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Assay and purity control of minocycline by thin-layer chromatography using UV and fluorescence densitometry — A comparison with liquid chromatography

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Abstract

A thin-layer chromatography (TLC) method using UV and fluoresecence densitometry is described for the assay and purity control of minocycline (MC). With a mobile phase dichloromethane–methanol–water (57:35:8, v/v/v) and a silica gel thin-layer, previously sprayed with 10% m/v sodium edetate adjusted to pH 9.0, 4-epiminocycline and 7-didemethylminocycline were well separated from MC and from each other, 7-monodemethylminocycline and 6-deoxy-6-demethyltetracycline (6-DODMTC) were not separated from each other and were only partially separated from minocycline. 6-DODMTC was selectively determined by fluorescence densitometry, while quantification of other impurities and the assay of MC were performed by UV densitometry. Results obtained with qualitative TLC were compared with those obtained by a liquid chromatography (LC) method using a poly(styrene–divinylbenzene) copolymer stationary phase. The correlation coefficient for TLC and LC results was >0.999. For TLC the relative standard deviation for the assay of MC at 1.25 mg ml^{-1} was <3.0% ($n = 4$), while for LC it was <1.0% ($n = 4$).

Keywords: Assay; LC; Minocycline; Purity control; Quantitative TLC

1. Introduction

The chemical structure of the antibiotic minocycline (MC) is shown in Fig. 1. Commercially, it is used as the hydrochloride salt (MC.HCl). MC is obtained by semisynthesis from demeclocycline [1]. 6-Deoxy-6-demethyltetracycline (6-DODMTC), 7-didemethylminocycline (7-DDM-MC) and 7-monodemethylminocycline (7-MDMMC) are described as intermediate compounds. 9-Minocycline or 9-dimethylamino-6-deoxy-6-demethyltetracycline (9-MC), is a side product while 4-epiminocycline (4-EMC) is a major degradation product. According to results obtained by liquid chromatography (LC), 9-MC is a minor impurity (<0.1% m/m) in commercial MC.HCl samples [2]. All these impurities can be separated from MC and from each other by LC on poly-(styrene-divinylbenzene) copolymer stationary phases [2]. A thin-layer chromatography (TLC) method for the separation of MC from its impurities has not appeared in literature.

Here the development of a TLC method is described for the assay and purity control of MC.HCl. The TLC method was derived from that previously developed for the identification of tetracyclines [3]. Similar TLC methods with

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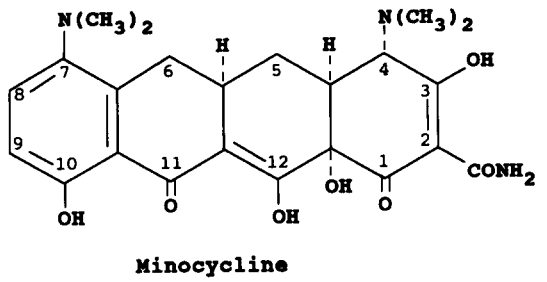

Fig. 1. Chemical structure of minocycline.

UV densitometry have been developed for the assay and purity control of oxytetracycline, doxycycline [4], chlortetracycline, demeclocycline [5], tetracycline [6,7] and metacycline [8]. Fluorescence densitometry was also used for the assay and purity control of metacycline [8]. The TLC method described here is fast, accurate and easy to perform. 6-DODMTC was selectively determined by fluorescence densitometry. Other compounds did not show fluorescence and were quantified by UV densitometry. Results obtained by TLC were compared with those obtained by using LC.

2. Experimental

2.1. 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 pro analysi quality (E. Merck, Darmstadt, Germany). Water was distilled in glass apparatus.

2.2. Reference substances and samples

The reference substance for MC.HCl (91.0% m/m) was available in the laboratory. For use as an internal standard, the reference substance for oxytetracycline (OTC) (91.7% m/m) was available from Janssen Chimica. The reference substances for 6-DODMTC.HCl (99.3% m/m), 7-DDMMC. HCl (content not specified) and 9-MC (92.1% m/m) were kindly donated by Cyanamid Lederle (Brussels, Belgium). 4-EMC was prepared from MC: a solution of MC was stored at pH 3, and the 4-EMC formed was isolated from the mixture by a published open column chromatographic method [9]. Small quantities of 7-MDMMC were obtained by methylation of 7-DDMMC.HCl and isolated by the TLC method described. Commercial samples of MC.HCl were obtained from different plants of manufacturer A and from manufacturers B and C.

