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Determination of metacycline and related substances by column liquid chromatography on poly(styrene–divinylbenzene)

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ABSTRACT

Isocratic column liquid chromatography on poly(styrene–divinylbenzene) copolymer allowed complete separation of metacycline, 4-epimetacycline, oxytetracycline, doxycycline and 6-epidoxycycline. 2-Acetyl-2-decarboxamidometacycline was eluted on the tail of metacycline. The mobile phase was 2-methyl-2-propanol–0.2 *M* phosphate buffer (pH 9.0)–0.01 *M* sodium ethylenediaminetetraacetate (pH 9.0)–water (2.5:10:10:77.5, m/v/v/v). The flow-rate was 1.0 ml/min and detection was performed at 254 nm. Official standards were compared and a number of commercial bulk samples and specialties were analysed. 2-Acetyl-2-decarboxamidometacycline, 6-epidoxycycline and doxycycline were the main impurities, while 4-epimetacycline and oxytetracycline were minor impurities.

INTRODUCTION

Metacycline (MTC) (Fig. 1) is a broad-spectrum, semi-synthetic antibiotic prepared from oxytetracycline (OTC) [1]. Although column liquid chromatography (LC) of several tetracyclines and their degradation products has been discussed extensively, the separation of metacycline and potential impurities which may be formed during synthesis has not been reported. Perhaps this is because information about the related substances of MTC has not been available. Microbiological assay is still the official method for determining MTC in the United States Pharmacopeia XXII [2]. In a companion paper we report on the isolation of 2-acetyl-2-decarboxamidometacycline (ADMTC), 6-epidoxycycline (6-EDOX) and doxycycline (DOX) from commercial MTC [3]. Analogues of ADMTC, such as 2-acetyl-2-decarboxamidooxytetracycline (ADOTC) or 2-acetyl-2-decarboxamidotetracycline (ADTC), only possess about 10% of the activity of the corresponding tetracycline [4,5]. DOX is an active antibiotic while 6-EDOX has only little activity [6,7].

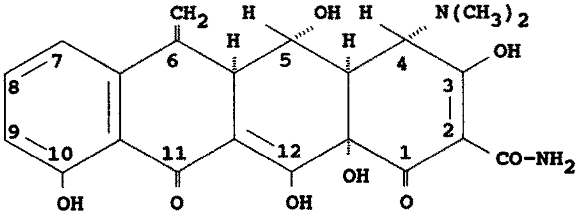

Fig. 1. Structure of metacycline.

In this paper, an isocratic method is described, using poly(styrene–divinylbenzene) (PSDVB) copolymer as the stationary phase. It enables the complete separation of 4-epimetacycline (EMTC), OTC, MTC, 6-EDOX and DOX. ADMTC is eluted on the tail of MTC. The method is based upon LC methods previously elaborated in this laboratory for the analysis of DOX [8–10], TC [11,12], OTC [13,14] and demeclocycline (DMCTC) [15]. The method has been used to compare official standards and to analyse a number of commercial samples.

EXPERIMENTAL

Reference substances and samples

The United States Pharmacopeia Reference Standard (U.S.P. - RS) Lot F (930 μg/mg) and Lot G, The European Pharmacopoeia Chemical Reference Substance (Ph. Eur. - CRS), which is not used for quantitative work, and the W.H.O. Second International Standard (W.H.O.-IS) were compared. House standards of MTC · HCl (95.3%, m/m) and DOX · HCl (93.1%, m/m) were available in the laboratory. 6-EDOX · HCl (96.5%, m/m) was obtained from the European Pharmacopoeia (Strasbourg, France). The content of DOX · HCl and 6-EDOX · HCl in the last two reference substances has been reported previously [8,10]. A reference sample of OTC (99.0%, m/m) was obtained from Janssen Chimica (Beerse, Belgium). Percentages are expressed as the hydrochloride salt. A small amount of EMTC · HCl was also prepared but its purity was not determined exactly since it is only a minor impurity of MTC. This sample was not used for quantitative work.

Bulk samples of MTC · HCl were obtained from different plants of one manufacturer. Capsules produced by the same manufacturer were obtained from the Belgium market.

Solvents and reagents

2-Methyl-2-propanol was from Janssen Chimica and was distilled before use. Other reagents were of *pro analysi* quality (Merck, Darmstadt, Germany). Water was freshly distilled twice from a glass apparatus.

