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MECHANISM OF REVERSED-PHASE SEPARATION OF TETRACYCLINES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Previously the most successful methods for the high-performance liquid chromatographic separation of tetracyclines have employed reversed-phase bonded packing materials and aqueous-organic eluents with pH in the range 1–2.5. Under these conditions a number of bonded packing materials are unstable so that column life can be short. Furthermore, most published methods, when applied to biological samples, require clean-up before high-performance liquid chromatography can be attempted.

We have now developed highly efficient separations in the presence of EDTA (10^{-3} *M*) under mildly acidic conditions with pH in the range 3–5. The general performance is further improved by the addition of salts. Two organic modifiers have been used, acetonitrile and dimethylformamide, of which the latter is far superior. The method has been applied to direct analysis of oxytetracycline in urine.

The results support the view that EDTA acts by forming zwitterion pairs with the bipolar forms of the tetracyclines which predominate in the pH range 3–5. The new conditions of analysis cause no noticeable deterioration in column performance over prolonged periods of use.

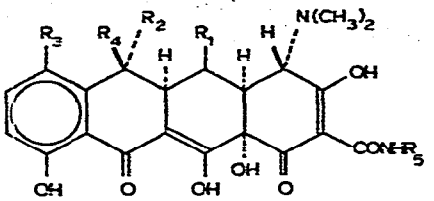
INTRODUCTION

During the past few years numerous papers have been published on the chromatographic separation of the tetracycline group of drugs[1–7] and of their common impurities and decomposition products[1,7–15]. A few papers have also appeared on their assay in biological material, urine and blood, following therapy[16–19].

The structures of the main tetracycline analogue drugs and of common impurities in tetracycline are presented in Tables I and II. It may be noted that the tetracycline skeleton is rigid; tetracycline and its analogues possess a great number of functional groups; the contaminants are often isomers with only minor structural differences from the original; the members of the group are soluble in aqueous or polar organic solvents but insoluble in non-polar organic solvents. These features, amongst others, make the chromatographic separation of the tetracyclines a major challenge to the separation scientist.

TABLE I

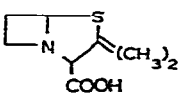
STRUCTURES OF TETRACYCLINE AND ANALOG DRUGS



Drug	R_1	R_2	R_3	R_4	R_5**
Tetracycline (TC)*	H	CH_3	H	OH	H
Oxytetracycline (OTC)*	OH	CH_3	H	OH	H
Chlortetracycline (ClTC)*	H	CH_3	Cl	OH	H
Minocycline (MC)	H	H	H	H	H
Methacycline (MeC)	OH	CH_2	H	—	H
Doxocycline (DoxoC)	OH	CH_3	H	H	H
Demeclocycline (DMC)*	H	H	Cl	OH	H
Penimocycline (PC)	H	CH_3	H	OH	P
Rolitetracycline (RTC)	H	CH_3	H	OH	R

* Samples available for this study.

** P = -CH_2NH-CH(C_6H_5)CONH



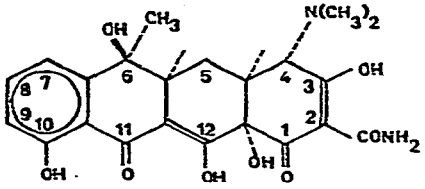
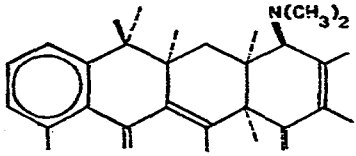
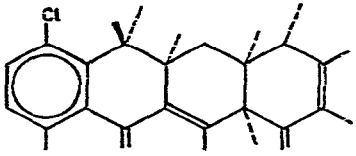
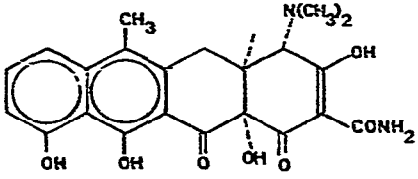
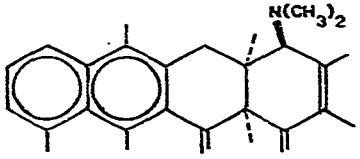
R = -CH_2-N

Although the separation of synthetic mixtures of tetracyclines might be regarded as an academic problem, knowledge of the chromatographic behaviour of each member of the group is important whenever several of the group are present in pharmaceutical preparations, and especially when it is desired to monitor impurities in order to meet the limits set by Pharmacopoeia Commissions. Examples of determinations required in such contexts are those of chlortetracycline in tetracycline[7–12], of tetracycline in rolitetracycline[4] or penimocycline[6], and of toxic analogues formed during storage. Thus the British Pharmacopoeia[20], for example, has set maximum levels of ATC and EATC in TC at 0.5% because of the serious toxic effects of these compounds, while the U.S. Food and Drug Administration (FDA)[21] has set slightly higher levels. The current method of assay approved by the British Pharmacopoeia uses thin-layer chromatography and is of relatively poor quantitative accuracy. There are good reasons for advocating that it be replaced by a high-performance liquid chromatographic (HPLC) method which can be shown to be reliable and reproducible from one laboratory to another.

HPLC has also been shown to be of great value in monitoring drugs in urine and blood especially when high-quality reversed-phase packing materials are used, for under many elution conditions the endogenous materials which are UV absorbing elute well before the drugs. In most biological analyses of tetracycline the samples have been subjected to a "clean-up" procedure prior to HPLC, such as treatment with methanol–trichloracetic acid[16], extraction of the calcium complexes with ethyl acetate followed by back extraction into acid[17,18], or complexation with calcium

TABLE II

STRUCTURES OF TETRACYCLINE AND ITS MAIN CONTAMINANTS

Name	*Structure*
Tetracycline (TC)	
Epitetracycline (ETC)	
7-Chlortetracycline (CITC)	
Anhydrotetracycline (ATC)	
Epianhydrotetracycline (EATC)	

ions and ion pairing with phenylbutazone[19]. Such techniques are laborious and likely to introduce errors. One of the main aims of this work was to see whether direct injections of body fluids could be used for assay of tetracyclines.

