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RETENTION MECHANISMS OF TETRACYCLINES ON A C_8 REVERSED-PHASE MATERIAL

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SUMMARY

The influence of eluent parameters on the retention of tetracyclines on a C_8 reversed-phase column has been investigated. Optimal performance was obtained using eluents containing organic acids at relatively low pH, where the solutes mainly exist in their cationic forms. In contrast to earlier studies, which suggested that ion-pair formation was the separation mechanism for tetracyclines, a more complex model is presented. Retention behaviour in the present system was not consistent with one single mechanism but was obviously caused by a mixture of phenomena. Essentially, several ionic interactions are likely to predominate over purely hydrophobic effects.

INTRODUCTION

The exact nature of separation mechanisms on reversed-phase materials in high-performance liquid chromatography (HPLC) is still subject to speculation[1]. Although the hydrophobic theory is well documented[2–6], more complex models, based on ionic interactions, are generally required to rationalize the puzzling retention behaviour of ionizable substances. Elucidation of the mechanisms involved in the chromatography of amphoteric compounds is a particular challenge. Tetracycline antibiotics can be considered as model compounds in this respect. Their highly unfavourable chromatographic properties have led to a proliferation of studies, all trying to develop more efficient HPLC systems for these drugs. An excellent review of the extensive literature available has recently been given by Knox and Jurand[7].

However, few studies have attempted to get more insight into the mechanisms underlying retention of tetracyclines on reversed phases. Knox and co-workers[8,9] initially suggested that a strongly acidic mobile phase, *e.g.*, one containing perchloric acid, was essential for efficient chromatography of these compounds. Retention was explained in terms of ion-pair formation with perchlorate anions. Partition of these ion-pairs was thought to occur between the mobile phase and a microlayer of acetonitrile, adsorbed on the packing material.

In more recent work, Knox and Jurand[7] preferred less acidic eluents (pH > 3),

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containing ethylenediaminetetraacetate (EDTA). They presented evidence which indicated zwitterion-pair formation between tetracyclines and this chelating compound, which itself would be adsorbed onto the hydrophobic surface. Maximum retention occurred at pH 3.3. Salt addition proved useful to reduce capacity ratios (k') and to improve the overall efficiency.

In two previous papers[10,11] we described new reversed-phase conditions consisting of RP-8 columns and a relatively strongly acidic mobile phase (pH 2.1), based on citric acid. Although originally used for the estimation of doxycycline and minocycline in biological materials, these systems show potential for the separation of different analogues and also of impurities. One system[10] was chosen to study the effect of eluent parameters on retention because it was characterized, unlike that of Knox and Jurand[7], by extremely fast equilibration. Our results indicate that for tetracyclines a RP-8 column probably acts via a mixture of ionic interactions rather than by one well-defined separation mechanism.

EXPERIMENTAL

The liquid chromatograph used was equipped with a syringe type pump (Model 8500; Varian, Palo Alto, CA, U.S.A.), a sample valve injector with a 20-μl loop (Model CV-6-UHP -N 60: Valco Instruments, Houston, TX, U.S.A.) and a variable-wavelength detector (Varichrom; Varian), operated at 350 nm. The column (10 × 0.2 cm) contained an octyl silica (5-μm LiChrosorb RP-8; E. Merck, Darmstadt, G.F.R.) and was packed using a previously described slurry technique[10]. Eluents were prepared from aqueous solutions of various organic acids or sodium dihydrogen phosphate (analytical grade; E. Merck) and acetonitrile (analytical grade; Carlo Erba, Milan, Italy). The specified pH (± 0.02 units) was adjusted with solid sodium hydroxide or phosphoric acid, respectively, before addition of the organic modifier and, hence, does not represent the "true" value in the eluent[12]. Salts (nitrates) were finally added whenever required. The eluent flow-rate was 0.5 ml/min (back pressure 1500–2000 p.s.i.) and the temperature ambient.

Three tetracycline derivatives, demeclocycline (DMC), chlortetracycline (CTC) (both from Lederle Laboratories, Brussels, Belgium) and doxycycline (DOX) (Pfizer, Brussels, Belgium), were chosen as test substances. Eluent parameters were varied and the k' values of these compounds in each system determined.

