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Note

Reversed-phase high-performance liquid chromatography of doxycycline

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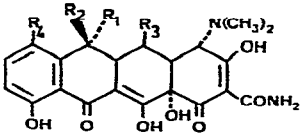
Of the tetracyclines, doxycycline is one of the most widely used in broad spectrum antibiotic therapy. It is particularly characterized by its greater chemical stability and chemotherapeutic activity compared with the parent molecule tetracycline. The structural formulae of some tetracyclines are presented in Table I.

TABLE I

STRUCTURES OF TETRACYCLINES



R_1	R_2	R_3	R_4	*Common name*
CH_3	OH	H	H	Tetracycline
CH_3	OH	OH	H	Oxytetracycline
H	OH	H	Cl	Demethylchlortetracycline
CH_3	H	OH	H	Doxycycline
$=CH_2$		OH	H	Methacycline

Microbiological[1,2] and fluorimetric techniques[3–6] are used to determine the levels of doxycycline in biological samples. However, both methods suffer from a lack of selectivity. To overcome this problem, a chromatographic system capable of separating the drug from interfering materials and analogues is required.

Up to now, only one paper has reported the use of gas–liquid chromatography (GLC) for separating tetracyclines[7]. However, owing to their considerable molecular weight and high degree of polarity, the GLC of these compounds suffers from several problems. Moreover, because these structures are chemically unstable in basic media, derivatization is extremely difficult. In contrast to GLC, high-performance liquid chromatography (HPLC) combined with UV detection seems to be more suitable. Several ion-exchange systems, using pellicular packing materials[8–12], have been described, but none of them was sufficiently sensitive for our purposes. Better results have been reported with various reversed-phase systems[13–19], although often

drastic pH conditions[15,16] or gradient elution[14,17] were required in order to provide acceptable resolution. Most of these methods have been applied only to relatively simple systems, *e.g.*, the determination of impurities in tetracycline formulations[13–17]. Only one paper has dealt with the separation of doxycycline from three other commonly used tetracyclines on a pellicular RP 18 column[19]. However, in common with other packing materials of this type a low efficiency was obtained.

Current interest in our laboratory in the determination of doxycycline in biological materials has led to the development of a new HPLC system, which permits the separation of the compound from four other tetracyclines.

EXPERIMENTAL

Apparatus

A Varian Model LC 8500 liquid chromatograph with gradient capability was used. Injections were made with the aid of a Valco sampling valve (Model CV-6-UHPa-C20), equipped with a 10-μl loop. A Vari-Chrom variable-wavelength detector was operated at 350 nm.

Chromatographic conditions

The following reversed-phase columns were studied.

(a) *Bonded phase: octadecyl chain.* (1) 50 cm × 2.1 mm I.D. (stainless steel), containing 35–50-μm Vydac RP 18 (Varian, Brussels, Belgium). (2) 25 cm × 4.6 mm I.D. (stainless steel), containing 5-μm RP 18 (R.S.L., St. Martens-Latem, Belgium).

(b) *Bonded phase: octyl chain.* (3) 25 cm × 4.6 mm I.D. (stainless steel), filled with 5-μm LiChrosorb RP 8 (Merck, Darmstadt, G.F.R.). (4) 25 cm × 3 mm I.D. (stainless steel), filled with 7-μm LiChrosorb RP 8 (Merck).

Eluent systems. The eluent systems consisted of mixtures of acidic buffer solutions (pump A) and acetonitrile or methanol (pump B), except for the Vydac column (No. 1), where pump A contained 0.15 *M* ammonium carbonate solution (adjusted to pH 8.4 with 13 *M* ammonia solution).

Chemicals and reagents

All tetracyclines were kindly provided by Pfizer (Brussels, Belgium), except demethylchlortetracycline, which we obtained from Lederle (Brussels, Belgium). Samples for injection were made by dissolving the tetracyclines (hydrochloride salts) in methanol to give concentrations of 1 mg/ml. The organic solvents acetonitrile and methanol, both from Merck, were of analytical grade. Freshly doubly distilled water was used to prepare buffer solutions, as follows. (1) A citrate–phosphate buffer of pH 2.2, according to McIlvaine[20], was prepared by mixing 980 ml of 0.1 *M* citric acid with 20 ml of 0.2 *M* disodium hydrogen orthophosphate solution. (2) Glycine buffers of pH 3.1 and 2.1 were prepared by adding 15 and 45 ml, respectively, of 0.2 *M* hydrochloric acid to 85 and 55 ml, respectively, of a solution containing 1.5 g of glycine and 1.17 g of sodium chloride per 100 ml.

RESULTS AND DISCUSSION

The high polarity of tetracyclines makes them unsuitable for adsorption chro-

matography on silica gel and, as confirmed in many studies reversed-phase chromatography appears to be more promising. Using simple solvent combinations such as acetonitrile–water or methanol–water, either no symmetrical doxycycline peak or a very poor separation from other tetracyclines was obtained. To enhance the selectivity of the chromatographic system, the incorporation of a "modifier" in the mobile phase seemed necessary.

