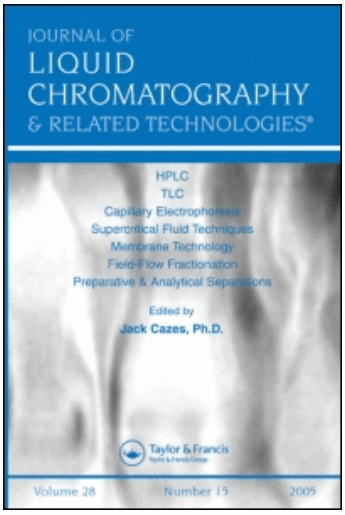

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COMPARATIVE STUDY OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF TETRACYCLINE ANTIBIOTICS

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ABSTRACT

This work reviewed all the past HPLC methods utilized for the detection of tetracyclines and created a model to study the most promising ones. Three general mobile phases were investigated. They included Mobile Phase I (0.05 M buffer (A)-acetonitrile (B) programmed from 85A:15B to 40A:60B), Mobile Phase II (0.05 M buffer (A)-acetonitrile (B)-methanol (C) programmed from 80A:0B:20C to 30A:50B:20C), and Mobile Phase III (0.05 M buffer (A)-acetonitrile (B)-dimethylformamide (C) programmed from 80A:OB:20C to 30A:50B:20C). Buffers made from citrate, oxalate, and phosphate, each prepared at pH 2.0, 4.5, and 7.0, were used with each mobile phase. Ethylenediaminetetraacetic acid (EDTA) at 0.005 M was added to the pH 4.5 and pH 7.0 buffers (it would not dissolve in pH 2.0 buffers). Tetramethylammonium chloride (TMA) was also added at 0.01 M to all of the buffers at each pH. The columns chosen for this study were a C18 bonded to silica (Supelco LC-18), a "deactivated" C18 bonded column (Supelco LC-18-DB), and a polymeric styrenedivinylbenzene copolymer column (Polymer

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Laboratories PLRP-S). The columns and mobile phases were evaluated using a mixed standard of oxytetracycline, tetracycline, and chlortetracycline. Comparisons were based on peak shape, separations, and comparative recoveries (integrated areas) of standards. The polymeric column was markedly superior in all respects. Generally optimum results were obtained with Mobile Phase I in pH 2.0 buffers. The oxalate buffer was best with the bonded columns while little difference was noted with the polymeric column. TMA markedly improved peak shape on the bonded columns. EDTA was of little benefit. Recoveries, especially of chlortetracycline, were higher from the polymeric column.

INTRODUCTION

There are numerous reports in recent years of the application of high performance liquid chromatography (HPLC) to analysis of tetracycline antibiotics in formulations (1-13), feeds (14,15), honey (16,17), milk (18-21) and other biological fluids (8,22-31), eggs (32) and tissues (22,26,30,33-44). A survey of the literature showed that a great variety of HPLC packings and mobile phases have been used. Reversed-phase HPLC has been the preferred mode although ion-exchange has also been used (8). The most widely used reversed-phase column type has been C18 bonded to silica (1,3,7-10,14,15,17, 19,21,23,28-30,32,33,40,43,44), followed by C8 bonded to silica (2,12,20,22, 24,35,36,41) and also methyl (3,8,39,42) or phenyl (31,38) bonded to silica. Several investigators have noted the advantages of polystyrenedivinyl-benzene copolymer packings (5,6,10,11,13, 25,29,34,37). Onji, et al. (42) also used a polystyrene packing.

A great variety of mobile phases has also been used. Phosphate buffers or phosphoric acid at acid pH (<2.6) have been most widely used (3,8,10,16,19,20,22,23,26,27,29-31,37,40,42). A few investigators have used phosphate buffers at neutral or alkaline pH (1,5,6,9). Oxalic acid or oxalate buffers at acid pH (2,15,21,33,35,36,38,41,43) and citrate buffers (8,14,17,24,39) have also been used frequently. Some others reported include

imidazole - Mg acetate (44), perchloric acid (7,11), trifluoracetic acid (18) and glycine adjusted to pH 12 (34).

