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REVERSED-PHASE ION-PAIR LIQUID CHROMATOGRAPHY OF TETRACYCLINES

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SUMMARY

Tetracyclines have been chromatographed on alkyl-bonded silica supports using reversed-phase ion-pair chromatography in mobile phases of acetonitrile–water in phosphoric acid. The capacity factors of the tetracyclines were strongly dependent upon the concentration of acetonitrile in the mobile phase. The selectivity of the chromatographic systems decreased with increasing concentration of the organic modifier in the mobile phase. Large variations of the retention were observed on supports from different manufacturers, although the nominal alkyl chain lengths were identical. Only minor differences in the selectivity were found with the different supports.

The retention of the tetracyclines can be regulated by the addition of organic ammonium compounds to the mobile phase. Retention models comprising adsorption of the solutes to different types of adsorption sites are discussed. The influence on the retention of the addition of inorganic anions (Cl^-, Br^- and ClO_4^-) as their sodium salts to the mobile phase has also been studied.

INTRODUCTION

Antibiotics in pharmaceutical formulations, sera and tissues are at present mainly determined by microbiological methods of assay[1]. Liquid chromatography using ion-exchange, normal or reversed-phase columns has also been used[2–5].

Retention mechanisms for chromatography of tetracyclines on reversed-phase materials have recently been proposed[6,7], such as ion-pair formation or a mixture of mechanisms including ion-pair formation, competition effects, deactivation of active sites, ion exchange and interaction with silanol groups. Very little attention has been paid to the possibilities of regulating the retention in order to optimize the chromatographic conditions[8].

In the present paper, tetracyclines (Fig. 1) have been chromatographed by reversed-phase liquid chromatography using alkyl-bonded silica phases. The retention of the compounds was strongly dependent on the acetonitrile concentration in the mobile phase.

Addition of alkylammonium compounds to the mobile phase has been re-

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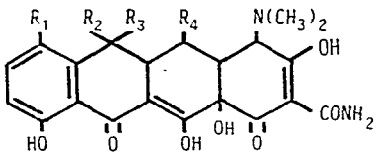

Compound	R_1	R_2	R_3	R_4
Chlortetracycline	Cl	CH_3	OH	H
Demeclomycine	Cl	H	OH	H
Doxycycline	H	CH_3	H	OH
Metacycline	H	$=CH_2$		OH
Oxytetracycline	H	CH_3	OH	OH
Tetracycline	H	CH_3	OH	H

Fig. 1. Structural formulae.

ported to decrease the retention of ammonium solutes in reversed-phase ion-pair chromatography[9–11], and in the present investigation was also found to be a powerful technique in regulating the retention of tetracyclines. The retention data obtained with tetrabutylammonium as additive have been compared with models based on the retention of tetracycline ion pairs on different types of adsorption sites.

The influence of the addition of inorganic anions (Cl^-, Br^- and ClO_4^-) as their sodium salts to the mobile phase on the retention and selectivity has also been studied.

EXPERIMENTAL

Apparatus

The pump used was of the LDC 711 solvent delivery system type. The stainless-steel columns (150 × 4 mm I.D. × 1/4 in. O.D.) were equipped with modified Swagelok® connectors and Altex stainless-steel frits (2 μm). The chromatographic detector was an LDC Spectromonitor III having a 10.0-mm pathlength and a cell volume of 8 μl, and operated at 357 nm. A Rheodyne (Model 70-10) injection valve with a sample loop of 100 μl was used.

Chemicals

The following compounds were used as test samples: tetracycline (ACO Läkemedel, Solna, Sweden), chlortetracycline (Glaxo Läkemedel, Mölndal, Sweden), demeclomycine (Cyanamid Nordiska, Stockholm, Sweden), doxycycline (Ferrosan, Malmö, Sweden), metacycline (Roerig, Täby, Sweden) and oxytetracycline (Pfizer, Täby, Sweden). Unless otherwise stated, 1 μg of each tetracycline dissolved in 10 μl of 10^{-2} *M* phosphoric acid was injected into the liquid chromatograph.

The following supports were used: LiChrosorb RP-2, RP-8 and RP-18 (E. Merck, Darmstadt, G.F.R.); Nucleosil C_8 and C_{18} (Macherey, Nagel & Co, Düren, G.F.R.) with a mean particle diameter of 5 μm; Zorbax BP-C8 and BP-ODS (DuPont, Wilmington, DE, U.S.A.) with a mean particle diameter of 7–8 μm; μBondapack C_{18} (Waters Assoc., Milford, MA, U.S.A.) with a mean particle diameter of 10 μm.

