Journal of Pharmaceutical & Biomedical Analysis
Vol. 8, Nos 8-12, pp. 891-898, 1990
Printed in Great Britain

0731-7085/90 $3.00 + 0.00
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Assay and purity control of oxytetracycline and doxycycline by thin-layer chromatography — a comparison with liquid chromatography*

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Abstract: A thin-layer chromatographic (TLC) method using densitometry is described for the assay and purity control of oxytetracycline and doxycycline. With a mobile phase of dichloromethane–methanol–water (59:35:6, 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 oxytetracycline or doxycycline are well separated from the main components and from each other. Results obtained with TLC are compared with those obtained by previously established liquid chromatography (LC) methods using poly(styrene-divinylbenzene) stationary phases. A good correlation was obtained ($r > 0.9999$). For TLC the relative standard deviation (RSD) for the assay of the main component was <2%, for LC the RSD was <1%.

Keywords: *Oxytetracycline; doxycycline; thin-layer chromatography (TLC); densitometry; liquid chromatography (LC); assay; purity control.*

Introduction

The chemical structures of the antibiotics oxytetracycline (OTC) and doxycycline (DOX) are shown in Fig. 1. Potential impurities of OTC are 4-epitetracycline (ETC), tetracycline (TC), 4-epioxytetracycline (EOTC), 2-acetyl-2-decarboxamidooxytetracycline (ADOTC), anhydrooxytetracycline (AOTC) and α- and β-apooxytetracycline (α-APOTC, β-APOTC). The latter three occur only in samples of the hydrochloride salt OTC·HCl. For DOX the potential impurities are 4-epidoxycycline (4-EDOX), 6-epidoxycycline (6-EDOX), metacycline (MTC) and 2-acetyl-2-decarboxamidodoxycycline (ADDOX). All these impurities can be separated from the corresponding main compound by LC on poly(styrene-divinylbenzene) stationary phases [1, 2]. Planar chromatographic methods for the separation of OTC from its impurities have appeared in literature. Originally, paper chromatography was used [3]. Difficulties with separations of tetracyclines by thin-layer chromatography (TLC) on silica gel were attributed to the formation of chelate complexes with metallic ions and to counter this, sequestering agents such as edetate, citrate, oxalate or phosphate had to be added. Better results were obtained with kieselguhr [4]. Early work on TLC of tetracyclines was reviewed by Vanderhaeghe [5]. At that moment it was already clear that the moisture content of the thin-layer was an important factor and not easy to control. Much experience was needed to obtain satisfactory separations. The suitability of cellulose layers for TLC of tetracyclines was also investigated. A method using cellulose is now prescribed by the European Pharmacopoeia (Ph.Eur.) for the identification and purity control of DOX [6]. This method requires less experience but still is much dependent on the moisture content of the thin-layer.

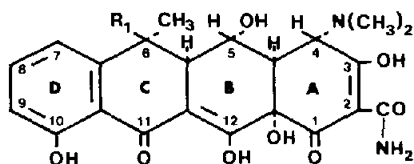

Figure 1
Structures of oxytetracycline (R_1 = OH) and doxycycline (R_1 = H).

*Presented at the Second International Symposium on "Pharmaceutical and Biomedical Analysis", April 1990, York, UK.

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In this paper we report on the development of a TLC method for the assay and purity control of OTC or DOX. The TLC method is derived from that previously developed for the identification of tetracyclines [7]. With a mobile phase of dichloromethane–methanol–water (59:35:6, v/v/v) and a silica gel thin-layer, previously sprayed with 10% solution of sodium edetate (m/v) adjusted to pH 9.0, all the impurities of OTC or DOX are well separated from the main component and from each other. The described TLC method is fast, accurate and easy to perform. Results obtained by TLC densitometry are compared with those obtained by using previously established liquid chromatography (LC) methods [1, 2].

Experimental

Chemicals

Methanol was obtained from Belgolabo (Overijse, Belgium) and redistilled in glass apparatus. Dichloromethane, 2-methyl-2-propanol and tetrabutylammonium hydrogen sulphate (TBA) were from Janssen Chimica (Beerse, Belgium). Other reagents were of *pro analysi* quality (E. Merck, Darmstadt, FRG). Water was freshly distilled in glass apparatus.