Organic modifiers used include acetonitrile (3,4,10,16,18, 19,22-24,29,30,32,34,37,39,42), methanol (1,28,31,42,44), acetonitrile-methanol (12,15,21,27,35-38,41,43), dimethylformamide (8,14,17,20), acetonitrile-dimethylformamide (26,40), t-butanol (6,11) and isopropanol (9).

Many investigators have found it beneficial to include EDTA in the mobile phase (1,2,5,6,8,9,13,14,17,28,39).

Other ion-pairs or counterions used include diethanolamine (9,40), ethanolamine (26), 1-esansulfonic acid (23), sodium heptane sulfonate (10), 1-hydroxy-2,3-diisobutylsulfonic acid (4), nitrate (8,14,17,22), and tetraalkylammonium compounds (3,5,10,37).

Knox and Jurand (8) concluded that optimum results were obtained at acid pH, preferably below 2.5. They noted, however, that bonded reversed-phase columns were not very stable under these conditions. Aszalos (45), on the other hand recommended a pH of above 5.5 for reversed-phase analysis to minimize oxidation and epimerization of tetracyclines. McCormick, et al. (46) found that epimerization occurred within the pH range of 2-6 in a variety of solvent systems.

In view of the wide range of conditions reported for chromatographic analysis of tetracycline antibiotics, the present study was undertaken in order to systematically compare chromatographic performance on bonded and polymeric columns with various commonly used buffers, organic modifiers and counterions.

MATERIALS AND METHODS

Chemicals and equipment

Acetonitrile and methanol were HPLC grades (EM Omnisolv or equivalent (Gibbstown, NJ). Tetramethyl ammonium chloride was

obtained from Eastman Kodak (Rochester, NY, U.S.A.). Potassium oxalate, oxalic acid, sodium citrate, citric acid, potassium dihydrogen phosphate, disodium phosphate, phosphoric acid, and ethylenediaminetetraacetic acid, sodium salt, were reagent grade chemicals obtained from several sources.

Oxytetracycline, tetracycline, and chlortetracycline were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and used as received. A mixed standard containing all three tetracyclines was prepared at 10 μg/ml in 0.01 M hydrochloric acid and was stable for up to a month refrigerated. A working dilution of 1 μg/ml was prepared from the standard.

The HPLC apparatus consisted of Varian Model 9010 pump (Sugarland, TX) a Waters 712 WISP autosampler utilizing a 2000 μl loop and 2500 μl syringe and a Waters 990 Photodiode array detector operated at 355 nm (Milford, MA, U.S.A.).

The HPLC columns used were a Supelco LC-18 column with LC-18 guard column, 150 x 4.6 I.D. mm in size, with a particle size of 5 μm (Bellefonte, PA), a Supelco LC-18-DB column with LC-18-DB guard column, 150 x 4.6 I.D. mm in size, with a particle size of 5 μm and a Polymer Laboratories PLRP-S with a special guard column, a styrenedivinylbenzene copolymer, 150 x 4.6 I.D. mm 100 Å pore diameter; 5 μm particle size (Amherst, MA, U.S.A.).

Preparation of buffers

Oxalate, citrate, and phosphate buffers of 0.05 M were prepared at pH's 2.0, 4.5, and 7.0. Also, each buffer contained either 0.005 M ethylenediaminetetraacetic acid (EDTA), 0.01 M tetramethylammonium chloride (TMA), or neither. Theoretical buffer compositions were calculated based on the equation:

$$pH = pKa + (\log \frac{salt}{acid}) \quad (47)$$

and are shown in Table I. The calculated pH's deviated somewhat from those measured with pH meter (Table II).

