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Separation of tetracyclines by liquid chromatography with acidic mobile phases and polymeric columns

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Abstract: The LC separation of selected tetracyclines has been studied using polymeric columns. Mobile phases containing acetonitrile–0.02 M sodium perchlorate, pH 2.0, were used. Asymmetry factor and number of theoretical plates were calculated for the tetracyclines investigated on four polymeric columns. The columns included: two polystyrene–divinylbenzene (PS–DVB) copolymeric columns, a PS–DVB column with octadecyl ligands and a polymethacrylate column with octadecyl ligands (PM–C_{18}). The PLRP-S (PS–DVB) column and the PM–C_{18} column were found to be the most suitable for the analysis of the selected tetracyclines. Resolutions between pairs of selected tetracyclines were calculated and compared for the latter two columns, with the PLRP-S (PS–DVB) columns showing the best results. The PM–C_{18} column has the advantage of allowing the use of higher flow rates, which minimized analysis times. The tetracyclines included minocycline, oxytetracycline, tetracycline, demeclocycline, chlortetracycline, methacycline, doxycycline and meclocycline. Representative degradation products and impurities for selected tetracyclines were also included.

Keywords: *LC; tetracycline; polymeric columns; acidic mobile phases; impurities.*

Introduction

Because tetracyclines are produced by fermentation or semi-synthetic processes and are unstable to light [1] and extremes of pH [2], analytical methods used on bulk drug and dosage forms are needed to separate a wide variety of closely related compounds. There have been LC methods reported for the analysis of tetracyclines on bonded-phase silica columns [3–8] where careful adherence to the pH 2–8 constraints are an essential requirement due to the instability of these columns outside this pH range. These methods are generally unsatisfactory for the analysis of tetracyclines because the common impurities and degradation products are not well separated from the parent drug, especially when the tetracycline is in great excess of the impurities or degradation products. Bonded-phase silica columns also have the distinct disadvantage that adjustments of the mobile phase pH is required to obtain the desired separations when changing column brands or even lots of the same column [8].

The use of polystyrene–divinylbenzene copolymer (PS–DVB) columns have eliminated some of these problems. Most methods using these columns for the analysis of tetracyclines employ alkaline mobile phases [9–17]. The use of acidic mobile phases and high viscosity organic modifiers with these columns have shown applicability to some tetracyclines, especially chlortetracycline, which is unstable in aqueous alkaline solutions [18–20]. There are still problems separating the various degradation products and impurities. For example, using PS–DVB columns, all of the common degradation products and impurities of tetracycline and/or chlortetracycline have not been separated in a single isocratic run [12, 19]. Some success has recently been reported in this regard for doxycycline using an alkaline mobile phase with a PS–DVB column [10]. Methods for the analysis of tetracyclines using polymeric columns other than PS–DVB have not been reported.

In this paper, four polymeric columns were evaluated for their ability to separate tetracyclines using acidic mobile phases with acetonitrile as the organic modifier. The column packing materials evaluated included two with a PS–DVB copolymer, a PS–DVB with octadecyl ligands (PS–DVB–C_{18}) and a polymethacrylate with octadecyl ligands (PM–C_{18}).

The tetracyclines selected for investigation

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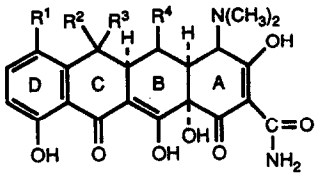
Table 1
Structure of selected tetracyclines



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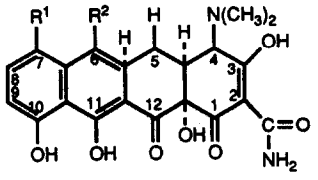