RESULTS AND DISCUSSION

The elution sequence of tetracyclines in the initially used "standard" mobile phase, *i.e.*, acetonitrile–0.1 *M* citric acid, was mainly in agreement with their relative degrees of lipophilicity (Table I). The basic structures of the derivatives involved are presented in Fig. 1. As predicted from their ability to partition into chloroform, anhydrotetracyclines are strongly retained. The other compounds, which are virtually insoluble in most organic solvents, elute approximately in the order of their increasing octanol–water partition coefficients. 6-*epi*-Doxycycline is, due to steric reasons, less lipophilic than doxycycline[13]. The 4-epimers are stronger bases[14] and more water soluble[15] than the "normal" derivatives and, hence, less strongly retained. Lymecycline, rolitetracycline and minocycline, all containing an additional nitrogen atom in their

TABLE I

ELUTION SEQUENCE OF TETRACYCLINES ON A RP-8 COLUMN

Column, LiChrosorb RP-8, 5 μm (10 × 0.2 cm); mobile phase, acetonitrile–0.1 *M* citric acid (25:75 v/v); temperature, ambient.

Compound	*Basic structure (Fig. 1)*	*Substituents*					*P**	*k'*
		R_1	R_2	R_3	R_4	R_5		
1 Lymecycline	I	CH_3	OH	H	H	b**		1.5
2 Rolitetracycline	I	CH_3	OH	H	H	a**		1.7
3 Minocycline	I	H	H	H	$N(CH_3)_2$	H	0	2.1
4 Oxytetracycline	I	CH_3	OH	OH	H	H	0.0035	2.3
5 4-*epi*-Tetracycline	II	CH_3	OH	H	H	H		2.8
6 Tetracycline	I	CH_3	OH	H	H	H	0.014	3.5
7 4-*epi*-Demeclocycline	II	H	OH	H	Cl	H		4.1
8 Demeclocycline	I	H	OH	H	Cl	H	0.13	5.2
9 Chlortetracycline	I	CH_3	OH	H	Cl	H	0.15	8.0
10 Methacycline	I	$=CH_2$		OH	H	H	0.69	9.0
11 4-*epi*-Doxycycline	II	CH_3	H	OH	H	H		9.1
12 6-*epi*-Doxycycline	I	H	CH_3	OH	H	H		9.3
13 Doxycycline	I	CH_3	H	OH	H	H	0.52	10.4
14 4-*epi*-Anhydrotetracycline	IV	—	—	—	—	—		13.5
15 Anhydrotetracycline	III	—	—	—	—	—		18.6

* P = Partition coefficient in octanol–water at pH 2.1[23].

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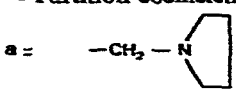
a =



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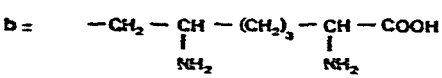

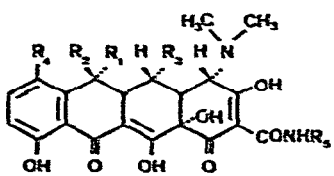

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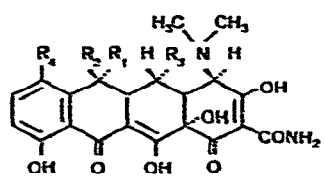

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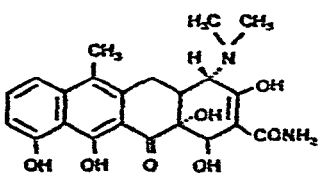

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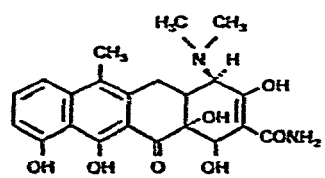

IV

Fig. 1. Basic structures of tetracyclines. Nature of substituents is given in Table I.

molecules, possess enhanced water solubility compared to other analogues. A typical separation of seven derivatives is illustrated in Fig. 2.