Reference substances and samples

Reference substances for OTC (91.7%), EOTC (84.5%), TC·HCl (91.6%), ETC·HCl (90.6%), α-APOTC (94.3%) and β-APOTC (95.2%) are available from Janssen Chimica. Reference substances for DOX·HCl (86.0%) and MTC·HCl (88.2%) were house standards, prepared from commercial samples. 6-EDOX·HCl (89.2%) was obtained from the European Pharmacopoeia (Strasbourg, France). The percentage content is always expressed as the free base. 4-EDOX was prepared from DOX by storing a solution of DOX at pH 3, the 4-EDOX formed was not isolated from the mixture. AOTC was prepared from OTC by storing a solution of OTC in acidified methanol but AOTC was not isolated. AOTC is not stable and is easily transformed into α- and β-APOTC. Small amounts of chromatographically pure ADOTC and ADDOX were isolated by column chromatography on silica gel and their structures were confirmed by mass spectrometry. The purity of these substances was not determined in detail. The column chromatographic method used in this isolation was derived from the TLC method described here. Commercial samples of oxytetracycline (OTC-S), oxytetracycline hydrochloride (OTC·HCl-S) and doxycycline hydrochloride (DOX·HCl-S1, DOX·HCl-S2 and DOX·HCl-S3) were obtained from Pfizer (Brussels, Belgium). Doxycycline hydrochloride samples always occur as the hyclate which is the hemihydrate, hemiethanolate (DOX·HCl·1/2 H_2O·1/2 C_2H_5OH). Minocycline hydrochloride (MC·HCl) was obtained from Cyanamid-Lederle (Brussels, Belgium).

Thin-layer chromatography

Laboratory-made silica gel layers on glass (20 × 20 cm) were prepared with kieselgel 60 H F_{254} (E. Merck, No. 11696) according to a previously described procedure [7]. Precoated silica gel layers on glass (20 × 20 cm) were obtained from Merck (No. 5715), Whatman (Maidstone, UK; No. 4861-820), Carlo Erba (Milan, Italy; No. 485321) and Macherey-Nagel (Düren, FRG; 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 42% 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 (110°C) for 1 h, shortly before use.

Aliquots of 2 μl of the sample solutions in methanol, containing 5 mg ml^{-1} of sample to be examined and 5 mg ml^{-1} (for assay) or 0.25 mg ml^{-1} (for purity control) of internal standard (IS), i.e. MC·HCl for OTC samples and OTC for DOX samples, were applied to the plate with a microsyringe (Hamilton, Bonaduz, Switzerland). Commercial grade of MC·HCl is pure enough to be used. For OTC as IS the use of a purified sample is preferred. Aliquots of 2 μl of the reference solutions in methanol, containing 5 mg ml^{-1} of standard substance and 5 mg ml^{-1} of IS (for assay) or 0.125 mg ml^{-1} of related substance and 0.25 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 days.

The chromatographic chamber was lined with paper and equilibrated with the mobile phase dichloromethane–methanol–water (59:35:6, 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: 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 = off; drift-line integration = 0.5. Mean values were obtained from the measurement of the spots in the chromatograms obtained with four reference solutions and three sample solutions for assay, or obtained with four reference solutions of related substances and three sample solutions for purity control.

Liquid chromatography

The LC system consisted of a L-6200 pump (Merck–Hitachi, Tokyo, Japan), an autosampler equipped with a 20-µl loop (Marathon, Emmen, The Netherlands), a model SM-3100 UV detector set at 254 nm (Milton–Roy, Riviera Beach, FL, 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-Alltech Europe, Eke, Belgium) maintained at 60°C through a water jacket. The flow rate was 1.0 ml min^{-1}.

OTC was analysed using a one-step gradient elution. The mobile phase was 2-methyl-2-propanol–0.2 M potassium phosphate buffer pH 7.5–0.02 M TBA pH 7.5–0.0001 M sodium edetate (EDTA) pH 7.5–water (5.8:10:5:10:69.2, m/v/v/v/v) for the isocratic part (15 min) and (8.3:10:5:10:66.7, m/v/v/v/v) for the gradient part (15 min). The mobile phase used for DOX was 2-methyl-2-propanol–0.2 M potassium phosphate buffer pH 8.0–0.02 M TBA pH 8.0–0.01 M EDTA pH 8.0–water (5.8:10:5:10:69.2, v/v/v/v). During preparation of the TBA or EDTA solutions, the pH was adjusted to the indicated values with sodium hydroxide solution. Mobile phases were 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 1 mg ml^{-1}. Reference solutions of related substances were prepared at a concentration of 0.0125 mg ml^{-1}. At about 5°C the solutions were stable for at least 2 days.

