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Assay and purity control of metacycline by thin-layer chromatography combined with UV and fluorescence densitometry — a comparison with liquid chromatography

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Abstract: A thin-layer chromatographic (TLC) method involving UV and fluorescence densitometry is described for the assay and purity control of metacycline. With a mobile phase dichloromethane–methanol–water (58:35:7, v/v/v) and a silica gel thin-layer, previously sprayed with 10% sodium edetate solution adjusted to pH 9.0, all the potential impurities of metacycline were well separated from the main component and from each other. Results obtained with UV densitometry (TLC–UV) and fluorescence densitometry (TLC–F) were compared with those obtained by a liquid chromatography (LC) method using a poly(styrene-divinylbenzene) stationary phase. The correlation coefficients (r) for TLC–UV and LC or TLC–F and LC were better than 0.9999. For TLC–UV the relative standard deviation (RSD) for the assay of the main component was <2%, for TLC–F <3.0% and for LC <1.0%.

Keywords: *Metacycline; thin-layer chromatography (TLC); UV densitometry; fluorescence densitometry; liquid chromatography (LC); assay; purity control.*

Introduction

The chemical structure of the antibiotic metacycline (MTC) is shown in Fig. 1. Metacycline which is used commercially as the hydrochloride salt (MTC.HCl) is obtained by semisynthesis from oxytetracycline (OTC). According to results obtained by liquid chromatography (LC), OTC and 4-epimetacycline (EMTC) are minor impurities (<0.02%) in commercial MTC.HCl samples [1]. Typical values for the three major impurities present in MTC.HCl samples are 2-acetyl-2-decarboxamidometacycline (ADMTC, ≤1%), 6-epidoxycycline (6-EDOX, ≤1%) and doxycycline (DOX, ≤0.5%) [1, 2]. All these impurities can be separated from MTC and from each other by LC on poly(styrene-divinylbenzene) stationary phases [1]. Thin-layer chromatographic (TLC) methods for the separation of MTC from its impurities have not been reported previously.

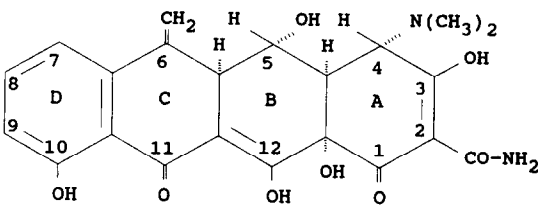

Figure 1
Structure of metacycline.

In this paper the development of a TLC method for the assay and purity control of MTC is reported. The TLC method was based on that previously developed for the identification of tetracyclines [3]. Similar TLC methods with UV densitometry (TLC–UV) have been developed for assay and purity control of OTC, DOX, chlortetracycline (CTC) and demeclocycline (DMCTC) [4, 5]. The described TLC method is fast, accurate and easy to perform. Results obtained by TLC are compared with those obtained by using LC [1].

Experimental

Chemicals

Methanol was obtained from Belgolabo (Overijse, Belgium) and redistilled in glass apparatus. Dichloromethane and 2-methyl-2-propanol were from Janssen Chimica (Beerse, Belgium). Other reagents were of analytical

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reagent quality (E. Merck, Darmstadt, Germany). Water was freshly distilled in glass apparatus.

Reference substances and samples

The reference substance of OTC (99.0%) was obtained from Janssen Chimica. Reference substances of DOX.HCl (92.8%) and MTC.HCl (95.3%) were house standards, obtained from commercial samples. 6-EDOX.HCl (96.2%) was obtained from the European Pharmacopoeia Commission (Strasbourg, France). The percentage content is expressed as the hydrochloride salt. EMTC was prepared from MTC by storing a solution of MTC at pH 3; the EMTC formed was isolated from the mixture by an open column chromatographic method [2]. A small amount of ADMTC was isolated from a commercial sample as described previously [2]. Commercial samples of metacycline hydrochloride (MTC.HCl-S1, MTC.HCl-S2, MTC.HCl-S3 and MTC.HCl-S4) were obtained from Pfizer, France.