II

Compound	Abbreviation	Base structure	R^1	R^2	R^3	R^4
chlortetracycline	CTC	I	Cl	CH_3	OH	H
tetracycline	TC	I	H	CH_3	OH	H
oxytetracycline	OTC	I	H	CH_3	OH	OH
demeclocycline	DMCTC	I	Cl	H	OH	H
doxycycline	DOX	I	H	CH_3	H	OH
minocycline	MIN	I	$(N(CH_3)_2$	H	H	H
methacycline	METH	I	H	$=CH_2$	—	OH
meclocycline	MECL	I	Cl	$=CH_2$	—	OH
anhydrotetracycline	ATC	II	H	CH_3	—	—
anhydrochlortetraycline	ACTC	II	Cl	CH_3	—	—

are shown in Table 1. They include tetracycline (TC), chlortetracycline (CTC), oxytetracycline (OTC), doxycycline (DOX), minocycline (MIN), methacycline (METH), demeclocycline (DMCTC) and meclocycline (MECL). The selected impurities and degradation products include 4-epi-tetracycline (ETC), 4-epi-anhydrotetracycline (EATC), anhydrotetracycline (ATC), 4-epi-chlortetracycline (ECTC), 4-epi-anhydrochlortetracycline (EACTC) and anhydrochlortetracycline (ACTC). The HPLC methods show improvements over existing procedures by using acidic mobile phases, ambient isocratic conditions and use of the common low viscosity organic modifier acetonitrile. This work was based on our previous bioanalytical work with CTC on polymeric columns with acidic mobile phases and was found to be applicable to other tetracyclines for purity control assays with only minor modifications [21].

Experimental

Materials

Reference standards for tetracycline HCl (lot J-1), oxytetracycline (lot I), doxycycline hyclate (lot H), minocycline HCl (lot H), chlortetracycline HCl (lot I) demeclocycline HCl (lot G-1) and methacycline HCl (lot G) were obtained from the United States Pharmacopeia (Rockville, MD). Meclocycline sulphosalicylate salt (MECL) (lot 50H-0682) was obtained from Sigma (St Louis, MO). 4-Epi-tetracycline HCl (lot 42832/2), anhydrotetracycline HCl (lot 47552/1), 4-epi-anhydrotetracycline HCl (lot 47553/1), 4-epi-chlortetracycline HCl (lot 45896/1), 4-epi-anhydrochlortetracycline HCl (lot 458971/1) and anhydrochlortetracycline HCl (lot 45900/1) were obtained from Janssen Chimica (Beerse, Belgium).

HPLC grade acetonitrile and perchloric acid (60–62%) were obtained from J.T. Baker (Phillipsburg, NJ). Sodium perchlorate (99% pure) was obtained from Janssen Chimica and in-house deionized and distilled water was used.

Instrumentation

The HPLC apparatus included a Model 110A pump (Beckman Instruments, Fullerton, CA) and a Model 7125 injector (Rheodyne, Cotati, CA) equipped with a 20 μl loop. The Model 2550 variable wavelength UV–vis detector (Varian Analytical Instruments, Sunnydale, CA) was set at 280 nm and data were recorded with a Model 4290 integrator (Spectra Physics, San Jose, CA). HPLC columns included: PRP-1, 15 cm × 4.6 mm i.d., 10 μm, PS–DVB (Hamilton, Reno, NV); PLRP-S, 25 cm × 4.6 mm i.d., 100Å, 5 μm, PS–DVB (Polymer Laboratories, Amherst, CA); ACT-1, 15 cm × 4.6 mm i.d. PS–DVB–C_{18} (Interaction Chemical, Mountain View, CA) and Polymer 18 PC-02-6, 15 cm × 4.6 mm i.d., 6 μm, PM–C_{18} (YMC, Wilmington, NC).

Separations were performed at ambient temperature (25 ± 2°C) using mobile phases containing 15–50% v/v organic modifier. Stan-

dard solutions were made by weighing approximately 1 mg of a specific tetracycline into a 10 ml volumetric flask and the addition of acetonitrile–sodium perchlorate (pH 2.0, 0.02 M) (15:85, v/v) volume. Fresh stock solutions were made daily. Mobile phases were filtered through a 0.45 μm Nylon filter and degassed by sonication before use.