Table I. Composition of experimental Buffers (1 liter)

0.05M Oxalate Buffers			
pH 2.0	0.0214M	$K_2C_2O_4$	(3.94g)
	0.0286M	$H_2C_2O_4$	(3.61g)
pH 4.5	0.0415M	$K_2C_2O_4$	(7.65g)
	0.0085M	$H_2C_2O_4$	(1.87g)
pH 7.0	0.0500M	$K_2C_2O_4$	(9.21g)
0.05M Citrate Buffers			
pH 2.0	0.0013M	$Na_3C_6H_5O_7$	(0.36g)
	0.0498M	$H_3C_6H_5O_7$	(10.25g)
pH 4.5	0.0120M	$Na_3C_6H_5O_7$	(3.53g)
	0.0380M	$H_3C_6H_5O_7$	(7.99g)
pH 7.0	0.0500M	$Na_3C_6H_5O_7$	(14.71g)
0.05M Phosphate Buffers			
pH 2.0	0.0260M	KH_2PO_4	(3.54g)
	0.0240M	H_3PO_4	(2.35g)
pH 4.5	0.0500M	KH_2PO_4	(6.81g)
pH 7.0	0.0330M	Na_2HPO_4	(4.69g)
	0.0170M	KH_2PO_4	(2.31g)

Table II. Actual pH Values of Experimental Buffers

		Actual pH		
	Calculated pH	Oxalate	Citrate	Phosphate
Buffer	2.0	2.30	2.39	2.32
Buffer	4.5	3.80	3.27	4.54
Buffer	7.0	7.20	7.63	7.14
Buffer w/TMA	2.0	2.29	2.38	2.32
Buffer w/TMA	4.5	3.78	3.27	4.53
Buffer w/TMA	7.0	7.20	7.63	7.14
Buffer w/EDTA	4.5	3.88	3.32	4.57
Buffer w/EDTA	7.0	5.60	6.73	6.83

High-performance liquid chromatographic procedures

Mobile Phase I contained buffer (A)-acetonitrile (B) programmed from 85A:15B (0-3 min) to 40A:60B (20 min). Mobile Phase II contained buffer (A)-acetonitrile (B)-methanol (C) programmed from 80A:OB:20C (0-3 min) to 30A:50B:20C (20 min). Mobile Phase III contained buffer (A)-acetonitrile (B)-dimethylformamide (C)-programmed from 80A:OB:20C (0-3 min) to 30A:50B:20C (20 min). All were run at a flow rate of 1 ml/min; injection volume - 1 ml. After completion of the gradient program, the column was flushed 5 min (20-25 min) with the final concentration and returned to initial conditions at 26 min. Loading of the next sample was started at 35 min. After use, the system was flushed 10 min. with water and 15 min. with 40:60 water-acetonitrile for storage.

RESULTS AND DISCUSSION

The initial conditions for the gradient programs were selected so that the tetracylines were essentially immobile. Under these conditions, tetracylines can be concentrated on-line from sample extracts and eluted with a gradient without distorting the peaks. An injection volume of 1ml was used to establish that the injection solvent did not affect peak shape. Conditions were selected which would just retain the tetracyclines so that an excessively long or excessively steep gradient was not required for elution. With gradient elution, all the tetracycline peaks were equally sharp.

As the model was systematically carried out, several complications arose and adjustments had to be made. One complication was the inability to dissolve EDTA in buffer solutions of pH 2.0. Even after placing the buffer in a sonicator for 30 minutes, the EDTA was still not dissolved. The

buffers were prepared based on mathematical calculations. Since the exact pH was not achieved, especially when EDTA or TMA was added, actual pH's were recorded using a pH meter and are listed in Table I. Difficulty was also observed with Mobile Phase III, water-acetonitrile-DMF. After obtaining several very poor chromatograms, it was decided to discontinue working with it. The problems included a drifting baseline, poor peak shape, and poor reproducibility, probably because the system failed to reequilibrate in a reasonable length of time with the gradient program used.

The focus was then turned solely to Mobile Phase I and Mobile Phase II for the duration of the study. In general, Mobile Phase I, buffer-acetonitrile, was much more effective than Mobile Phase II, buffer-acetonitrile-methanol. Effectiveness was based on separation, peak shape, and recovery. Recoveries were based on raw integration data of peak areas in Absorbance units X minute (AU·min) for tetracycline antibiotic standards. Recovery data is important because peak shapes may differ and visual determination of recoveries may be inaccurate.