Thin-layer chromatography

Laboratory-made silica gel layers on glass (20 × 20 cm) were prepared with Kieselgel 60 H (E. Merck, No. 11695) according to a previously described procedure [3]. Precoated silica gel layers on glass (20 × 20 cm) were obtained from Merck (No. 5721), Whatman (Maidstone, UK, No. 4861-820), Carlo Erba (Milan, Italy, No. 485321), Baker (Phillipsburg, USA, No. 1301121), Woelm (Eschwege, Germany, No. 04613), Riedel-de-Haën (Seelze, Germany, No. 91940) and Macherey–Nagel (Düren, Germany, No. 809013). Before use, the silica gel plates were sprayed with a 10% m/v solution of sodium edetate (EDTA), the pH of which had been adjusted to 9.0 with 40% m/v solution of sodium hydroxide. The plates were dried in a horizontal position for at least 1 h at room temperature and then in an oven at 110°C for 1 h, shortly before use.

For UV densitometry (TLC–UV), aliquots of 2 μl of the sample solutions in methanol, containing 1.25 mg ml^{-1} (for assay) or 5 mg ml^{-1} (for purity control) of sample to be examined and 1.25 mg ml^{-1} (for assay) or 0.5 mg ml^{-1} (for purity control) of OTC as internal standard (IS) were applied to the plate with a microsyringe (Hamilton, Bonaduz, Switzerland). For OTC as IS the use of a purified sample is preferred. Aliquots of 2 μl of the reference solutions in methanol, containing 1.25 mg ml^{-1} of standard substance and 1.25 mg ml^{-1} of IS (for assay) or 0.1 mg ml^{-1} of related substance and 0.5 mg ml^{-1} of IS (for purity control) were also applied to the same plate. At about 5°C the solutions were stable for at least 2 d.

For fluorescence densitometry (TLC–F), aliquots of 2 μl of the sample solutions in methanol, containing 0.1 mg ml^{-1} (for assay) or 0.2 mg ml^{-1} (for purity control) of sample to be examined and 0.1 mg ml^{-1} (for assay) or 0.01 mg ml^{-1} (for purity control) of OTC were applied to the plate. Aliquots of 2 μl of the reference solutions in methanol, containing 0.1 mg ml^{-1} of standard substance and 0.1 mg ml^{-1} of IS (for assay) or 0.004 mg ml^{-1} of related substance and 0.01 mg ml^{-1} of IS (for purity control) were also applied to the same plate.

The chromatographic chamber was lined with paper and equilibrated with the mobile phase dichloromethane–methanol–water (58:35:7, v/v/v) for at least 1 h prior to use. The plate was developed at room temperature over a distance of 15 cm. The developed plate was flushed with a stream of nitrogen to remove the solvents and the spots were measured with a CS-930 TLC scanner (Shimadzu, Kyoto, Japan) using the following parameters for TLC–UV: zigzag swing width = 10 mm; scan step in the *y*-direction = 0.1 mm; beam size = 1.2 × 1.2 mm; absorption–reflection mode with λ = 280 nm; linearizer SX = 3; background correction = on; drift-line integration = 0.5. The following parameters were used for TLC–F: linear scanning; scan step in the *y*-direction = 0.1 mm; beam size = 1.2 × 6 mm; fluorescence mode with λ = 400 nm; filter No. = 3; linearizer = off; background correction = on; drift line integration = 0.5.

Liquid chromatography

The LC system consisted of a L-6200 pump (Merck–Hitachi, Tokyo, Japan), a Marathon autosampler equipped with a 20 μl loop (Spark Holland, Emmen, The Netherlands), a Waters model 440 detector set at 254 nm (Waters Associates, Milford, MA, USA), an integrator model 3393 A (Hewlett–Packard, Avondale, PA, USA) and a 25 × 0.46 cm i.d. column packed with poly(styrene-divinylbenzene) (PSDVB) (RoGel, 7–9 μm, RSL-BioRad, Eke, Belgium) maintained at 60°C in an oven. The flow rate was 1.0 ml min^{-1}.

