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SEPARATION OF TETRACYCLINES BY HIGH-SPEED LIQUID CHROMATOGRAPHY

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SUMMARY

The separation of tetracycline and its four common impurities has been studied by high-speed liquid chromatography. A preliminary study of the effectiveness of ion-exchange, adsorption, liquid–liquid partition and reversed-phase ion-pair chromatography indicated that only the last method showed promise. By developing special hydrocarbon-bonded stationary phases a rapid and complete resolution of all five tetracyclines has been obtained within 10 min. Plate heights using a derivatised 18-μm Partisil are in the range 0.15–0.3 mm. The method can be used to quantify the impurities in tetracycline at around the 1% level.

INTRODUCTION

Pharmaceutical preparations of tetracycline (compound I, TC) contain small quantities of related compounds as impurities. The most important are 4-epitetracycline (II, ETC), 7-chlorotetracycline (III, ClTC), anhydrotetracycline (IV, ATC) and 4-epianhydrotetracycline (V, EATC). Their permitted concentrations are listed in the British Pharmacopoeia[1]. The need for an improved assay method for tetracycline and its main impurities prompted us to investigate, in detail, conditions under which these components could be separated and quantitated by high-speed liquid chromatography (HSLC).

Several chromatographic techniques have in the past been applied to the analysis of the tetracyclines. Thin-layer chromatography[2,3], paper chromatography[4] and column chromatography followed by UV spectrophotometric assay[5,6] have proved laborious, often require sample concentration[5], and are generally not sufficiently sensitive or precise. A recent gas chromatographic method[7] requires prior formation of the trimethylsilyl derivative under carefully controlled conditions, while a method using HSLC[8] with a hydrocarbon stationary phase gave poor resolution and plate height. The last method did, however, suggest that reverse-phase methods might offer the most promising approach.

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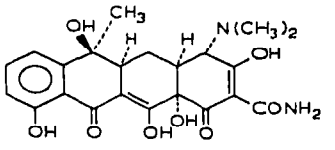
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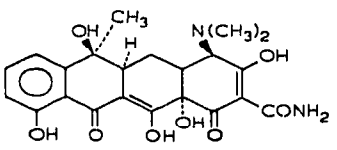
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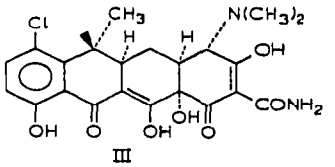
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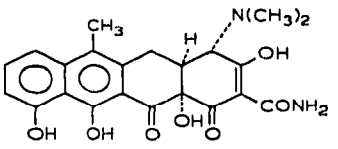
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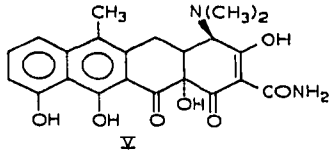
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This was confirmed (see below) by a wide-ranging study using adsorption, ion exchange, liquid–liquid partition and reversed-phase ion-pair partition chromatography, of which the last proved the most satisfactory. However, in contrast to Tsuji *et al.*[8], we used hydrocarbons chemically bonded to silica instead of a hydrocarbon polymer coated on to silica. We thereby hoped to avoid many of the undesirable features commonly associated with polymeric stationary phases deposited on a silica surface, *viz.* residual adsorption due to the presence of silanol groups and slow mass transfer in the stationary phase. By studying a number of different bonded hydrocarbon supports, prepared in the Wolfson Liquid Chromatography Unit (WLCU), we have established that the best material for the separation of the five tetracyclines is a short-chain hydrocarbon bonded to a high-quality commercial silica gel such as Partisil 20 (Reeve Angel, London, Great Britain).

EXPERIMENTAL

The high-speed liquid chromatograph comprised the pump of a Model 830 liquid chromatograph (DuPont) operated at 500–3000 p.s.i., a variable wavelength UV photometer (Cecil Instruments, Cambridge, Great Britain, Type 212) fitted with an 8-μl flow cell and operated at 280 nm. Columns were either 2 mm bore and 500 mm long (Column A) or 5 mm bore and 125 mm long (Column B) of internally polished 316S stainless steel with fittings made according to a design described elsewhere[9]. They were terminated by 6-μm porosity frits (B.S.A., Birmingham, Great Britain). Columns were packed by the rotate, bounce-and-tap method[10] and were operated at ambient temperature.