CRITIQUE OF PREVIOUS WORK

The HPLC methods used for tetracycline group analysis are summarized in Tables III and IV. They can be divided into those employing ion exchangers[1,3,4,6,8,13,18] and those employing reversed-phase bonded materials[2,5–7,9–12,14,15,17,19]. The favoured ion exchangers are cation exchangers although two groups have used anion exchangers[1,18]. Most investigators working with bonded materials have used octadecyl silicas although it has been stressed[6,7,9,11] that all such materials are not equally

TABLE III

ION-EXCHANGE SEPARATIONS OF TETRACYCLINES

Authors	*Year*	*Ref.*	*Compounds**	*Packing*	*Column length (mm)*	*Eluent*	*pH*	*EDTA*	*Plate height (mm)*
Loeffler	1972	8	OTC + impurities	Zipax SCX	1500	0.01 *M* EDTA, 0.01 *M* KH_2PO_4	7	Yes	20
Butterfield *et al.*	1973	1	TC, ETC, ATC, EATC	Pellionex CP	2250	0.1 *M* Na^+, 0.002 *M* EDTA in water–ethanol (70:30)	4.6	Yes	>10
			DMCC, MC, CITC, EMC, ECITC, TC, ETC	Zipax SAX	1000	0.05 *M* NO_3^-, 0.004 *M* EDTA in water–ethanol (90:10)	9.0	Yes	> 5
Butterfield *et al.*	1975	4	RTC and TC in RTC preparations	Pellionex CP 128	2250	0.1 *M* Na^+, 0.003 *M* EDTA in water–ethanol (60:40)	4.4	Yes	6–10
Lotsher *et al.*	1975	3	OTC, TC, Doxo C, CITC	Pellionex SCX	1500	0.1 *M* NH_4NaHPO_4	8.2	No	>36
Chevalier *et al.*	1976	6	PC, TC, ETC	Pellionex SCX	2000	0.001 *M* EDTA in methanol–water gradient	5.0	Yes	
Lindauer *et al.*	1976	13	EATC, ETC in TC preparations	Zipax SCX 35°	2100	Saturated EDTA	7.0	Yes	13–34
Sharma *et al.*	1977	18	TC, OTC in urine extracts	Ion-X SA (Perkin-Elmer)	not given	0.005 *M* EDTA, 0.05 *M* NaCl in water–methanol (95:5 and 70:30)	9.9	Yes	140–400 plates

* DMCC = Demethylchlortetracycline; EMC = epiminocycline; ECITC = epichlortetracycline.

suitable. Knox and Jurand[11], for example, demonstrated the importance of using "capped" materials which contain a negligible proportion of accessible hydroxyl groups.

Several groups have advocated using bonded materials with shorter chains than octadecyl. Thus Knox and Jurand[11] used TMS silica containing short alkyl chains, and De Leenheer and Nelis[5] preferred a C_8 to a C_{18} material.

Tables III and IV compare the results obtained with ion-exchange and reversed-phase packing and show that the latter gave much better performance in terms of plate number, plate height and resolution generally (approximate plate height values have been calculated from the chromatograms presented by the authors of the papers cited). Most of the studies on ion exchangers employed pellicular materials of fairly large particle size (*ca.* 40 μm) but even so the plate heights ranging from 4 to 40 mm are very poor, corresponding to reduced plate heights in the range 100 to 1000. While the results obtained with microparticulate reversed-phase materials (*ca.* 5 μm diameter) are much better with plate heights from 0.1 mm corresponding to reduced plate heights of 20 or more, the performance is still relatively poor when compared to that obtainable from sample test mixtures (reduced plate heights of 2–5).

All previous workers using reversed-phase systems have added an organic modifier to the predominantly aqueous eluent. Three modifiers have been used, methanol, propanol and acetonitrile. The results obtained by Knox and Jurand[11] and Tsuji *et al.*[9] indicated that acetonitrile gave more symmetrical peaks and higher plate efficiencies, but the reason for this was not understood.

Many investigators stress that with reversed-phase systems the highest efficiencies are obtainable only when the pH is 2.5 or less[5,9–11] especially when separating epimers. Knox and Jurand[11] maintained that the function of the acid was to form ion pairs between the cationic form of the tetracycline and the anion of the acid, but others[5] claimed that the hydrogen ion concentration was critical rather than that of the acid anion, which according to the hypothesis of Knox and Jurand could just as well have been provided by a neutral salt of the acid.

Fig. 1 shows the stages of ionization of the tetracyclines[22] within the pH range 1–12. The first ionization stage with $pK_a = 3.3$ involves ionization of the acidic OH group at the 3-position, the dimethyl amino group being protonated in acid pH. The amino proton is lost with $pK_a = 7.5$ while the OH group at position 12 loses a proton with $pK_a = 9.4$. In strongly acidic pH (for example 1–2.5) the TC molecule exists in the fully protonated form as a singly charged cation. Between pH 3.3 and 7.5 it exists predominantly in the zwitterion form with the dimethylamino group protonated and the hydroxyl group at the 3-position ionized. Thus the apparent requirement for strongly acidic eluents could well arise from the necessity for the TC molecule to be in the cationic form which could be paired with a suitable anion such as ClO_4^-. Thus for successful ion-pair chromatography of the TC group a strongly acidic eluent is required to ensure that the cationic forms of the TCs are the predominant species.

An unfortunate consequence of this requirement is that column life is often short since many reversed-phase packing materials are unstable at pH < 2–3 (depending upon the chain length). Our second aim in this work was to establish conditions for the liquid chromatography of the TC group which were not so destructive to bonded packing materials.

A further controversial aspect of previous work is the role of ethylenediamine-

REVERSED-PHASE SEPARATIONS OF TETRACYCLINES

Authors	*Year*	*Ref.*	*Compounds**	*Packing*	*Column length (mm)*	*Eluent*	*pH*	*EDTA*	*Plate height (mm)*
Tsuji *et al.*	1974	9	TC, ETC, CITC, EATC, ATC	Zipax HCP Polymer	1000	0.001 *M* EDTA, 0.01 *M* H_3PO_4 in water–methanol (87:13, v/v)	2.5	Yes	1–2
White *et al.*	1975	2	OTC, DoxoTC, DMCC, TC	ODS-SIL-X-1 (Perkin-Elmer)	1000	0.005 *M* EDTA, 0.05 *M* $(NH_4)_2CO_3$ in water–methanol (92:8)	7.5	Yes	>7
Knox and Jurand	1975	11	TC, ETC, CITC, EATC, ATC	TMS-bonded Partisil	125	0.10 *M* $HClO_4$ in water–acetonitrile (85:15 to 70:30)	1.5–2	No	0.1–0.3
Knox and Pryde	1975	12	TC, ETC, CITC, EATC, ATC	TMS-bonded WLCU silica gel	125	0.10 *M* $HClO_4$ in water–acetonitrile (85:15 to 70:30)	1.5–2	No	0.1–0.3
Tsuji and Robertson	1976	10	TC, ETC, CITC, EATC, ATC	μBondapak C_{18}	300	0.02 *M* Phosphate buffer in water–acetonitrile gradient	2.5	No	
De Leenheer and Nelis	1976	5	OTC, TC, DMCC, MeC, DoxoC	LiChrosorb C_8	250	Citrate–phosphate buffer in water–acetonitrile (65:35)	2.1	Pre-treatment essential	0.15
				Vydac RP C_{18}	500	$(NH_4)_2CO_3$ in methanol	8.4	Pre-treatment essential	>4
Chevalier *et al.*	1976	6	PC, ETC, TC, CITC, EPC, APC, EAPC	MicroPak CH	250	0.001 *M* EDTA, 0.02 *M* phosphate buffer in water–methanol (gradient, 25–50%, v/v methanol)	7.6	Yes	
Sharma *et al.* and Sharma and Bevill	1977 1978	17 19	OTC, TC, CITC in extracts of urine and plasma	μBondapak C_{18}	250	0.01 *M* NaH_2PO_4 in water–acetonitrile (60:40)	2.4	Pre-treatment essential	>0.5
Mack and Ashworth	1978	7	ETC, OTC, EATC, TC, MeC, DoxoC, ATC, CITC, MC	Vydac RP C_{18}	250	1 m*M* $(NH_4)_4$ EDTA, 0.05 *M* diethylamine in 4% isopropyl alcohol	7.4	Yes	0.2
Ali and Strittmatter	1978	14	ETC, TC, EATC, ATC	ODS-SIL-X-1	250	Water–acetonitrile–perchloric acid (76:22:1.8) or (61:37:1.8)	1.2	No	0.22–0.3
Steinbach and Strittmatter	1978	15	ETC, TC, EATC, ATC	ODS-SIL-X-1	250	Water–acetonitrile–perchloric acid (76:22:1.8) or (61:37:1.8)	1.2	No	0.22–0.3

