBondapak C_{18}. Mobile phase: acetonitrile–phosphate buffer (35:65), pH 7.5, with $5 \cdot 10^{-3}$ *M* TBA.

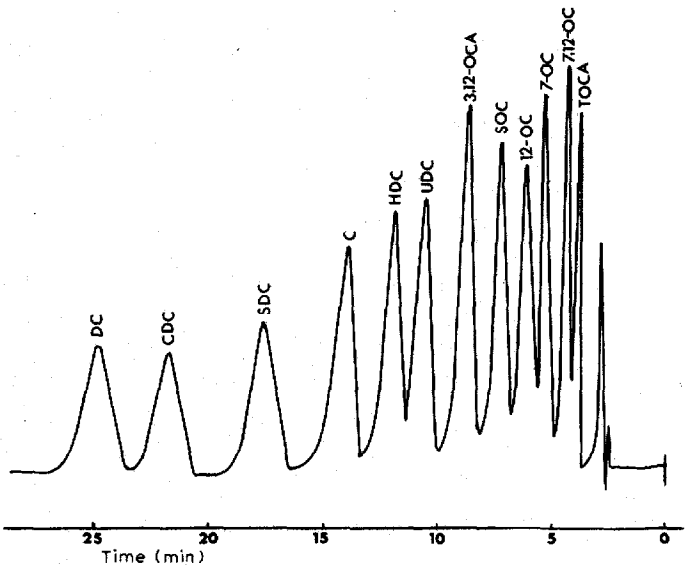

Fig. 8. Chromatogram showing the separation of bile acids by IPC. Column: Nucleosil C_{18}. Mobile phase: methanol–phosphate buffer (70:30), pH 7.5, with $5 \cdot 10^{-3}$ *M* TBA.

methanol–phosphate buffer containing TBA as the mobile phase at high pH. All the bile acids are separated and the time required for the separation is shorter.

Influence of negatively charged species. It is known that the addition of negatively charged species has a considerable influence on the retention of acids[14] and thus could modify their separation and selectivity. Fig. 9 illustrates the effect of hexanesulphonate ion concentration on the retention of bile acid anions at high pH. In each instance k' decreases slightly between 0 and $2.5 \cdot 10^{-3}$ *M* hexanesulphonate concentration, then increases or tends to remain relatively constant with further increase in

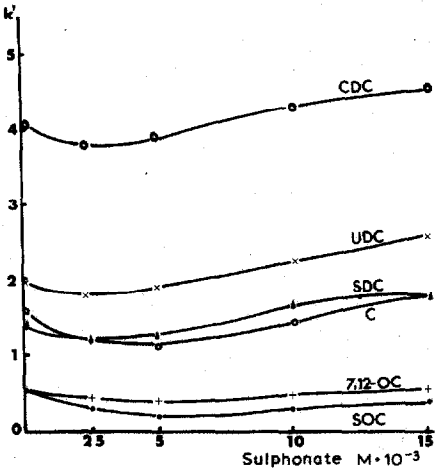

Fig. 9. Effect of hexanesulphonate concentration in the eluent on the retention of bile acids. Column: Nucleosil C_{18}. Mobile phase: acetonitrile–phosphate buffer (35:65), pH 7.5.

concentration. This effect of negatively charged species on the k' values of the bile acids is different from the effect of octanesulphonate on the retention of negatively charged solutes as reported by Bidlingmeyer *et al.*[14]. This may be due to the opposite effects of electrostatic force and solubility. Electrostatic repulsions cause a decrease in retention on adding sulphonate (of any kind) to the eluent, whereas it has been observed that the addition of hexanesulphonate decreases the solubility of the bile acids in the mobile phase. This results in an increase in the affinity of the solutes for the stationary phase which exceeds the electrostatic forces when the alkylsulphonate content increases. However, in general, the control of the retention and selectivity of bile acids as anions by hexanesulphonate is very limited.