The mobile phases contained acetonitrile (E. Merck; Uvasol grade) as organic

modifier and phosphoric acid (E. Merck, p.a. grade) in a final concentration of 10^{-2} *M*. The following organic ammonium compounds were used as additives to the mobile phase: desmethyldesipramine, desipramine, imipramine and chloroimipramine, obtained as chlorides from Ciba-Geigy (Basel, Switzerland). They were extracted as bases into methylene chloride from alkalized aqueous solutions and re-extracted into phosphoric acid.

Tetrabutylammonium iodide, tetrapropylammonium iodide and tetraethylammonium iodide (Kebo, Stockholm, Sweden) and N-methylimipramine iodide (synthesized from imipramine[12] and kindly supplied by Dr. P.-O. Lagerström, Hässle, Mölndal, Sweden) were converted into phosphates by treatment with silver oxide in phosphoric acid[13].

Chromatographic technique

The chromatographic columns were packed by the slurry packing technique using glycerol–methanol (1:3) as the suspension medium[14]. The slurry was forced into the column at a flow-rate of 9 ml/min or a pressure of 5000 p.s.i., whichever was the limiting factor. The mobile phases were passed through the chromatographic system until constant retention was obtained. Usually less than 50 ml were required. The interstitial volume, V_m, of the columns was determined by the injection of 0.1 *M* phosphoric acid (10 μl).

A 7-cm pre-column, packed with the same support as the separation column, was connected between the pump and the injector. The pre-columns were replaced every second day. In this way the lifetime of the separation column was considerably increased when the mobile phase contained ammonium additives (*cf.*, ref. 11).

All capacity factors (k) given in figures and tables are averages from at least three determinations. The mobile phase flow-rate was 1.0 ml/min throughout this study. The experimental temperature was 25.0 $\pm$ 0.5°C.

RESULTS AND DISCUSSION

Liquid chromatography on alkyl-bonded supports without ammonium additives in the mobile phase

The k' values of the tetracyclines are strongly dependent upon the concentration of acetonitrile in the mobile phase. Fig. 2 shows the relationship between k' (log scale) and percentage (v/v) of acetonitrile in the mobile phase on solid phases with different alkyl chain lengths (LiChrosorb RP-2, RP-8, RP-18). The solutes were highly retarded on LiChrosorb RP-8 and RP-18 and only the RP-2 support permitted isolation of all tetracyclines at optimal separation speed (*cf.*, ref. 8). The selectivity decreased with increasing concentration of acetonitrile in the mobile phase and almost no differences in retention of the tetracyclines were observed at an acetonitrile content of 90%. The retention on RP-8 and RP-18 passes through a minimum at 40–50% acetonitrile. This is rather unusual since most samples show a more or less linear decrease of log k' with increasing concentration of organic modifier in the mobile phase, expressed as a percentage[15–18] or as the logarithm of the molar concentration[19–21].

Similar chromatographic behaviour (Fig. 2) has however been observed for anthraquinone glycosides on LiChrosorb RP-2, RP-8 and RP-18[22].