Results and Discussion

Figure 1 shows the effects of changing acetonitrile concentration in the mobile phase on the capacity factors of the various tetracyclines chromatographed on the PLRP-S (PS–DVB) polymeric column. The results using the two PS–DVB columns (PLRP-S and PRP-1) were nearly identical in terms of capacity factor versus per cent acetonitrile in the mobile phase. The PM–C_{18} column generally gave slightly larger capacity factors than the other columns but was similar to the PS–DVB columns. On the basis of the capacity factors obtained with the PS–DVB–C_{18} column, the tetracyclines were divided into two groups: the anhydrotetracyclines, which lack a C6 hydroxyl group and have an aromatic 'C' ring; and the tetracyclines containing only one aromatic ring. The anhydrotetracyclines showed similar behaviour to that seen for these compounds on the PS–DVB columns. The tetracyclines containing only one aromatic ring showed little retention on the PS–DVB–C_{18} column, even at the lowest amount of acetonitrile possible in the mobile phase. They also showed a steeper slope on the PS–DVB–C_{18} than on the other three columns investigated.

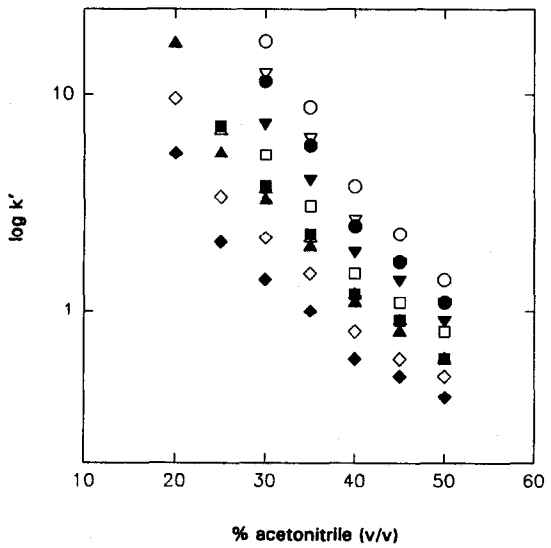

Figure 1
Effects of per cent acetonitrile on the capacity factor of tetracyclines chromatographed on a PLRP-S 25 cm × 4.6 mm i.d., PS–DVB column. Acetonitrile–sodium perchlorate (pH 2.0, 0.02 M), 1.0 ml min^{-1}, 280 nm. ○ = ACTC, ▽ = ATC, ● = MECL, ▼ = DOX, □ = METH, ■ = CTC, △ = DMCTC, ▲ = TC, ◇ = OTC, ◆ = MIN.

Ambient column temperatures (25 ± 2°C) were chosen in order to simplify the HPLC apparatus required. Also, in our previous work with CTC using polymeric columns, elevated column temperatures caused a degradation in peak shape and a loss of resolution due to band broadening [21].

Table 2 shows the asymmetry factor and plates m^{-1} for the tetracyclines when the capacity factor was adjusted between 3 and 8. The most symmetrical peaks were usually obtained on the PM–C_{18} column, while the PLRP-S (PS–DVB) column was the most efficient, giving the largest number of plates m^{-1}. In general, the PRP-1 (PS–DVB) and PS–DVB–C_{18} columns gave more asymmetric peaks and were less efficient than the latter two columns, with the PS–DVB–C_{18} column showing the worst overall performance. The lower efficiency of the PRP-1 column could be due to the fact that the particle size (10 μm) for this column is larger than the PLRP-S (5 μm) and the PM–C_{18} (6 μm) columns. In addition to low efficiency, the PS–DVB–C_{18} column also has the drawback that at least 20% organic modifier is recommended in the mobile phase with this column. Also, high back pressures are obtained when lower amounts of organic modifier were used in the mobile phase. One advantage of the PM–C_{18} column over the others investigated is that higher flow rates could be used, allowing for shorter retention times.