The mobile phase was 2-methyl-2-propanol–potassium phosphate buffer (pH 9.0; 0.2 M)–EDTA solution (pH 9.0; 10 mM)–water (2.5:10:10:77.5, m/v/v/v). During preparation of the EDTA solution, the pH was adjusted to 9.0 with sodium hydroxide solution. The mobile phase was degassed by sonication. Solutions for injection were prepared in 0.01 M hydrochloric acid. Solutions to be examined and reference solutions for assay were prepared at a concentration of 0.5 mg ml^{-1}.

Reference solutions of related substances were prepared at a concentration of 0.005 mg ml^{-1}. At about 5°C the solutions were stable for at least 2 d.

Results and Discussion

Development of the TLC method

It is known that for the identification of tetracyclines on silica gel, EDTA should be incorporated in the stationary phase to avoid the formation of tetracycline–metal complexes [3]. This can be achieved by spraying EDTA solutions on the layer. This technique is fast and applicable to all layers. The variation of the pH of the edetate permits fine-tuning of the separations. The concentration of the edetate solution sprayed is less critical. The results obtained at different pH values, using Macherey–Nagel stationary phases and a mobile phase dichloromethane–methanol–water (59:35:6, v/v/v) are shown in Table 1. The reported values are the mean of several experiments. At pH 9.0 all the impurities of MTC are well separated from the main component and from each other. In order to obtain good repeatability, an internal standard (IS) had to be used. OTC was found to be suitable as IS for the mobile phase above. With a mobile phase dichloromethane–methanol–water (58:37:7, v/v/v), the separation was even better and therefore this mobile phase was used for further work. Small amounts of 2-acetyl-2-decarboxamidooxytetracycline (ADOTC) present in OTC, did not interfere with the determination of MTC but might affect the accurate determination of 6-EDOX in MTC samples. Therefore two levels of concentration of internal standard were used, one for assay equal to the concentration of sample and the other for purity control, equal to 10% of the sample concentration. At this low concentration, ADOTC in OTC no longer interfered with the quantitation of 6-EDOX. UV spectra of the spots showed maximal absorbance at about 280 nm for all tetracycline derivatives and therefore this wavelength was chosen for the quantitation by TLC–UV. An excitation wavelength of 400 nm was suitable for TLC–F since the highest signal-to-noise ratio was obtained at 400 nm and potential interference arising from extraneous matter was excluded at this wavelength [6].

Macherey–Nagel plates were used for further validation of the method. The stability of the peak areas measured in TLC–UV or TLC–F was examined by measuring seven times the chromatograms of a sample solution obtained on three plates. The results are shown in Table 2. The low relative standard deviations (RSD) for the peak areas are an indication of the good stability of the signals. The even lower RSD for ratios of MTC/OTC peak areas indicate that part of the variation was compensated by using an internal standard possessing similar chemical properties. The overall mean figures reflect the good repeatability of the method. The results for the calibration curves are summarized in Table 3. Good proportionality was obtained in the ranges examined. In comparison with TLC–UV a much broader linear range was obtained for TLC–F. ADMTC was quantified with reference to a standard reading of MTC and

Table 1
Influence of the pH of the stationary phase on the Rf values

	Rf × 100					
Substance	pH 5.0	pH 6.0	pH 7.0	pH 8.0	pH 9.0	pH 10.0
EMTC	22	21	17	13	7	4
OTC	20	21	17	15	13	10
MTC	28	26	24	22	18	19
6-EDOX	27	26	27	27	25	24
DOX	32	32	32	33	30	27
ADMTC	41	41	39	39	33	29

Mobile phase: dichloromethane–methanol–water (59:35:6, v/v/v). Stationary phase: Macherey–Nagel, sprayed with 10% m/v EDTA solutions at different pH. See Experimental for other conditions. The values reported are the means of several experiments.