Separation could always be achieved with Mobile Phase I using any of the columns and at any pH. However, this could be achieved only at pH 7.0 with Mobile Phase II when using the LC-18 or LC-18-DB columns. This is illustrated in Figure 1 using the LC-18 column. At pH 2.0 and pH 4.5 oxytetracycline and tetracycline were not separated from one another but eluted at the same time and appeared as one large peak. Therefore, only two peaks were observed. The first peak contained oxytetracycline and tetracycline fused together and the second was chlortetracycline. However, at a pH of 7.0 this separation was achieved and all three peaks were resolved. Mobile Phase I will be discussed below in more detail.

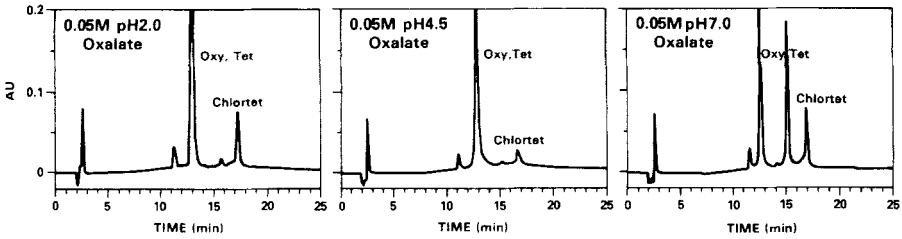

Figure 1. Separation difficulty with Mobile Phase II - (0.05 M buffer (A)-acetonitrile (B)-Methanol (C)- 80A:0B:20C (0-3 min) - 30A:50B:20C, LC-18 column 355 nm.

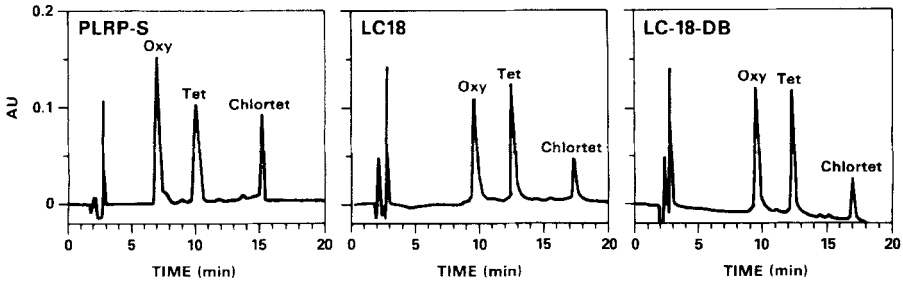

Figure 2. Comparison of chromatograms obtained with PLRP-S, LC-18, and LC-18-DB columns using 0.05 M pH 2.0 oxalate buffer (A)-actonitrile (B)-from 85A:15B (0-3 min) - 40A:60B (20 min) (Gradient 1), 1 ml/min, 355 nm.

The present data indicate comparable efficiency for the PLRP-S column when using columns packed with the same particle size (5 μm) materials. Poor recoveries of tetracyclines from silica-based HPLC packing materials have been observed before. Some workers have resorted to lengthy conditioning procedures including saturating the column with a tetracycline in order to avoid such losses (19,30). However, the problem was easily avoided by switching to the polystyrenedivinylbenzene packing. While the oxalate buffer was slightly better with standards, the

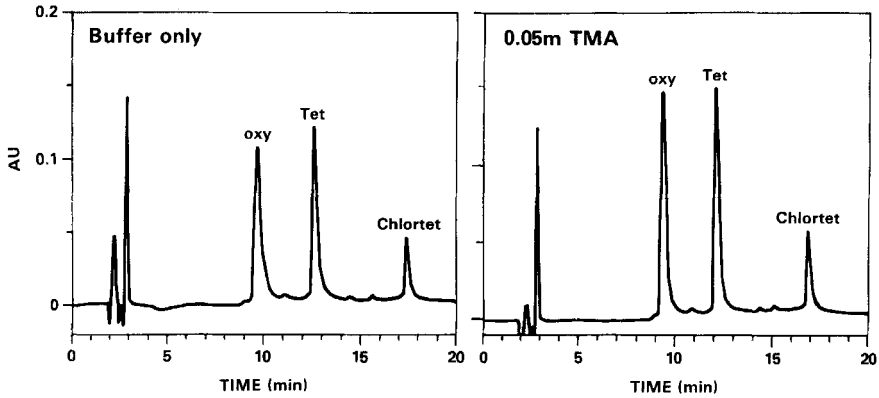

Figure 3. Effect of tetramethylammonium chloride (TMA) addition on chromatograms, pH 2.0 oxalate buffer, LC-18 column. Conditions as in Figure 2.