* EPC = Epipenimocycline; APC = anhydropenimocycline; EAPC = epianhydropenimocycline.

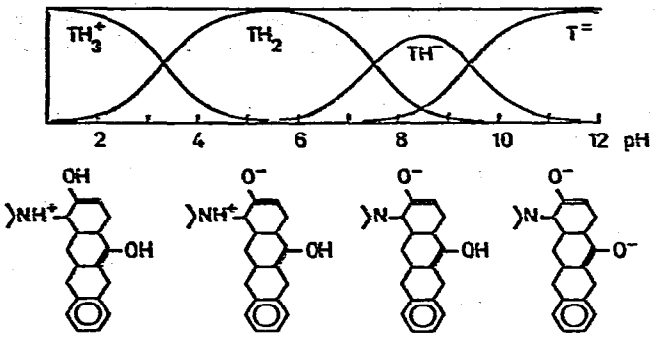


Fig. 1. Ionization stages, and proportions of different forms of tetracyclines as a function of pH[22]. Formulae show ionizable groups only. For full formulae see Table I.

tetraacetic acid (EDTA) which many authors claim to be essential for successful separations[2,5] while others find no such requirement[11,12]. It has been claimed that EDTA increases retention by chelation with the tetracyclines, and improves the partition isotherms by chelation with metallic impurities in the eluent or with the stainless-steel surfaces of the equipment. Examination of Tables III and IV shows that, with a few exceptions, EDTA seems to be necessary whenever the pH of the eluent exceeds about 3 and unnecessary when the eluent is more strongly acidic.

The stages in the ionization of EDTA[23,24] are given in Fig. 2, from which it is seen that the zwitterionic form predominates at pH below 3.0, that the doubly charged anionic form predominates between pH 3.0 and 6.5 and that for pH exceeding 6.5 various higher anionic forms predominate. Accordingly it might be deduced from the data available that interaction of the zwitterionic forms of EDTA and the TCs could be important under mildly acidic conditions.

From the published work the following generalizations can be made:

(1) Reversed-phase bonded materials give the best separations.

(2) Short-chain reversed-phase materials are preferable to octadecyl materials.

(3) An organic modifier must be present in the otherwise aqueous eluent. Acetonitrile is superior to alcohols.

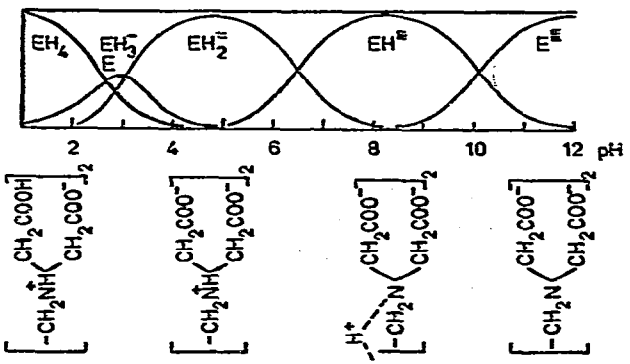


Fig. 2. Ionization stages, and proportions of different forms of EDTA as a function of pH[23,24].

(4) The best separations are achieved at pH in the range 1–2. Under weakly acidic conditions pH 3–7 separations are less good and the addition of EDTA to the eluent appears to be necessary.

(5) Under conditions of low pH, which give the best separations, many bonded materials are unstable and column life can be short.

Further work is desirable to establish: (1) the optimum organic modifier; (2) the role of EDTA in the separation especially at pH 3–7; (3) the role of pH; (4) whether good separations can be achieved at pH > 3 when short-chain bonded materials appear to have good long-term stability; and (5) whether direct injections of biological samples can be employed for bio-assays of tetracyclines.

EXPERIMENTAL

The high-performance liquid chromatograph comprised the pump from a DuPont (Wilmington, Del., U.S.A.) Model 830 liquid chromatograph or a piston pump (Orlita type DMP 1515, Giessen, G.F.R.), and a variable wavelength UV photometer (CE 212, Cecil Instruments, Cambridge, Great Britain) with an 8-μl flow cell. Columns were 100 or 125 mm long and 5 mm bore of internally polished stainless steel (Shandon Southern Products, Runcorn, Great Britain). Injection was by syringe, using a Shandon pattern injector.

Packing materials were ODS-Hypersil (a C_{18} bonded silica gel) and SAS-Hypersil (a C_1 bonded silica gel) with nominal particle sizes of 5 μm (obtained from Shandon).

Specimens of tetracyclines were kindly gifted by Harris Pharmaceuticals (London, Great Britain) and originated from the European Pharmacopoeia Commission. They were: tetracycline hydrochloride, ammonium salt of 4-epitetracycline, 7-chlortetracycline, anhydrotetracycline hydrochloride, epianhydrotetracycline hydrochloride, oxytetracycline and demeclocycline.

Samples for chromatography were made by dissolving the tetracyclines in aqueous methanol to give solutions of 1–5 mg/cm^3. Volumes of 0.1–1 μl of these solutions were injected into the column. Urine samples were provided by a volunteer who had taken a therapeutic dose of OTC over 2 days.

Eluents were made from distilled water and acetonitrile (HPLC grade from Rathburn Chemicals, Walkerburn, Great Britain), and dimethylformamide (DMF) (AnalaR grade from BDH, Poole, Great Britain). Analytical-reagent salts were added as required.

