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Analysis of tetracycline antibiotics and their common impurities by high-performance liquid chromatography using a polymeric column*

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Abstract: The chromatographic separation of tetracycline, oxytetracycline, 6-demethylchlortetracycline, methacycline and minocycline, and the common impurities of tetracycline, oxytetracycline and chlortetracycline has been optimised on a Hamilton PRP-1 column using citrate-phosphate buffer with propan-2-ol and tetrahydrofuran as organic modifiers. Column efficiency was approximately doubled by the inclusion of 1% v/v dichloromethane in the mobile phase.

Keywords: *Tetracyclines and impurities; polymeric column; high-performance liquid chromatography.*

Introduction

The tetracyclines are a group of broad spectrum antibiotics that have been in use for over 25 years in the treatment of both human and animal infections. About 10 derivatives are now in frequent clinical use, all of which are based on the molecular framework and functionality array 1, shown in Table 1, which dominates the physical properties, appearance and electronic and vibrational spectroscopic features of the group. This means that the more common analytical approaches are of little value for the identification of individual members, and their common impurities [1]. This report forms part of a combined high-performance liquid chromatographic (HPLC) and ^{1}H and ^{13}C nuclear magnetic spectroscopic investigation [2, 3] of the analysis, identification and stereochemical study of tetracycline antibiotics and their common impurities. An HPLC system was required with sufficient selectivity to monitor the purity of all the compounds subjected to the NMR study, and also to provide a semi-preparative procedure for the isolation of individual compounds.

The importance of using end-capped reversed-phase materials was stressed in 1975 [4] and recently [5] the addition of tetramethylammonium chloride as a silanol-blocking agent has been shown to further improve peak shape. In that study a polymeric column was also used to determine oxytetracycline (OTC), tetracycline (TC) and chortetra-

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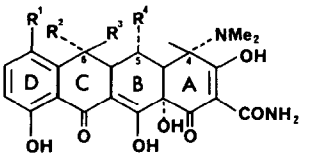
Table 1
Structural features of some tetracycline antibiotics



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Generic name	Code	R^1	R^2	R^3	R^4
Tetracycline	TC	H	Me	OH	H
Chlortetracycline	CTC	Cl	Me	OH	H
6-Demethylchlortetracycline	DMCTC	Cl	H	OH	H
Minocycline	MINO	NMe_2	H	H	H
Oxytetracycline	OTC	H	Me	OH	OH
Methacycline	METHA	H	C_6	$=CH_2$	OH
Doxycycline	DOXY	H	Me	H	OH
Lymecycline	LIME	Complex of TC with CH_2O and lysine			

cycline (CTC) in animal tissues using a gradient elution system. The separation of five tetracyclines that represent this group of antibiotics, namely OTC, TC, 6-demethylchlortetracycline (DMCTC), methacyline (METHA) and minocycline (MINO) on a commercially available polymeric column, PRP-1 has been optimised. Under isocratic conditions, the selectivity of propan-2-ol, acetonitrile and tetrahydrofuran has been studied in a 7-point mixture design [6] and the effects of buffer pH, and ion-pair reagents have also been examined. The organic modifier content was then reduced to improve the resolution required for the detection of common impurities in tetracycline, oxytetracycline and chlortetracycline, and column efficiency was improved by the addition of dichloromethane to the mobile phase. The mobile phases described are suitable for semi-preparative as well as analytical applications.

Experimental

Chemicals and solutions

All the solvents were HPLC grade, buffer components were analytical grade, both from Fisons (Loughborough, UK) and water was prepared by a Milli Q water purification system (Millipore, Harrow, UK). Teorell and Stenhagen's citrate–phosphate–borate buffers [7] in the pH range 2–12 were used. No further pH adjustment was made after the addition of organic solvent.

Apparatus

Two systems were used: (1) a modular HPLC system assembled from a Constametric III pump (Milton Roy, Stone, UK), a Rheodyne 7125 injection valve with 10 μl sample loop, a UV detector (Du Pont, Stevenage, UK) and BBC Servogor SE 120 recorder (Milton Roy, Stone, UK). The column, valve and solvent reservoir were immersed in a water bath maintained at 42°C by a thermostirrer (Gallenkamp, London, UK); (2) a Spectra Physics SP8100 liquid chromatograph with autosampler, oven and 10 μl sample loop, SP8440 UV–VIS detector and SP4200 computing integrator.

Chromatography

Tetracycline antibiotics and crude samples of their common impurities, all generally as their hydrochlorides, were dissolved in aqueous buffer, passed through a 0.2 μm Acro LC13 filter assembly (Anachem, Luton, UK) and chromatographed at either 0.8 ml or 1.0 ml min^{-1} and monitored at 272 nm, 0.08 a.u.f.s., with a chart speed of 5 mm min^{-1}. Aqueous buffer containing potassium bichromate was used to determine the retention time of an unretained compound.