differences were not great on the PLRP-S packing and one of the others may be better for analysis of some types of samples.

Mobile Phase I - Oxalate buffers

For the oxalate buffers, both the chromatograms (Figures 2-4) and integration data (Tables III and IV) for the oxytetracycline and chlortetracycline peaks are shown. The peak height gives a measure of column efficiency and peak area gives a measure of recovery of the material from the column. Comparison of the chromatograms at pH 2.0 (Figure 2) suggests that results were reasonably satisfactory with all three types of column. Separations were good with slight tailing on the bonded columns. However, integration data (Tables III and IV) show substantially higher recoveries for oxytetracycline and especially chlortetracycline from the PLRP-S column. This is evident visually for chlortetracycline. At pH 2.0, addition of TMA markedly improved peak shape in the LC-18 column (Figure 3) and slightly increased recoveries of oxytetracycline. At pH 4.5,

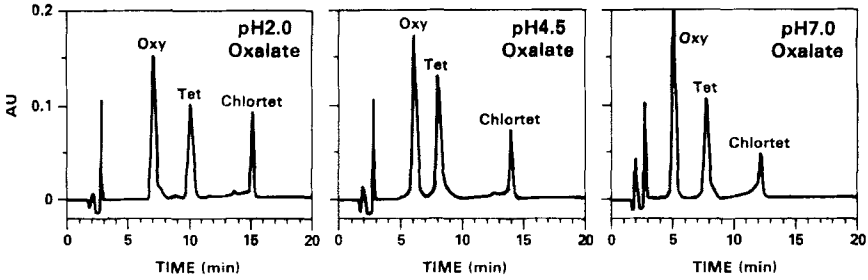

Figure 4. Effect of pH on Retention Times in oxalate buffers, PLRP-S column. Conditions as in Figure 2.

chlortetracycline was barely detectable on the LC-18-DB column. Peak shape and recoveries were improved by addition of TMA. Addition of EDTA also improved recoveries but not peak shape (Tables III and IV) at this pH. At pH 7, TMA improved recoveries but had little effect on peak shape on the LC-18-DB column. Figure 4 illustrates the effect of pH on retention time on the PLRP-S column. The peak shape was poorer at higher pH, especially pH 7, and apparent recoveries of chlortetracycline were lower (Table IV).

The fact that recoveries were so low with the LC-18-DB column was quite unexpected. Since the LC-18-DB column is supposedly deactivated it would be expected to produce higher recoveries than the LC-18 column without the addition of a silanol blocking agent. However, under these conditions, recoveries were the same for both columns at pH 1.0 and lower for the LC-18-DB at pH's 4.5 and 7.0. Also, the addition of TMA would be expected to improve recoveries with the LC-18 column, however, it had little effect. Many authors have added EDTA or TMA (10,37) to their mobile phases. However, although the addition of EDTA and, especially, TMA seemed to

Table III. Comparative Recoveries of Oxytetracycline in Oxalate Buffers (Mobile Phase I)

	Column					
	LC-18-DB		LC-18		PLRP-S	
Buffer + Additive	H(AU)	A(AU·min)	H(AU)	A(AU·min)	H(AU)	A(AU·min)
Oxalate, pH 2.0						
None	0.129	0.0463	0.105	0.0433	0.101	0.0616
TMA[a]	0.155	0.0455	0.145	0.0515	0.108	0.0600
Oxalate, pH 4.5						
None	0.086	0.0285	0.101	0.0421	0.125	0.0630
TMA	0.130	0.0372	0.120	0.0425	0.120	0.0625
EDTA[b]	0.096	0.0377	0.144	0.0431	0.114	0.0600
Oxalate, pH 7.0						
None	0.108	0.0256	0.110	0.0446	0.103	0.0603
TMA	0.117	0.0366	0.136	0.0451	0.097	0.0607
EDTA	0.104	0.0376	0.132	0.0479	0.127	0.0609