Table 2
The stability of the peak areas after development. Peak areas and ratios of peak areas are recorded

	Plate 1			Plate 2			Plate 3			Overall mean
	MTC	OTC	MTC/OTC	MTC	OTC	MTC/OTC	MTC	OTC	MTC/OTC	
UV densitometry: (Time range covered: 190 min) $n = 7$										
Mean	1595	1251	1.275	1581	1217	1.299	1590	1248	1.274	1.283
RSD (%)	0.68	1.00	0.46	0.48	0.89	0.56	0.38	0.16	0.38	1.10
Fluorescence densitometry: (Time range covered: 100 min) $n = 7$										
Mean	1115	1098	1.015	1104	1056	1.045	1259	1224	1.028	1.029
RSD (%)	1.16	1.37	0.76	0.36	0.53	0.19	0.48	0.52	0.14	1.46

Mobile phase: dichloromethane–methanol–water (58:35:7, v/v/v). Stationary phase: Macherey–Nagel, sprayed with 10% m/v EDTA solution of pH 9.0. Solution applied: 2 μl of 1.25 mg ml^{-1} MTC and 1.25 mg ml^{-1} OTC in methanol (TLC–UV) or 0.1 mg ml^{-1} MTC and 0.1 mg ml^{-1} OTC in methanol (TLC–F). RSD = Relative Standard Deviation.

Table 3
Calibration curves for metacycline hydrochloride and its related substances obtained with the TLC method

	Intercept	Slope (area μg^{-1})	r	$S_{y,x}$	R (μg)	n
UV densitometry:						
MTC	1850	49334	0.9998	694	2–3	12
MTC	219	57414	0.9997	277	0.02–0.38	15
6-EDOX	81	49205	0.9999	159	0.02–0.38	15
DOX	348	50267	0.9991	425	0.02–0.38	15
Fluorescence densitometry:						
MTC	20	85690	0.9999	155	0.001–0.32	24
6-EDOX	3	70678	0.9999	16	0.001–0.028	15
DOX	−1	71820	0.9991	43	0.001–0.026	15

R: range examined, expressed as the mass (μg) of substance examined loaded onto the plate. n = number of determinations performed, $S_{y,x}$ = standard error of estimate. Mobile phase: dichloromethane–methanol–water (58:35:7, v/v/v). Stationary phase: Macherey–Nagel, sprayed with EDTA solution of pH 9.0

Table 4
Influence of the origin of the stationary phase on the Rf values

	Rf × 100					
Stationary phase	EMTC	MTC	6-EDOX	DOX	ADMTC	OTC (IS)
MN	7	20	26	32	35	12
M	11	17	21	26	27	13
W	8	16	20	25	28	10
B	19	29	32	36	39	23
CE	10	19	22	28	30	12
WM	12	23	25	30	32	18
RH	12	24	28	32	35	15
LM	13	26	30	35	38	19

Mobile phase: dichloromethane–methanol–water (58:35:7, v/v/v). Stationary phase: MN = Macherey–Nagel, M = Merck, W = Woelm, B = Baker, CE = Carlo Erba, WM = Whatman, RH = Riedel-de-Haën, LM = Laboratory-made; all sprayed with EDTA solution of pH 9.0. IS = internal standard. The values reported are the means of several experiments.

expressed as MTC. The limit of quantitation of TLC–UV and TLC–F was 20 and 0.8 ng for each compound, respectively, both corresponding to 0.2% of the sample load.

The TLC separation, developed on Macherey–Nagel plates as described above was also examined by using thin-layer plates from other manufacturers. The results shown in Table 4 demonstrate that the TLC method is equally well applicable to silica gel layers from different manufacturers. The time needed for development over 15 cm varied between 40 and 80 min, depending upon the origin of the plate.