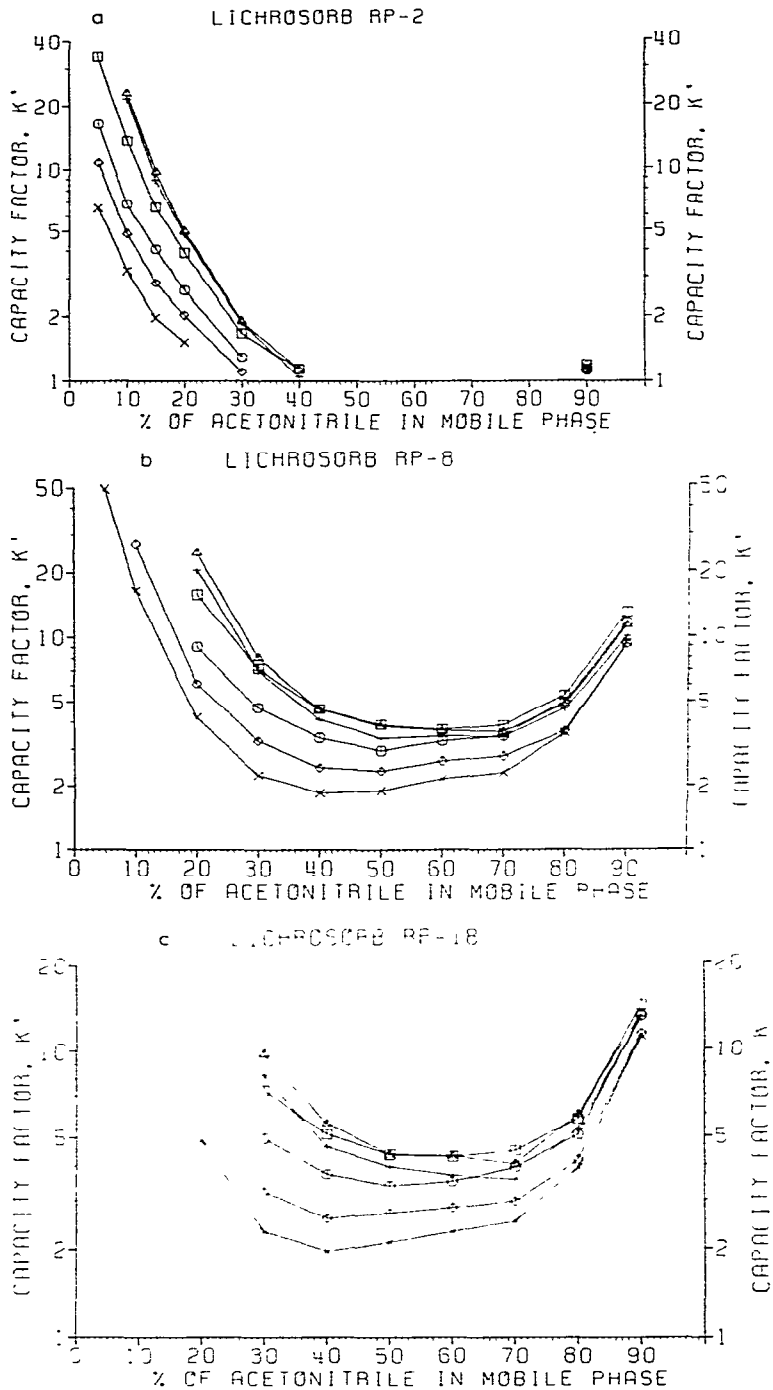

Fig. 2. Retention and concentration of acetonitrile in mobile phase. Mobile phase: phosphoric acid (10^{-2} *M*) in acetonitrile–water; flow-rate 1.0 ml/min. Sample: 1 μg of each solute in 10 μl of 10^{-2} *M* phosphoric acid. Solutes: □, chlortetracycline; ○, demeclomycin; △, doxycycline; +, metacycline; ×, oxytetracycline; ◇, tetracycline. Supports: a, LiChrosorb RP-2; b, LiChrosorb RP-8; c, LiChrosorb RP-18.

Large differences in the retention of the tetracyclines were observed using support materials from different suppliers (Tables I and II). Using a mobile phase containing 30% of acetonitrile, LiChrosorb and Zorbax BP supports gave k' values about fifteen times higher than did Nucleosil supports, for supports of identical nominal alkyl chain lengths. On the μBondapak C_{18} support the retention was very low. Increase of the acetonitrile concentration from 30% to 90% resulted in an increase of the retention on all supports except Nucleosil C_8 and μBondapak C_{18}. For identical mobile phase compositions, only minor variations in selectivity were observed for the different alkyl-bonded supports.

Several authors[14,22–24] have reported an increase in retention with increasing nominal alkyl chain length. In this study an increase in retention of the tetracyclines was found when changing the LiChrosorb RP-2 column for one of LiChrosorb RP-8 (Fig. 2a and b). However, a further increase of the alkyl chain length, by use of a LiChrosorb RP-18 column, only resulted in a very slight increase of k' for the tetracyclines (Fig. 2b and c, *cf.*, ref. 22). A similar slight increase of the retention was observed when comparing Zorbax BP-C_8 and C_{18}, and Nucleosil C_8 and C_{18}, respectively (Tables I and II).

Only minor differences in selectivity due to differences in alkyl chain length were observed, which is in accordance with results obtained for anthraquinone glycosides on LiChrosorb supports[22]. An increase in selectivity with increasing alkyl chain length has been reported previously[24,25].

Liquid chromatography on alkyl-bonded supports with ammonium additives in mobile phase