2.3. TLC

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. 7000–04), 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 was adjusted to 9.0 with a 10% (m/v) solution of sodium hydroxide; about 20 ml was used for a 20 cm × 20 cm plate. 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.

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 0.125 mg ml^{-1} (for purity control) of internal standard (IS), i.e. OTC, were applied to the plate with a microsyringe (Hamilton, Bonaduz, Switzerland). The following 2-μl aliquots of reference solutions in methanol, containing 1.25 mg ml^{-1} of standard substance and 1.25 mg ml^{-1} of IS (for assay), or 0.05 mg ml^{-1} of MC.HCl (for determination of non-fluorescent impurities), or of 6-DODMTC.HCl (for determination of fluorescent impurities), together with 0.125 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 1 day.

The chromatographic chamber was lined with paper and equilibrated with the mobile phase, dichloromethane–methanol–water (57:35:8, 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 nitrogen to remove the solvents and the spots were measured with a CS-930

TLC scanner (Shimadzu, Kyoto, Japan) using the following parameters: zigzag swing width = 10 mm; scan step in the y direction = 0.1 mm; beam size = 1.2 mm × 1.2 mm; absorption–reflection mode with $\lambda = 280$ nm; linearizer SX = 3; background correction = on; drift-line integration = 0.5. The lanes used for the purity control were then scanned by fluorescence densitometry using the following parameters: linear scanning; scan step in the y direction = 0.1 mm; beam size = 1.2 mm × 6 mm; fluorescence mode with excitation $\lambda = 400$ nm; filter = 3; linearizer = off; background correction = on; drift-line integration = 0.5.

2.4. LC

The LC system consisted of an L-6200 pump (Merck–Hitachi, Tokyo, Japan), a Marathon autosampler equipped with a 20-μl loop (Spark Holland, Emmen, The Netherlands), a Merck–Hitachi L-4000 UV detector set at 254 nm, an integrator model 3393 A (Hewlett–Packard, Avondale, PA, USA) and a 25 cm × 0.46 cm i.d. column packed with poly(styrene–divinylbenzene) copolymer (PSDVB) PLRP-S 100 Å, 8 μm (Polymer Labs, Church Stretton, Shropshire, UK) maintained at 60 °C in an oven. The flow rate was 1.0 ml min^{-1}.

The mobile phase was 2-methyl-2-propanol–phosphate buffer (pH 10.5; 0.2 M)–tetrabutylammonium (TBA) sulphate (pH 10.5; 0.02 M)–EDTA (pH 10.5; 0.01 M)–water (7:10:10:10:63, m/v/v/v/v). During preparation of TBA and EDTA solutions the pH was adjusted to 10.5 with sodium hydroxide solution. The mobile phase was degassed by sonication. Solutions for injection were prepared in 0.01 N sodium hydroxide containing 0.1% m/v Na_2SO_3. Solutions to be examined and reference solutions for assay were prepared at a concentration of 1.0 mg ml^{-1}. Reference solutions of related substances were prepared at a concentration of 0.01 mg ml^{-1}. At about 5 °C the solutions were stable for at least 1 day.

3. Results and discussion

3.1. Development of the TLC method

In order to obtain good separation of tetracyclines on silica gel, it was necessary to incorporate EDTA into the stationary phase to avoid the formation of tetracycline–metal complexes [3]. The adjustment of the EDTA pH permits fine tuning of the separations. The concentration of the EDTA solution is less critical. The results obtained at different pH values, using Macherey–Nagel stationary phases and the mobile phase dichloromethane–methanol–water (57:35:8, v/v/v) are shown in Table 1. The reported values are the means of two experiments, which showed differences in hRf of 2 units at most. At pH 9.0, 4-EMC and 7-DDMMC are well separated from MC and from each other. 7-MDMMC and 6-DODMTC are not separated and are only partially separated from MC. 6-DODMTC was selectively determined by fluorescence densitometry as discussed below. 9-MC was not separated from MC. However, according to the results obtained by LC, 9-MC is only a minor impurity in commercial MC.HCl samples; the amount found was always less than 0.1% m/m [2].