LC equipment and operating conditions

The LC apparatus consisted of an L-6200 pump (Merck-Hitachi, Darmstadt, Germany), a Marathon autosampler equipped with a 20-μl loop (Spark Holland, Emmen, Netherlands), a Waters Model 440 detector set at 254 nm (Waters Assoc., Milford, MA, USA) and a Model 3393 A integrator (Hewlett-Packard, Avondale, PA, USA). The PSDVB copolymers PLRP-S, 8 μm, 100 Å (Polymer Labs, Church Stretton, UK) and RoGel, 7–9 μm (RSL - BioRad, Eke, Belgium) were packed in 250 mm × 4.6 mm I.D. columns following a previously described method [16]. The column was maintained at 60°C in an oven. The flow-rate was 1.0 ml/min. The back-pressure was between 45 and 70 bar, depending on the brand of packing material. For some experiments, a Waters 990 photodiode array detector was used. The mobile phases finally used contained 2% (m/v) and 2.5% (m/v) 2-methyl-2-propanol as the organic modifier for PLRP-S and RoGel, respectively. The required amounts were weighed and rinsed into a volumetric flask. All mobile phases contained 10% (v/v) 0.2 *M* potassium hydrogenphosphate buffer (pH 9.0) and 10% (v/v) 0.01 *M* sodium ethylenediaminetetraacetate (EDTA). During preparation of the latter solution, the pH was adjusted to 9.0 with sodium hydroxide solution. The volume was made up with water. The mobile phase was degassed by sonication.

Sample preparation and stability

About 25.0 mg of bulk samples were precisely weighed, dissolved in 0.01 *M* hydrochloric acid and diluted to 50.0 ml with the same solvent. For capsules, the sample was weighed to obtain the equivalent of about 25.0 mg of MTC · HCl and diluted to 50.0 ml with 0.01 *M* hydrochloric acid. The mixture was sonicated for 5 min at room temperature and then centrifuged at 2500 *g* for 5 min. The supernatant liquid was filtered through a membrane filter with 1.5-μm pores. The solutions were stable for at least 2 days at 6°C.

RESULTS AND DISCUSSION

Development of the chromatographic method

Experience obtained with previous LC analysis [8–16] on PSDVB stationary phases was used for the present studies. In preliminary experiments, it was observed that tetrabutylammonium (TBA) hydrogensulphate, used in the mobile phases for analysis of DOX, TC, OTC and DMCTC, had to be omitted in the mobile phase for analysis of MTC, otherwise poor separation of ADMTC and 6-EDOX was obtained. In the former methods, TBA was needed to obtain satisfactory separation of the corresponding 4-epimer or 6-epimer (for DOX).

As for the other tetracyclines, 2-methyl-2-propanol was chosen as the organic modifier. The influence of the mobile phase pH and the amount of organic modifier on the separation is shown in Fig. 2. At pH 7.0, ADMTC is eluted after 6-EDOX, while at higher pH this order is reversed. At pH 9.0, not only