RESULTS AND DISCUSSION

Comparison of acetonitrile and dimethylformamide as modifiers

In previous work[11] acetonitrile (ACN) was shown to be a better modifier than methanol for the separation of tetracycline and its main impurities (see Table II). We now report that dimethylformamide (DMF) has significant advantages over ACN. The comparison is seen directly in Figs. 3 and 4 where the performance under equivalent conditions with DMF and ACN is shown. In both cases a standard eluent was used containing 10% by volume of organic modifier. To this various other substances are added as described below.

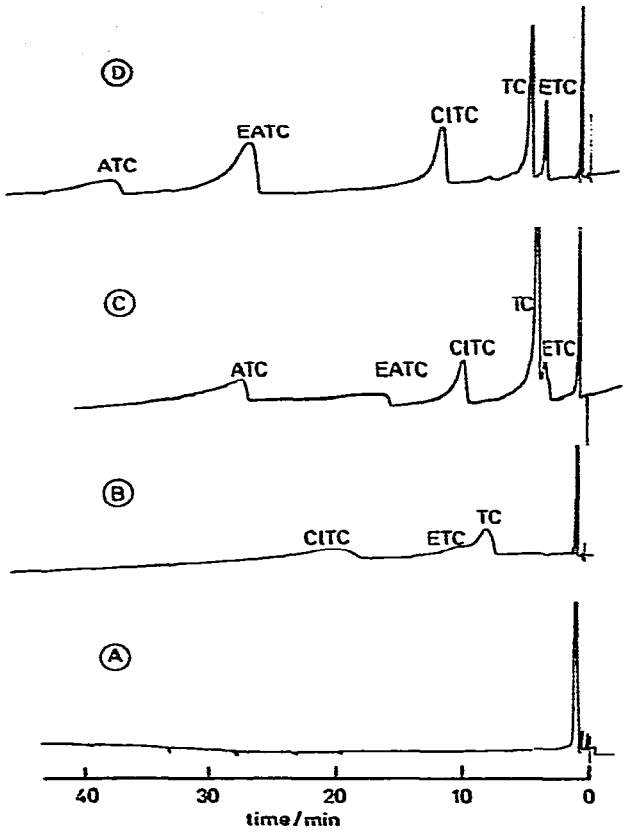


Fig. 3. Chromatograms of tetracyclines on SAS-Hypersil with various eluents containing acetonitrile as organic modifier. Column: 100 × 5 mm. Eluents: (A) water–acetonitrile (90:10, v/v); (B) as A but 10^{-3} *M* in Na_2EDTA; (C) as B but 0.12 *M* in KNO_3; (D) as C but with HNO_3 added to give pH 3.2. Detector: UV, 280 nm, 0.2 a.u.f.s.

(a) Standard eluents alone. When the organic modifiers are used only with water, highly unsatisfactory results are obtained. With ACN (Fig. 3A) no peaks were observed while with DMF (Fig. 4A) two broad bands were eluted corresponding to the benzenoid and naphthalenic tetracyclines respectively. In both cases it was observed that the tetracyclines were irreversibly adsorbed at the top of the column giving a yellow discolouration. With DMF as modifier the retention of the solutes increased with successive samples as more of the tetracyclines were adsorbed by the column.

(b) Standard eluents + 10^{-3} M EDTA. Addition to the standard eluent of Na_2EDTA at a concentration of 10^{-3} *M*, buffers the pH at around 5 and effects a dramatic improvement in chromatographic performance. With ACN as modifier (Fig. 3B) peaks were now observed for the benzenoid tetracyclines although they showed severe tailing. With DMF on the other hand (Fig. 4B) all five tetracyclines were resolved and even the naphthalenic compounds gave moderately well-shaped

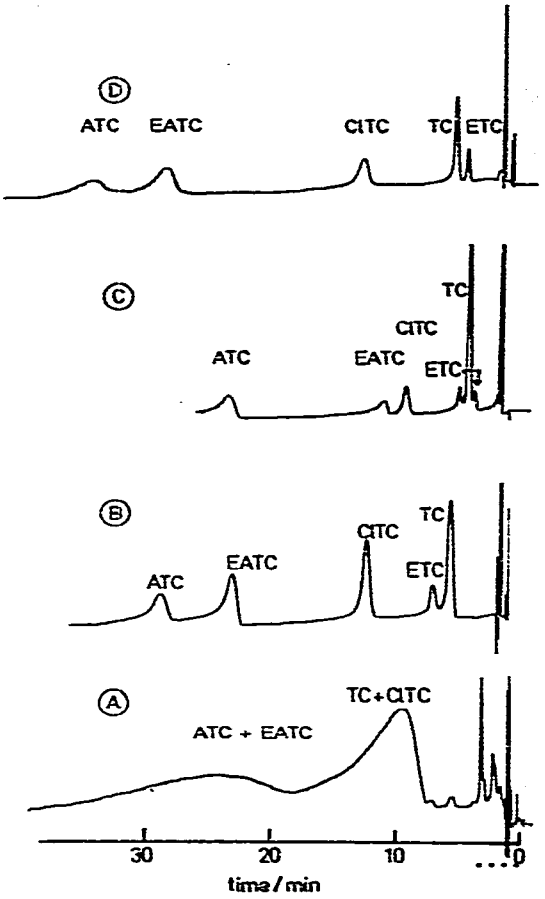


Fig. 4. As Fig. 3 except dimethylformamide used as organic modifier.

peaks. Peak shapes were not significantly improved by increasing the proportion of organic modifier.

(c) Standard eluents + 10^{-3} M EDTA + salt. On addition of salt, for example potassium nitrate, to EDTA-containing eluent retention was reduced and peak shape somewhat improved. With ACN as modifier the naphthalenic tetracyclines were now eluted as badly tailed peaks but in a reasonable time. With DMF as modifier fairly good peak shape was now achieved. ETC now eluted before TC while EATC eluted just after CITC. By increasing the salt concentration further EATC could be eluted before CITC.

(d) Standard eluent + EDTA + salt + acid. On addition of nitric acid to reduce the pH from 5 to 3 retention was increased while peak symmetry was improved (Figs. 3D and 4D). With ACN as modifier EATC and ATC still showed strongly tailed peaks while with DMF peaks were more or less symmetrical and resolution of all five components was satisfactory. With ACN as modifier some improvement in peak shape and resolution could be achieved by using a higher modifier concentration.

(e) Optimum conditions for resolution. Whereas sections (a)–(d) show qualitatively the effects of successive additions to standard eluents of EDTA, KNO_3 and HNO_3 and demonstrate the clear superiority of DMF as modifier, the conditions shown are not optimum for either modifier. To achieve the best resolution when using ACN it is necessary to use the lowest pH consistent with stability of packing materials and the highest concentrations of ACN and salt which allow adequate retention. Fig. 5 shows a reasonable compromise which provided about the best resolution, in spite of some tailing, which we could achieve using ACN as modifier. The eluent is buffered at pH = 3 by acetate–acetic acid.