Table 1
Influence of the pH of the EDTA spray for the stationary phase on separation obtained by TLC

Substances	hRf					
	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10
4-EMC	42	38	34	33	30	24
7-DDMMC	30	33	31	30	32	29
7-MDMMC	50	49	44	42	39	32
6-DODMTC	52	52	48	44	41	35
9-MC	50	53	50	46	44	38
MC	55	56	52	48	45	37

Mobile phase: dichloromethane–methanol–water (57:35:8, v/v/v). Stationary phase: silica gel (Macherey–Nagel), sprayed with 10% m/v of EDTA solutions at different pH values. See Section 2 for other conditions. The values reported are the means of two experiments.

In order to obtain good repeatability, an IS had to be used. OTC was found to be a suitable IS. Small amounts of 2-acetyl-2-decarboxamidooxytetracycline (ADOTC) present in OTC did not interfere with the determination of MC, but may have affected the accurate quantification of 7-DDMMC in MC.HCl samples. Therefore two levels of concentration of IS were used, one 1.25 mg ml^{-1} for assay and the other 0.125 mg ml^{-1} for purity control. At this low concentration, ADOTC no longer interfered with the quantification of 7-DDMMC.

UV spectra measured on the spots showed maximal absorbance at about 280 nm for all tetracycline derivatives of interest, and there-

Table 2
Influence of the origin of the stationary phase on separation obtained by TLC

Stationary phase	*h*R*f*						
	IS	4-EMC	7-DDMMC	7-MDMMC	6-DODMTC	9-MC	MC
MN	18	30	32	39	41	44	45
M	19	28	33	40	41	42	45
W	16	27	29	37	38	40	42
B	26	32	35	39	40	40	42
CE	15	26	32	38	39	40	42
WM	28	34	38	42	42	45	46
RH	22	33	38	43	44	47	49
LM	22	28	33	39	41	42	46

Mobile phase: dichloromethane–methanol–water (57:35:8, v/v/v). Stationary phase: silica gel; MN, Macherey–Nagel; M, Merck; W, Woelm; B, Baker, CE, Carlo Erba; WM, Whatman; RH, Riedel-de-Haën; LM, laboratory-made. All were sprayed with EDTA solution at pH 9.0. See Section 2 for other conditions. IS = internal standard (OTC). The values reported are the means of two experiments.

fore 280 nm was chosed as the wavelength for UV densitometry. For fluorescence densitometry, an excitation wavelength of 400 nm was chosen, as the highest signal-to-noise ratio was obtained at this wavelength.

Macherey–Nagel plates were used for further validation of the method. The stability of MC and OTC spots after development was measured by scanning the same lane ten times consecutively over a period of 4 h. The relative standard deviations (RSDs) for peak areas thus obtained were 0.8% and 0.6% for MC and OTC, respectively, indicating the good stability of tetracyclines during the scanning procedure. For assay, the developed plates could be stored in the dark for at least 1 week. However, for purity control, the developed plates had to be scanned within 2 days, since the fluorescence of 6-DODMTC decayed significantly after this period. The linearity of the method was examined and the following results were found, where y = peak area, x = amount of the hydrochloride salt spotted in μg, r = correlation coefficient, $S_{y.x}$ = standard error of estimate, and CR = range of spotted mass. MC.HCl (purity control): $y = 20719 + 13\,671\,x$, $r = 0.9982$, $S_{y.x} = 1924$, CR = 2–3 μg: MC.HCl (assay): $y = 1115 + 47722x$, $r = 0.9913$, $S_{y.x} = 354$, CR = 0.02–0.4 μg. 6-DODMTC.HCl: $y = -28 + 103\,975\,x$, $r = 0.9985$, $S_{y.x} = 354$, CR = 0.0008 – 0.13 μg. Results for MC were obtained by UV densitometry and results for 6-DODMTC were obtained by fluorescence densitometry. Except for 6-DODMTC, related substances were expressed as MC. The limit of quantification was 0.8 ng for 6-DODMTC and 20 ng for other impurities, corresponding to 0.008% m/m and 0.2% m/m, respectively.

The TLC separation developed on Macherey–Nagel plates as described above was also examined using thin-layers of material from other sources. The results shown in Table 2 demonstrate that the TLC method is applicable to silica gel materials from different origins. The reported values are the means of two experiments, which showed differences in *h*R*f* of 2 units at most.

3.2. Comparison of TLC and LC

Typical chromatograms obtained by TLC or LC are shown in Figs. 2 and 3 respectively. The TLC chromatograms in the three panels in Fig. 2 are intended respectively for assay (I), purity control of impurities (II) and selective purity control of fluorescent impurities (III). The need for an IS is a major disadvantage of quantitative TLC. The LC method shown in Fig. 3 allows the simultaneous determination of MC and impurities.