Selectivity

The selectivities obtained with the different chromatographic conditions tested are compared by calculating the separation factors, α, for three pairs of bile acids, 12-OC–TOCA, UDC–HDC and CDC–DC, which are the worst separated. The results, which correspond to different chromatographic systems as listed in Table III, are shown in Table II. Using acetonitrile as the mobile phase, the separation factors are almost identical, near 1.0, on all the stationary phases, except for CDC–DC. With the methanolic mobile phase the selectivity is better for 12-OC–TOCA and UDC–HDC, but not for CDC–DC. The results indicate that methanol is preferable to acetonitrile as an organic modifier in the mobile phase in all the chromatographic systems to improve the separation selectivity of the bile acids. For example, from Figs. 3 and 4, it can be seen that using acetonitrile the peaks of 7-OC and 12-OC on the one hand, and those of UDC and HDC on the other, coincide, but using methanol only 7-OC and SOC have the same retention, and a good separation of UDC and HDC is obtained.

There are no significant differences in selectivity between the two octadecyl-bonded silicas tested whereas, as could be expected owing to the higher lipophilic

TABLE II

SEPARATION FACTORS FOR THREE PAIRS OF BILE ACIDS USING DIFFERENT CHROMATOGRAPHIC CONDITIONS

*Chromatographic system**	α		
	12-OC–TOCA	*UDC–HDC*	*CDC–DC*
1	0.98	0.97	0.89
5	0.98	0.97	0.89
9	0.99	0.98	0.99
3	0.98	0.95	0.85
7	0.98	0.91	0.88
11	0.98	0.97	0.99
2	1.19	0.75	0.94
6′	1.07	0.88	0.93
10	1.06	0.82	1.00
4′	1.12	0.77	0.89
8′	1.06	0.88	0.92
12	1.13	0.89	1.00

* See Table III.

affinity of long alkyl chains, the selectivity with octadecyl-bonded phases is higher than with octyl-bonded phases.

Discussion of retention mechanism

The retention of bile acids alters sharply with the form in which they exist in the chromatographic system, *i.e.*, neutral (undissociated), anionic or ion pair. The organic solvent used in the mobile phase may have a marked effect on these changes in the retention. From the various experimental conditions tested it can be seen that the most efficient separation can be obtained either by ion suppression at low pH or by ion pairing at high pH with TBA as the counter ion. As could be expected, the separation of the bile acids as anions at high pH is very poor because their retentions are lower. A discussion of the possible retention according to the molecular structures of these compounds is presented below.

Table III lists twelve chromatographic systems differing in the stationary phase, the nature of the mobile phase and the type of chromatography (ISC or IPC), and the retentions of fifteen bile acids using these systems are given in Table IV. To avoid changes in the measured retention of a compound due to the evolution of the column with time and to make easier the comparison when using different mobile phases, the retention is expressed as the relative capacity factor, rk', defined in relation to cholic acid as the reference compound, for which the k' values are also given in Table IV.

At high pH, although the carboxylic group is ionized, some retention can be obtained, as shown in Fig. 1. The molecule of a bile acid contains a carboxylic group bound to a hydrocarbonaceous ring system with a non-polar β side and a polar α

TABLE III

CHROMATOGRAPHIC SYSTEMS USED

No.	*Stationary phase*	*Mobile phase*		
		Composition	*pH*	*TBA concentration (M)*
1	Nucleosil C_{18}	CH_3CN–H_2O (45:55)	3	0
2	Nucleosil C_{18}	CH_3OH–H_2O (72.5:27.5)	3	0
2′	Nucleosil C_{18}	CH_3OH–H_2O (70:30)	3	0
3	Nucleosil C_{18}	CH_3CN–H_2O (35:65)	7.5	$5 \cdot 10^{-3}$
3′	Nucleosil C_{18}	CH_3CN–H_2O (45:55)	7.5	$5 \cdot 10^{-3}$
4	Nucleosil C_{18}	CH_3OH–H_2O (75:25)	7.5	$5 \cdot 10^{-3}$
4′	Nucleosil C_{18}	CH_3OH–H_2O (70:30)	7.5	$5 \cdot 10^{-3}$
5	μBondapak C_{18}	CH_3CN–H_2O (45:55)	3	0
6	μBondapak C_{18}	CH_3OH–H_2O (75:25)	3	0
6′	μBondapak C_{18}	CH_3OH–H_2O (72.5:27.5)	3	0
7	μBondapak C_{18}	CH_3CN–H_2O (35:65)	7.5	$5 \cdot 10^{-3}$
8	μBondapak C_{18}	CH_3OH–H_2O (75:25)	7.5	$5 \cdot 10^{-3}$
8′	μBondapak C_{18}	CH_3OH–H_2O (70:30)	7.5	$5 \cdot 10^{-3}$
9	Nucleosil C_8	CH_3CN–H_2O (45:55)	3	0
10	Nucleosil C_8	CH_3OH–H_2O (72.5:27.5)	3	0
11	Nucleosil C_8	CH_3CN–H_2O (35:65)	7.5	$5 \cdot 10^{-3}$
12	Nucleosil C_8	CH_3OH–H_2O (65:35)	7.5	$5 \cdot 10^{-3}$