Comparison of TLC–UV, TLC–F and LC

Typical chromatograms, obtained by TLC and LC, are shown in Figs 2 and 3, respectively. The TLC chromatograms in Fig. 2, I and II, were obtained for assay or for purity control of MTC, respectively. The advantages of TLC–F over TLC–UV are its greater sensitivity, its faster scanning speed and its broader linear range. The need for an IS is a major disadvantage of quantitative TLC. The LC method shown in Fig. 3 allows the simultaneous determination of main component and impurities.

Table 5 shows all the quantitative results for MTC samples, obtained by TLC–UV, TLC–F and LC. The RSD values for MTC were <2% for TLC–UV, <3% for TLC–F and <1% for LC. The RSD values for the main component were analysed by an F-test ($P = 0.05$) [7]. It was found that only for these results obtained by TLC–F and by LC for samples MTC.HCl-S2 and MTC.HCl-S3 did the F-test show that there was a significant difference in precision. It should be emphasized that the amount of sample used for each assay was different, viz. for TLC–UV, 2.5 μg; for TLC–F, 0.2 μg and for LC, 10 μg. The poorer repeatability of the TLC–F method can be explained by the smaller sample loading. In no case did the Student's t-test show that there was a significant difference between the mean

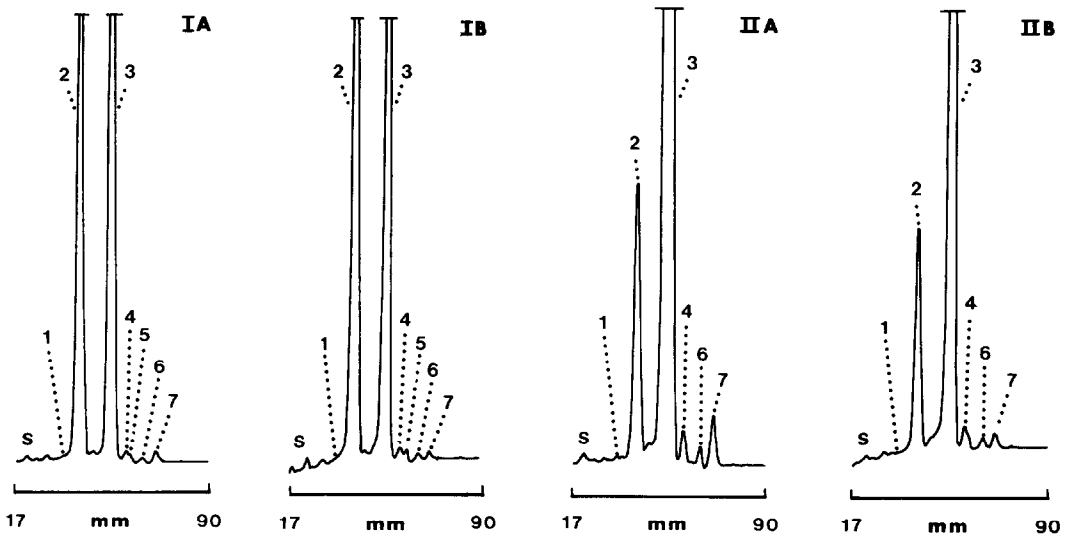

Figure 2
Typical chromatograms obtained by the proposed TLC method for assay (I) or for purity control (II) of metacycline hydrochloride by UV densitometry (A) or fluorescence densitometry (B). Stationary phase: silica gel (Macherey–Nagel), previously sprayed with 10% m/v edetate solution at pH 9.0. Mobile phase: dichloromethane–methanol–water (58:35:7, v/v/v). See the Experimental section for other conditions. Sample: MTC.HCl-S2. Peak identity: 1 = EMTC, 2 = OTC (internal standard, IS), 3 = MTC, 4 = 6-EDOX, 5 = ADOTC, 6 = DOX, 7 = ADMTC, S = origin.