Results and Discussion

Development of the TLC methods

It was known that for the identification of tetracyclines on silica gel, EDTA was required to be incorporated in the stationary phase to avoid the formation of tetracycline–metal complexes [7]. This can be achieved by addition of EDTA to the slurry used for the preparation of the layers, but this technique then excludes the use of precoated plates. Predevelopment with an aqueous edetate solution is rather time consuming. Moreover, this technique is not applicable to some brands of ready-made layers or laboratory-prepared layers since it leads to cracks in these layers. Therefore, we chose to spray the edetate solution onto the layer. This technique is fast, applicable to all layers, and leads to a more homogeneous distribution on the layer, while predevelopment probably results in a gradient partition. It was also considered important to use EDTA solutions of defined pH values, preferably slightly alkaline, since this improves the chelating properties and avoids the formation of 4-epimers of the tetracycline antibiotics on the thin-layer. The adjustment 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 are shown in Table 1. The reported values are the mean of several experiments. At pH 9.0 all the impurities of OTC or DOX are well separated from the main component and from each other.

Table 1
Influence of the pH of the stationary phase on the separations obtained by TLC

Substance	$R_f \times 100$			
	pH 7.0	pH 8.0	pH 9.0	pH 10.0
EOTC	8	5	4	3
ETC	14	7	6	6
OTC	18	13	10	12
α-APOTC	18	15	16	20
TC	30	22	21	23
ADOTC	31	24	23	26
β-APOTC	43	31	27	32
4-EDOX	25	23	16	11
MTC	26	23	21	18
6-EDOX	27	26	25	27
DOX	32	31	30	30
ADDOX	42	41	40	38

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

A mixture of dichloromethane–methanol–water (59:35:6, v/v/v) was found to be the most suitable mobile phase. The same mobile phase was also proposed for identification of tetracyclines [7]. Lower contents of water in the mobile phase gave insufficient migration while higher contents resulted in increased diffusion of the spots. The activation conditions of the plates (110°C, 1 h) described in the Experimental section are minimal conditions. Prolonged activation up to 3 h did not alter the quality of the separations. After activation the plates could be stored for several hours in the laboratory atmosphere before use. This delay did not affect the separations. In order to obtain a good repeatability an internal standard had to be used. MC (R_f = 0.39) was chosen as internal standard for analysis of OTC and OTC (R_f = 0.10) was selected as internal standard for DOX since on all brands of plates the separation of MC and ADDOX was insufficient. The use of purified OTC as IS is preferred since ADOTC, if present in considerable amounts, may affect proper integration of the DOX peak. ADOTC is present up to 2% in commercial samples. The use of OTC instead of OTC·HCl as the IS excludes the presence of acid decomposition products (α- and β-APOTC). Small amounts (<2%, m/m) of other impurities in these internal standards did not interfere with the determination of the main component but some of these impurities might affect the accurate determination of impurities in OTC or DOX samples. Therefore two levels of concentration of internal standards were used, one (5.0 mg ml^{-1}) for assay and the other (0.25 mg ml^{-1}) for purity control. At this low concentration the impurities of the internal standards no longer interfere with the quantitation of the impurities in the sample to be examined. UV spectra measured on the spots showed maximal absorbance at about 280 nm for all tetracycline derivatives of interest and therefore 280 nm was chosen as the wavelength for densitometry.

Macherey–Nagel plates were used for further validation of the method. The stability of OTC, DOX and MC spots after development was measured by scanning the same lane nine times consecutively over a period of 4 h. The relative standard deviations (RSD) for peak areas thus obtained were 0.16, 0.40 and 0.55% for OTC, DOX and MC, respectively, indicating the good stability of spots during the scanning procedure. Developed plates could be stored in the dark for 1 week at least. The coefficient of correlation, r, was calculated for calibration curves determined in the range of 8–12 μg for the main component and up to 0.25 μg for each related substance, corresponding to 80–120% or 2.5% of the total amount of substance to be examined that was spotted on the plate. Results are summarized in Table 2. This means that good linearity is obtained in the range of interest. ADOTC and ADDOX are expressed as OTC and DOX, respectively. AOTC is expressed as α-APOTC. 4-EDOX is expressed as 6-EDOX. The limit of quantitation was 0.02 μg for each compound, corresponding to 0.2%. The repeatability expressed as the RSD was calculated for 11 lanes, obtained with the same solution of OTC or DOX on the same plate.