[a]TMA = Tetramethylammonium chloride
[b]EDTA = Ethylenediaminetetraacetate

Table IV. Comparative Recoveries of Chlortetracycline in Oxalate Buffers (Mobile Phase I)

	Column					
	LC-18-DB		LC-18		PLRP-S	
Buffer + Additive	H(AU)	A(AU·min)	H(AU)	A(AU·min)	H(AU)	A(AU·min)
Oxalate, pH 2.0						
None	0.043	0.0120	0.042	0.0131	0.088	0.0208
TMA[a]	0.050	0.0109	0.050	0.0135	0.088	0.0211
Oxalate, pH 4.5						
None	0	0	0.008	0.0043	0.066	0.0174
TMA	0.010	0.0033	0.011	0.0040	0.063	0.0175
EDTA	0.019	0.0055	0.010	0.0042	0.055	0.0153
Oxalate, pH 7.0						
None	0.010	0.0036	0.009	0.0052	0.043	0.0154
TMA	0.024	0.0061	0.024	0.0067	0.039	0.0147
EDTA	0.005	0.0025	0.014	0.0063	0.047	0.0136

[a]See Table II for footnotes.

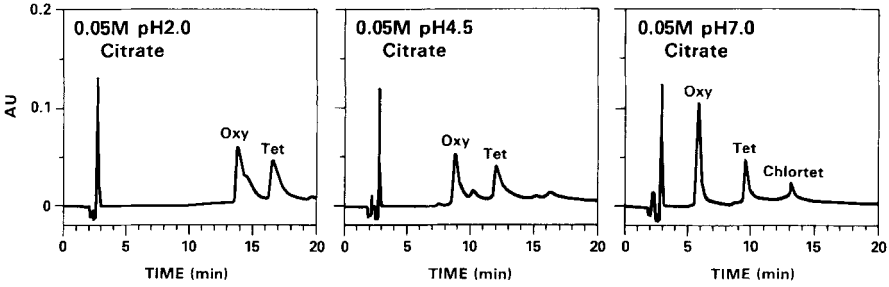

Figure 5. Effect of pH on separation in 0.05 M citrate buffers, LC-18 column. Conditions as in Figure 2.

improve peak shape, recoveries remained poor. The EDTA and TMA would seemingly compete with tetracyclines for interfering metal ions and residual silanols, respectively, on the column. The fact that this occurred with the LC-18-DB column rather than the LC-18 column was the opposite of what was expected.

Mobile Phase I - Citrate Buffer

Results were not quite as good with the citrate buffer as compared with the oxalate buffer, especially on the bonded columns. The addition of EDTA and TMA had little effect on separation, peak shape, or recovery when using the PLRP-S column.

When the LC-18 column was utilized, separation was very poor at pH 2.0 and improved only slightly at pH 4.5 and pH 7.0 (Figure 5). This also illustrates, once more, the effect of pH on retention time. The addition of TMA improved peak shape markedly at pH 2.0 as well as noticeably improving recovery. Improvement was slight at pH 4.5 and no improvement was noted at pH 7. This indicates that the TMA may be acting as a silanol blocking agent as previously suggested. The addition of EDTA had little effect. Separation with the LC-18-DB