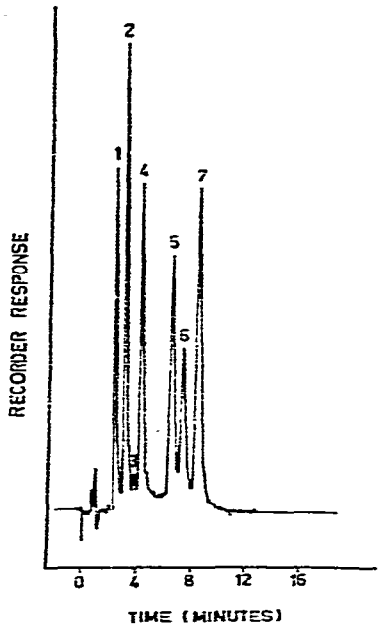

Fig. 2. Separation of seven tetracyclines. Column 10 × 0.2 cm LiChrosorb RP-8 (5 μm). Eluent: 0.1 *M* citric acid–acetonitrile (76:24, v/v); flow-rate, 0.33 ml/min. Temperature, 20°C. Pressure, 1800 p.s.i. Peaks: 1 = oxytetracycline; 2 = tetracycline; 3 = doxycycline by-product; 4 = demeclocycline; 5 = chlortetracycline; 6 = methacycline; 7 = doxycycline.

Eluents based on citric acid

Effect of pH. The effect of pH upon retention (Fig. 3) is similar to that generally observed in reversed-phase ion-pair[16] or cation-exchange[17] chromatography. In an acidic medium, the cationic form of tetracyclines is predominant, so that interactions with negative counter-ions or anionic sites on the packing material are favoured. The pH of the standard eluent provided maximal k' values (Fig. 3 and Table II) and optimal column performance.

Effect of ionic strength. Increasing amounts of citric acid were incorporated in the eluent and the pH adjusted to 2.1 with sodium hydroxide. Consequently, the sodium ion concentration was increased simultaneously. Capacity ratios were inversely proportional to the molar citric acid concentration (Fig. 4). Such a relationship usually offers a reliable criterion for the occurrence of ion-exchange phenomena[7,9]. When the pH was not kept constant, a significant decrease in k' was also observed upon increasing the citric acid concentration (Table II). These effects conflict with a retention model based on hydrophobic interactions[4]. Citrate can be supposed to adsorb onto the packing and thus would act as a deactivator by displacing the solute from active sites. It is doubtful whether a dynamic ion-exchange system is generated because citrate is mainly uncharged under the conditions used.

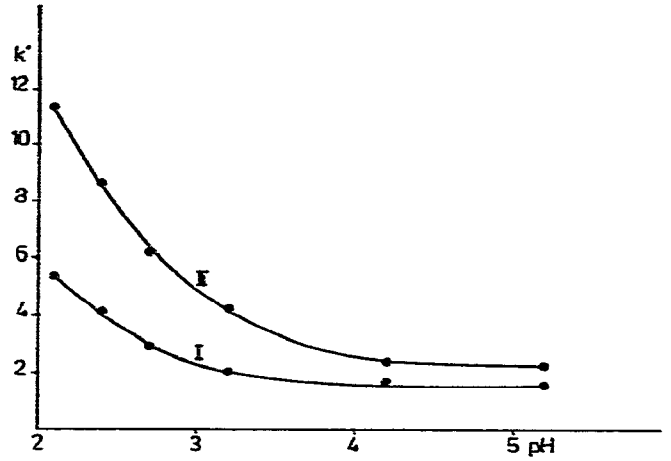

Fig. 3. Effect of pH on the capacity factors of demeclocycline (I) and doxycycline (II). Column, 10 × 0.2 cm LiChrosorb RP-8 (5 μm). Eluent, 0.1 *M* citric acid–acetonitrile (76:24, v/v). pH adjusted with sodium hydroxide.

TABLE II

EFFECT OF CITRIC ACID CONCENTRATION ON THE CAPACITY RATIO OF DOXYCYCLINE

Mobile phase, water–acetonitrile (76:24, v/v), containing increasing amounts of citric acid.