Table 3 compares resolution between pairs of selected tetracyclines and/or degradation products. Closely eluting pairs with similar capacity factors were chosen for comparison. The PLRP-S column consistently provided greater resolution than the PM–C_{18} column. Even though the PM–C_{18} column generally gave more symmetrical peaks, the greater efficiency of the PLRP-S column allowed for better resolution of the analytes. Even when chromatographic conditions were chosen such that resolutions were compared by similar retention times, greater resolutions were obtained with the PLRP-S column.

Table 2
Asymmetry factor and plates m^{-1} for selected tetracyclines

	PRP-1 (PS–DVB)		PLRP-S (PS–DVB)		ACT-1 (PS–DVB–C_{18})		YMC–C_{18} (PM–C_{18})	
	Asymmetry factor*	Plates m^{-1}†	Asymmetry factor	Plates m^{-1}	Asymmetry factor	Plates m^{-1}	Asymmetry factor	Plates m^{-1}
ATCT	1.4	2900	2.2	19,900	2.9	5900	0.9	16,300
ATC	4.5	3100	0.9	32,800	2.0	4300	0.9	19,900
METH	1.7	4600	1.3	28,500	1.9	2300	0.8	13,000
DOX	2.0	3700	1.4	23,600	1.9	2400	0.9	13,900
MECL	3.1	2900	1.5	26,600	2.5	6000	1.0	14,500
CTC	3.2	3800	1.4	18,900	2.2	1800	0.7	11,300
DMCTC	3.6	3700	1.5	20,200	4.0	900	1.0	10.700
TC	2.4	4000	1.1	20,800	3.3	1100	1.0	12,700
OTC	3.3	3500	1.2	29,600	5.0	1000	1.3	9000
MIN	2.6	1700	1.4	13,000	4.9	800	1.4	6300

*From ref 22.
†From ref. 22 using $N = 5.54\ (t_r/w_b)^2$.

Table 3
Comparison of resolution between selected tetracycline pairs using PLRP-S (PS–DVB) and PM–C_{18} (YMC–C_{18}) polymeric columns

	PLRP-S (PS–DVB)						YMC–C_{18} (PM–C_{18})					
Pair*	Mobile phase†	Δt (min)	w_b (min)	t_r (min)	k'	R_s‡	Mobile phase†	Δt (min)	w_b (min)	t_r (min)	k'	R_s‡
ACTC	40:60	2.58	0.68	11.38	2.82	3.28	45:55	1.46	1.20	8.40	3.65	1.40
EACTC			0.89	8.80					0.89	6.94		
ATC	30:70	6.45	1.84	24.64	7.11	4.01	40:60	1.21	0.85	7.73	3.22	1.64
EATC			1.38	18.19					0.63	6.52		
CTC	25:75	6.96	1.73	21.15	4.87	4.87	30:70	2.86	1.52	11.36	4.55	2.13
DMCTC			1.13	14.19					1.17	8.50		
METH	30:70	1.74	1.70	14.62	4.31	1.03	30:70	0.22	1.72	13.50	7.75	0.02
DOX			1.65	12.88					1.69	13.28		
ECTC	25:75	6.03	1.13	15.12	5.48	4.22	25:75	2.18	1.01	11.36	4.73	1.72
CTC			1.73	21.15					1.52	9.18		
TC	25:75	1.68	0.85	9.68	2.32	2.32	25:75	1.34	1.25	9.20	2.99	1.21
ETC			0.60	8.00					0.96	7.86		
OTC	20:80	3.05	1.05	12.65	3.21	3.21	20:80	4.42	1.67	12.51	3.40	2.95
MIN			0.85	9.60					1.33	8.09		

*EACTC = 4-epi-anhydroCTC, EATC = 4-epi-TC, ECTC = 4-epi-CTC, ETC = 4-epi-TC.
†Acetonitrile–0.02 M $NaClO_4$, pH 2.0 v/v.
‡From ref. 22 using $R_s = \Delta t/[(w_{b1} + w_{b2})/2]$.