The influence of organic ammonium additives on the chromatographic behaviour of tetracyclines was studied using LiChrosorb RP-8 as support with mobile phases of 0, 20 and 95% of acetonitrile in 10^{-2} *M* phosphoric acid. The addition of tetrabutylammonium (TBA) to the mobile phases resulted in a strong decrease in retention of the tetracyclines (Fig. 3), but did not significantly change the selectivity of the chromatographic systems. A decrease in retention was obtained even with 10^{-5} *M* TBA, Fig. 3b and c. When the mobile phase contained 20% of acetonitrile the curves describing the influence of the concentration of TBA on the retention tended to flatten out at higher concentrations of TBA, and only minor changes in the retention were observed when increasing $[TBA]_m$ from 10^{-2} *M* to 10^{-1} *M* (Fig. 3b). The retention of the tetracyclines was still strongly decreased by an increase of $[TBA]_m$ from 0.1 *M* to 1.0 *M* when no acetonitrile was present in the mobile phase (Fig. 3a). When the mobile phase contained 95% of acetonitrile all tetracyclines were non-retarded at $[TBA]_m \geqslant 10^{-2}$ *M* (Fig. 3c). The curves in Fig. 3 indicate differences in retention mechanisms dependent upon the concentration of acetonitrile in the mobile phase, *e.g.*, the behaviour in Fig. 3b may be due to the presence of at least two types of binding sites having quite different affinities for ammonium ion pairs, while the behaviour in Fig. 3c most likely is the result of competition for one type of adsorption site only (*cf.*, ref. 10).

Retention model

A retention model for reversed-phase ion-pair chromatography of ammonium

TABLE I

RETENTION ON REVERSED-PHASE SUPPORTS FROM DIFFERENT SUPPLIERS

Concentration of acetonitrile in mobile phase: 30%. Other conditions as in Fig. 2.

Compound	*Capacity factor, k'*						
	LiChrosorb RP-8	*Zorbax BP-C8*	*Nucleosil C_8*	*LiChrosorb RP-18*	*Zorbax BP-ODS*	*Nucleosil C_{18}*	*μBondapak C_{18}*
Chlortetracycline	7.2	6.2	0.38	7.3	12.1	1.00	0.67
Demeclomycine	4.7	4.1	0.16	4.9	8.5	0.54	0.33
Doxycycline	8.0	7.1	0.52	9.7	15.5	1.25	0.92
Metacycline	7.0	6.0	0.44	8.1	13.5	1.08	0.83
Oxytetracycline	2.3	1.48	<0.05	2.3	3.0	0.10	<0.05
Tetracycline	3.3	2.6	<0.05	3.2	5.6	0.33	0.17

TABLE II

RETENTION ON REVERSED-PHASE SUPPORTS FROM DIFFERENT SUPPLIERS

Concentration of acetonitrile in mobile phase: 90%. Other conditions as in Fig. 2.

Compound	*Capacity factor, k'*						
	LiChrosorb RP-8	*Zorbax BP-C8*	*Nucleosil C_8*	*LiChrosorb RP-18*	*Zorbax BP-ODS*	*Nucleosil C_{18}*	*μBondapak C_{18}*
Chlortetracycline	13.0	12.1	<0.05	14.2	12.7	1.76	<0.05
Demeclomycine	11.6	10.5	<0.05	13.1	10.7	1.75	<0.05
Doxycycline	11.2	13.4	<0.05	13.1	13.9	1.54	<0.05
Metacycline	10.0	13.1	<0.05	12.9	11.0	1.73	<0.05
Oxytetracycline	9.3	11.0	<0.05	11.1	9.3	1.63	<0.05
Tetracycline	9.3	10.4	<0.05	11.3	10.7	1.63	<0.05