The packing materials used in the preliminary study were: DuPont Zipax strong cation exchanger (SCX), Waters Ass. Corasil (both 37–44-μm particle size), Reeve Angel Partisil (18 μm), Perkin-Elmer Sil-X (13 μm), Zipax (37–44 μm) coated with 1% polyethylene glycol (PEG) 400 and Partisil (18 μm) reacted with octadecyltrichlorosilane to give an octadecyl-bonded silica (ODS-silica, WLCU No. 171).

In the subsequent more detailed study two further bonded support materials were examined. The first was ODS-silica which had been further treated to substitute any residual ≡Si–OH groups with short-chain trialkylsilyl groups (ODS/TAS silica WLCU No. 263). The second was a sample of silica gel (Partisil) which had been completely silanized to replace the highest possible proportions of ≡Si–OH groups by ≡Si–O–SiR_3 groups where R was a short-chain alkyl group (SC-TAS-silica, WLCU No. 264).

The specimens of tetracyclines, kindly gifted by Harris Pharmaceuticals (London, Great Britain) were: tetracycline·HCl, 7-chlorotetracycline·HCl, anhydrotetracycline·HCl, the ammonium salt of 4-epitetracycline and 4-epianhydrotetracycline·HCl.

Specimens were dissolved in the mobile phase or in water immediately before use to give solutions containing 1–5 mg/ml of each component. 0.1–1 μl of these solutions were injected into the chromatograph through an EPR septum (Waters Ass., Stockport, Great Britain) using a 1-μl Type B syringe (SGE, London, Great Britain).

RESULTS

Presentation of data

The chromatographic behaviour of individual components is expressed by means of two main parameters, the capacity ratio, and the plate height. The capacity ratio, k', which measures the degree of retention of a solute, is obtained from the elution chromatogram by

$$k' = (t_R - t_0)/t_0 \quad (1)$$

where t_R and t_0 are the elution times of a retained and unretained solute respectively. In our study acetone was used as "unretained solute".

The plate height, H, which is a measure of the dispersive capacity of the column, is obtained from the elution chromatogram by

$$H = (L/16)\,(w_t/t_R)^2 \quad (2)$$

where L is the column length, w_t is the peak width at the baseline.

Chromatographic behaviour of tetracyclines using different chromatographic methods

The results of the initial study using different chromatographic methods are summarised in Table I, and show that none of the classical methods gave efficient resolution of tetracyclines. With ion-exchange chromatography using Zipax SCX, tetracyclines could be eluted with alkaline, neutral or acidic solvent, the last giving slowest elution; anhydrotetracyclines were consistently eluted before the corresponding tetracyclines but the epi- and normal forms could not be separated. In all cases column efficiencies and peak shapes were poor. With adsorption chromatography using Partisil (18 μm) the tetracyclines could be eluted with aqueous acidic solvents, but peaks were broad, badly tailed and overlapping. Peak shape was particularly poor for the anhydro forms. The addition of solvating agents such as acetonitrile accelerated elution without improving efficiency. Somewhat better results were ob-

TABLE I

CHROMATOGRAPHIC PARAMETERS FOR TETRACYCLINES IN VARIOUS CHROMATOGRAPHIC SYSTEMS USED IN HSLC

*Chromatographic system and column**	*Support*	*Mobile phase*	*k'*					*H (mm) at 1000 p.s.i.*	*Comments*
			ETC	*TC*	*CITC*	*EATC*	*TC*		
Ion exchange A	Zipax SCX	0.4 *M* $K_2B_4O_7$ 0.01 *M* EDTA 1% isopropyl alcohol (pH 9.8)	0.25	0.22	0.19	0.07	0.05	0.5 – 1.4	Poor column efficiency, only ATC/TC separation feasible
		0.1 *M* citric acid 0.001 *M* EDTA pH adjusted to 5.2	6.8	6.3	8.7	2.9	1.2	1.2 –15	Very poor column efficiency and peak shape
Adsorption chromatography B	Partisil (18 μm)	Aqueous 0.08 *M* $HClO_4$	1.8	3.8	4.3	4.3	7.5		Very broad overlapping peaks
	Sil-X (13 μm)	0.1 *M* $HClO_4$ 1.9 *M* $NaClO_4$ 0.005 *M* citric acid–acetonitrile (2:1)	0.2	0.2	1.3	1.8	1.8	0.35	Good separation of TC, CITC and ATC
Liquid–liquid partition chromatography A	Corasil coated with PEG 400	Dioxane-pentane (1.5:1)	11.0	8.0	unknown	1.6	0.6	0.62– 3.0	Poor column efficiency poor reproducibility
Reversed-phase partition chromatography B	ODS chemically bonded on to Partisil (18 μm)	0.1 *M* $HClO_4$ 0.4 *M* $NaClO_4$ 0.0025 *M* citric acid–acetonitrile (85:15 v/v)	3.56	6.0	17.0	long adsorption		0.8	Possible separation of ETC, TC, CITC but with poor efficiency