Table 5
Comparison of assay or purity control of metacycline hydrochloride by TLC with UV densitometry or fluorescence densitometry or by LC

		Per cent m/m expressed as the hydrochloride salt			
Sample	Method	MTC	ADMTC	6-EDOX	DOX
MTC.HCl-S1	TLC–UV	99.1 (1.1)	0.6 (6)	0.4 (13)	<0.2
	TLC–F	100.1 (2.7)	0.3 (4)	0.3 (7)	<0.2
	LC	99.9 (0.8)	0.3 (2)	0.3 (5)	0.1 (17)
MTC.HCl-S2	TLC–UV	99.3 (0.9)	0.9 (5)	0.7 (4)	0.4 (6)
	TLC–F	100.2 (2.8)	0.8 (4)	0.8 (10)	0.3 (7)
	LC	99.5 (0.4)	0.5 (8)	0.7 (4)	0.3 (11)
MTC.HCl-S3	TLC–UV	97.6 (1.9)	1.2 (2)	0.5 (7)	0.2 (11)
	TLC–F	98.6 (2.6)	0.6 (5)	0.5 (5)	<0.2
	LC	97.8 (0.4)	0.7 (1)	0.3 (8)	0.1 (5)
MTC.HCl-S4	TLC–UV	98.4 (1.1)	0.8 (5)	<0.2	<0.2
	TLC–F	97.2 (0.6)	0.5 (5)	<0.2	<0.2
	LC	97.8 (0.8)	0.6 (3)	<0.1	<0.1

The values reported are the means of four experiments and were calculated on an "as is" basis, i.e. uncorrected for residual solvents; ADMTC is expressed as MTC; RSD values are given in parenthesis. Levels of EMTC and OTC are always below the detection limit (TLC <0.2, LC <0.02).

assay values of MTC obtained by TLC–UV, TLC–F or LC. The results in Table 5 show that good agreement also existed between the means for the related substances, except for ADMTC, for which TLC–UV gave higher values. This was due to the fact that, for TLC–UV, 280 nm was used as the wavelength of detection whereas for LC it was 254 nm. In both cases ADMTC was quantified with reference to a standard loading of MTC. The ratio of absorbance ADMTC/MTC is about 1.5 times higher at 280 nm than at 254 nm. For small amounts of related substance, the precision of the TLC methods is poorer than that obtained by LC as indicated by the higher RSD values. The correlation coefficients r, calculated from the results obtained in TLC–UV, TLC–F and LC for the main component and for the related substances are all greater than 0.9999.

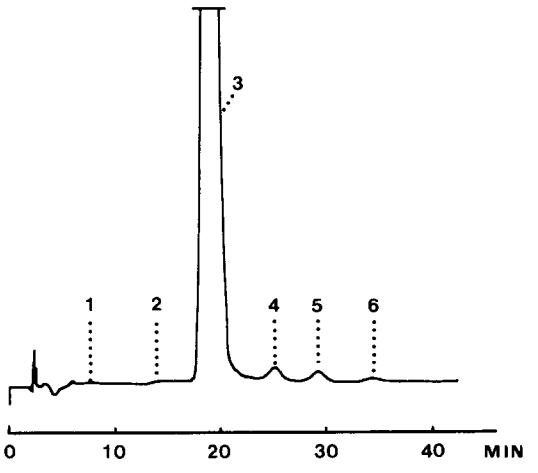

Figure 3
Typical chromatogram of metacycline hydrochloride obtained by LC. See the Experimental section for chromatographic conditions. Sample: MTC.HCl-S2. Peak identity: 1 = OTC, 2 = EMTC, 3 = MTC, 4 = ADMTC, 5 = DOX, 6 = 6-EDOX.

It can be concluded that TLC, in combination with either UV densitometry or fluorescence densitometry, is a valuable alternative method to LC for the assay and purity control of MTC.

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