With DMF a much wider range of elution conditions can be used giving typical plate heights of around 50 μm (reduced plate height 8 to 10). Chromatograms obtained under two such sets of conditions are shown in Fig. 6 for pH = 3.1 and 4.8. It may be noted that the position of the EATC peak between those of CITC and ATC is considerably altered by this change of pH.

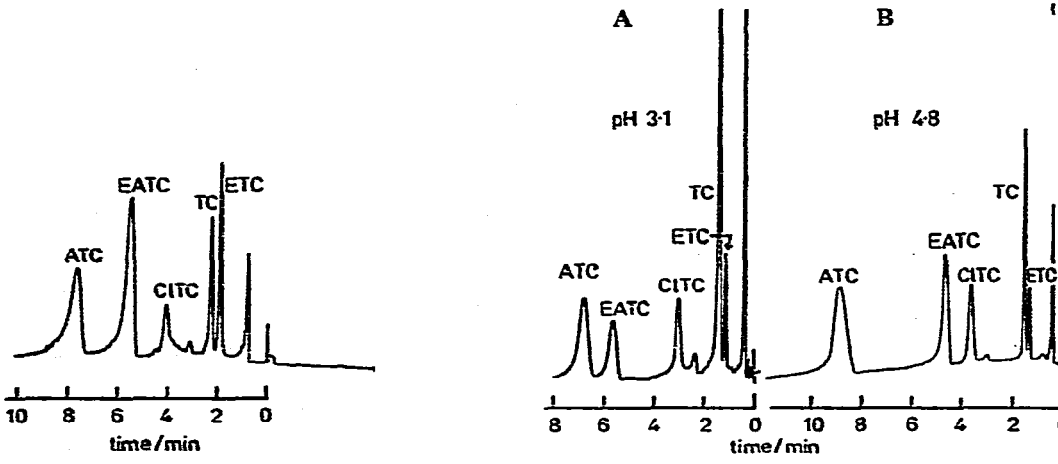


Fig. 5. Chromatogram of tetracyclines on SAS-Hypersil showing near-optimum conditions with acetonitrile as modifier. Eluent: water–acetonitrile–acetic acid (71:18.5:10.5, v/v/v) containing $2.8 \cdot 10^{-3}$ *M* Na_2EDTA, 0.011 *M* KNO_3 and 0.06 *M* sodium acetate giving pH 3.0. Detector: UV, 272 nm; 0.1 a.u.f.s.

Fig. 6. Chromatograms of tetracyclines on SAS-Hypersil showing near-optimum conditions with dimethylformamide as modifier. Eluent: (A) water–dimethylformamide (90:10, v/v) containing 0.001 *M* Na_2EDTA, 0.01 *M* citric acid, 0.01 *M* sodium citrate giving pH 4.8; (B) water–dimethylformamide (82:18, v/v) containing 0.001 *M* Na_2EDTA, 0.05 *M* KNO_3, citric acid giving pH 3.1. Column and detector as in Fig. 3.

(f) Quantitation. Without addition of EDTA the TCs are irreversibly adsorbed and quantitation is impossible with eluents in the pH range 3–5. EDTA is not only essential for satisfactory chromatography but also enables the TCs to be eluted quantitatively as shown by Fig. 7A and B, where the elution conditions are the same as for Figs. 5 and 6A, respectively.

(g) Modifier concentration. The effect of modifier concentrations in the presence of 10^{-3} *M* Na_2EDTA (but without added salt or acid) is shown for both modifiers in Fig. 8. While capacity ratios (k') are similar for the monoaromatic TCs

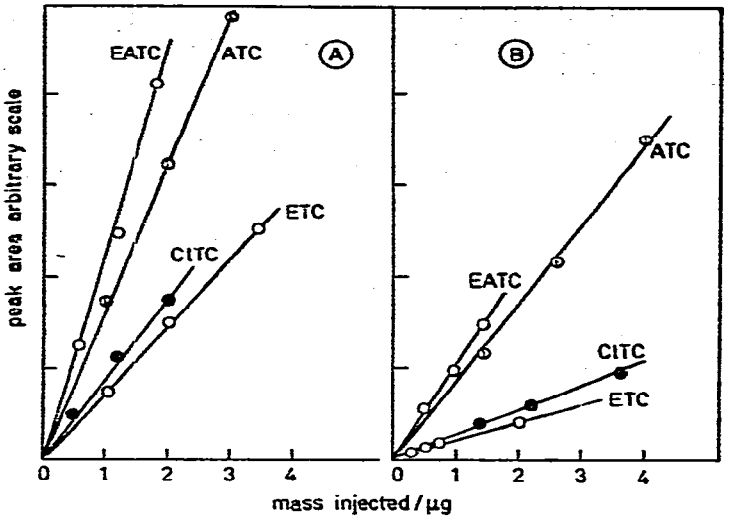


Fig. 7. Plots of peak area *versus* quantity for elution of tetracyclines. Packing: SAS-Hypersil. (A) Eluent: as for Fig. 5; detector: UV, 272 nm. (B) Eluent: as for Fig. 6A; detector: UV, 280 nm.

with the two modifiers there is a striking difference for the naphthalenic members EATC and ATC. These are very strongly retained with ACN as modifier ($k' > 50$ with 25% ACN) but with DMF k' values for EATC and ATC are only about four times those of their monoaromatic analogues. In the absence of salt and acid, but with both modifiers, the order of elution of ETC and TC changes at a concentration of about 20% modifier. Below 20% modifier TC elutes first, while above 20% modifier ETC elutes first.

The experiments described in sections (a)–(g) show the clear superiority of DMF over ACN as organic modifier. They also show that in the pH range 3–5 addition of EDTA is essential for reasonable peak shape. The peak shape is further improved, especially with ACN as modifier, by adding acid buffer or KNO_3 and sufficient acid to reduce the pH from 5 to 3.

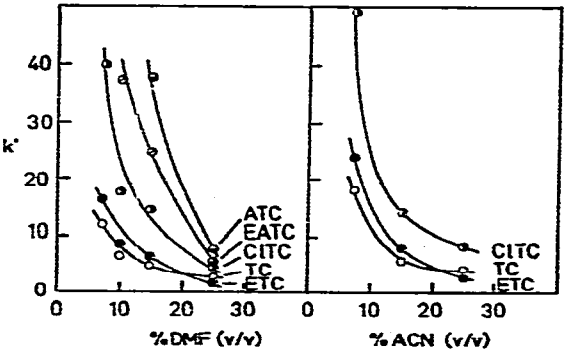


Fig. 8. Dependence of k' of tetracyclines upon proportion of organic modifier. Packing: SAS-Hypersil; eluent: water–organic modifier containing 0.001 *M* EDTA.