As shown in Fig. 2 (panel III), in the fluorescence mode MC appears as a minor negative peak adjacent to the 6-DODMTC peak. The influence of the amount of MC on the quantification of 6-DODMTC was examined. An MC.HCl sample containing 0.56% m/m of 6-DODMTC.HCl, as determined by the LC method, was spiked with 0.5% m/m or 1.0% m/m of 6-DODMTC.HCl. The recovery by fluorescence densitometry was 92% ($n = 3$, RSD = 2.2%) and 101% ($n = 3$, RSD = 5.6%), respectively. With 8 μg, 10 μg and 12 μg of the

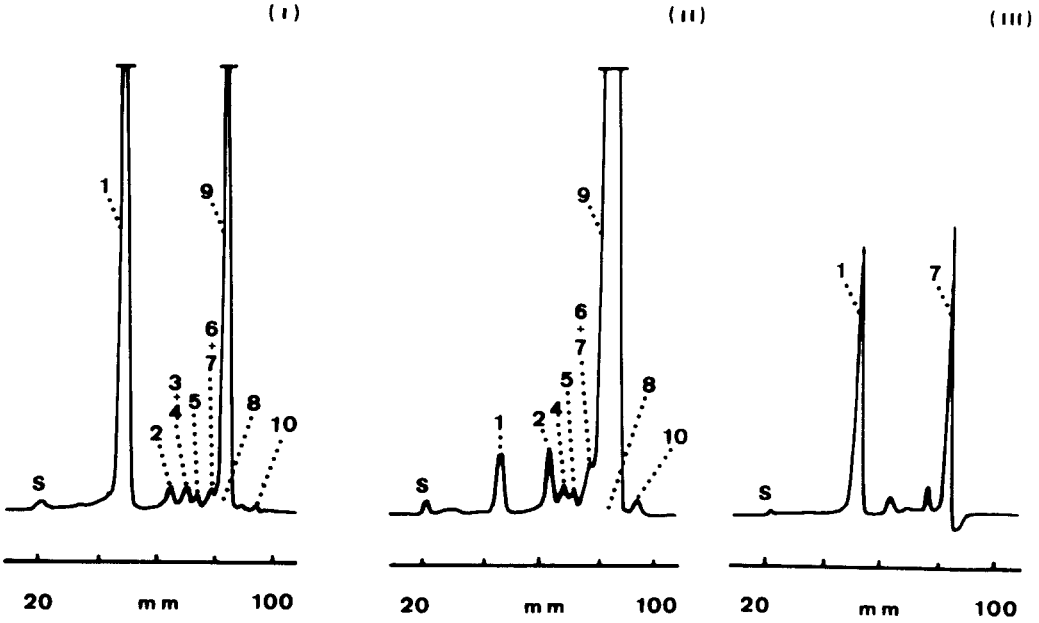

Fig. 2. Typical chromatograms obtained by the proposed TLC method: (I) for assay by UV; (II) for purity control by UV; and (III) for selective determination of 6-DODMTC by fluorescence. Stationary phase: silica gel (Macherey–Nagel), previously sprayed with 10% m/v of EDTA at pH 9.0. Mobile phase: dichloromethane–methanol–water (57:35:8, v/v/v). See Section 2 for other conditions. Sample: MC.HCl-S1. Peak identity: (1) OTC (internal standard); (2) 4-EMC; (3) ADOTC; (4) 7-DDMMC; (5) UNK1; (6) 7-MDMMC; (7) 6-DODMTC; (8) 9-MC; (9) MC; (10) UNK2; (S) point of application.

above-mentioned MC.HCl sample loaded on the plate, the amount of 6-DODMTC was found to be 0.57% m/m ($n = 4$, RSD = 3.3%), 0.58% m/m ($n = 4$, RSD = 2.7%) and 0.52% m/m ($n = 4$, RSD = 4.9%), respectively. The above results showed that quantification of 6-DODMTC was not adversely affected by MC. Two minor impurities (<0.02% m/m for each impurity, expressed as 6-DODMTC) were found between OTC and 6-DODMTC, as shown in Fig. 2 (panel III). Their retention time in LC was not investigated further.