* A and B refer respectively to 2 × 500 mm and 5 × 125 mm columns.

tained on Sil-X (13 μm), and with 33% acetonitrile in an aqueous perchlorate buffer a good separation of TC, CITC and ATC could be obtained within a few minutes (Fig. 1). However, the epiforms were not resolved from the normal forms.

With liquid–liquid partition chromatography on Corasil coated with PEG 400 more symmetrical peaks were obtained but retention times were irreproducible. Here the anhydrotetracyclines were consistently eluted before the tetracyclines. The irreproducibility was thought to arise from the dissociation of hydrochlorides and retention of HCl by the column packing. The effect was similar to that previously observed in the analysis of tricyclic antidepressants[11].

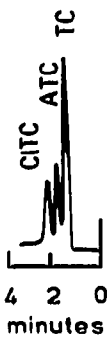

Fig. 1. Separation of TC, ATC and CITC on Perkin-Elmer Sil-X (13 μm). Column, 5 × 125 mm. Eluent, (0.1 *M* $HClO_4$ + 1.9 *M* $NaClO_4$ + 0.005 *M* citric acid)–acetonitrile (2:1) (v/v). Eluent velocity, 2.6 mm/sec. Detector, UV photometer, wavelength 280 nm, sensitivity 0.2 absorbance units full scale deflection (a.u.f.s.). Sample about 100 ng of each component.

Using ODS-silica and aqueous acetonitrile containing perchloric acid as eluent, separations of ETC and CITC from TC (Fig. 2) could be achieved with low percentages of acetonitrile in mobile phase but the anhydro compounds were strongly retained and gave very broad peaks. Their retention could be reduced and their peaks sharpened by addition of higher concentrations of acetonitrile but then the separation of the first three components disappeared. Nevertheless the last system was the only one which gave separation of epitetracycline from tetracycline and encouraged the

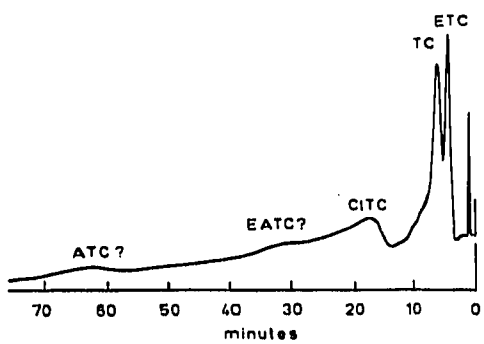

Fig. 2. Separation of ETC, TC and CITC on Partisil (18 μm) reacted with trichlorooctadecylsilane. Column, 5 × 125 mm. Eluent, (0.1 *M* $HClO_4$ + 0.4 *M* $NaClO_4$ + 0.0025 *M* citric acid)–acetonitrile (85:15). Velocity, 2.1 mm/sec. Detector as in Fig. 1, sensitivity 0.1 a.u.f.s.

belief that a bonded hydrocarbon stationary phase could be developed to give a really satisfactory separation of the tetracyclines.

From the preliminary survey the following conclusions may be drawn:

(1) The poor peak shape obtained in conventional ion-exchange, adsorption and liquid–liquid partition chromatography (particularly for well-retained tetracyclines) seems to be associated with the presence of free Si–OH groups on the support surface. Even in the presence of water, which must be very strongly adsorbed, there apparently remain a small number of sites at which the polar multifunctional tetracycline molecules can displace water and become strongly adsorbed so that even slightly retained tetracyclines give badly tailed peaks.