TABLE IV

RELATIVE CAPACITY FACTORS (rk') OF BILE ACIDS WITH CHROMATOGRAPHIC SYSTEMS IN TABLE III

Bile acid	*Substituents*	*Chromatographic system*											
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
7,12-OC	3α-OH, 7,12=O	0.54	0.18	0.48	0.24	0.56	0.24	0.49	0.23	0.83	0.41	0.70	0.41
12-OC	3α,7α-OH, 12=O	0.64	0.45	0.62	0.49	0.67	0.49	0.62	0.52	0.84	0.63	0.74	0.64
7-OC	3α,7α-OH, 7=O	0.66	0.38	0.64	0.44	0.69	0.46	0.63	0.47	0.86	0.59	0.72	0.56
TOCA	3,7,12=O	0.79	0.16	0.70	0.21	0.77	0.21	0.70	0.19	1.11	0.41	0.98	0.35
SOC	12α-OH, 7=O, 3-S	1.17	0.40	0.76	0.58	1.15	0.34	0.80	0.33	1.22	0.91	0.82	0.56
C*	3α,7α,12α-OH	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
		(4.07)	(5.40)	(4.08)	(2.25)	(3.88)	(2.89)	(3.96)	(1.86)	(2.06)	(1.35)	(2.53)	(2.66)
UDC	3α,7β-OH	1.58	0.81	1.32	0.81	1.41	0.78	1.30	0.71	1.85	1.28	1.80	1.17
HDC	3α,6α-OH	1.60	1.08	1.39	0.87	1.44	1.05	1.43	0.83	1.92	1.56	1.87	1.33
3,12-OCA	3.12=O	2.74	0.69	2.76	0.62	2.00	0.68	2.66	0.57	2.72	1.15	3.16	0.85
CDC	3α,7α-OH	3.04	2.19	3.18	1.50	2.56	2.08	3.03	1.71	2.77	2.19	3.19	1.67
DC	3α,12-OH	3.43	2.31	3.68	1.80	2.95	2.19	3.53	1.82	2.78	2.19	3.23	1.67
SDC	12α-OH, 3-S	6.76	2.47	3.56	1.08	5.64	2.26	3.35	1.15	2.83	2.11	3.19	1.30
LC	3α-OH				3.15		4.30		3.25	10.76	5.48		
SLC	3-S				2.47		5.67		2.26	12.00	6.15		
3-OCA	3=O				2.71		3.55			11.95	4.85		

* The numbers in parentheses are the capacity factors (k') of cholic acid.

side[7]. The polarity of the non-ionic part of the molecule depends on the number and position of polar substituents such as hydroxyl groups fixed on this hydrocarbonaceous moiety. The retention observed even at high pH indicates the strong influence of the lipophilic part of the bile acid molecule, which is sufficient to create an interaction between the bile acid and a lipophilic stationary phase even with a relatively large amount of organic solvent in the mobile phase. This would explain the already published results obtained for the separation of such ionizable compounds within unusual conditions for the chromatography of acids.

When using the same mobile phase, all the bile acids are eluted in the same order on the different stationary phases. On Nucleosil C_{18} and μBondapak C_{18}, the rk' values are closely related and differ from those on Nucleosil C_8 where the interactions between the C_8 alkyl groups and the lipophilic molecules are lower, leading to lower retention times, particularly for the reference cholic acid (Table IV). However, the retentions of monosubstituted bile acids on Nucleosil C_{18} are slightly higher than on μBondapak C_{18}. This may be due to the differences in the siliceous supports, mainly their specific surface areas, and to the concentration of bonded alkyl groups or remaining hydroxyl sites on the silica[20]. The higher retention observed on Nucleosil C_{18} compared with that on μBondapak C_{18} is enhanced when using a methanolic mobile phase.