Results and Discussion

In order to reduce the number of compounds examined in the solvent optimisation step, representative compounds were chosen. Tetracycline (TC) and 6-demethylchlortetracycline (DMCTC) represent compounds without an −OH group at C-5, and with different functionalities at C-6 and C-7; oxytetracycline (OTC) and methacycline (METHA) represent compounds possessing an −OH group at C-5, and also having different functionalities at C-6; minocycline (MINO) was chosen because its dimethylamino group at C-7, in addition to the ionizable functional groups common to all the tetracyclines (Table 1), would strongly influence its retention. These ionizable groups are the dimethylamino group at C-4 (pK_a = 9.69), and acidic −OH groups at C-3 (pK_a = 3.30) and C-12 (pK_a = 7.68). The common impurities are the 4-epi derivatives and anhydro derivatives. The 4-epi derivatives have no significant antibacterial activity, and can arise by reversible epimerisation in aqueous solutions, particularly between pH 3.0–4.5 via an enol form. Their chromatographic behaviour is very similar to that of their parent tetracycline. The anhydro derivatives may be found in out-of-date samples, of, for example, TC, and, although they may have some *in vitro* antibacterial activity, are not used clinically. Anhydro-TC may also be formed by dehydration at C-5a, 6 in aqueous solutions at pH values below 2.0, especially on heating. DMCTC is more stable than OTC or TC because its C-6 group has a secondary −OH group, whilst MINO cannot degrade in this way because it does not possess an −OH group in this position. OTC dehydrates in the same way as TC but the anhydro form is less stable and ring B opens to produce α and β apo-OTC.

A general purpose chromatographic system for the resolution of so many closely related compounds therefore needed to be as selective as possible, and so a wide range (pH 2.0–12.0) buffer (Teorell and Stenhagen, [7]) was chosen to permit the pH-stability (pH 1–13) of the polymeric column to be fully exploited, if required. Initially a buffer pH of 5.0 was chosen because at this pH tetracycline would predominantly be a stable, neutral, internal zwitter ion. The styrene–divinylbenzene polymeric material was selected because its inert, hydrophobic surface lacks any active adsorption sites, such as the residual uncapped acidic silanol groups present in silica-based reversed-phase materials.

Initially the column gave very broad peaks for all solutes when aqueous–methanol phases at room temperature were used, but changing to propan-2-ol and 42°C produced a considerable improvement. It has been suggested recently that restricted diffusion of some solutes in the polystyrene-based packing can occur, and that this mass transfer dispersion mechanism can be reduced by raising the temperature [8]. Buffer:propan-2-ol (78:22; v/v) gave capacity ratios (k′ values) in the range of 1–5 although peak resolution was poor; isoeluotropic equivalent compositions were calculated [9] for acetonitrile and tetrahydrofuran (THF) to be (80:20; v/v) and (85.8:14.2; v/v) respectively. Four more

mixtures were prepared with these compositions according to the selectivity triangle proposed by Glajch *et al.* [6]. Three were obtained by mixing equal volumes of any two of the original compositions, and the fourth was prepared by mixing the three original compositions in equal volumes. The seven mobile phases were then examined for their selectivity using a test mixture of the five tetracycline antibiotics. Figure 1a shows that good resolution and peak shapes were obtained with buffer:propan-2-ol:THF (82:11:7; v/v/v), although the k′ values for TC and OTC were too low for their separation. Combinations of binary mobile phases gave better results than any single system, whilst combinations containing acetonitrile increased the retention of minocycline excessively. There were no changes in retention order.

The selectivity of this mobile phase was then examined over the pH range 3.2–6.9, and the results in Fig. 2a show that selectivity is good between 5.0 and 6.0, with the shortest analysis time at pH 5.0. The retention order can be explained by a consideration of the relative hydrophobicities of the solutes at the various pH values. The additional −OH group at C-5 in OTC should permit its earlier elution than TC when the total percentage of organic modifier in the mobile phase is reduced. The additional hydrophobicity caused by the Cl atom (about 1 logP unit) in 6-demethyl CTC is partly offset by the loss of the C-6 $-CH_3$ (about 0.7 logP unit). The addition of the non-polar $=CH_2$ group and absence of the polar −OH group at C-6 increase the retention of METHA. For all these solutes, there is a tendency for retention to increase as pH falls, due to suppression of the acidic −OH group at C-3. The $-NMe_2$ group at C-4 remains fully protonated over the pH range studied and tends to increase solute solubility as it is released from its zwitter ion complex with the −OH group at C-3. MINO has an additional $-NMe_2$ at C-7 which greatly increases its solubility, lowering retention. This interpretation of retention was confirmed by repeating the pH study in the presence of 1-heptanesulphonic acid, sodium salt (Fig. 2b) which demonstrated that retention is increased as protonated $-NMe_2$ groups become available for ion-pair formation. The phosphate ($H_2PO_4^-$) and citrate