A previously described mobile phase[19], containing 8% (v/v) of methanol in 0.05 *M* ammonium carbonate solution, proved to be unsuccessful on the Vydac RP 18 column (No. 1), the doxycycline appearing as a broad, strongly tailing peak. However, we considerably improved the peak shape by adding 13 *M* ammonia solution to a final pH of 8.4 as a tailing suppressor. In addition, we obtained an increased efficiency with more concentrated solutions, *e.g.*, 0.15 *M* ammonium carbonate, and replacement of methanol with acetonitrile also gave a similar effect. The separation of oxytetracycline, doxycycline, demethylchlortetracycline and tetracycline was achieved with as little as 4% (v/v) of acetonitrile in the mobile phase, as shown in Fig. 1.

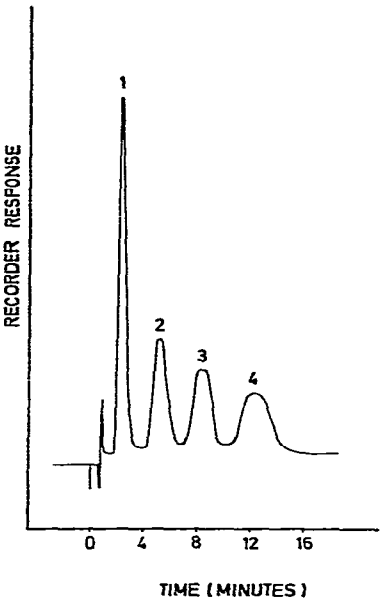


Fig. 1. Separation of four tetracyclines. Column: 50.0 cm × 2.1 mm I.D., Vydac RP 18. Eluent: 0.15 *M* ammonium carbonate–acetonitrile (96:4) + 13 *M* ammonia solution to pH 8.4. Flow-rate: 60 ml/h. Temperature, 20°; pressure, 500 p.s.i. Peaks: 1 = oxytetracycline; 2 = doxycycline; 3 = demethylchlortetracycline; 4 = tetracycline.

Unfortunately, the low efficiency of the pellicular packing material limits the sensitivity of detection. We therefore applied the modified solvent system to a totally porous RP 18 column (No. 2). Again, the peaks tailed badly and very high concentrations of organic solvents (up to 95% of methanol) were necessary in order to elute the required compounds. This effect was thought to be due to secondary adsorption on the porous silica[15]. Some workers have assumed that the latter phenomenon is due to

presence of divalent cations, such as calcium[18], and a small amount of a chelating agent, *e.g.*, EDTA is therefore often added to the mobile phase[7–9,11–13,17–19]. We also performed chromatography with 0.005 *M* disodium EDTA incorporated in an acetonitrile–water eluent. Although we obtained no better results with the RP 18 column, a shorter bonded alkyl chain, *i.e.*, an octyl chain (column No. 3) proved to be more suitable. In fact, on the latter column system doxycycline chromatographed as a symmetrical peak, but could not be separated from four other tetracyclines. This effect was probably due to non-optimization of the pH.

Knox and co-workers[15,16] studied the addition of inorganic acids (*e.g.*, perchloric acid) to the eluent, giving pH values of 1.0–1.5. We examined various buffers, containing organic acids such as citric and tartaric acid, which also serve as chelating agents. Initially, maximum retention of tetracyclines on an apolar stationary phase would be expected near the isoelectric point (pH 5), where they exist in their most lipophilic form. However, using a citrate buffer of this pH, a particularly poor resolution between five tetracyclines was obtained on the RP 8 column. The retention times increased considerably when the pH was decreased. Maximum resolution was achieved with a citrate–phosphate buffer of pH 2.20, which is the lower limit of this buffer system (Fig. 2). Doxycycline and methacycline, whose structures are closely related, are still incompletely resolved. The two small peaks (2 and 4) are probably caused by some degradation products, perhaps epimers at the C_4 position. Analogous results were obtained with the latter eluent on a second RP 8 column (No. 4). No significant improvement in resolution was obtained when the pH was decreased

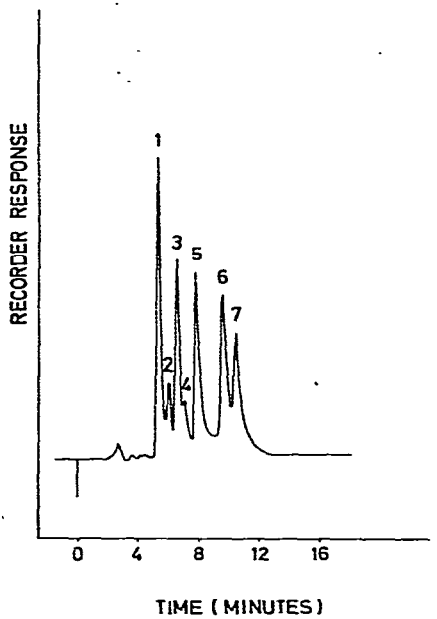


Fig. 2. Separation of five tetracyclines. Column: 25.0 cm × 4.6 mm I.D., LiChrosorb RP 8 (5 μm). Eluent: citrate–phosphate buffer (pH 2.20)–acetonitrile (65:35). Flow-rate: 80 ml/h. Temperature, 20°; pressure, 3900 p.s.i. Peaks: 1 = oxytetracycline; 3 = tetracycline; 5 = demethylchlortetracycline; 6 = methacycline; 7 = doxycycline; 2 and 4 = unidentified.