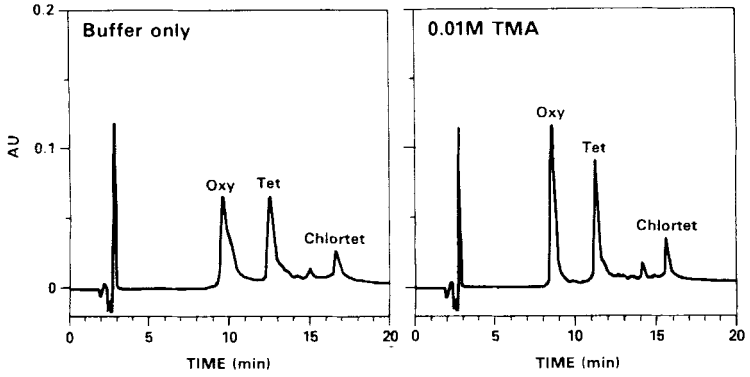

Figure 6. Effect of TMA addition to pH 2.0 phosphate buffer, LC-18 column. Conditions as in Figure 2.

column was also poor. TMA once again improved peak shape and recovery. Overall, the addition of EDTA had little effect.

Mobile Phase I - Phosphate Buffer

The results obtained with the phosphate buffer were somewhat poorer than those with the citrate buffer, especially on the bonded columns. The addition of EDTA and TMA had little effect on separation, peak shape, or recovery of tetracyclines on the PLRP-S column. When utilizing the LC-18 column, improvement was observed with the addition of TMA at a pH of 2 (Figure 6). At pH 4.5, chlortetracycline was not recovered even in the presence of EDTA or TMA. The addition of EDTA had no effect. TMA had little effect on recoveries when using the LC-18-DB column but improved peak shape at pH 2.0. At other pH's, the effects of TMA and EDTA on recoveries and peak shape were variable with the LC-18-DB column.

These results are consistent with those of other investigators who reported optimum chromatographic results at pH 2.5 or below. The role of EDTA is unclear. It has been

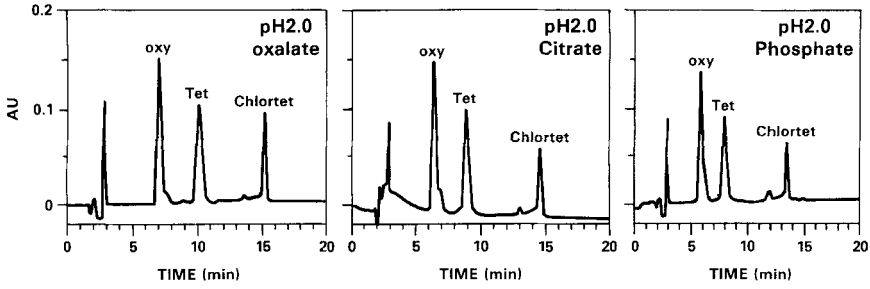

Figure 7. Comparison of oxalate, citrate, and phosphate buffers and their effect on recovery on a PLRP-S column. Conditions as in Figure 2.

claimed that it binds metal ions thus improving chromatography. Knox and Jurand (8) concluded that it acted as an ion-pair with the tetracyclines in the pH range 3-5. They also observed that it was necessary to prevent irreversible binding of tetracyclines to silica-based reversed-phase columns. With oxalate or citrate buffers which also complex with metal ions, addition of EDTA as a metal ion complexing agent would seem superfluous and our results support this conclusion. Addition of amines to improve peak shape has been reported by a number of investigators (3,5,9,10,26,40). The tetramethyl ammonium ion has been used as a silanol blocking agent to improve peak shape in chromatography of compounds with basic functions (10). It was generally more effective than EDTA in improving peak shape on silica-based reversed-phase columns. The lack of effect on the polymeric packing confirms that it is acting as a silanol blocking agent.

In summary, apparent recoveries were found to be the greatest when using Mobile Phase I, especially at a pH of 2.0.

the differences in appearance of the chromatograms on the PLRP-S column in the respective pH 2.0 buffers was slight (Figure 7). The PLRP-S column was the column of choice as compared with the LC-18 and LC-18-DB columns. Recoveries of tetracyclines were conspicuously better, especially chlortetracycline. Although peak shapes on the bonded columns could be markedly improved by adding TMA to the mobile phase, recoveries were still less than from the polymeric column. TMA is believed to block unreacted silanols. Addition of EDTA slightly improved recoveries from the bonded columns in some cases but was of little benefit otherwise. The column efficiency of some other brands of polymeric columns was inferior to that of the PLRP-S column used in the present study.

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