(2) Coating a siliceous support with hydrocarbon either by physical deposition as in Zipax HCP[8] or by chemical bonding produces increased retention of the tetracyclines but does not change the order of retention. The order of retention is just the opposite of that found with ion exchange and normal phase liquid–liquid partition chromatography. Peak tailing while still present in the reverse phase system is reduced particularly when the surface of a silica is reacted with octadecyl trichlorosilane followed by hydrolysis. We attribute the residual tailing to those original Si–OH groups which remain unreacted or, for silanized silicas, to new Si–OH groups produced by hydrolysis of the Si–Cl groups remaining after silylation.

Since both silica gel and octadecyl-silica (ODS-silica) give the same order of retention, we appear to be dealing with the same form of chromatography in both cases. Since peak shape is apparently adversely affected by the heterogeneity of Si–OH adsorption sites, a procedure for removing all such hydroxyl groups should improve both retention and peak shape.

(3) While adsorption chromatography gives a reasonable spread of k'-values but very badly tailed peaks, chromatography with similar eluents on ODS-silica gives a very wide range of k'-values, *i.e.* high selectivity. To reduce the selectivity of the bonded silica without producing peak tailing it may therefore be useful to reduce the chain-length of the hydrocarbon radicals bonded to the surface.

Chromatographic behaviour of tetracyclines on bonded hydrocarbon stationary phases

To test these ideas we have compared the retention of tetracyclines on the following four supports using the same eluent with each:

(1) Untreated silica gel (Partisil).

(2) Partisil exhaustively treated with octadecyltrichloro silane and then hydrolyzed (ODS-silica, WLCU No. 171).

(3) The material of (2) further treated in order to trialkylsilylate the remaining Si–OH groups (ODS/TAS-silica, WLCU No. 263).

(4) Partisil exhaustively silanized to substitute all Si–OH groups with short-chain trialkylsilyl groups (SC-TAS-silica, WLCU No. 264).

The results of these experiments are presented in Table II. They may be summarised as follows:

(1) Using untreated Partisil and an aqueous eluent 0.08 M in perchloric acid containing 20% acetonitrile, retentions were lower than on any other support while peaks were more badly tailed.

(2) The packings in order of increasing retention were: untreated silica gel, SC-TAS-silica, ODS-silica, ODS/TAS-silica.

TABLE II

CHROMATOGRAPHIC PARAMETERS OF TETRACYCLINES USING DIFFERENT CHEMICALLY BONDED STATIONARY PHASES

Mobile phase, water–acetonitrile (80:20, v/v), 0.08 *M* overall in $HClO_4$; support, Partisil (18 μm); columns, 5 × 125 mm.

Column number	*Stationary phase*	*k'*					*H (mm) at 1000 p.s.i.*	*Comments*
		ETC	*TC*	*CITC*	*EATC*	*ATC*		
1	None	0	0	0	0	0	Peaks broad and tailed	All components emerge essentially unretained
2	ODS only	2.80	5.00	16.0	31.0	61.0	1.0 –1.5	Poor efficiency particularly of the well retained anhydrotetracyclines
3	ODS/TAS	5.26	7.45	31.0	44.0	68.0	0.25–0.60	Good separation of first three components
4	SC-TAS	2.50	3.80	11.0	23.0	35.0	0.20–0.30	Good separation of all five components

(3) The packings in order of increasing peak symmetry and decreasing plate height were: Silica, ODS-silica, ODS/TAS-silica, SC-TAS-silica, with the last two being roughly equivalent at the same *k'* value.

These results confirm our previous conclusions. With 20% aqueous acetonitrile as eluent, tetracyclines are more strongly retained by hydrocarbon-like groups than by Si–OH groups. This is presumably because the Si–OH groups are highly hydrated. Nevertheless, whenever Si–OH groups are present, a proportion can apparently still adsorb the tetracyclines strongly in spite of hydration, and it is these groups, with their range of affinity for TC's, which cause tailing even when their total number is small. Once the residual Si–OH groups are completely removed by vigorous silanization, as in the ODS/TAS and SC-TAS-silica's, their effect is more or less eliminated and good peak shape and retention are obtained. Strong retention by a hydrocarbon phase is, of course, quite unexpected since tetracyclines either as bases or simple salts are virtually insoluble in hydrocarbon solvents. As argued in the Discussion we believe this is explained if the partitioning phase is acetonitrile dissolved in the bonded hydrocarbon and if the species being extracted are perchlorate ion-pairs which are solvated by the acetonitrile associated with the support surface. It then follows that the greater the loading of alkyl groups the greater the retention.