In previous work with CTC, 0.2% perchloric acid was used in the aqueous portion of the mobile phase [21]. It was found that greater resolution between peaks could be achieved if sodium perchlorate was added to the aqueous portion of the mobile phase before the pH was adjusted to 2.0. As the sodium perchlorate concentration increased from 0 to 0.02 M in the mobile phase, a slight increase in peak symmetry was noted with the peak width remaining relatively constant. At molarities equal or greater than 0.02 M sodium perchlorate, the peak symmetry remained constant. The increase in peak symmetry caused the resolution between peaks to improve. The increased peak symmetry could be due to a decrease in the amount of diketo tautomerization on column [18]. A decrease in the amount of diketo tautometer was noted when chlortetracycline solutions containing 100 μg ml^{-1} were prepared with increasing amounts of sodium perchlorate adjusted to pH 2.0. The solutions were allowed to come to equilibrium (approx. 30 min) [21]. Each solution was chromatographed and the ratio of diketo to enol tautometer was calculated. A slight decrease in the amount of diketo tautometer formed was observed for solutions containing 0.02 M sodium perchlorate and greater.

Figure 2 shows the separations achieved by the PLRP-S [Fig. 2(A)] and the PM–C_{18} [Fig. 2(B)] columns. Using the conditions shown in the legend of Fig. 2, the PLRP-S column provided a separation of all the tetracyclines

