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A collaborative study of the analysis of doxycycline hyclate by high-performance liquid chromatography on polystyrene-divinylbenzene packing materials

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Abstract: An improved method for the analysis of doxycycline hyclate by high-performance liquid chromatography using polystyrene-divinylbenzene column packings is described. The separation obtained with this method was compared with that of other, recently described methods. The improved method was examined in a collaborative study involving five separate laboratories, using 11 different columns and four discrete samples. The main component and impurities were determined. An analysis of variance showed absence of consistent laboratory bias and presence of significant laboratory–sample interaction. Estimates for the repeatability and reproducibility of the method, expressed as relative standard deviations (RSD) of the result of the determination of doxycycline, were found to be 0.9 and 1.2%, respectively.

Keywords: *Doxycycline; high-performance liquid chromatography (HPLC); polystyrene-divinylbenzene stationary phase; collaborative analytical study.*

Introduction

Doxycycline (DOX) is a tetracycline antibiotic obtained by semi-synthesis from oxytetracycline (OTC). Metacycline (MTC) is an intermediate. During the transformation of MTC into DOX, some 6-epidoxycycline (6-EDOX) also can be formed. In solution and upon storage DOX is prone to epimerization, resulting in the formation of 4-epidoxycycline (4-EDOX). The 4-epimer of 6-EDOX (4,6-EDOX) is a derivative of minor importance. Doxycycline hyclate is the hydrochloride hemiethanol hemihydrate of DOX.

A method for analysis of DOX by high-performance liquid chromatography (HPLC) on polystyrene-divinylbenzene (PSDVB) columns has been described previously [1].

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Since then, the method has been improved in order to decrease the analysis time. This was achieved mainly by replacing the organic modifier tetrahydrofuran by 2-methyl-2-propanol. Separations obtained by means of recently published [2, 3] methods for analysis of DOX using classical reversed-phase materials are compared in the present study with separations obtained using PSDVB. The HPLC method involving PSDVB columns has been examined by means of a multicentre study. Since the results obtained are very satisfactory, this method is being proposed for the testing of related substances and for the standard assay for doxycycline hyclate to be incorporated in the European Pharmacopoeia (Ph. Eur.). The method is also applicable to derivatives other than the hyclate, however as this is the most frequently used form of the drug, the present study was limited accordingly.

Experimental

The conditions adopted for use in the collaborative study are as specified.

Apparatus and columns

The equipment consisted of a pump set at a flow rate of 1.0 ml min^{-1}, a fixed loop injector with a loop of about 20 µl, a column heating device maintained at 60°C, a UV detector set at 254 nm and an integrator allowing peak area measurements.

All columns were 25 × 0.46 cm i.d. Four of the five laboratories participating in the collaborative study were provided with a column packed in the organizing laboratory with 8 µm PLRP-S (Polymer Laboratories, Church Stretton, Shropshire, UK). The laboratories further used columns of their own choice, i.e. pre-packed and home-packed PLRP-S columns and PRP-1 columns (Hamilton, Reno, NV, USA). Some experiments were carried out with 7–9 µm ROGEL (RSL-Alltech Europe, Eke, Belgium).

Mobile phase

The required amount of 2-methyl-2-propanol was weighed and rinsed into a volumetric flask with 200 ml of water. Depending upon the column employed 5–6% m/v of 2-methyl-2-propanol was required to achieve satisfactory separations. To this mixture was added 100 ml of 0.2 M potassium hydrogen phosphate buffer pH 8.0, 50 ml of 0.02 M tetrabutylammonium (TBA) hydrogen sulphate and 10 ml of 0.1 M sodium edetate (EDTA). During preparation of the latter two solutions, the pH was adjusted to 8.0 with sodium hydroxide solution. The mixture was made up to 1000 ml with water. Mobile phases were degassed by sonication. The phosphate buffer was prepared by mixing 0.2 M potassium monohydrogen phosphate and 0.2 M potassium dihydrogen phosphate.