Concentration (M)	*pH*	k'_{DOX}
0.1*	2.1	11.4
0.15	1.9	8.9
0.25	1.7	7.5

* Standard eluent.

Effect of sodium nitrate. Table III summarizes the variations in k' following sodium nitrate incorporation in the standard eluent. Initially, salt addition dramatically reduced retention, but subsequently a slight increase was again noted. The effect was independent of the nature of the cation, *i.e.*, sodium, potassium or lithium. This behaviour initially seemed to be in agreement with the hydrophobic theory[4]. However, it has previously been shown that the solubility of doxycycline in water is considerably enhanced by sodium nitrate[18], so that a "salting out" effect, which would explain the secondary increase in k', is unlikely.

Eluents based on other organic acids

Citric acid could be replaced by other organic acids (Table IV). Their relative elution strengths seemed to parallel their dissociation constants, but also could partially be ascribed to the presence of variable concentrations of sodium ions. The effect of acids with increasing aliphatic chain lengths was more relevant (Table V). Differences in cation concentration could no longer account for the great divergency of k' values. For example, it is striking that the eluent based on 2,2-dimethylglutaric acid, which was free of sodium ions, afforded much smaller k' values than succinic acid, containing sodium. If ion-pair formation in the mobile phase is predominant, the order of elution strength should be correlated with the hydrophobicity of the

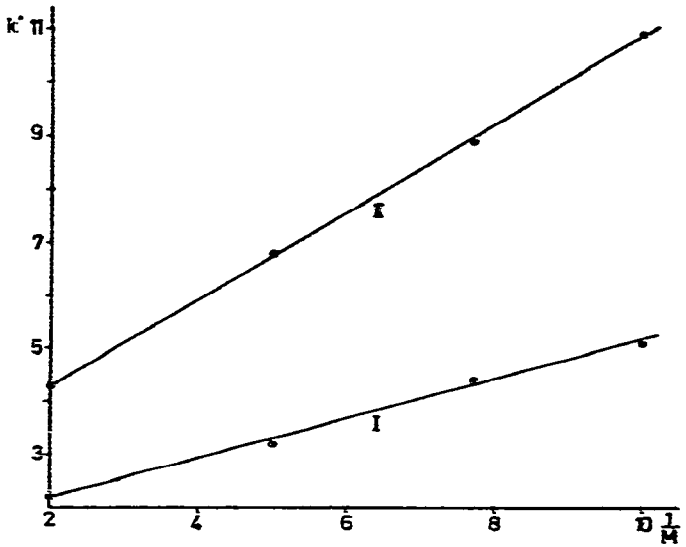

Fig. 4. Plot of capacity factors of demeclocycline (I) and doxycycline (II) *vs.* the reciprocal value of the molar citric acid concentration ($1/M$). Column, 10 × 0.2 cm LiChrosorb RP-8 (5 μm). Eluent: water–acetonitrile (76:24, v/v), containing increasing concentrations of citric acid. pH adjusted to 2.1 with sodium hydroxide.

TABLE III

EFFECT OF SODIUM NITRATE ON THE CAPACITY RATIO OF DOXYCYCLINE

Mobile phase, 0.1 *M* citric acid–acetonitrile (76:24, v/v), containing increasing amounts of sodium nitrate, pH 2.1.

$NaNO_3$ concentration (M)	*k'_{DOX}*
—	11.4
0.1	6.3
0.2	6.6
0.5	7.1
1.0	7.9

TABLE IV

CAPACITY RATIOS (k') AND PLATE NUMBER (N), OBTAINED WITH ELUENTS CONTAINING DIFFERENT ORGANIC ACIDS

Mobile phase, water–acetonitrile (77:23, v/v), containing different organic acids (0.1 *M*). pH adjusted to 2.1 with sodium hydroxide.