Fig. 3A shows the excellent resolution and peak shape attainable with the ODS/TAS material for the separation of ETC, TC and CITC. With this material, however, the anhydro forms are very strongly retained. Fig. 3B shows a typical chromatogram in which all five tetracyclines are resolved in under 10 min on the SC-TAS-silica. Both columns have efficiencies of around 500 theoretical plates in a 125-mm column giving values of *H* between 0.25 and 0.35 mm.

Comparative experiments in the ODS/TAS and SC-TAS-silica show that the latter is less selective and so more suitable for separation in a single run of all five tetracyclines. Subsequent experiments on the effects of eluent composition were therefore carried with SC-TAS-silica.

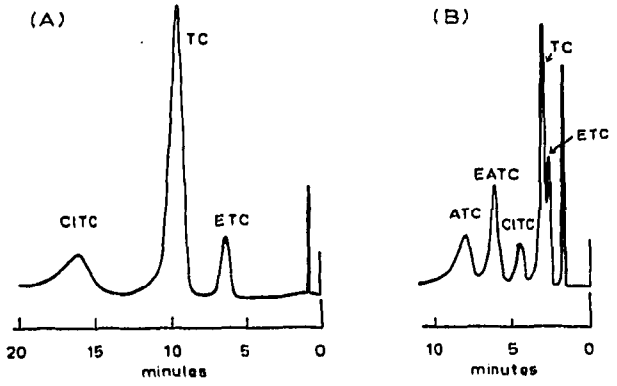

Fig. 3. Separations of tetracyclines on ODS/TAS and SC-TAS-silica's. (A) Packing, ODS/TAS-silica. Column, eluent and detector as for Fig. 2. Velocity, 2.4 mm/sec. (B) Packing, SC-TAS-silica. Column, 5 × 125 mm. Eluent, water–acetonitrile (3:1), 0.1 *M* overall in $HClO_4$. Velocity 1.3 mm/sec. Detector as in Fig. 2.

Effects of eluent composition on the performance of SC-TAS-silica

Experiments were carried out using (a) different solvating agents, (b) different acids (Table III), and (c) different concentrations of acetonitrile, the preferred solvating agent (Fig. 4). The main conclusions were as follows:

(a) Of the four solvating agents examined, *viz.* methanol, propanol, dioxane and acetonitrile, acetonitrile gave the lowest plate heights and the best resolution.

(b) Up to threefold changes of acid concentrations, as shown by Table III, had little effect on k' values although there was a slight tendency for k' to increase with acid concentration. Changes in the nature of the acid as shown in Table III had much more effect. The greatest retention was obtained with perchloric acid. Nitric acid at the same concentration gave about half the retention and sulphuric acid about a quar-

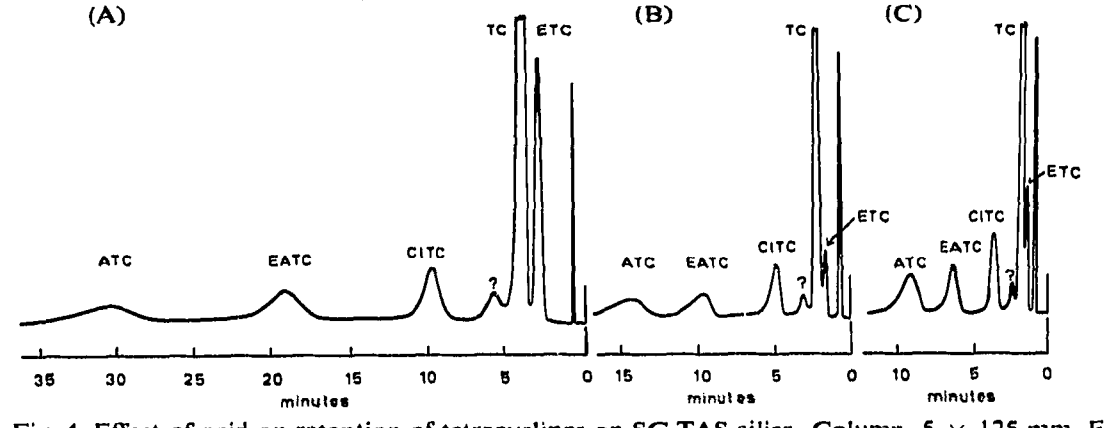

Fig. 4. Effect of acid on retention of tetracyclines on SC-TAS-silica. Column, 5 × 125 mm. Eluent, aqueous acid–acetonitrile (85:15). Overall acid concentrations: (A), 0.1 *M* $HClO_4$; (B), 0.1 *M* H_2SO_4; (C), 0.05 *M* HNO_3. Velocity 2.6 mm/sec. Detector as in Fig. 1, sensitivity 0.1 a.u.f.s. Unknown peak is impurity in CITC formed on standing.