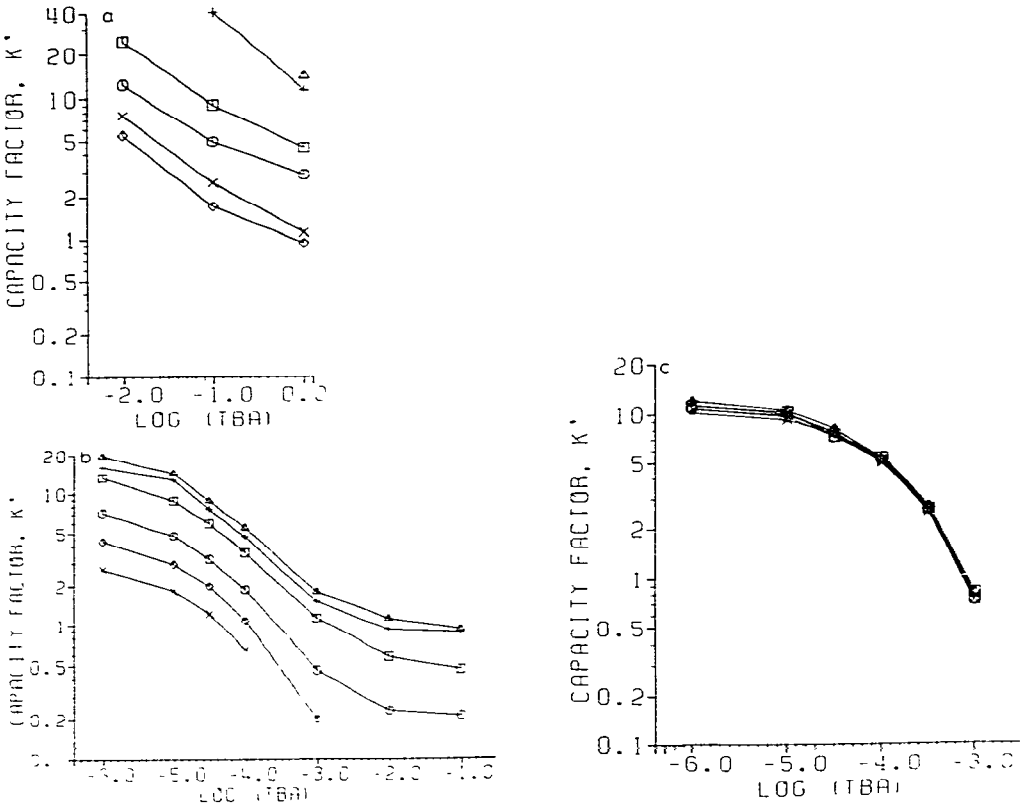

Fig. 3. Retention and concentration of aliphatic ammonium additive in mobile phase. Support: Li-Chrosorb RP-8. Ammonium additive: TBA. Concentration of acetonitrile in mobile phase: a, 0 %; b, 20 %; c, 95 %. Other conditions as in Fig. 2.

compounds has been postulated by Melin *et al.*[10] and by Sokolowski and Wahlund[11]. The capacity factor of the ammonium solute, $k'_{Q,(A^*)}$, is described by

$$k'_{Q,(A^*)} = \frac{W_s K_0^* K_{QZ}^* [Z^-]_m}{V_m (1 + K_{BZ}^* [B^+]_m [Z^-]_m)} \quad (1)$$

where W_s/V_m is the ratio of solid phase to mobile phase in the column, expressed in g/l, and K_0^* is the adsorption capacity, *i.e.*, the maximum concentration of sample that can be adsorbed on the adsorption sites A*, assuming that all ion pairs occupy the same areas as the adsorption site. K_{QZ}^* and K_{BZ}^* are equilibrium constants for adsorption of solute and ammonium ion pairs respectively, defined by

$$K_{QZ}^* = \frac{[QZA^*]_s}{[Q^+]_m [Z^-]_m [A^*]_s} \quad (2)$$

and

$$K_{BZ}^* = \frac{[BZA^*]_s}{[B^+]_m [Z^-]_m [A^*]_s} \quad (3)$$

where $[Q^+]_m$, $[B^+]_m$ and $[Z^-]_m$ are the molar concentrations of solute, ammonium additive and counter ion in the mobile phase, respectively. $[QZA^*]_s$, $[BZA^*]_s$ and $[A^*]_s$ are the concentrations, expressed in moles per gram of solid phase, of adsorbed ion pairs of solute and ammonium additives, and of free adsorption sites of the solid phase at equilibrium, respectively.

Eqn. 1, which is valid in the case of low sample concentrations[11], describes how the concentration and adsorption characteristics of B^+ influence the retention of Q^+ as a result of a competition between the two ammonium compounds for the limited number of adsorption sites on the surface of the support. It can be rewritten as:

$$\frac{1}{k'_Q} = \frac{V_m (1 + K^*_{BZ} [B^+]_m [Z^-]_m)}{W_s K^*_0 K^*_{QZ} [Z^-]_m} \tag{4}$$

With tetrabutylammonium as additive to a mobile phase containing 95% of acetonitrile, a linear relationship was found when plotting $1/k'$ *versus* $[B^+]$ (Fig. 4), which supports the validity of the retention model described by eqn. 1. The plots for the different tetracyclines almost coincided as a result of the very low selectivity of this chromatographic system. Values for $K^*_0 K^*_{QZ} [Z^-]_m$ were calculated from the intercepts [= 24.8 ± 1.2 (mean value ± S.D.)] and $K^*_{BZ} [Z^-]_m$ [= $1.55 \times 10^4 \pm 1.33 \times 10^3$ (mean value ± S.D.)] from the slopes and intercepts.