For the same capacity factor to be obtained, it has been already seen that the percentage of methanol needed in the mobile phase would be higher than that of acetonitrile. It is well known that the acetonitrile eluent strength in RP HPLC is higher than that of methanol. However, the solubility of the bile acids in acetonitrile is much lower than that in methanol[8]. Our results indicate that the retentions are controlled first by the solvent strength, not by the solubility.

On the basis of their retentions, measured as k' or rk', the compounds studied may be divided in three groups corresponding to the number of substituents on the cholanic molecule: trisubstituted bile acids are eluted first, followed by di- and then monosubstituted bile acids. The behaviour of succinyl compounds with two carboxylic groups is different because they are considerably influenced by pH: at low pH the lipophilic character of their molecules is strongly enhanced and in IPC their retention is also much affected by the concentration of TBA, but compared with that in ISC it strongly decreases, mainly with a methanolic eluent (Table IV).

Bonded-phase chromatography generally separates on the basis of the type and number of functional groups within the solute molecule, the changes in retention being caused by the effect of the different substituents on the molecular polarity. For the free bile acids, the elution order varies according to the organic eluent in the mobile phase: with methanol the order is UDC > HDC > C > CDC > DC $\gg$ LC in IPC or UDC > UDC > C > HDC > CDC > DC $\gg$ LC in ISC; with acetonitrile it is C > UDC > HDC > CDC > DC $\gg$ LC in all instances. In general, it can be stated that the lipophilic character of these solutes decreases when the number of hydroxyl groups increases and that axial α-hydroxylated bile acids are eluted later than the β-hydroxylated bile acids with the same substituents. The retention also increases according to the position of the hydroxyl groups in the order $7\beta < 6\alpha < 7\alpha < 12\alpha$.

The presence of oxo groups, similarly to hydroxyl groups, reduces the hydrophobic area of a molecule and consequently reduces the affinity of the species for the

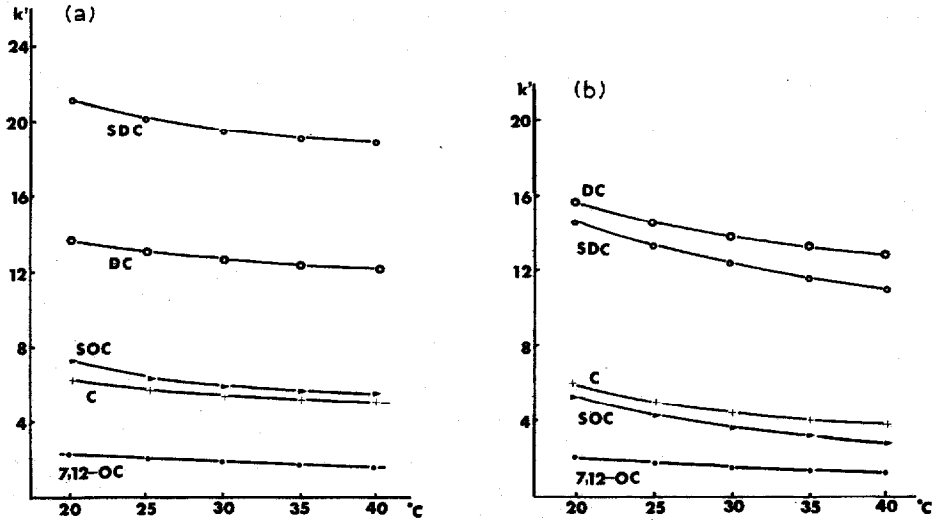

Fig. 10. (a) Effect of temperature on the retention of bile acids in ISC. Column: Nucleosil C_{18}. Mobile phase: acetonitrile–phosphate buffer (45:55), pH 3. (b) Effect of temperature on retention of bile acids in IPC. Column: Nucleosil C_{18}. Mobile phase: acetonitrile–buffer (35:65), pH 7.5, containing $5 \cdot 10^{-3}$ *M* TBA.

bonded stationary phase. The data in Table IV indicate some differences according to the organic solvent used in the mobile phase; the decrease in retention is enhanced with methanol for compounds with both oxo and hydroxyl substituents, and higher hydrogen bonding with a methanolic mobile phase increases the solubility of the bile acids with only oxo groups, and therefore considerably reduces their retention compared with acetonitrile.