further by using pure citric or tartaric acid solutions as the eluent. As shown in Table II, the capacity ratios increased until a maximum value was reached, whereas doubling the citric acid concentration resulted in less retention. Changing the nature of the organic acid had little or no effect, as shown by the replacement of citric acid with tartaric acid.

TABLE II

CAPACITY RATIOS (k') OF DOXYCYCLINE AT DIFFERENT pH VALUES

Column: 25 cm × 3 mm I.D., LiChrosorb RP 8 (7 μm). Flow-rate: 80 ml/h. Acetonitrile: 29% (v/v) (pump B).

Aqueous eluent (pump A)	*pH*	*k'*
Citrate–phosphate buffer	2.20	4.6
Citric acid (0.1 *M*)	2.15	6.9
Tartaric acid (0.1 *M*)	2.15	6.9
Citric acid (0.2 *M*)	1.98	5.9

To elucidate the factors that influence retention and resolution, another acidic buffer system combined with a chelating agent, *viz.*, a glycine buffer containing 0.005 *M* penicillamine hydrochloride (pH 3.1), was examined. This eluent gave a very similar chromatographic pattern on the first RP 8 column (No. 3) in comparison with a citrate–phosphate buffer of identical pH. Furthermore, to establish whether the chelating agent is obligatory, penicillamine was omitted, and no significant differences in peak shape, resolution or retention time were observed. The separation could also be optimized by decreasing the pH to 2.1, as shown in Fig. 3.

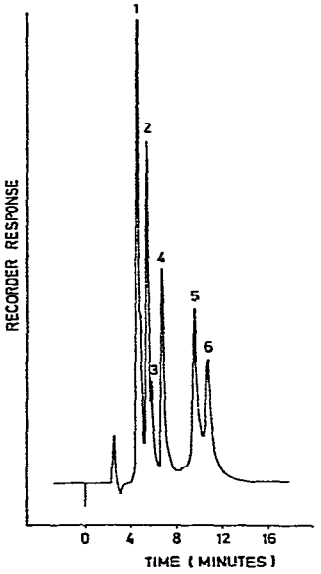


Fig. 3. Separation of five tetracyclines. Column: 25.0 cm × 4.6 mm I.D., LiChrosorb RP 8 (5 μm). Eluent: glycine buffer (pH 2.1)–acetonitrile (72:28). Flow-rate: 80 ml/h. Temperature, 20°; pressure, 3700 p.s.i. Peaks: 1 = oxytetracycline; 2 = tetracycline; 4 = demethylchlortetracycline; 5 = methacycline; 6 = doxycycline; 3 = unidentified.

CONCLUSION

The elucidation of the mechanism of the retention of tetracyclines in a reversed-phase chromatographic system involves several problems. Complex formation with metal ions should not be considered crucial, as eluents without any chelating agent (*i.e.*, glycine buffers) also yield acceptable resolution. However, satisfactory results with the latter solvents were obtained only on "old" columns that had previously been used with EDTA and citrate–phosphate buffers. Apparently, some "modification" of the packing material takes place. Re-filling the top of a column with fresh packing material resulted in a total loss of resolution. Therefore, pre-equilibration with a chelating agent is probably required.

The pH of the mobile phase was found to be most important. Under the experimental conditions, tetracyclines exist mainly in their cationic form. The ion-pair approach of Knox and co-workers[15,16] provides an acceptable explanation of why positively charged molecules are retained by hydrocarbon stationary phases. According to this theory, the nature of the counter ion should be the key to the chromatographic behaviour. In contrast to the results of Knox and co-workers[15,16], the retention times and resolution were considerably affected by pH variations, rather than by the nature of the anions (citrate, phosphate, tartrate or glycinate). It is not clear why the retention decreases when the acid concentration (or the pH) reaches a certain limit.

The method described permits the rapid and simple separation of five tetracyclines. This system seems to be sufficiently selective and provides a basis for the analysis of doxycycline in biological materials.

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NOTE ADDED IN PROOF

After submission of this paper, another article on the same subject appeared in this journal[21].

A reversed-phase liquid chromatographic system using a RP 18 column to separate oxytetracycline, tetracycline and chlortetracycline was described. No chelating agent was incorporated in the mobile phase consisting of 40% of acetonitrile in 0.01 *M* phosphate buffer of pH 2.4.

As we have discussed above, the latter is in contradiction with several former experiments[18,19], as well as with some of our own observations. This confirms once more the existing controversy about this problem.

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