The reason for the superiority of DMF over ACN is by no means clear. It was at first thought that this could be due to the presence of dimethylamine formed by hydrolysis of DMF rather than to DMF itself. However, addition of diethylamine to eluent containing ACN had no beneficial effect. We therefore concluded that the presence of free or ionized amine could not be the reason for the improvement in going from ACN to DMF as modifier. It seems most likely that a greater ability of DMF to solvate the tetracyclines and their ion pairs as compared to ACN must be the cause of its superiority as an organic modifier.

Role of EDTA

The experiments described under *Comparison of acetonitrile and dimethylformamide as modifiers* establish that within the pH range 3–5 the presence of EDTA is essential for satisfactory chromatography of tetracyclines whereas our previous work[11] established that EDTA was not necessary at pH in the range 1–2. We attempted to determine whether EDTA was adsorbed by the column packing material by observing the changes in k' for the tetracyclines when standard eluent was replaced by eluent containing EDTA. Significantly the improvement in separation occurred quite slowly. With 25% DMF as modifier a gradual increase in k' was observed as soon as discrete peaks were observed after about 80 ml of 10^{-3} *M* EDTA containing eluent had been passed. This subsequent increase in k' is shown in Fig. 9. In previous work[25] we observed similar but more dramatic changes in the k' values of acidic paracetamol metabolites on adding ion pairing agents to the eluent and we deduced that significant amounts of such materials were adsorbed by the packing. In the present case the changes in k' are much smaller but nevertheless appear to indicate that to be effective EDTA must be adsorbed by the packing material. Either a substantial quantity must be adsorbed (we estimate up to $1.5 \cdot 10^{-4}$ mol or 45 mg of EDTA) or the equilibration with EDTA is slow and requires the passage of about 400 ml of eluent before it is complete. Our conclusions are not firm since with 10% DMF as modifier there was no change in k' although again passage of about 400 ml of eluent was required before the peak shape became stable from one injection to the next.

In a second attempt to determine whether the surface of the packing material had been altered, we determined the change in k' of acetophenone as EDTA-containing eluent was fed to the column. k' gradually fell by about 25% with DMF as modifier and by 8% with ACN as modifier. It is suggested that this confirms the hypothesis that EDTA is adsorbed since the highly polar EDTA would have little affinity for a hydrophobic species such as acetophenone and would be expected to reduce its retention.

The mode of operation of EDTA must be considered in relation to its pattern of ionisation taken in conjunction with that of the tetracyclines (Figs. 1 and 2). Within the pH range 3–5 the TCs exist as zwitterrions while EDTA exists predominantly as dipolar anions with net charges of one or two units although at pH 3 a significant proportion of EDTA still exists in the zwitterionic form. Since the hydrophobic surface of the SAS-Hypersil will prefer neutral zwitterions it is reasonable to suppose that a high proportion of any adsorbed EDTA will be in this form but that the proportion present as zwitterions will fall as the pH increases from 3 to 5. It seems to us most probably that the main mode of retention in the pH range 3–5 is by

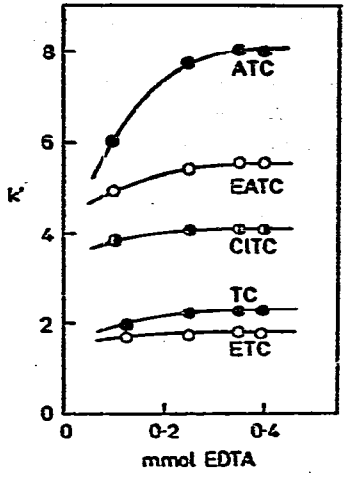


Fig. 9. Increase of k' of tetracyclines with passage of eluent containing EDTA. Packing: SAS-Hypersil; eluent: initially water–dimethylformamide (75:25, v/v) followed by water–dimethylformamide (75:25, v/v) containing 0.001 *M* EDTA.

formation of zwitterion pairs between the zwitterionic form of the TCs and the adsorbed zwitterions of EDTA.

In the strongly acidic region of pH the TCs exist as cations and a zwitterionic pairing agent is no longer relevant. What is now required is a pairing anion. In the intermediate pH range around 3 it is probable that both a pairing anion and a pairing zwitterion are required.

Role of pH and added salt

Variation of pH with EDTA present. Variation of pH in the range from 5.1 (the pH of 0.001 *M* Na_2EDTA) to 2.9 results in considerable changes in k' of TC as shown in Fig. 10. Independent of the modifier or acid used retention is a maximum at pH $\approx$ 3.3. This pH is close to the pK_a for the first ionization stages of TC and EDTA. At pH $\approx$ 3 in aqueous solution there is a change on increasing pH from the zwitterion of EDTA to the doubly charged dipolar anion, while for TC there is a change from the cation to the zwitterion (Figs. 1 and 2). Thus the probability of forming zwitterion pairs between the EDTA zwitterion and the TC zwitterion will be a maximum at around pH 3. This correlates well with the observed maximum k' at around pH 3.3 (Fig. 10). Since the eluent contains 25% organic modifier and since the organic bonded phase will preferentially extract EDTA zwitterions it is very reasonable that the maximum in k' should be at slightly higher pH than predicted by considering a purely aqueous environment.

Effect of change of salt concentration with EDTA present. In a situation where an ion-pairing agent, P^-, is strongly adsorbed by the column packing it will be associated, in the absence of solute, S^+, with a counter ion C^+. During elution S^+ must displace C^+ according to the equation

$$S^+ + C^+P^-_{ads} \rightleftharpoons C^+ + S^+P^-_{ads}$$

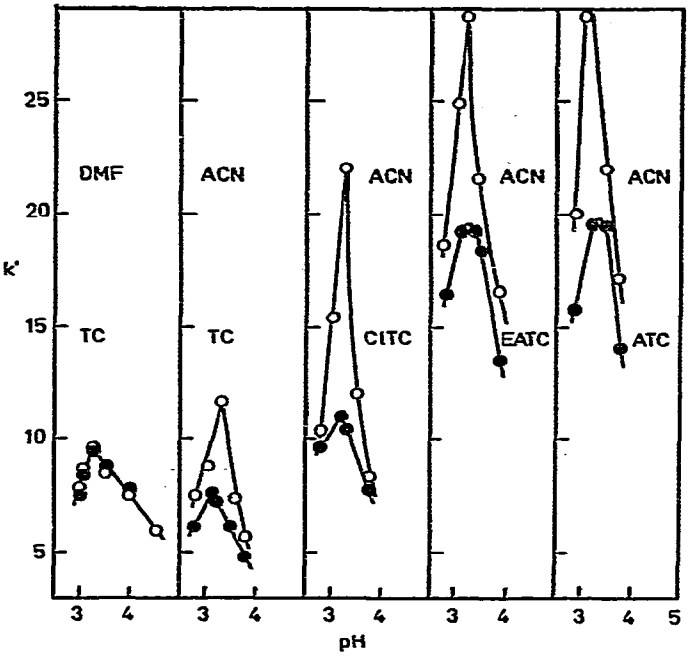


Fig. 10. Dependence of k' of tetracyclines on pH. Packing: SAS-Hypersil; eluent: water–organic modifier (75:25, v/v) containing 0.001 *M* EDTA. pH adjusted by nitric acid (●) and citric acid (○).

as in normal ion exchange where the ion exchanging group P^- is chemically bonded to the surface rather than adsorbed on to it. One then expects a linear relationship between k' and $1/[C^+]$.