Samples, chemicals and solvents

Ph. Eur. Chemical Reference Substances (CRS) for metacycline hydrochloride (MTC·HCl) and for 6-epidoxycycline hydrochloride (6-EDOX·HCl) were used. These were accepted to contain 91.9% m/m of MTC base and 89.2% m/m of 6-EDOX base, respectively, account being taken of solvents and small amounts of impurities. The reference sample for doxycycline hyclate (DOX·HCl·0.5 C_2H_5OH·0.5 H_2O) DOX-R, prepared by the organizing laboratory was accepted to contain 86.0% m/m of DOX base as determined by HPLC against the home standard previously described [1]. No official standard of DOX was used as the reference sample since relatively large amounts had to

be distributed and since the aim of the study was not to determine exact contents but to examine the repeatability within each laboratory and the reproducibility of the method between laboratories. Three of the samples examined were of commercial origin (DOX-S1, DOX-S2, DOX-S3). The Ph. Eur. CRS for doxycycline hyclate Lot I was also examined (DOX-Eur).

Chemicals complied with Ph. Eur. requirements. Water was freshly distilled from glass apparatus. Hydrochloric acid (0.01 M) was used as the solvent for the samples. For quantitative analysis, solutions were prepared to contain 1.0 mg of doxycycline hyclate/ml. Sample solutions were found to be stable for at least 1 day at about 20°C and for at least 2 days at about 5°C.

Laboratories participating in the collaborative study

Experiments for the collaborative study were carried out by:

(i) the organizing laboratory, the Laboratorium voor Farmaceutische Chemie, K.U. Leuven, Belgium;
(ii) two plants, Delft and Meppel, of Gist-Brocades, The Netherlands;
(iii) Pfizer, Amboise, France;
(iv) the European Pharmacopoeia Laboratory, Strasbourg, France.

Method development and preliminary experiments were carried out by the organizing laboratory. Attributed laboratory numbers do not correspond to the order of laboratories cited above.

Results

Development of an improved HPLC method for doxycycline hyclate

The development of an improved assay method was based upon previous experience gained with the analysis of DOX [1]. The unknown substance previously reported to be eluted after the DOX peak has now been identified as 2-acetyl-2-decarboxamidodoxycycline (ADDOX). A small amount of ADDOX was isolated by preparative thin-layer chromatography as described before for the OTC analogue [4]. The ADDOX structure was confirmed by mass spectrometry.

It was observed that by replacing the original organic modifier tetrahydrofuran by 2-methyl-2-propanol, it was possible to reduce the analysis time from about 50 min to about 20 min. Moreover, 2-methyl-2-propanol is stable against oxidation, and mobile phases containing this organic modifier do not form gas bubbles when used at 60°C.

The influence of the amount of 2-methyl-2-propanol and of the pH of the mobile phase is shown in Fig. 1. The quality of the separation at pH 8.0 or 7.0 seems to be comparable, but at the latter pH the peaks are less symmetrical. At pH 9.0 MTC, 4-EDOX and 6-EDOX are not separated from each other. Finally, pH 8.0 was retained as the pH of choice where OTC, 4,6-EDOX, 4-EDOX, MTC, 6-EDOX and DOX are separated completely and ADDOX is sufficiently separated from DOX. The influence of the amount of phosphate buffer or of TBA in the mobile phase had already been investigated [1]. The presence of some EDTA in the mobile phase improves the column efficiency [1].

Figure 2 shows a typical chromatogram. Comparable separations are obtained with other PSDVB gels, e.g. Hamilton PRP-1 and ROGEL. The latter material was not available at the time when the collaborative study was commenced.