TABLE III

CHROMATOGRAPHIC PARAMETERS OF TETRACYCLINES ON SC-TAS-SILICA USING DIFFERENT ACIDS IN THE MOBILE PHASE

Acid	*Overall concentration of acid*	*Concentration of acetonitrile (%, v/v)*	*k'*					*H (mm) at 1000 p.s.i.*
			ETC	*TC*	*CITC*	*EATC*	*ATC*	
H_2SO_4	0.1 *M*	15	0.33	0.70	2.30	5.70	8.70	
	0.2 *M*	15	0.33	0.78	2.80	5.90	9.0	0.3
HNO_3	0.05 *M*	15	1.00	1.50	5.00	10.7	16.5	0.3
	0.1 *M*	15	1.30	1.90	6.90	14.0	19.5	0.3
$HClO_4$	0.03 *M*	15	2.00	3.30	9.00	18.2	29.0	0.4–0.8
	0.06 *M*	15	2.70	3.50	8.60	20.5	33.0	0.30
	0.1 *M*	15	2.50	3.80	11.20	23.1	36.5	0.30
	0.1 *M*	25	1.50	1.80	6.20	11.5	17.5	0.25
Citric acid	0.05 *M*	25	0.22	0.43	1.38	2.40	3.40	0.40
	0.01 *M*	25	0.22	0.44	1.33	2.33	3.34	0.40
	0.15 *M*	25	0.20	0.41	1.30	2.30	3.50	
Formic acid	0.3 *M*	25	0.35	0.40	1.00	1.40	3.30	0.5

ter to a fifth of the retention. Citric and formic acids gave about one fifth of the retention obtained with perchloric acid.

The addition of sodium perchlorate to perchloric acid gave somewhat poorer resolution and higher plate heights, and was discontinued.

(c) Increasing acetonitrile concentration from 15% to 35%, as shown in Fig. 5, reduced retention between 5 and 8.5 times, the decrease being greatest for the most retained solutes. With the lowest concentration of acetonitrile the separation of the first three components was optimum but the anhydro forms were then very strongly retained.

The experiments whose results are shown in Fig. 5 were carried out in a single series. After washing the column overnight for approximately 16 h with water, each

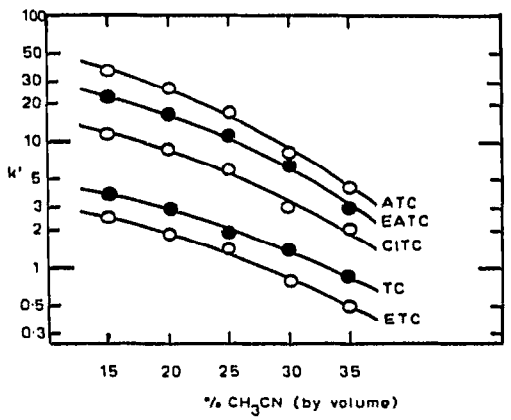

Fig. 5. Effect of proportion of acetonitrile on column capacity ratio of tetracyclines on SC-TAS-silica. All eluents 0.1 *M* in $HClO_4$ overall.

solvent was run for 30 min before determining column parameters starting with that containing 15% acetonitrile. In this time approximately 30 column volumes of eluent were passed. Later experiments suggested that this procedure may not have resulted in complete column equilibration. Thus in the long series of calibration experiments in which a 25% acetonitrile solution was used the k' values were similar to those obtained in the present series with 35% content of acetonitrile. The reason for this slow equilibration of the column, if indeed equilibration was slow, is not clear at this stage.

Quantitative analysis of tetracyclines

According to the British Pharmacopoeia[1] the allowed impurity limits in tetracycline are as follows, ETC 4%, CITC 2%, EATC 0.5%, ATC 0.5%.