Table 2
Calibration curves for oxytetracycline, doxycycline and the corresponding related substances, as obtained with the TLC method

	Intercept	Slope	r	$S_{y,x}$	CR (μg)
OTC	15794	42342	0.9990	4667	8–12
OTC	259	69301	0.9998	170	0.06–0.25
EOTC	992	68020	0.9988	370	0.06–0.25
ETC	585	71521	0.9999	100	0.06–0.25
α-APOTC	1086	70431	0.9992	321	0.06–0.25
TC	202	71376	0.9999	106	0.06–0.25
β-APOTC	1514	72063	0.9952	795	0.06–0.25
DOX	69978	44884	0.9999	891	8–12
DOX	2994	71571	0.9908	690	0.06–0.25
MTC	1804	78625	0.9996	265	0.06–0.25
6-EDOX	1323	67877	0.9998	154	0.06–0.25

Mobile phase: dichloromethane–methanol–water (59:35:6, v/v/v). CR = concentration range examined, expressed as the number of μg applied to the plate.
Stationary phase: Macherey–Nagel, sprayed with EDTA solution of pH 9.0.

Table 3
Influence of the TLC plate brand on the separation obtained

Stationary phase	$R_f \times 100$													
	EOTC	ETC	OTC	α-APOTC	TC	ADOTC	β-APOTC	MC(IS)	OTC(IS)	4-EDOX	MTC	6-EDOX	DOX	ADDOX
MN	4	6	10	16	21	23	27	39	10	16	21	25	30	40
M	2	4	8	11	17	20	23	34	8	14	16	20	25	32
CE	3	5	8	12	16	19	23	35	8	13	16	20	26	32
WM	4	8	13	18	22	25	27	38	13	16	18	21	26	35
LM	5	9	13	20	26	29	33	49	13	16	20	24	33	46

Mobile phase: dichloromethane–methanol–water (59:35:6, v/v/v).
Stationary phase: MN = Macherey–Nagel, M = Merck, CE = Carlo Erba, WM = Whatman, LM = Laboratory-Made; all sprayed with EDTA solution of pH 9.0. IS = internal standard. The values reported are the means of several experiments.

The RSD values were 1.3 and 1.0% for OTC and DOX, respectively.

The TLC separation, developed on Macherey–Nagel plates as described above was also examined by using thin-layers of other origin. The results shown in Table 3 demonstrate that the TLC method is equally well applicable to silica gel layers of different origin. The time needed for development over 15 cm varied between 40–80 min, depending upon the origin of the plate.

Comparison of TLC and LC

Typical chromatograms, obtained by TLC densitometry or LC, are shown in Figs 2 and 3, respectively. The TLC chromatograms in Fig. 2(I) are intended for assay of the main peak and therefore contain an amount of IS corresponding to the substance to be determined. This improves the repeatability. The small impurities present in the IS, corresponding to peak 5 in Fig. 2(IA) and to peaks 1 (EOTC) and 6 (ADOTC) in Fig. 2(IB), do not interfere with the determination of the main peak. However, for the quantitation of the related substances important interference by the impurities of the IS would occur and therefore separate chromatograms, containing 20 times less IS, are used for the purity control. Such chromatograms are shown in Fig. 2(II). The need for an IS is a major disadvantage of quantative TLC methods. The HPLC chro-