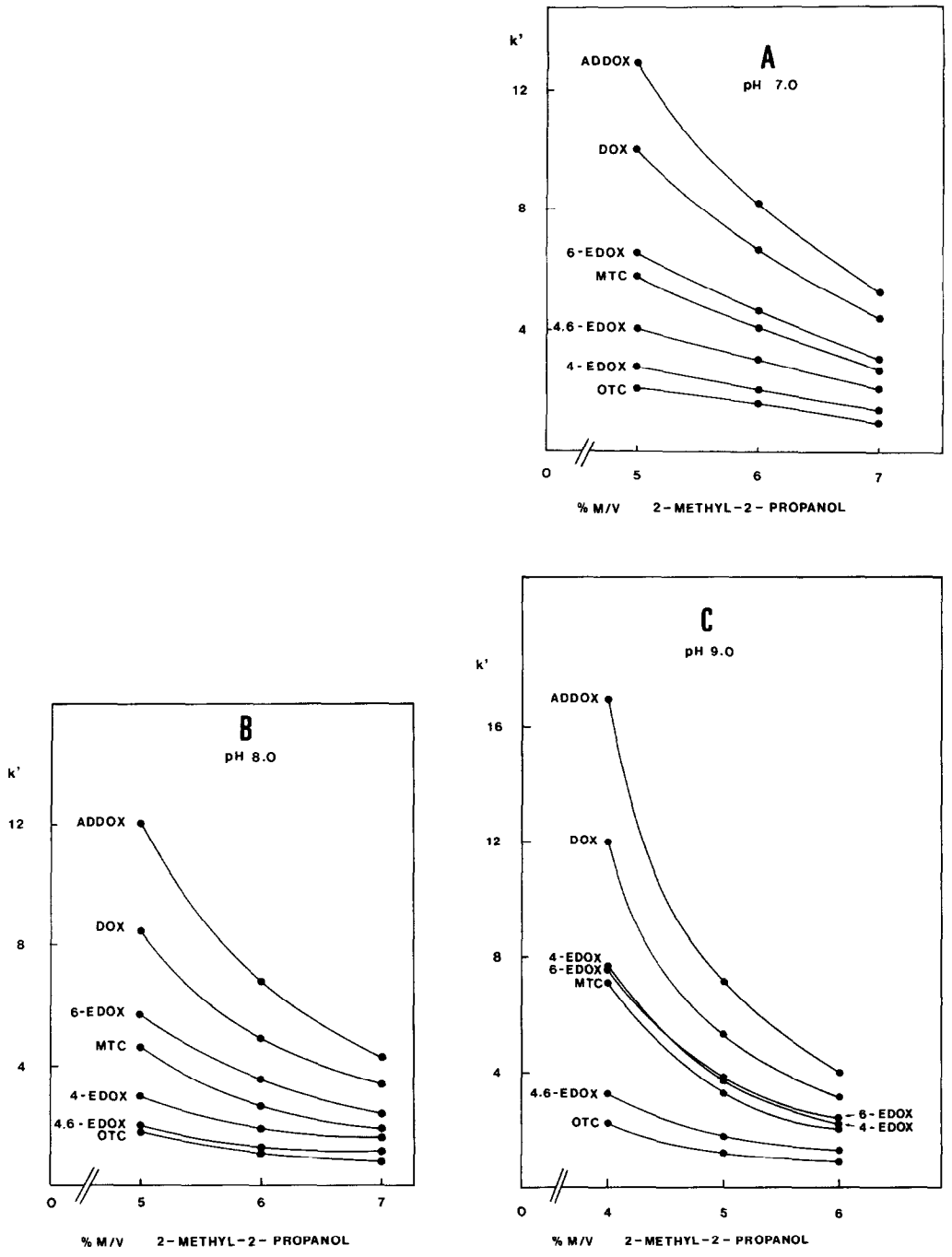

Figure 1
Influence of the amount of organic modifier and of the pH of the mobile phase. Column: PLRP-S, 25 × 0.46 cm i.d. at 60°C. Mobile phase: 2-methyl-2-propanol (x, g)–0.2 M potassium phosphate buffer pH z (10.0 ml)–0.02 M tetrabutylammonium sulphate pH z (5.0 ml)–0.1 M EDTA pH z (1.0 ml)–water (up to 100.0 ml). See abscissa for x, the pH (z) is indicated. Flow rate: 1.0 ml min^{-1}. Detection wavelength: 254 nm.

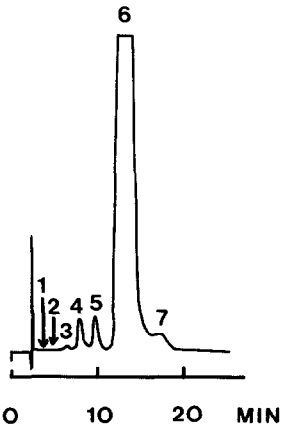

Figure 2
Typical chromatogram of doxycycline hyclate obtained by the proposed method. Column: PLRP-S, 25 × 0.46 cm i.d. at 60°C. Mobile phase: 2-methyl-2-propanol (6.0 g)–0.2 M phosphate buffer pH 8.0 (10.0 ml)–0.02 M tetrabutylammonium sulphate pH 8.0 (5.0 ml)–0.1 M EDTA pH 8.0 (1.0 ml)–water (up to 100.0 ml). Flow rate: 1.0 ml min^{-1}. Detection wavelength: 254 nm. Peak identity: 1, OTC; 2, 4,6-EDOX; 3, 4-EDOX, 0.05%; 4, MTC 0.5%; 5, 6-EDOX, 0.7%; 6, DOX; 7, ADDOX, 0.2%.