Effect of temperature

In order to examine the influence of temperature on retention, the changes in

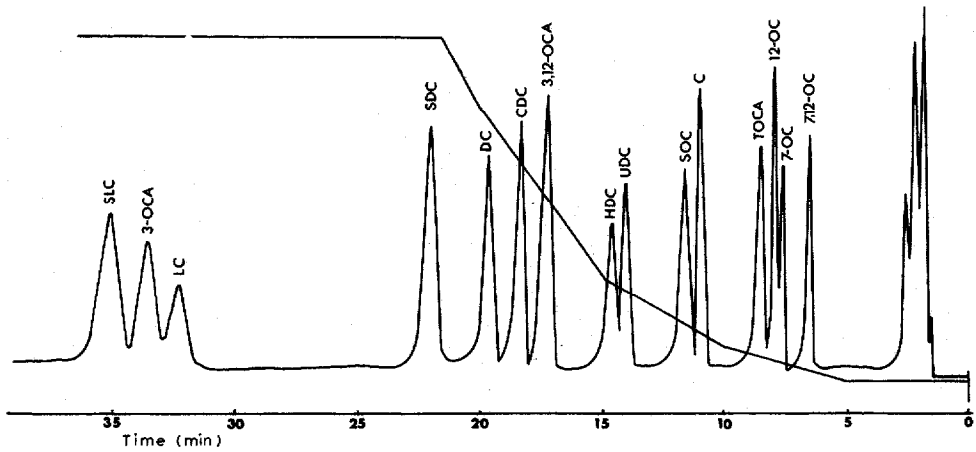

Fig. 11. Chromatogram showing the separation of bile acids with one, two and three substituents in ISC using gradient elution. Column: μBondapak C_{18}. Mobile phase: (A) acetonitrile–phosphate buffer (40:60), pH 3; (B) acetonitrile–phosphate buffer (60:40), pH 3.

the k' values of the bile acids with the column temperature were determined (Fig. 10). The data show that the retentions are little affected by the column temperature. In ISC the k' values decrease with increasing temperature by about 0.5 % per °C change. In ion-pair chromatography the k' values decrease for the trisubstituted bile acids by about 0.6 % per °C increase, and for di- and monosubstituted compounds by about 1–1.3 % per °C increase. No significant change in the selectivity was obtained with temperature increase as is generally found in liquid chromatography[21].

Gradient elution

The polarity of the monosubstituted bile acids is much lower than that of the di- and trisubstituted acids and therefore their retention times are so much higher than they cannot be eluted within a reasonable time under isocratic conditions; the elution time is about 100 min. Under isocratic conditions such that all the bile acids are well separated, the k' values differ widely and the last compounds eluted give tailing peaks with wide band spreading. A solution to the problem is to carry out a gradient elution as shown in Fig. 11 for the separation of a sample mixture containing 15 different bile acids by ISC. In this instance all the bile acids are eluted and separated within 45 min. The k' values lie in the range 3–20. Fig. 12 shows the separation of the same mixture in IPC with gradient elution; the k' values are 3.8–21. To obtain a stable baseline, the initial mobile phase A must be selected so as not to elute the first bile acid too rapidly and a relatively flat gradient was selected; the difference in the content of acetonitrile between the mobile phases A and B, as mentioned in Figs. 11 and 12, was not too great and the absorbance changes due to small acetonitrile concentration changes would prevent the baseline from rising gradually with gradient elution. Because of the higher UV cut-off of methanol compared with acetonitrile it was not possible to carry out gradient elution with a methanolic mobile phase at 205 nm.