Fig. 11 shows plots of k' *versus* the reciprocal of potassium nitrate concentration using ACN as modifier. Fig. 12 shows a similar plot using DMF as modifier. k' is plotted against reciprocal ionic strength of citrate buffer whose composition was such as to maintain the pH at 5.35 irrespective of the total ionic strength. Both figures show the characteristic shape previously observed with the ionized or ionizable paracetamol metabolites[25] with a trend towards linearity at high ionic strengths and near-zero intercepts at infinite ionic strength. The behaviour shown in Figs. 11 and 12 is highly characteristic of an ion-pairing mechanism of retention. Unfortunately it gives no indication as to the nature of the pairing species and in particular whether or not zwitterion pairs could be involved.

While the addition of salts can be used to control retention it also improves peak symmetry as mentioned under *Comparison of acetonitrile and dimethylformamide as modifiers*. This improvement is particularly dramatic when using ACN as modifier.

Chain length of bonded stationary phase

Comparative data obtained with identical eluents using ODS-Hypersil and SAS-Hypersil are given in Table V. ODS-Hypersil contains $C_{18}H_{37}$ groups bonded to the surface and has residual silanol groups capped with trimethylsilyl groups. The carbon content is about 9% (w/w). SAS silica has CH_3 groups bonded to the surface and a carbon content of about 3% (w/w).

TABLE V

EFFECT OF BONDED CHAIN LENGTH ON RETENTION USING ODS- AND SAS-HYPERSILS

Eluent	*Bonded material*	*k'*							*Plate height (mm)*	
		ETC	*TC*	*OTC*	*DMC*	*CITC*	*EATC*	*ATC*	*TC*	*ATC*
Water–dimethylformamide (90:10, v/v) containing $5 \cdot 10^{-4}$ *M* EDTA, $5 \cdot 10^{-2}$ *M* KNO_3, $1.5 \cdot 10^{-2}$ *M* citric acid, and $2 \cdot 10^{-2}$ *M* sodium citrate, pH 5.0	SAS-Hypersil	3.6	4.9	6.5	7.5	15.6	18.6	49	0.04	0.05
	ODS-Hypersil	8.0	10.2	14.1	17.8	–	–	–	0.04	0.05
Water–acetonitrile (91.5:18.5, v/v), containing $3 \cdot 10^{-3}$ *M* EDTA, $1.1 \cdot 10^{-2}$ *M* KNO_3, and acetic acid to give pH 3.0	SAS-Hypersil	1.5	1.9	–	–	4.0	6.0	7.8	0.05	0.1
	ODS-Hypersil	1.7	2.3	–	–	7.0	14.7	22	0.07	0.3

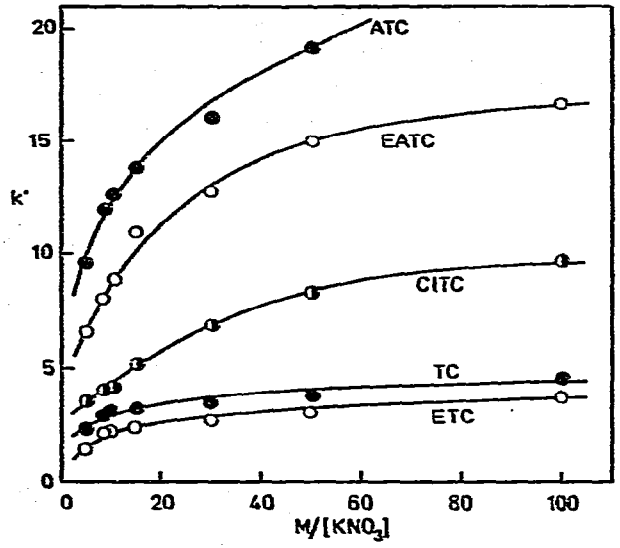


Fig. 11. Dependence of k' for tetracyclines on KNO_3 concentration. Packing: SAS-Hypersil; eluent: water–acetonitrile (75:25, v/v) containing 0.001 *M* EDTA and HNO_3 added to give pH 3.25.

Using DMF as modifier retention on ODS-Hypersil is just over twice that on SAS-Hypersil and the plate height obtained for both materials for all solutes is about 0.05 mm. Using ACN as modifier retention of the monoaromatic TCs is roughly the same on the two materials and the plate height is just over 0.1 mm with both. For the naphthalenic components retention on ODS-Hypersil is about twice that on SAS-Hypersil and the peaks on ODS-Hypersil are more asymmetric than on SAS-Hypersil. Typical chromatograms of some monoaromatic TCs on the two materials are shown in Fig. 13 where the difference in retention using DMF as modifier is clearly seen.

Evidently with ACN as modifier ODS-Hypersil gives greater selectivity than does SAS-Hypersil of monoaromatic TCs from the naphthalenic TCs but poorer

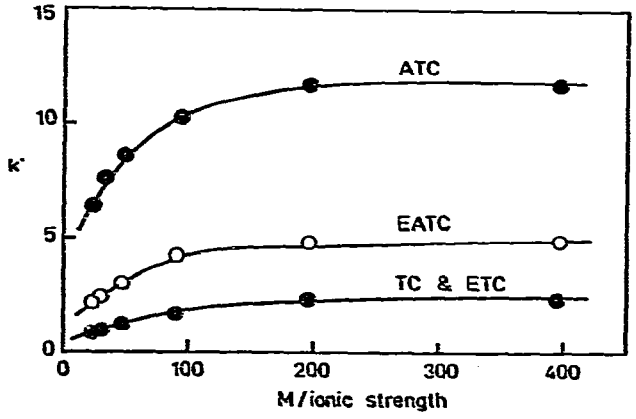


Fig. 12. Dependence of k' for tetracyclines on concentration of citrate buffer adjusted to pH 5.4. Packing: SAS-Hypersil; eluent: water–dimethylformamide (75:25, v/v) containing 0.001 *M* EDTA.