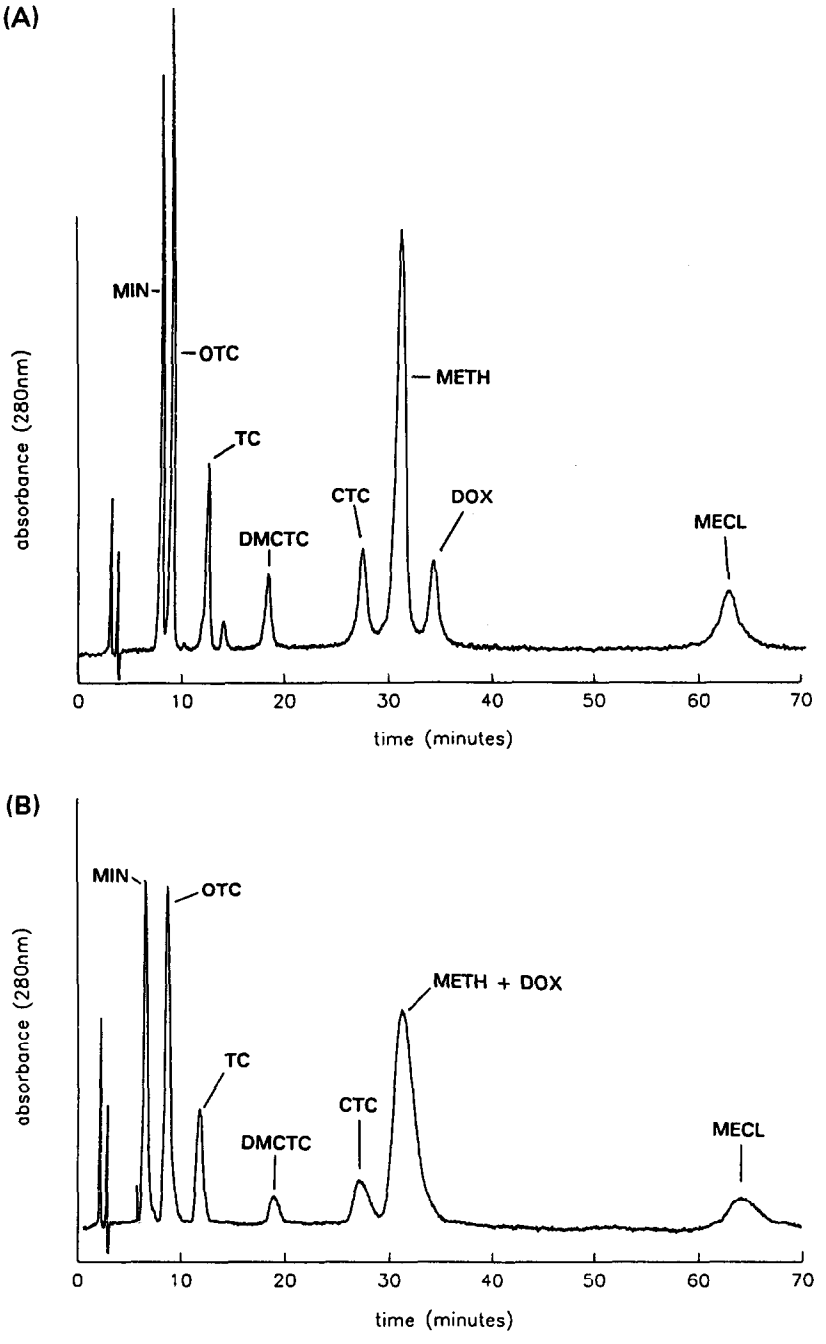

Figure 2
Separation of selected tetracyclines. (A) PLRP-S, 25 cm × 4.6 mm i.d., PS–DVB column. Acetonitrile–sodium perchlorate (pH 2.0, 0.02 M) (25:75, v/v) 1.0 ml min^{-1}, 280 nm. (a) MIN (6.89 min); (b) OTC (7.74 min); (c) TC (11.01 min); (d) DMCTC (16.15 min); (e) CTC (24.42 min); (f) METH (29.61 min); (g) DOX (30.68 min); (h) MECL (61.50 min). (B) PM–C_{18}, 15 cm × 4.6 mm i.d., acetonitrile–sodium perchlorate (pH 2.0, 0.02 M) (25:75, v/v) 1.0 ml min^{-1}, 280 nm. (a) MIN (5.36 min); (b) OTC (7.27 min); (c) TC (10.14 min); (d) DMCTC (16.66 min); (e) CTC (24.10 min); (f), (g) METH and DOX (27.68 min); (h) MECL (57.63 min).

investigated, while the PM–C_{18} gave similar results except for the coelution of METH and DOX. The elution order using the acetonitrile–sodium perchlorate pH 2.0 mobile phase was similar to that obtained with alkaline mobile phases except that because MIN was in its very polar dicationic form, it was the first tetracycline to elute under the described conditions. From this data, it was concluded that the PLRPS-S column with the mobile phase

described in the legend of Fig. 2 would be useful for the general screening or identification of unknown tetracycline samples.

The general advantages of these LC methods are that by using an acidic mobile phase with acetonitrile as the organic modifier, all the tetracyclines described herein can be separated in a single isocratic run. This includes CTC where acidic conditions are necessary for its separation. Because of the rapid formation of the isochlortetracycline analogue under alkaline conditions, the CTC peak becomes a broad and tailing peak. Also, using a mobile phase at pH 2, formation of the C4 epimer of the tetracyclines and the formation of the anhydrotetracyclines are minimized.

Some methods reported for the analysis of tetracyclines use elevated column temperatures because of the need to increase the mass transfer of analytes between a high viscosity mobile phase and the stationary phase [9–20]. By using the low viscosity organic modifier acetonitrile, ambient temperatures can be used and other mobile phase additives such as EDTA and tetrabutylammonium ion pairing agents are not required.

Using acetonitrile–sodium perchlorate pH 2.0 mobile phases, the best polymeric columns for the LC separation of tetracyclines are the PLRP-S (PS–DVB) and the PM–C_{18} columns. Even though the PM–C_{18} column gave more symmetrical peaks, the PLRP-S column showed greater resolution of the tetracyclines investigated because of its greater efficiency. The ideal column for the HPLC separation of tetracyclines would depend on the resolution required between compounds of interest and the time required for analysis. The ACT-1 (PS–DVB–C_{18}) column was the least desirable column because of poor peak shape, low efficiency, high back pressure and limitations on the amount of organic modifier that can be used in the mobile phase. The methodology reported herein will be used to obtain optimum HPLC conditions for the separation of tetracyclines, their degradation products and impurities in bulk drug substance and dosage forms.

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