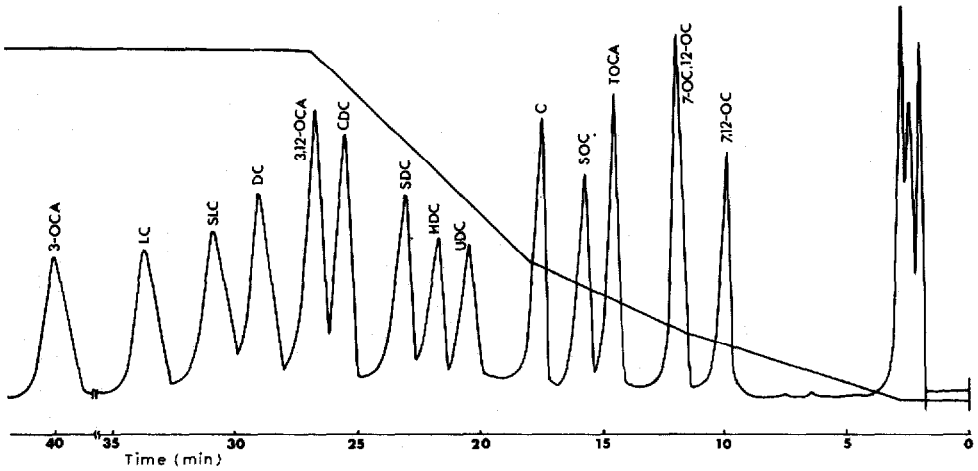

Fig. 12. Chromatogram showing the separation of bile acids with one, two and three substituents in IPC using gradient elution. Column: Nucleosil C_{18}. Mobile phase: (A) acetonitrile–phosphate buffer (30:70), pH 7.5, containing $5 \cdot 10^{-3}$ *M* TBA; (B) acetonitrile–phosphate buffer (65:35), pH 7.5, containing $5 \cdot 10^{-3}$ *M* TBA.

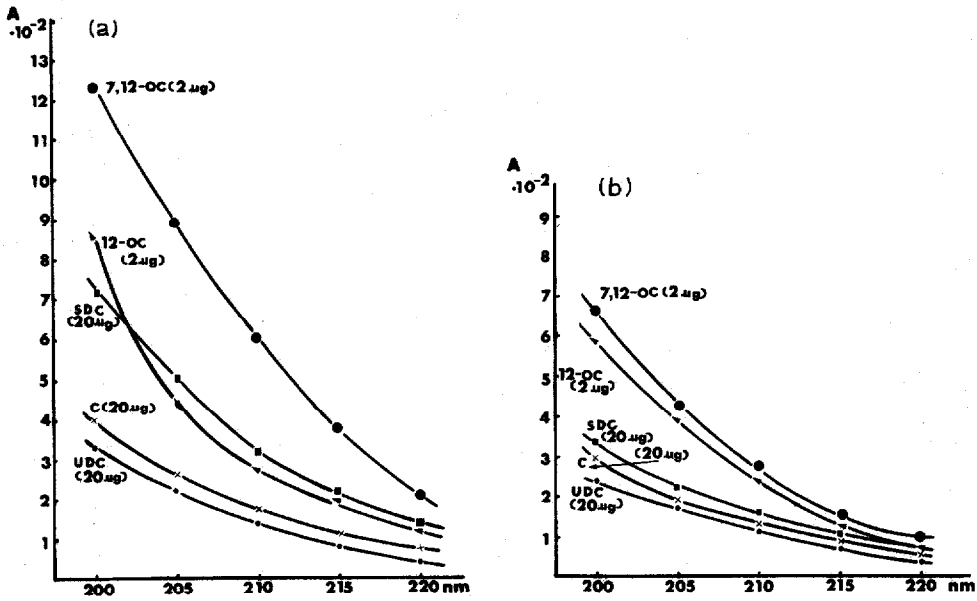

Fig. 13. (a) Influence of UV wavelength on the detection response for bile acids in IPC. Column: Nucleosil C_{18}. Mobile phase: acetonitrile–phosphate buffer (45:55), pH 7.5, with $5 \cdot 10^{-3}$ *M* TBA. (b) Influence of UV wavelength on the detection response for bile acids in ISC. Column: Nucleosil C_{18}. Mobile phase: acetonitrile–phosphate buffer (45:55), pH 3.