Table 3 combines quantitative results for MC.HCl samples, obtained by TLC and LC. The RSD values for MC at 1.25 mg ml^{-1} were <3% for TLC ($n = 4$) and <1% for LC ($n = 4$). The RSD values and the mean results obtained for MC were analyzed by an F-test and a Student's t-test ($P = 0.05$) [10]. For samples MC.HCl-S1 the F-test was significant, but this was probably due to the very low RSD value (0.2%) obtained with the LC method. In no case did the Student's t-test show significant differences between the main components as determined by TLC and LC. The results in Table 3 show that good agreement exists between the means obtained for the related substances by TLC or LC. For small amounts of related substances the precision of the TLC method was less than that obtained by LC. The correlation coefficient r, calculated with the results obtained in TLC and LC for MC and for related substances, was greater than 0.999.

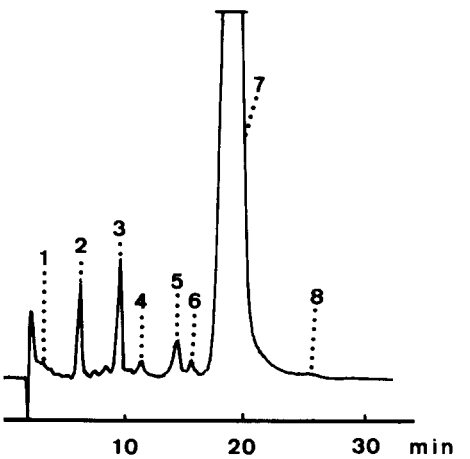

Fig. 3. Typical chromatogram of minocycline hydrochloride obtained by LC on 8-μm PSDVB 100 Å. See Section 2 for chromatographic conditions. Sample: MC.HCl-S1. Peak identity: (1) 7-DDMMC; (2) 7-MDMMC; (3) 4-EMC; (4) UNK1; (5) 6-DODMTC; (6) UNK2; (7) MC; (8) 9-MC.

Table 3
Comparison of assay and purity control data [a] for minocycline by TLC or LC

Sample method	% m/m [b]							
	4-EMC	7-DDMMC	UNK1	7-MDMMC	6-DODMTC	9-MC	MC	UNK2
S1 TLC	1.32	0.19	0.20	0.92 [c]	0.45 [d]		86.9	0.28
	(20)	(36)	(7.9)	(6.9)	(5.6)		(2.0)	(40)
LC	1.24	0.15	0.19	0.65	0.51	<0.05	86.9	0.16
	(5.1)	(2.7)	(5.8)	(4.6)	(0.8)		(0.2)	(9.7)
S2 TLC	1.24	<0.2	<0.2	0.62 [c]	0.55 [d]		91.6	0.22
	(14)			(13)	(1.0)		(2.6)	(22)
LC	1.04	0.03	0.08	0.18	0.59	0.07	90.6	0.14
	(5.0)	(17)	(25)	(6.5)	(2.1)	(21)	(0.4)	(5.3)
S3 TLC	0.82	<0.2	<0.2	0.83 [c]	0.28 [d]		92.3	<0.2
	(13)			(0.7)	(2.6)		(1.9)	
LC	0.70	0.12	<0.02	0.43	0.34	<0.05	91.4	0.13
	(4.6)	(5.6)		(1.3)	(22)		(0.4)	(1.5)
S4 TLC	1.06	<0.2	<0.2	0.42 [c]	0.22 [d]		92.7	0.29
	(4.1)			(12)	(5.9)		(1.0)	(27)
LC	0.89	0.02	0.09	0.15	0.32	0.07	91.9	0.26
	(11)	(36)	(29)	(12)	(18)	(35)	(0.4)	(0)

[a] The values reported are the means of four experiments. All impurities except 6-DODMTC are expressed as MC. RSD values in percentage are given in parentheses.
[b] Concentration, % m/m, calculated on an "as is" basis, expressed as the hydrochloride salt.
[c] 7-MDMMC + 6-DODMTC.
[d] Determined by fluorescence densitometry.

4. Conclusion

A quantitative TLC method has been developed for the assay and purity control of MC. 4-EMC and 7-DDMMC were well separated from MC and from each other. 7-MDMMC and 6-DODMTC were not separated from each other and only partially separated from minocycline. The latter was selectively determined by fluorescence densitometry. Results obtained with TLC compared favourably with those obtained by LC using a PSDVB stationary phase. The correlation coefficient for TLC and LC was >0.999. It can be concluded that the TLC method described is a valuable alternative to LC for the assay and purity control of MC.

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