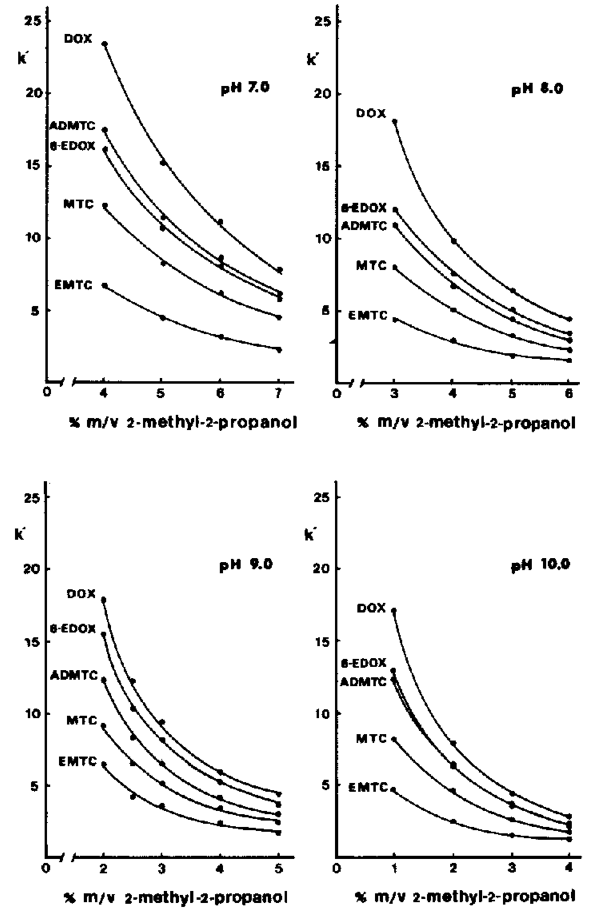

Fig. 2. Influence of the concentration of the organic modifier and the pH of the mobile phase on the separation of metacycline and related substances. Column: RoGel. Mobile phase: 2-methyl-2-propanol (x g)–0.2 M phosphate buffer of the pH indicated (10.0 ml)–0.01 M EDTA (10.0 ml), the pH of which was brought to the pH indicated with a solution of sodium hydroxide–water (up to 100.0 ml). See Experimental section for other conditions. EMTC = 4-epimetacycline; MTC = metacycline; ADMTC = 2-acetyl-2-decarboxamidometacycline; 6-EDOX = 6-epidoxycycline; DOX = doxycycline.

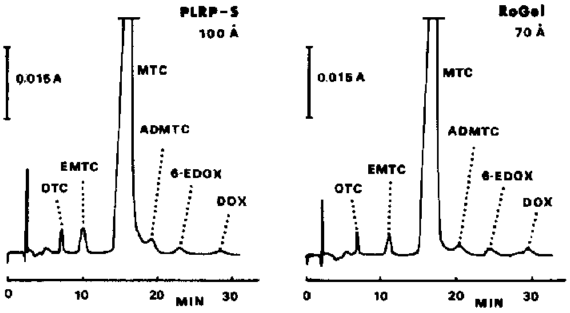

Fig. 3. Typical chromatogram of metacycline hydrochloride spiked with OTC and EMTC. Mobile phase: 2-methyl-2-propanol (x g per 100 ml)–0.2 M phosphate buffer pH 9.0 (10.0 ml)–0.01 M EDTA pH 9.0 (10.0 ml)–water (up to 100.0 ml). PLRP-S, 100 Å (8 μm): x = 2.0; RoGel, 70 Å (7–9 μm): x = 2.5. Amount injected: 10 μg. Flow-rate: 1.0 ml/min. Detection: UV at 254 nm. Temperature: 60°C. Content (%, m/m): OTC, 0.9; EMTC, 0.8; ADMTC, 0.4; 6-EDOX, 0.5; DOX, 0.2.

was the selectivity better than that at pH 8.0, but also the symmetry improved and the plate number increased. Detailed results for OTC were not added since preliminary tests revealed that OTC was always eluted well before EMTC. The influence of the phosphate buffer concentration was also investigated. Retention increased with increasing concentration. The separation of ADMTC and 6-EDOX decreased when more than 15% (v/v) 0.2 M phosphate buffer was used. In order to have sufficient buffer capacity, a content of 10% (v/v) was chosen. The presence of EDTA in the mobile phase is necessary, otherwise the separation of MTC and ADMTC rapidly deteriorates. A concentration of 10% (v/v) 0.01 M EDTA was used throughout.

A column temperature of 60°C was maintained throughout the study. This temperature was also found to be suitable for the analysis of other tetracyclines [8–16]. The stability of MTC during analysis was checked by repeated analysis of MTC · HCl house standard at 50 or 60°C. The areas of the MTC peak were not found to differ significantly. The good repeatability of the method (see below) was also an indication of MTC stability. Peak homogeneity was determined by on-line photodiode array detection.

Fig. 3 shows chromatograms obtained on PLRP-S, 100 Å and RoGel. The physical properties of the packing materials and chromatographic characteristics are indicated in Table I. All further analyses were performed on a RoGel column.

Calibration curves, repeatability and limits of quantitation

Calibration curves were constructed with the house standards MTC · HCl and DOX · HCl and with the reference substance 6-EDOX · HCl. The following relationships were found, where y = peak area, x = mass (μg), corrected for its content of

TABLE I

CHROMATOGRAPHIC CHARACTERISTICS OF THE STATIONARY PHASES

See Fig. 2 for chromatographic conditions.