Acid	*pK_a*	*Demeclocycline*		*Doxycycline*	
		k'	*N*	*k'*	*N*
Oxalic acid	1.26	2.5	646	5.3	990
Maleic acid	1.92	3.1	430	6.6	510
Malonic acid	2.86	3.6	922	7.8	1674
Tartaric acid	3.04	5.3	1247	10.7	1260
Citric acid	3.13	5.9	1220	12.2	1628

TABLE V

EFFECT OF ACIDS WITH INCREASING ALIPHATIC CHAIN LENGTHS

Mobile phases, water–acetonitrile (80:20, v/v) containing organic acids (0.1 *M*). pH adjusted to 2.7 with sodium hydroxide. DMC = Demeclocycline; CTC = chlortetracycline.

Acid	pK_a	k'_{DMC}	k'_{CTC}
Oxalic acid	1.27	2.2	4.2
Malonic acid	2.86	2.9	5.3
3,3-Dimethylglutaric acid	3.70	4.6	8.0
2,2-Dimethylglutaric acid	>4.32	6.5	11.4
Glutaric acid	4.32	10.8	19.3
Succinic acid	4.21	11.4	20.4

counter-ion. The substituted glutaric acids would be expected to yield the highest k' values. However, this was not the case. Alternatively, saturation of active sites could again contribute to the observed effects. The affinity of each acid for the non-polar surface will be mainly governed by its hydrophobicity and degree of ionization. However, because the nature of the ionic species in the eluent plays an essential rôle, ionic rather than purely hydrophobic interactions must be operative.

Eluents based on phosphoric acid

When a mobile phase containing a phosphate buffer was employed, the chromatographic efficiency and capacity ratios of tetracyclines were perfectly comparable to those obtained with organic acids. Phosphate has little or no affinity for hydrophobic surfaces and is a poor counter-ion in ion-pair chromatography[19]. Therefore, ionic interactions, as outlined above, become less probable in this medium.

Sodium nitrate again considerably affected retention. Table VI compares k' values in the presence and absence of this salt. By relating these to the data given in Table III, some conclusions may be drawn with respect to the rôle of cations. After addition of the salt to an eluent which is originally free of sodium ions, a decrease followed by a slight increase of the capacity ratios occurs. In contrast, if sodium ions are already present, *e.g.*, in the form of sodium phosphate, there is no initial decrease in k'. Since salting out is unlikely the phenomenon might be alternatively explained

TABLE VI

EFFECT OF SODIUM NITRATE ADDITION TO AN ELUENT BASED ON A PHOSPHATE BUFFER

Mobile phase, 0.1 *M* sodium dihydrogen phosphate–acetonitrile (78:22, v/v). pH adjusted to 2.1 with phosphoric acid.

NaH_2PO_4 *concentration (M)*	$NaNO_3$ *concentration (M)*	k'_{DMC}	k'_{DOX}
—*	—	5.2	12.5
0.1	—	2.5	5.8
0.1	0.05	3.1	7.2
0.1	0.1	3.3	7.7
0.1	0.2	3.4	8.3
0.1	0.75	3.7	9.7

* Phosphoric acid only.

by assuming participation of residual silanol groups. This has been documented for other substances[20,21]. The effect of sodium ions on the interaction of polar compounds with such groups has also been rationalized[21]. The initial decrease in k' can be considered to arise from a competition between sodium ions and tetracycline cations for accessible polar sites. Ion-pair formation with nitrate ions could account for the subsequent retention increase. This theory is supported by the fact that an octyl phase should be richer in unreacted silanol groups than octadecyl materials[5]. In addition, it has been pointed out that RP-8 silica occupies a unique position in the chromatography of tetracyclines[22]. Indeed, similar eluents containing citric acid, as used in this study, failed to chromatograph tetracyclines on RP-18 materials.

CONCLUSIONS

In the present paper, evidence is given which suggests that retention of tetracyclines on a RP-8 column can not be explained in terms of one single class of interactions. Our results indicate that probably a complex mixture of mechanisms, including ion-pair formation, competition effects, deactivation of active sites and possibly ion-exchange and interaction with silanol groups, underlies the behaviour on this particular reversed-phase material. The eluent composition determines the relative importance of each effect. Nevertheless, the group of ionic interactions as a whole obviously predominate over hydrophobic effects, if present at all.

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