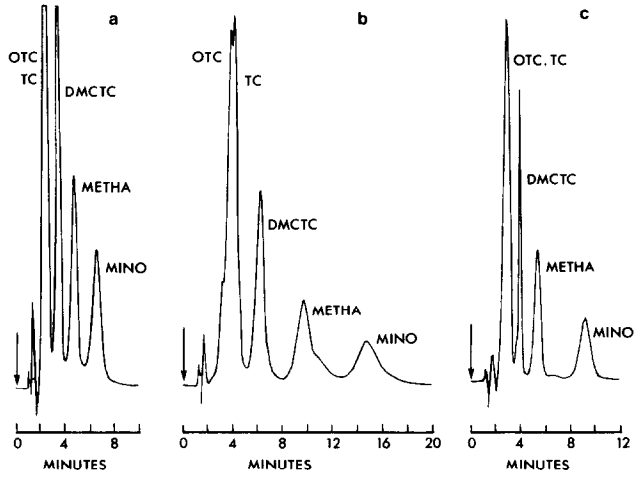

Figure 1
Comparison of chromatograms obtained for five tetracycline antibiotics using related mobile phases. (a) Mobile phase = citrate–phosphate buffer (pH = 5):propan-2-ol:THF (82:11:7, v/v/v); (b) mobile phase = as a but (87:8:5, v/v/v); (c) mobile phase = as b but containing 1% v/v/v/v dichloromethane, namely (86:8:5:1, v/v/v/v). Other chromatographic conditions as in Experimental.

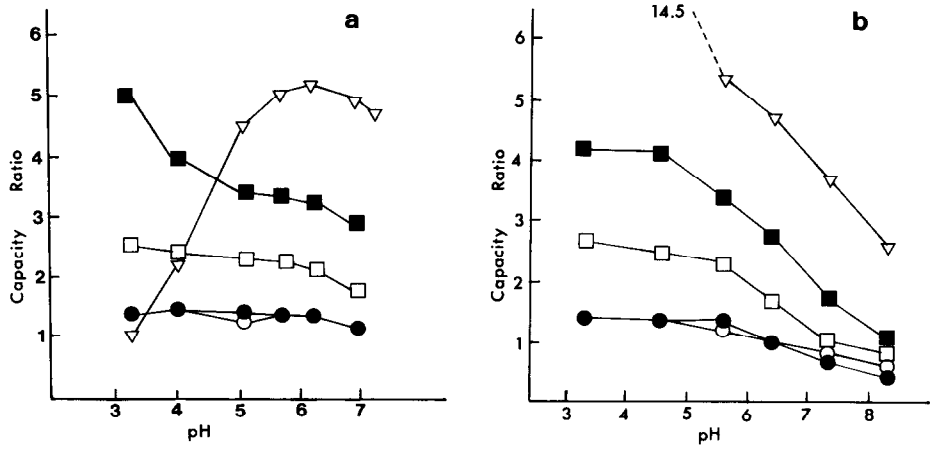

Figure 2
Influence of pH on the resolution of some tetracycline antibiotics, using citrate–phosphate–borate buffer:propan-2-ol:THF (82:11:7, v/v/v). (a) Other chromatographic conditions as in Experimental; (b) as in 2a but in the presence of 5 mM 1-heptansulphonic acid, Na salt. ○, oxytetracycline; ●, tetracycline; □, 6-demethylchlortetracycline; ■, methacycline; ▽, minocycline.

($HCit^{2-}$ and H_2Cit^-) anions of the buffer system must also form ion-pairs and influence retention, although their individual contributions were not studied. McIlvaine's citric acid (0.025 M):disodium phosphate (0.05 M) buffer gave identical separations at pH 5.0 and is recommended for routine use instead of the more aggressive citrate–phosphate–borate buffer initially used. The possibility of employing ion-pair formation was examined and its influence on solute retention found to be identical for the polymeric material as would be expected from a reversed-phase material. Since ion-pair reagents were eventually found not to be required for improving selectivity, these results are not considered further.

The solvent strength of the propan-2-ol:THF combination was optimised by varying the total volume of organic modifier in the mobile phase, and the results are summarised in Table 2. By increasing the aqueous buffer from 82% (mobile phase 1) to 90% (mobile phase 2) the k′ values increased by a factor of about 6, but produced broad peaks.