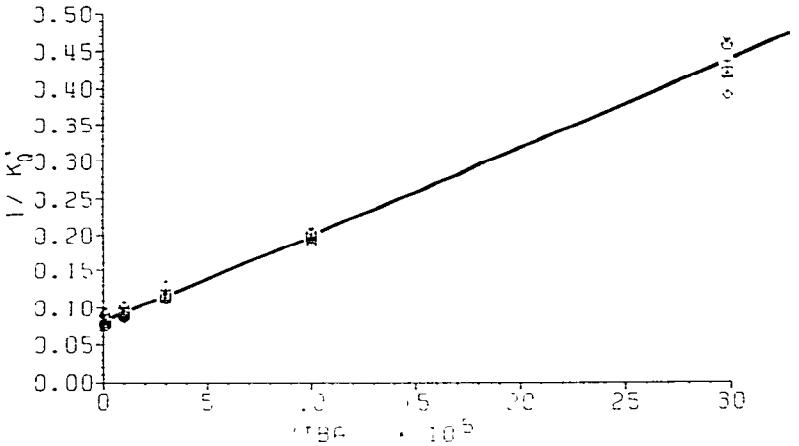

Fig. 4. Fittings of model to retention data. Chromatographic data from Fig. 3c plotted according to eqn. 4. Symbols as in Fig. 2.

When the mobile phases contained 20% of acetonitrile, plots according to eqn. 4 deviated from linearity, indicating that this equation gives a too simplified picture of the retention mechanism in this case.

Expansion of eqn. 1 to give a retention model comprising two different types of adsorption sites has been suggested[10,11]:

$$k'_Q = k'_{Q,(A)} + k'_{Q,(A^*)} = \frac{W_s K_0 K_{QZ} [Z^-]_m}{V_m} + \frac{W_s K^*_0 K^*_{QZ} [Z^-]_m}{V_m (1 + K^*_{BZ} [B^+]_m [Z^-]_m)} \tag{5}$$

Eqn. 5 is valid under the assumption that the tetracyclines are retained as ion pairs with a buffer anion, Z^-, on two different types of adsorption sites. The retention on adsorption site A_s ($k'_{Q,(A)}$) is unaffected by the addition of ammonium additives to the mobile phase, while the solute ion pair and the ion pairs of the ammonium additives are competitively bound to the adsorption site A_s^*, *cf.*, eqn. 1. The equilibrium constant K_{QZ} is defined as in eqns. 2 and 3, and K_0 is the adsorption capacity of the adsorption site unaffected by the addition of ammonium additives.

The hypothesis of two binding sites for the retention of the tetracycline ion pairs on the RP-8 support using mobile phases containing 20% of acetonitrile was tested by a graphical estimation of some of the equilibrium constants. Eqn. 5 can be rewritten as

$$\frac{1}{k'_Q - A} = \frac{V_m}{W_s K_0^* K_{QZ}^* [Z^-]_m} + \frac{V_m K_{BZ}^* [Z^-]_m [B^+]_m}{W_s K_0^* K_{QZ}^* [Z^-]_m} \quad (6)$$

where $A = k_{Q,(A)}$. It was possible to choose the values of A so as to obtain straight lines when plotting retention data according to eqn. 6 for all tetracyclines, when $[CH_3CN]_m$ was 20% and $[B^+]_m$ was within the range 10^{-6}–10^{-4} M, Fig. 5. The correlation coefficients obtained by the least-squares fitting of the lines were in all cases better than 0.9995. The precision of the estimated values of A was *ca.* 5%. Larger variations in the estimated A values resulted in drastic deviations from linearity with a concomitant decrease of the correlation coefficients. From each line, the values of $K_0^* K_{QZ}^* [Z^-]_m$ and $K_{BZ}^* [Z^-]_m$ were calculated from the slopes and the intercepts, and the results are summarized in Table III. The values of $K_{BZ}^* [Z^-]_m$, which contain the equilibrium constant for the adsorption of TBA on the support, obtained from retention values of the different samples, are fairly constant, which supports the validity of the retention model, *cf.* ref. 11.

Deviation from linearity was found when retention data from chromatographic experiments with $[B^+]_m \geq 10^{-3}$ M were included in the plot given in Fig. 5. This suggests that A in fact consists of at least two retention terms whose magnitudes are

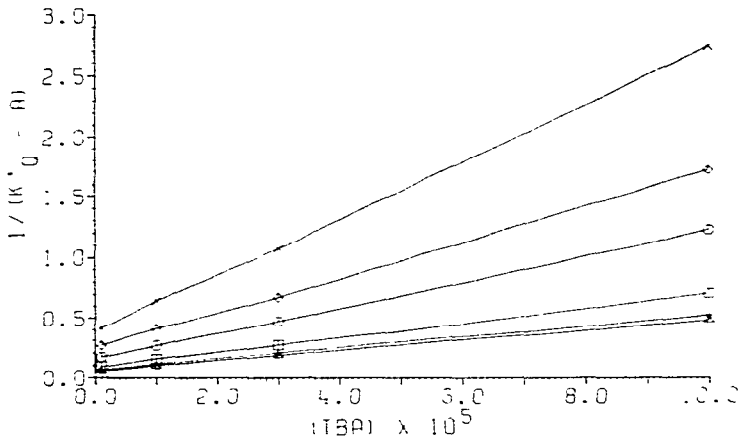

Fig. 5. Fitting of model to retention data. Chromatographic data from Fig. 3b plotted according to eqn. 6. Symbols as in Fig. 2.