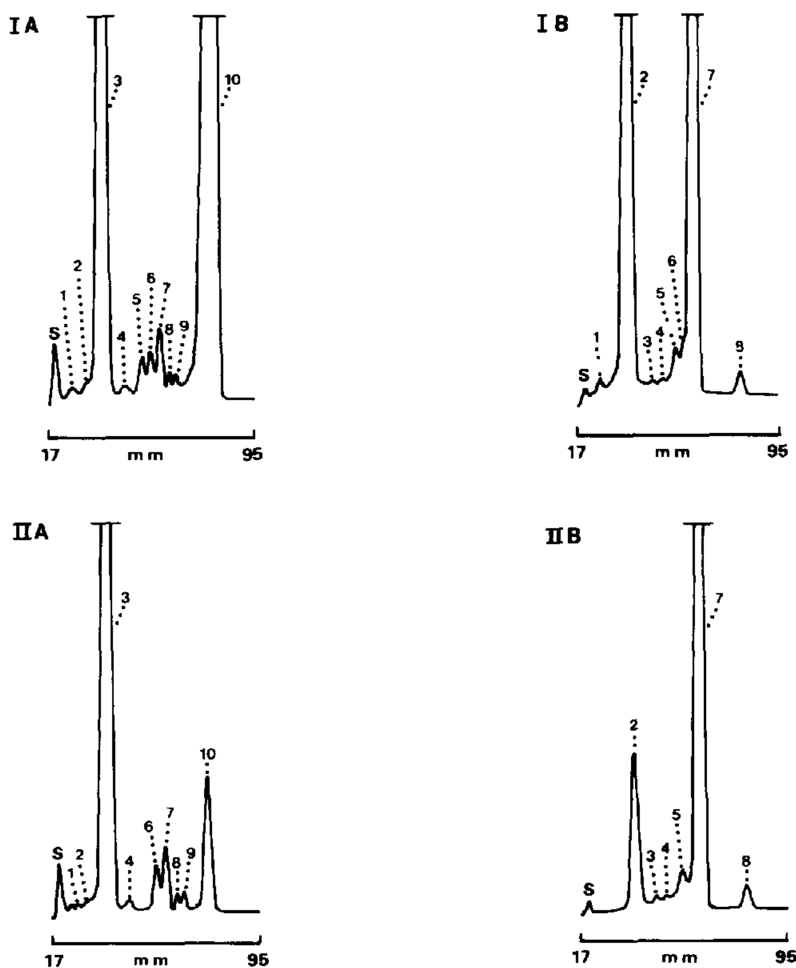

Figure 2
Typical chromatograms obtained by the proposed TLC method for assay (I) or for purity control (II) of oxytetracycline hydrochloride (A) or doxycycline hyclate (B). Stationary phase: silica gel (Macherey–Nagel), previously sprayed with 10% edetate solution at pH 9.0. Mobile phase: dichloromethane–methanol–water (59:35:6, v/v/v). See Experimental section for other conditions. Samples: OTC·HCl-S and DOX·HCl-S2. Peak identity: (A) 1 = EOTC, 2 = ETC, 3 = OTC, 4 = α-APOTC, 5 = impurity from MC, 6 = TC, 7 = ADOTC, 8 = AOTC, 9 = β-APOTC, 10 = MC (internal standard, IS); (B) 1 = EOTC, 2 = OTC (internal standard, IS), 3 = 4-EDOX, 4 = MTC, 5 = 6-EDOX, 6 = ADOTC, 7 = DOX, 8 = ADDOX, S = start point.

Table 4
Comparison of assay and purity control by TLC and LC of oxytetracycline and doxycycline

		OTC	*EOTC*	*ETC*	*α-APOTC*	*TC*	*ADOTC*	*AOTC*	*β-APOTC*
OTC-S	TLC	88.4 (0.9)	0.9 (7.8)	0.7 (53)	<0.2	2.4 (2.9)	1.8 (2.7)	<0.2	<0.2
	LC	88.5 (1.0)	0.7 (1.5)	0.6 (0.6)	<0.1	2.0 (1.4)	1.2 (2.2)	<0.2	<0.1
OTC·HCl-S	TLC	88.9 (0.9)	0.2 (17)	<0.2	0.3 (18)	1.4 (32)	0.9 (31)	0.2 (4.8)	0.3 (26)
	LC	89.1 (0.1)	0.1 (5.3)	0.2 (12)	0.4 (23)	1.2 (1.2)	0.6 (3.9)	0.1 (30)	0.2 (15)