	Column	
	PLRP-S, 8 μm, 100 Å	RoGel 7–9 μm, 70 Å
Amount (%, m/v) of 2-methyl-2-propanol in mobile phase	2.0	2.5
Plate number per per column (MTC)	2340	2950
Peak symmetry (MTC), $b_{0.05}/2a$[a]	1.09	1.03
Resolution		
OTC–EMTC	4.6	5.6
EMTC–MTC	5.4	5.1
MTC–ADMTC	2.5	2.8
ADMTC–6-EDOX	2.1	2.3
6-EDOX–DOX	2.0	1.9
Capacity factor (k′)		
OTC	2.20	2.21
EMTC	3.39	4.07
MTC	5.70	6.50
ADMTC	7.30	8.35
6-EDOX	8.89	10.27
DOX	11.00	12.34

[a] $b_{0.05}$ = Width of the peak at one-twentieth of the peak height. a = Distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height.

hydrochloride salt injected, r = correlation coefficient, $S_{y,x}$ = standard error of estimate, R = range of injected mass examined, n = number of analyses. MTC · HCl: $y = 96913 + 194907x$; $r = 0.9979$; $S_{y,x} = 21566$; R = 8–12 μg; n = 12. DOX · HCl: $y = 1767 + 162902x$; $r = 0.9997$; $S_{y,x} = 680$; R = 0.05–0.4 μg; n = 15. 6-EDOX · HCl: $y = -500 + 168447x$; $r = 0.9970$; $S_{y,x} = 2053$; R = 0.05–0.4 μg; n = 15. MTC · HCl: $y = 625 + 197631x$; $r = 0.9999$; $S_{y,x} = 356$; R = 0.05–0.4 μg; n = 15. The calibration curves were not used to calculate the content of the samples but only to check the linearity. The calculations for the content of the main component were based on the results obtained for the MTC · HCl house standard in each series of analyses. The limits of quantitation (LOQ) were 0.02% (m/m) for OTC and EMTC and 0.1% (m/m) for ADMTC, 6-EDOX and DOX. The house standard was analysed 54 times over a period of 7 days. The relative standard deviation (R.S.D.) for MTC was 1.0%.

Comparison of metacycline hydrochloride standards

The MTC · HCl house standard was titrated with perchloric acid in non-aqueous conditions. A total of five titrations gave a mean of 95.5% (m/m) MTC · HCl (R.S.D. = 0.5%). A total of three Karl Fischer titrations gave a mean of 0.9% (m/m) water (R.S.D. = 5.6%). The standard contained also 2.8% (m/m) acetone (n = 5, R.S.D. = 1.1%) and 0.7% (m/m) n-butanol (n = 4, R.S.D. = 8%), as determined by gas chromatography (GC). The total content of MTC · HCl house standard was therefore accepted to be 95.5% (m/m), and this figure was corrected by means of chromatography. The total concentration of chromatographic impurities corresponded to 0.2% (m/m). Therefore, the MTC · HCl house standard was assigned a purity of 95.3% (m/m).

Using the MTC · HCl house standard, the content of the official standards was compared by LC. Table II summarizes the results obtained. The MTC · HCl content was determined by comparison with the chromatograms for the MTC · HCl house standard, obtained on the same day. Reference solutions of MTC · HCl, 6-EDOX · HCl and DOX · HCl prepared at a concentration of 0.005 mg/ml, corresponding to 0.5% (m/m), were used to determine the content of ADMTC · HCl, 6-EDOX · HCl and DOX · HCl, respectively. Since an ADMTC · HCl reference sample was not available, it was expressed in terms of MTC · HCl. The R.S.D. values given in parentheses are within acceptable limits for all the determinations. The declared content for the U.S.P. Lot F of 930 μg/mg activity, determined by microbiological assay, is very close to the content of 92.0% (m/m), expressed as MTC base, and determined by the LC method.

Analysis of commercial samples

The commercial samples were analysed as described above for the standards. Table III shows results for the bulk samples of MTC · HCl. The repeatability of the assay is good. The content of OTC · HCl and EMTC · HCl is lower than the LOQ for all the samples. Since MTC carries a hydroxyl

TABLE II

COMPOSITION OF METACYCLINE HYDROCHLORIDE STANDARDS

Values (%, m/m) are expressed in terms of the hydrochloride salt; n = number of analyses; R.S.D. values (%) are given in parentheses; ND = not determined owing to the limited amount of sample.

Chromatography	House standard[a]	Ph. Eur.-CRS	U.S.P.-RS, Lot F, 930 μg/mg	U.S.P.-RS, Lot G	W.H.O.-IS
Number of solutions	54	4	4	3	2
Number of analyses	54	8	6	6	4
Number of days	7	2	2	2	1
OTC	<0.02	<0.02	<0.02	<0.02	<0.02
EMTC[b]	<0.02	<0.02	<0.02	<0.02	<0.02
ADMTC[b]	<0.1	0.5 (9)	0.4 (3)	<0.1	0.5 (3)
DOX	0.1 (28)	0.2 (18)	<0.1	<0.1	<0.1
6-EDOX	0.1 (26)	0.2 (9)	<0.1	<0.1	<0.1
MTC	95.3 (1.0)	97.7 (0.9)	99.3 (0.9)	99.9 (0.9)	99.2 (0.9)
Subtotal	95.5	98.6	99.7	99.9	99.7
Titration	95.5	ND	ND	ND	ND
n (R.S.D., %)	5 (0.5)				
Water determined	0.9	ND	ND	ND	ND
n (R.S.D., %)	3 (5.6)				
Water declared		0.9 Ref. 17	0.6 Ref. 18	0.2 Ref. 18	0.5 Ref. 19
Total	99.9	99.5	100.3	100.1	100.2