TABLE III

EQUILIBRIUM CONSTANTS FROM CHROMATOGRAPHIC RETENTION DATA OF TETRACYCLINES

Ammonium additive: TBA. Other conditions as in Fig. 3b.

Compound	$K_0^* K_{QZ}^* [Z^-]_m$	$K_{BZ}^* [Z^-]_m \times 10^{-4}$	*A*
Chlortetracycline	28.3	7.2	2.1
Demeclomycin	15.5	6.8	1.03
Doxycycline	43.4	7.6	3.3
Metacycline	36.7	6.9	2.7
Oxytetracycline	6.0	5.9	0.28
Tetracycline	9.5	5.7	0.49
Mean ± S.D.		6.7 ± 0.7	

affected differently by the concentration of the ammonium additives. The tendency of the curves in Fig. 3b to flatten out at $[B^+]_m \geqslant 10^{-2}$ *M* indicates that one of these retention terms is unaffected by the addition of ammonium additives, *cf.*, refs. 10 and 11. Fitting of a suitable model to the retention data from chromatographic experiments without acetonitrile in the mobile phase was not possible, as only capacity factors at $[TBA]_m \geqslant 10^{-3}$ *M* were sufficiently low to be determined within a reasonable time, *i.e.*, under conditions where neither the counter-ion concentration, $[Z^-]_m$, nor the ionic strength was constant.

Structure of ammonium additive: influence on retention and selectivity

The capacity factors decreased with increasing carbon number when the additive was a quaternary alkylammonium compound (Table IV). Hence, an increase of the hydrophobicity of the additives[26] results in increased ability to compete for binding sites on the supports.

The effect of ammonium additives with aromatic structures can also be studied since liquid chromatographic detection of tetracyclines is carried out at 357 nm[27]. This is of fundamental interest, since only alkylammonium additives have so far been used for the regulation of the retention of cationic solutes[10,11]. The effect of additives

TABLE IV

RETENTION AND CARBON NUMBERS OF THE AMMONIUM ADDITIVE

Solute: chlortetracycline. TEA = Tetraethylammonium; TPrA = tetrapropylammonium; TBA = tetrabutylammonium. Other chromatographic conditions as in Fig. 3b.

$[B^+]_m$	*Capacity factor, k'*		
	TEA	*TPrA*	*TBA*
10^{-4}	10.4	7.5	3.54
10^{-3}	7.6	3.36	1.12
10^{-2}	3.83	1.50	0.57
10^{-1}	3.00	1.00	0.46

TABLE V

RETENTION AND N-SUBSTITUTION OF AMMONIUM ADDITIVES WITH AROMATIC STRUCTURE

Concentrations: acetonitrile in mobile phase, 20%; ammonium additive in mobile phase, 10^{-4} *M*. Other chromatographic conditions as in Fig. 2b.

Compound	*Capacity factor, k'*			
	Desmethyldesipramine	*Desipramine*	*Imipramine*	*N-Methylimipramine*
Chlortetracycline	2.68	2.50	2.12	2.24
Demeclomycine	1.36	1.10	1.07	1.19
Doxycycline	4.09	3.38	3.26	3.36
Metacycline	3.45	2.90	2.76	2.81
Oxytetracycline	0.44	0.33	0.30	0.35
Tetracycline	0.75	0.62	0.56	0.62
log K_{ex}^{*}	0.14**	0.34	2.14	0.64**

* Organic phase: chloroform. Counter ion: chloride[26].
** Constants estimated according to the rules given in ref. 28.

with aromatic structure (tricyclic antidepressant drugs) on the retention was almost identical to that of TBA. An increase of the hydrophobic character of the additives, as measured by the extraction constants, K_{ex}, of their chloride ion pairs into chloroform, resulted in an increase of the masking of the adsorption sites on the support (Table V).

The selectivity of the chromatographic system was almost unaffected by the concentration of the ammonium additives, as well as by the substitution of the ammonium group. The differences in selectivity observed when using imipramine and chlorimipramine as additives are illustrated in Fig. 6. They indicate that the retention of the tetracyclines is not only due to differences in hydrophobic character but also to a specific interaction between the support and functional groups of the tetracyclines and ammonium additives, respectively.