While separation of the five tetracyclines using a single solvent (for example 25% aqueous acetonitrile 0.1 *M* in perchloric acid) is readily achieved using the SCTAS column, if the five components are present in comparable amounts (Fig. 3), analysis of the four main impurities at the 1% level requires two solvents or gradient elution. For the least retained components, ETC, TC and CITC 10–16% aqueous acetonitrile is required to obtain adequate resolution of ETC and CITC from the massive TC peak, but under these conditions EATC and ATC are so strongly retained that they can hardly be detected at the trace level. It is necessary for their elution and determination to use a richer eluent containing 25–35% acetonitrile. Typical calibration curves for the five tetracyclines under such conditions are shown in Fig. 6. Chromatograms of a TC sample to which small amounts of impurities had been added are shown in Fig. 7.

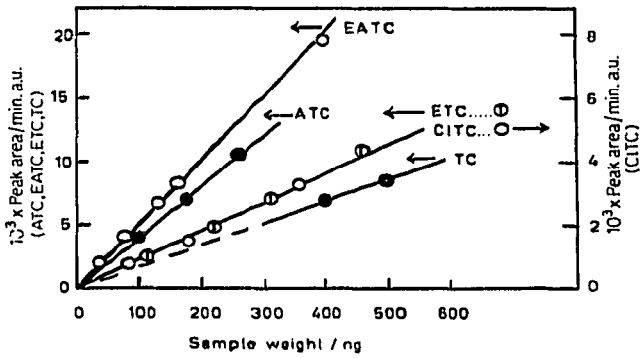

Fig. 6. Calibration curves for determination of tetracyclines. Column and detector conditions as for Fig. 1. Eluent for EATC and ETC in TC, water–acetonitrile (3:1), 0.1 *M* overall in $HClO_4$; for ETC and CITC in TC, previous eluent diluted with half its volume of water.

DISCUSSION

The results of the present work show that tetracyclines can be rapidly and precisely determined by HSLC on siliceous supports whose surface hydroxyl groups have been completely substituted with trialkylsilyl groups. Such materials give excellent peak symmetry and good retention. The plate heights using 18-μm Partisil were around 0.2 0.3 mm at linear velocities in the region of 5 mm/sec. Considering

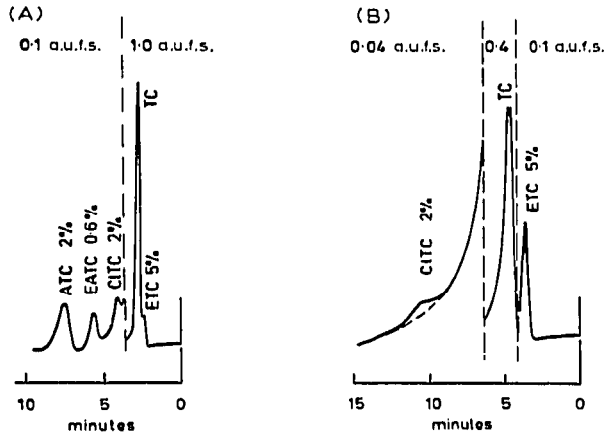

Fig. 7. Chromatograms of 10 μg of a TC sample with impurity contents made up to values shown. Chromatographic conditions as for Fig. 6. Eluents: (A) water–acetonitrile (3:1) 0.1 *M* overall in $HClO_4$; (B) previous eluent diluted with half its volume of water.

the viscosity of the solvent and the low diffusion coefficient of the tetracyclines or their derived ion pairs, these values are reasonably good, but better and faster resolution nevertheless might be obtained using smaller particles although the pressure capability of the available equipment might then become limiting.

Our results show clearly that the degree of retention is dependent upon the hydrocarbon content of the bonded packing material (≈15% for the ODS/TAS silica and ≈5% for the SC-TAS-silica). As already noted it is surprising that substances such as tetracyclines which are virtually insoluble in hydrocarbon solvents can be retained by a hydrocarbon stationary phase. We believe that the retaining phase is in fact acetonitrile which is extracted from the aqueous phase into the bonded hydrocarbon phase to give a thin surface layer which contains a high mole fraction of acetonitrile and a low mole fraction of water. In this way the hydrocarbon layer generates what amounts to a liquid–liquid partition system in which partitioning occurs between an aqueous mobile phase and a predominantly organic, but nevertheless polar, stationary phase. Acetonitrile may be particularly suitable substance for this self-partitioning role since it cannot form hydrogen bonds with itself yet is both hydrophilic and organophilic. Because the hydrocarbon layer is saturated with acetonitrile, increasing the proportion of acetonitrile in the eluent improves the solubility of the tetracycline in the eluent but has little or no effect upon its solubility in the stationary phase. Increasing the acetonitrile content of the eluent therefore reduces retention.