Comparison with recent methods described in the literature

The United States Pharmacopeia (USP) prescribes reversed-phase HPLC on C_{18} packing materials for the assay of DOX [2]. The method is based upon that previously published by Hermansson *et al.* [5]. It was evaluated in the organizing laboratory, using different types of C_{18} packing materials. Results are shown in Fig. 3. The order of elution of MTC and 6-EDOX is reversed, compared with that obtained with the proposed method using PSDVB materials. As can be seen the separations differ with the type of C_{18} phase used, thus in Figs 3A and 3B the separation is distinctly better than those in Figs 3C and 3D. With the latter, correct integration is hindered by incomplete separation and tailing of the main peak. ADDOX is not separated by this method. Moreover, the relatively high pH of 8.0 can affect the lifetime of the packing material. In the USP this is resolved by the use of a guard column.

A recently published paper [3] describes a method for the separation of DOX, OTC, TC, MTC and 6-EDOX using a cyano reversed-phase column. This method was also evaluated in the organizing laboratory, the results being shown in Fig. 4. 6-EDOX and MTC are separated from DOX, but 6-EDOX and 4-EDOX are not completely separated. ADDOX is not separated by this method.

Results of the collaborative study

In all, 11 columns were used in five laboratories. Table 1 includes information regarding the columns, conditions used and the results of performance checks carried out by each laboratory. Ph. Eur. instructions were followed for calculation of characteristics of the separations. The symmetry factor S and the theoretical plate number n were calculated for the DOX peak. Resolutions (R_s) were calculated for the pair MTC and 6-EDOX and for the pair 6-EDOX and DOX. The repeatability expressed as the relative standard deviation (RSD) was calculated for five consecutive injections of different solutions of DOX. The coefficient of correlation r was calculated for a calibration curve determined in the range of 16–24 μg of doxycycline hyclate injected, corresponding to 80–120% of the prescribed amount.

The individual results expressed as % m/m DOX base in the different samples are reported in Table 2. Means and RSD values are given in Table 3. Means and RSD values for the impurities are reported in Table 4. All results are expressed in terms of the base. 4-EDOX and ADDOX are expressed as 6-EDOX.