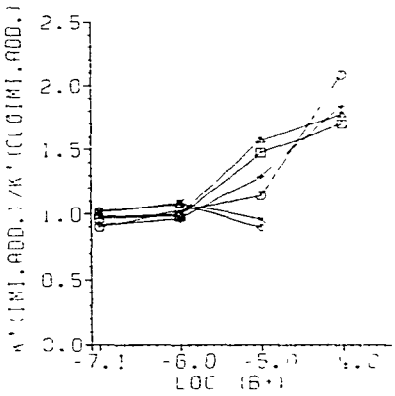

Fig. 6. Selectivity and chlorosubstitution on the ammonium additive. Support: LiChrosorb RP-8. Ammonium additives: imipramine or chlorimipramine. Other conditions as in Fig. 2.

TABLE VI

RETENTION AND SODIUM SALTS IN MOBILE PHASE

Support: LiChrosorb RP-8. Concentrations: acetonitrile in mobile phase, 20%; sodium salt added, 10^{-2} *M*. Other conditions as in Fig. 2b.

Compound	*k' without ammonium additive*			*k' with 10^{-2} M TBA as ammonium additive*		
	NaCl	*NaBr*	*$NaClO_4$*	*NaCl*	*NaBr*	*$NaClO_4$*
Clortetracycline	7.4	7.8	11.4	0.40	0.47	1.01
Demeclomycin	4.04	4.24	6.0	0.12	0.15	0.48
Doxycycline	11.7	12.1	16.9	0.84	0.98	1.37
Metacycline	9.8	10.2	14.1	0.80	0.87	1.31
Oxytetracycline	1.42	1.49	2.22	<0.10	<0.10	<0.10
Tetracycline	2.34	2.47	3.56	<0.10	<0.10	<0.10

Anions in mobile phase: influence on retention and selectivity

The retention and the selectivity of the chromatographic system used for the separation of tetracyclines should, according to the model, be influenced both by the concentration and nature of the anions present in the mobile phase (eqn. 5).

The capacity factors of the tetracyclines increased with increasing extraction ability of the inorganic anions added to the mobile phase as their sodium salts (Table VI). Despite the large variations found in the extraction ability of the anions in liquid–liquid extraction systems[26], only small differences were noticed in the chromatographic system.

A linear increase of the capacity factor with increasing anion concentration in the mobile phase is predicted from eqn. 5. However, in chromatographic systems with no organic ammonium additive present in the mobile phases, the retention of the tetracyclines decreased with increasing concentration of sodium chloride within the range 10^{-5}–10^{-1} *M* (Fig. 7a, *cf.*, ref. 29). A small increase in retention was observed on further increasing the sodium chloride concentration. In a chromatographic system where the adsorption sites on the support had to a great extent been masked

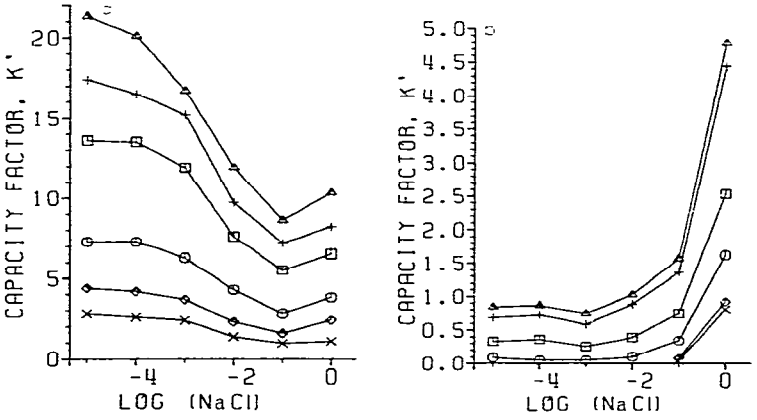

Fig. 7. Retention and counter ion concentration. Support: LiChrosorb RP-8. Concentrations: acetonitrile in mobile phase, 20%; TBA in mobile phase, none (a); 10^{-2} *M* (b). Other conditions as in Fig. 2.

by using a mobile phase containing 10^{-2} *M* TBA as ammonium additive (*cf.*, Fig. 3b), an increase of k' with increasing sodium chloride concentration was observed at $[NaCl]_m \geqslant 10^{-3}$ *M*, (Fig. 7b). Fig. 7 indicates that NaCl and TBA are competitively bound to the same types of adsorption sites on the support.

The selectivity of the chromatographic system was almost independent of the concentration of NaCl in the mobile phase when no TBA was present, but decreased with increasing $[NaCl]_m$ in a mobile phase containing 10^{-2} *M* TBA.

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