Detection response and detection limit

As free bile acids do not have strong UV absorbance, it is often necessary, in order to obtain sufficient sensitivity, to prepare derivatives with a convenient absorbance[22] or to use UV-absorbing counter ions[10]. Parris[8] examined the influence of wavelength on the UV detection of bile acids. The UV detection responses at 200–220 nm for some bile acids with acetonitrile as the mobile phase were determined and are shown in Fig. 13. Acetonitrile as an organic modifier allows higher responses than methanol owing to its lower UV cut-off point. It has been observed that the maximum detection response is at 200 nm but, because of the high noise level at low wavelengths, measurements have been performed at 205 nm where the signal-to-noise ratio

TABLE V

UV DETECTION LIMITS (ng) FOR BILE ACIDS AT 205 nm WITH CHROMATOGRAPHIC SYSTEMS IN TABLE III

Bile acid	*Chromatographic system*			
	1	*3′*	*2′*	*4′*
7,12-OC	9	4	11	9
12-OC	10	9	18	17
C	195	130	380	340
UDC	200	130	320	270
CDC	300	160	450	350
SDC	160	95	300	280

is better. A considerable increase in the detection response is given by using TBA as the counter ion in ion-pair chromatography, as shown in Fig. 13a. A comparison of the various bile acids indicates that the trisubstituted acids with one or two oxo groups have the highest response and, among the disubstituted acids, a higher response is obtained by replacing the hydroxyl group by a succinyl group; the response of SDC is higher than that of DC and CDC.

The detection limits at 205 nm, based on the response of twice the noise level, are shown in Table V for some bile acids. They vary widely from about 0.02 to 1.0 nmole.

REFERENCES

1 D. E. Güveli and B. W. Barry, *J. Chromatogr.*, 202 (1980) 323.
2 K. Imai, Z. Tamura, F. Mashige and T. Osuga, *J. Chromatogr.*, 120 (1976) 181.
3 R. Edenharder and J. Slemr, *J. Chromatogr.*, 222 (1981) 1.
4 H. Jaeger, W. Nebelung, H. U. Kloer, H. Ditschaneit and H. Frank, *Chromatogr. Sci.*, 15 (1981) 365.
5 A. Guffroy, D. Baylocq, F. Pellerin and J. P. Ferrier, *C.R. Acad. Sci., Sér. C*, 290 (1980) 393.
6 W. H. Elliot and R. Shaw, *Chromatogr. Sci.*, 16 (1981) 1.
7 R. Shaw, M. Rivetna and W. H. Elliott, *J. Chromatogr.*, 202 (1980) 347.
8 N. A. Parris, *J. Chromatogr.*, 133 (1977) 273.
9 J. Crommen, B. Fransson and G. Schill, *J. Chromatogr.*, 142 (1977) 283.
10 N. Parris, *Anal. Biochem.*, 100 (1979) 260.
11 J. L. M. Van de venne and J. L. H. M. Henrikx, *J. Chromatogr.*, 167 (1978) 1.
12 C. Prandi and T. Venturini, *J. Chromatogr. Sci.*, 19 (1981) 303.
13 B. A. Bidlingmeyer, *J. Chromatogr. Sci.*, 18 (1980) 525.
14 B. A. Bidlingmeyer, S. N. Deming, W. P. Price, B. Sachok and M. Petrusek, *J. Chromatogr.*, 186 (1979) 419.
15 B. L. Karger, J. N. Lepage and N. Takara, in Cs. Horvath (Editor), *High Performance Liquid Chromatography, Advances and Perspectives*, Vol. 1, Academic Press, New York, 1980, p. 129.
16 A. Tilly-Melin, Y. Askemark, K. G. Wahlund and G. Schill, *Anal. Chem.*, 51 (1979) 976.
17 E. Tomlinson, T. M. Jefferies and C. M. Riley, *J. Chromatogr.*, 159 (1978) 315.
18 J. H. Knox and R. A. Hartwick, *J. Chromatogr.*, 204 (1981) 3.
19 R. Gloor and E. L. Johnson, *J. Chromatogr. Sci.*, 15 (1977) 413.
20 N. H. C. Cooke and K. Olsen, *J. Chromatogr. Sci.*, 18 (1980) 512.
21 R. K. Gilpin and W. R. Sisco, *J. Chromatogr.*, 194 (1980) 285.
22 F. Stellaard, D. L. Hachey and P. D. Klein, *Anal. Biochem.*, 87 (1978) 359.