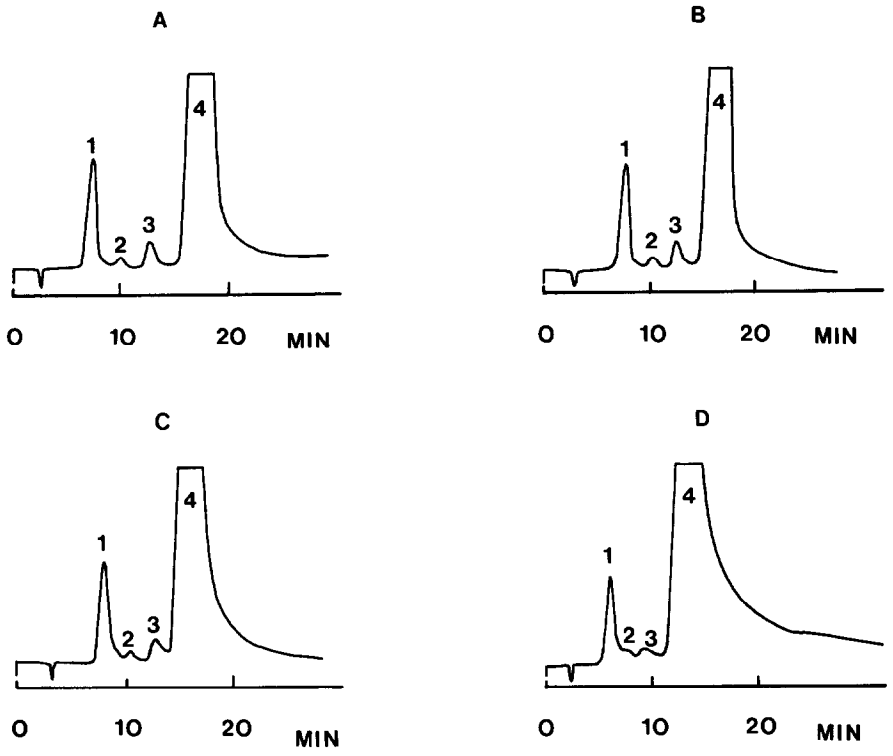

Figure 3
Typical chromatograms obtained by the USP method. Columns: 25 × 0.46 cm i.d. A, Hypersil C_{18} 5 μm; B, LiChrosorb C_{18} 10 μm; C, μ-Bondapak C_{18} 10 μm; D, Zorbax C_{18} 7 μm. Mobile phase: methanol (x ml)–0.1 M sodium dihydrogen phosphate [(1000-x)ml]–*N,N*-dimethyl-*n*-octylamine (3.0 ml), 5 N sodium hydroxide to pH 8.0. x is A, 470 ml; B, 450 ml; C, 460 ml; D, 510 ml. Flow rate: 1.0 ml min^{-1}. Detection wavelength: 254 nm. Peak identity: 1, 4-EDOX; 2, 6-EDOX; 3, MTC; 4, DOX. The sample is spiked with 4-EDOX.

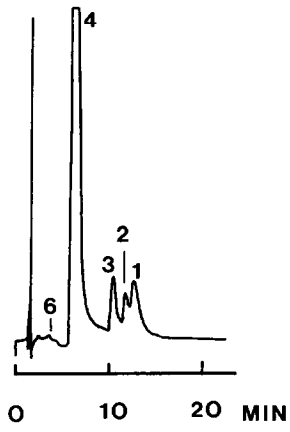

Figure 4
Typical chromatogram obtained using a cyano reversed-phase column. Column: 25 × 0.46 cm i.d. packed with RSiL CN 10 μm (RSL-Alltech Europe). Mobile phase: tetrahydrofuran–dimethylformamide–acetic acid–water–EDTA, 72:10:16:2:0.0015 (v/v/v/v/m). Flow rate: 2.0 ml min^{-1}. Detection wavelength: 280 nm. The sample is spiked with 4-EDOX. Peak identity: 1, 4-EDOX; 2, 6-EDOX; 3, MTC; 4, DOX; 6, OTC.

Table 1
General information on columns and method performance

Laboratory	Column	Stationary phase	Particle size (μm)	Packing	2-methyl-2-propanol % m/m in mobile phase	S DOX	n DOX	R_s MTC 6-EDOX	R_s 6-EDOX DOX	Repeatability: DOX surface RSD	Repeatability: Retention time DOX (min) Mean	Repeatability: Retention time DOX (min) RSD	r DOX
1	a	PLRP-S	8	home-packed	6.0	1.14	979	2.4	2.8	0.2	13.6	0.8	0.9999
	b	PLRP-S	8	home-packed	5.7	1.09	1026	2.3	2.7	0.4	13.4	0.2	0.9998
	c	PRP-1	10	home-packed	5.5	1.16	943	1.8	2.3	0.4	13.5	0.3	0.9997
2	a	PLRP-S	8	home-packed	5.5	1.11	692	1.9	2.4	0.8	18.1	1.2	0.9998
	b	PLRP-S	8	pre-packed	5.5	1.16	965	2.3	3.3	0.4	18.4	0.2	0.9997
	c	PRP-1	10	pre-packed	5.0	1.06	681	1.8	2.7	0.7	17.6	0.7	0.9995
3	a	PLRP-S	8	home-packed	6.0	1.31	717	1.7	2.8	1.2	14.5	16.0	0.9930
	b	PLRP-S	8	pre-packed	6.0	1.25	1035	2.1	2.9	1.3	15.9	6.4	0.9989
	c	PRP-1	10	pre-packed	6.0	1.14	735	1.3	2.9	1.9	13.5	9.8	0.9951
4	a	PLRP-S	8	home-packed	6.0	1.07	808	2.1	2.7	0.9	11.9	1.5	0.9962
5	b	PLRP-S	8	pre-packed	6.0	1.08	1019	2.7	2.9	0.1	13.9	0.2	0.9999

S = symmetry factor, n = theoretical plate number; R_s = resolution; RSD = relative standard deviation; r = coefficient of correlation.