We believe that the species which are partitioned between the mobile and stationary phase are almost certainly tetracycline–perchlorate ion-pairs and not unionized tetracycline molecules. This is indicated by a number of observations. In the first place chromatography is only possible when the aqueous phase is acidified to a pH of 1.5–2.0. Under these conditions the tetracyclines exist in their ammonium forms. The widely differing effects of different acids and the very slight effect of hydrogen ion concentration strongly suggests that the nature of the anion is the key to retention for if undissociated amine molecules were being partitioned then pH would have a major effect on retention and the nature of the anion little effect. Since it is exceedingly

improbable that the ammonium ions themselves could be extracted it is almost certain that ion pairs are the important species in the partitioning. Accordingly, we believe that the mode of chromatography which we have employed is best termed "reverse phase ion-pair partition chromatography". This conclusion is supported by the results of Wachlund and Groningsson[12] who have shown that the extraction coefficients of perchlorate ion pairs of some hydrophilic amines are several times greater than those of sulphate ion pairs in accord with our observations on retention.

The order of retention in general correlates with the structure of the main part of the TC molecules and with their polarity. The anhydro forms which possess two aromatic rings are more strongly retained than TC and ETC which possess only one, and ClTC possessing a polar Cl group is retained more than the unsubstituted TC. Such polar effects will determine the affinity of molecules or ion pairs for the stationary phase which is rich in acetonitrile. By contrast they will have little effect in the aqueous phase where the major solvating forces arise from hydrogen bonding of water to the oxygen and nitrogen atoms. Thus the elution order follows the polarity of the parent molecules. The opposite order of retention is obtained in liquid–liquid partition chromatography on Corasil where hydrogen bonding determines the affinity for the stationary phase (PEG 400) and polarity the affinity for the mobile phase.

The extraction of ammonium compounds as ion-pairs with inorganic anions is now well documented[13–25] both in batch extraction and in partition chromatography. Recently this principle of separation has been applied successfully to the HSLC of catecholamines[26] and tricyclic antidepressants[11] using an organic eluent and an aqueous stationary phase. Although reversed-phase ion-pair systems are now beginning to be used, the systems published to date[12,27] have all used stationary phases containing hydrophobic aliphatic alcohols. Hydrocarbon stationary phases on the other hand have been mostly applied to reversed-phase partition chromatography of unionized compounds[28] although in a few cases they have been used for ionizable bases such as the ergot[29] and hashish alkaloids[28] and librium[29]. Our work and that of Tsuji *et al.*[8] appear to be the only cases where a hydrocarbon stationary phase has been used to separate strongly hydrophilic amines as ion pairs and in this context it seems that the presence of an acid in the aqueous phase is essential for the successful elution.

An important advantage of reversed-phase ion-pair partition chromatography over normal ion-pair partition chromatography is that the counter anion is continually replaced rather than being gradually removed from the stationary phase by successive samples, as occurs in normal phase ion-pair systems, and can also be varied much more readily. Thus the reversed-phase mode has much greater flexibility under practical operating conditions.

CONCLUSIONS

The results of the present study demonstrate that bonded hydrocarbon stationary phases can be used with great effect in the reversed-phase ion-pair partition chromatography of strongly hydrophilic bases such as the tetracyclines.

The SC-TAS-silica developed especially for this particular problem has been shown to be a high quality column packing for HSLC which can be used for quantitative micro-analysis. An important aspect of the present technique is that no pre-

conditioning of the column and fittings was required such as the washing with EDTA which was considered essential by previous authors[8]. In the present work there was no evidence of loss of tetracyclines from the formation of metal derivatives. We believe this is because the pH of the mobile phase was kept low (pH 1.5–2).

The technique can be used for the separation and accurate quantitation at the sub-microgram level of the tetracycline group, and has considerable potential as a basis for the development of a standard method for the assay of pharmaceutical preparations of tetracyclines and for the determination of tetracyclines in biological specimens.

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