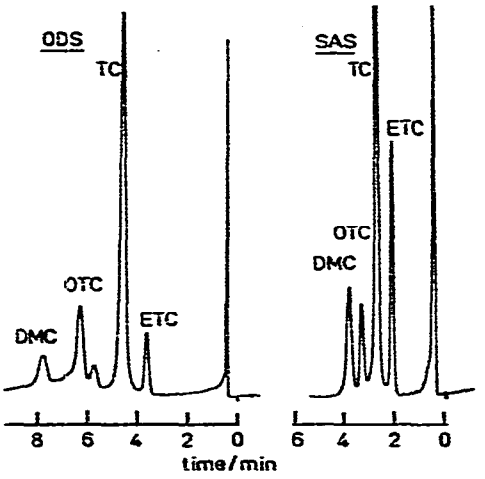


Fig. 13. Chromatograms of tetracyclines on ODS- and SAS-Hypersils. Eluent: water–dimethylformamide (90:10, v/v) containing 0.0005 *M* EDTA, 0.015 *M* citric acid, 0.02 *M* sodium citrate and 0.05 *M* KNO_3. Column and detector as in Fig. 3, except sensitivity: 0.1 a.u.f.s.

plate efficiency. SAS-Hypersil is undoubtedly preferable especially if it it desired to determine members of both classes under isocratic conditions. However, if DMF is used as modifier the selectivity and plate heights are the same with both materials and there is little reason to prefer one over the other since the degree of retention is readily adjusted by changing the proportion of modifier.

Determination of oxytetracycline in urine

Both SAS and ODS-Hypersils can be used with direct injections of urine as has been demonstrated previously[25,26]. This is clearly advantageous since possible errors arising from clean-up procedures are avoided. To test the applicability of the present methods to determination of tetracyclines in urine, samples were provided by

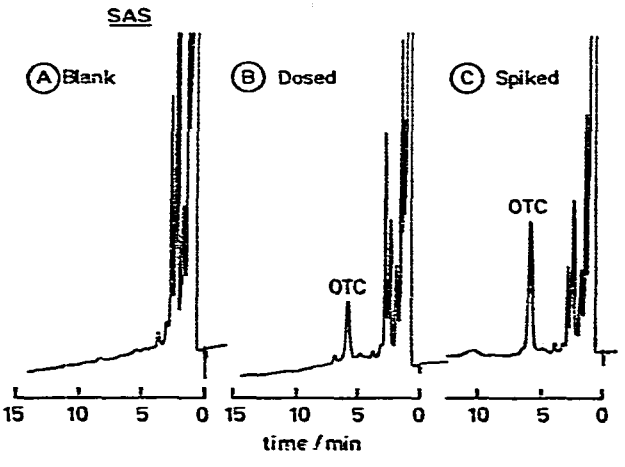


Fig. 14. Chromatograms of 5 μl of urine samples on SAS-Hypersil. Conditions as in Fig. 13. (A) normal sample, (B) sample following 2 days therapeutic dose of OTC, (C) as B but spiked with OTC.

a volunteer who had taken a therapeutic dose of oxytetracycline over two days. Figs. 14 and 15 show analyses of a urine blank, a urine following the therapeutic dose of OTC, and a urine spiked with additional OTC. Figs. 14 and 15 show chromatograms obtained on SAS and ODS-Hypersils, respectively. The OTC peak following the therapeutic dose is clearly seen. While there is always the possibility that an endogenous component could elute at the same time as OTC one could readily check this by carrying out analyses under different conditions of acidity or salt content when the OTC peak would be expected to move in a different way from any endogenous peak. It may be noted that the entire analysis takes no more than 8 min when using SAS-Hypersil.

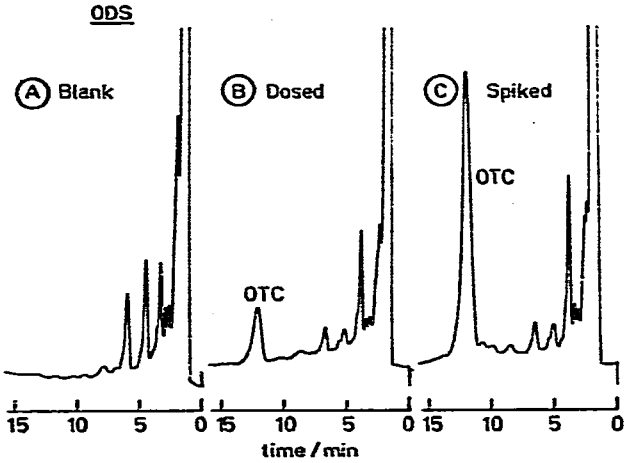


Fig. 15. Chromatograms of 5 μl urine samples on ODS-Hypersil. Eluent: water–dimethylformamide (82:18, v/v) containing 0.001 *M* EDTA, 0.05 *M* citric acid, 0.013 *M* sodium citrate and 0.1 *M* KNO_3, pH 3.5. Other conditions as in Fig. 13.

In general, the injection of 20 urine samples caused no deterioration in column performance. However, as an precaution against potential deterioration we adopted the practice of replacing the first 3 mm of column packing at the start of each day's analyses. In routine applications it would be desirable to use a short throw-away precolumn.

CONCLUSIONS

(1) The tetracycline group may be separated with high efficiency (plate heights around 0.05 mm) using SAS- and ODS-Hypersils in the pH range 3–5. Under these conditions there is no loss of bonded layer and columns are stable for long periods.

(2) Within the pH range 3–5, the presence of EDTA at a concentration of about 10^{-3} *M* is essential for satisfactory chromatography. EDTA is thought to act as a zwitterion pairing agent with the zwitterionic form of the tetracyclines.

(3) The optimum eluent must contain an organic modifier. Extensive comparisons of acetonitrile and dimethylformamide show that dimethylformamide in a volume proportion of 10–25% is much superior in terms of peak symmetry and ability to resolve the complete range of TCs under isocratic conditions.

(4) In addition to EDTA it is desirable to add a salt such as potassium nitrate to the eluent at a concentration of around $5 \cdot 10^{-2}$ M, and to stabilize the pH by the addition of nitric, acetic, or citric acid or suitable buffers.

(5) Retention of TC shows a maximum at pH 3.3. This pH is close to the pK_a for the first ionization step of the TCs and may be indicative of the ion-pairing mechanism of separation.

(6) Added salt reduces retention and improves the plate height in agreement with the hypothesis of an ion-pairing mechanism.

(7) Under the above conditions good quantitation of the tetracyclines can be carried out.

(8) Under the above conditions oxytetracycline can readily be assayed in urine by direct injection of urine. The analysis takes less than 8 min when using SAS-Hypersil as column packing. The column performance does not deteriorate if the top 3 mm is replaced daily.

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