		DOX	*4-EDOX*	*MTC*	*6-EDOX*	*ADDOX*
DOX·HCl-S1	TLC	84.0 (0.8)	<0.2	0.4 (35)	0.5 (33)	<0.2
	LC	83.0 (0.5)	0.1 (10)	0.5 (4.0)	0.8 (1.7)	<0.2
DOX·HCl-S2	TLC	84.5 (1.1)	<0.2	<0.2	0.5 (57)	0.8 (5.2)
	LC	85.5 (0.6)	0.1 (20)	0.2 (16)	0.4 (2.3)	0.5 (4.5)
DOX·HCl-S3	TLC	84.8 (0.8)	<0.2	<0.2	0.4 (23)	0.7 (2.2)
	LC	84.7 (0.6)	0.1 (5.7)	0.1 (8.9)	0.6 (0.8)	0.3 (4.1)

Values in % m/m, expressed as the base, are the mean of three experiments; RSD values are given in parentheses.

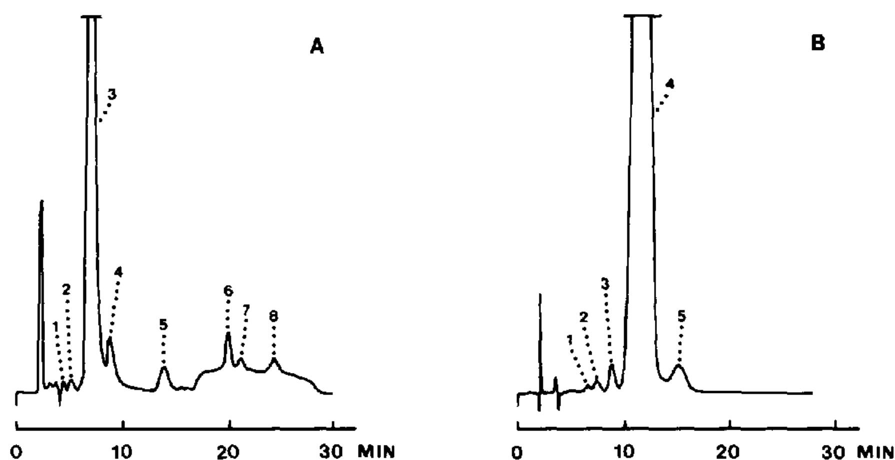

Figure 3
Typical chromatograms of oxytetracycline hydrochloride (A) and doxycycline hyclate (B) obtained by LC. See Experimental section for chromatographic conditions. Samples: OTC·HCl-S and DOX·HCl-S2. Peak identity: (A) 1 = EOTC, 2 = ETC, 3 = OTC, 4 = ADOTC, 5 = TC, 6 = α-APOTC, 7 = AOTC, 8 = β-APOTC; (B) 1 = 4-EDOX, 2 = MTC, 3 = 6-EDOX, 4 = DOX, 5 = ADDOX.

matograms shown in Fig. 3 allow the simultaneous determination of main component and impurities. It was observed that the different components are better separated by HPLC.

Table 4 combines quantitative results for OTC and DOX samples, obtained by TLC and LC. The RSD values for the main component was <2% for TLC results, and for LC this was <1%. The RSD values and the mean results obtained for the main component were analysed by an F-test and a student's t-test ($P = 0.05$) [8]. For sample OTC·HCl-S the F-test was significant, but this was probably due to the unusually low RSD value (0.1%) obtained with the LC method. In no case was the student's t-test for difference between the means of the main components as obtained by LC and TLC found to be significant. The results in Table 4 show that a good agreement existed between the means obtained for the related substances by TLC or LC, except for the 2-acetyl-2-decarboxamido-derivatives, for which LC gave lower values. This was probably due to the fact that for TLC these derivatives were measured at 280 nm while for LC they were measured at 254 nm. In both cases the acetylderivatives have been expressed as the corresponding tetracycline. The ratios of absorbances ADOTC/OTC or ADDOX/DOX are about 1.5 times higher at 280 nm than at 254 nm. For small amounts of related substances the precision of the TLC method was less than that obtained by LC as indicated by the frequently higher RSD values. The correlation coefficient, r, calculated with the results obtained in TLC and LC for the main component and for the related substances was >0.9999.

It can be concluded that the TLC method described is a valuable alternative for the assay and purity control of OTC or DOX by LC.

Acknowledgements — The authors thank Dr G. Janssen for mass spectrometric identifications and Mrs A. Decoux for fine secretarial assistance.

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[*Received for review 4 April 1990*]