However, 87% aqueous buffer (mobile phase 3) was a good compromise between resolution and retention that permitted better separation of impurities as well as parent compounds, Fig. 1b. Chromatograms with all these mobile phases produce very symmetrical peaks, and any deviations strongly indicate the presence of other compounds. Recently, a similar study by Reeuwijk *et al.* [10] required quantitative determinations of a number of tetracyclines and some of their degradation products in plasma samples. PRP-1 was selected because the strong adsorption of tetracyclines onto silica-based materials resulted in non-linear calibration graphs. A mobile phase was developed comprising acetonitrile:dichloromethane:0.05 M acetate buffer (10:1:90; v/v/v) containing 0.025 M EDTA. The dichloromethane apparently caused the polymeric material to swell and resulted in improved column efficiency, whilst the EDTA prevented TC complexation with metal ions from the steel column, and acted as an ion-pair reagent. At the buffer pH chosen (3.3) EDTA is in the doubly charged anionic form and tetracycline antibiotics occur either as a zwitter ion or a cation. The elution order was the same as reported here with good selectivity although peak-tailing was evident.

Table 2
Capacity ratios of some tetracycline antibiotics and their common impurities

Solute	Mobile phase 1	2	3	4	5
4-ETC			1.22	0.18,* 0.65	
TC	0.58	3.8	1.59	0.22,* 1.06	
ATC					6.9,† 27.0
4-EOTC			1.30	0.21,* 0.70	
OTC	0.62	4.2	1.83	0.22,* 0.98	
α-APO-OTC			1.64	0.21,* 0.86	
β-APO-OTC				15.20	
ICTC			1.23	1.38	
4-ECTC			0.66	0.55,* 2.19	
CTC			5.10	2.86	
ACTC					10.5,† 47.0
DMCTC	1.28	8.6	3.40	2.20	
METHA	2.12	16.7	6.11	3.56	
MINO	3.31	>24.0	9.90	6.22	
DOXY			7.20	3.56	
6-EDOXY			6.13	2.86	
LIME			1.57	0.54, 1.11	

Mobile phase compositions: 1 = citrate–phosphate buffer (pH = 5):propan-2-ol:THF (82:11:7, v/v/v) at 1 ml min^{-1}; 2 = as 1 but (90:6:4, v/v/v), at 0.8 ml min^{-1}; 3 = as 1 but (87:8:5, v/v/v), at 0.8 ml min^{-1}; 4 = citrate–phosphate buffer (pH = 5):propan-2-ol:THF:dichloromethane (86:8:5:1, v/v/v/v), at 0.8 ml min^{-1}; 5 = as 4 for 0–4 min, then a linear gradient to (81:11:7:1, v/v/v/v), 4 to 6 min, and isocratic until end of run, at 0.8 ml min^{-1}.

* Impurity.

† Probably epi-anhydro-derivative.

The effect of adding dichloromethane (1% v/v) to mobile phase 3 (Table 2) was examined, and the results are summarised in Table 2 (under mobile phase 4). The stability of the column was monitored by using potassium bichromate to measure the retention of an unretained compound. The column had stabilised after 1 h at 0.8 ml min^{-1} with a 16% increase in back-pressure and a 14% reduction in V_0, the volume of mobile phase within the column. For most compounds, column efficiency was almost doubled and retentions were reduced by a factor of 0.55–0.65. Comparison of column efficiency for peaks of similar retention showed an increase by a factor of about 1.3–2.0. Peak symmetry was excellent with no tailing evident, and impurity "standard mixtures" were completely resolved into two or more major peaks compared to one peak obtained with the same mobile phase without dichloromethane, Table 2. Its effect on the test mixture is shown in Fig. 1c. This system is recommended for the analysis of all the compounds listed in Table 2, except the anhydro-derivatives which require more organic modifier to reduce retention, as obtained with mobile phase 5, Table 2. Minor adjustments to the total content of organic modifier should enable any tetracycline antibiotic in this Table to be resolved from its common impurities, and most tetracycline antibiotics to be resolved from one another. Gradient elution would permit good resolution of all components including anhydro derivatives, within a short time.

The PRP-1 column can also be used semi-preparatively. A sample of β-apo OTC, prepared in the laboratory, was required to be purified before NMR studies. The mobile phase was modified to 0.07 M phosphate buffer (pH 5.0):propan-2-ol:THF (82:11:7;

v/v/v) because traces of phosphate buffer would not be detected in a ^{13}C NMR spectrum. Good resolution of β-apo OTC was obtained from OTC and other impurities and fractions collected from 100 μl injections of a saturated solution of the sample (0.74 mg ml^{-1}) with the column at 50°C and a flow rate of 5 ml min^{-1}.

In conclusion, the inert styrene-divinylbenzene polymeric material, when used with combinations of propan-2-ol and THF, was found to provide excellent chromatography of the tetracycline antibiotics and their common impurities. The inclusion of 1% v/v dichloromethane in the mobile phase almost doubled column efficiency and is well worth considering whenever this column material is used.

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