[a] This sample also contains 2.8% (m/m) acetone (n = 5, R.S.D. = 1%) and 0.7% (m/m) n-butanol (n = 4, R.S.D. = 8%).

[b] Expressed in terms of MTC · HCl.

group at C-5 it is, like OTC and DOX, more stable to epimerization in C-4. This is probably because of hydrogen bonding of the C-5 hydroxyl with the C-4 dimethylamino group [20]. The absence of a hydroxyl at C-6 excludes the possibility of acid degradation to the corresponding anhydro derivative [21]. The ADMTC · HCl or 6-EDOX · HCl contents are always lower than 1% (m/m); the DOX · HCl content is always lower than 0.5% (m/m). The water content for all the bulk samples is well within the U.S.P. limit of 2% (m/m) [2].

Table IV gives the results obtained for capsules.

TABLE III

COMPOSITION OF BULK SAMPLES OF METACYCLINE HYDROCHLORIDE

Values (%, m/m) are expressed in terms of the hydrochloride; four independent chromatographic analyses and four Karl Fischer titrations were performed for each sample within one day; R.S.D. values (%) are given in parentheses.

Sample	OTC	EMTC[a]	MTC	ADMTC[a]	6-EDOX	DOX	Water content	Total
31256701	<0.02	<0.02	98.2 (0.3)	0.8 (5)	0.3 (4)	0.1 (10)	0.6 (4)	100.0
088-0672	<0.02	<0.02	98.8 (0.9)	0.3 (8)	0.3 (6)	0.1 (5)	0.6 (7)	100.1
21256707	<0.02	<0.02	97.5 (0.5)	0.5 (4)	0.6 (3)	0.2 (21)	0.6 (5)	99.4
836-56007	<0.02	<0.02	97.8 (0.9)	0.3 (8)	0.4 (7)	0.2 (3)	0.6 (7)	99.3
21256705	<0.02	<0.02	99.2 (0.6)	0.4 (2)	0.5 (2)	0.2 (5)	0.6 (9)	100.9
21256708	<0.02	<0.02	97.1 (1.0)	0.6 (1)	0.4 (5)	0.3 (5)	0.6 (5)	99.0
31256702	<0.02	<0.02	98.4 (0.7)	0.5 (1)	0.2 (8)	0.2 (4)	0.5 (11)	99.8
51256004	<0.02	<0.02	98.4 (0.7)	0.4 (2)	0.4 (2)	0.2 (31)	0.2 (20)	99.6

[a] Expressed in terms of MTC · HCl.

TABLE IV

COMPOSITION OF CAPSULES

Values (%, m/m) are expressed in terms of the hydrochloride salt as claimed on the label; four independent analyses were performed for each sample within one day; R.S.D. values (%) are given in parentheses.

Sample	Sample age in months	OTC	EMTC[a]	MTC	ADMTC[a]	6-EDOX	DOX
A	60	<0.02	0.05 (12)	97.8 (1.2)	0.2 (2)	1.1 (1)	0.4 (11)
B	60	<0.02	0.05 (23)	99.7 (0.8)	0.2 (3)	1.1 (0.5)	0.4 (15)
C	60	<0.02	0.05 (30)	100.1 (1.4)	0.1 (5)	0.8 (1)	0.2 (19)

[a] Expressed in terms of MTC · HCl.

MTC · HCl in capsules is quite stable. Only trace amounts of EMTC · HCl (0.05%, m/m) were measured for all the capsules. The contents of DOX and 6-EDOX seem to be somewhat higher than those in bulk samples. However, these impurities, which are obtained by reduction of the methylene group, are formed during the semi-synthesis rather than upon storage.

CONCLUSION

The results show that the described LC method is suitable for the determination of MTC in bulk samples and in preparations. An important advantage of the method is its applicability to the different polymer stationary phases available on the market. This is not often obtained with silica-based reversed-phase materials, for which it is known that important differences in selectivity can exist between brands.

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