Table 2
Individual values (% m/m) for DOX

Laboratory	Column	Samples: DOX-S1				DOX-S2				DOX-S3				DOX-Eur			
1	a	84.9	84.9	84.9	84.9	84.3	84.2	84.6	84.6	85.7	85.7	85.8	86.0	85.9	85.8	85.9	86.1
	b	84.8	85.1	84.3	85.1	84.8	85.1	84.7	84.7	85.9	86.2	86.0	86.1	85.9	85.7	85.9	85.8
	c	85.6	84.8	85.5	85.0	85.3	84.9	84.7	84.9	85.5	84.8	85.3	84.9	85.3	85.7	84.9	84.8
2	a	85.2	85.7	84.2	85.2	85.8	86.1	85.6	85.8	86.1	85.5	84.9	84.4	85.5	85.6	85.6	85.5
	b	83.9	85.7	85.5	84.9	86.2	86.2	85.2	85.6	86.4	85.6	86.3	86.0	85.7	84.7	85.1	84.3
	c	84.7	85.7	85.3	85.3	85.1	84.9	85.4	85.7	86.7	85.7	86.1	85.9	84.5	84.8	84.7	85.9
3	a	83.1	86.0	81.8	85.6	82.8	83.8	84.8	86.6	85.2	84.3	85.8	85.0	84.7	85.2	84.0	86.6
	b	82.8	83.5	82.9	84.0	86.1	86.1	86.3	86.1	84.1	83.7	84.8	85.3	86.0	85.9	85.5	86.0
	c	85.5	84.8	82.5	82.8	84.4	84.7	84.0	85.2	83.6	83.5	79.2	84.7	84.8	85.0	84.6	85.0
4	a	83.6	83.6	84.9	83.2	83.9	84.2	82.4	84.6	85.4	84.7	85.1	85.2	82.2	83.9	81.7	85.7
5	b	85.5	84.7	85.5	85.1	85.3	85.1	85.5	85.3	86.0	85.2	85.9	85.7	85.5	84.6	85.4	85.5

Table 3
Mean values (% m/m) for DOX

Laboratory	Column	Samples DOX-S1	DOX-S2	DOX-S3	DOX-Eur.
1	a	84.90	84.43	85.80	85.93
		(0)	(0.2)	(0.2)	(0.2)
	b	84.83	84.83	86.05	85.83
		(0.5)	(0.2)	(0.2)	(0.1)
	c	85.23	84.95	85.13	85.18
		(0.5)	(0.3)	(0.4)	(0.5)
2	a	85.08	85.83	85.23	85.55
		(0.7)	(0.2)	(0.9)	(0.1)
	b	85.00	85.80	86.08	84.95
		(0.9)	(0.6)	(0.4)	(0.7)
	c	85.25	85.28	86.10	84.98
		(0.5)	(0.4)	(0.5)	(0.7)
3	a	84.13	84.50	85.08	85.13
		(2.4)	(1.9)	(0.7)	(1.3)
	b	83.30	86.15	84.48	85.85
		(0.7)	(0.1)	(0.8)	(0.3)
	c	83.90	84.58	82.75	84.85
		(1.8)	(0.6)	(2.9)	(0.2)
4	a	83.83	83.78	85.13	83.38
		(0.9)	(1.2)	(0.4)	(2.2)
5	b	85.20	85.30	85.70	85.25
		(0.5)	(0.2)	(0.4)	(0.5)
Mean of means		84.60	85.04	85.23	85.17
RSD		0.8	0.8	1.1	0.8

RSD are given in parentheses.

Table 4
Mean of mean values (% m/m) for related substances

	Samples DOX-S1	DOX-S2	DOX-S3	DOX-Eur.
4-EDOX	0.15	0.09	0.08	0.11
	(33)	(43)	(31)	(35)
MTC	0.12	0.44	0.06	0.07
	(40)	(8)	(23)	(26)
6-EDOX	0.78	0.69	0.35	0.61
	(8)	(6)	(16)	(17)
ADDOX	0.13*	ND	0.25†	0.13‡
	(3)		(30)	(32)

RSD are given in parentheses.
ND, not detected; * Detected on one column; † detected on seven columns; ‡ detected on three columns. See Fig. 2 for abbreviations.

Discussion

The HPLC method developed in the organizing laboratory was retained for the collaborative study since it enables the separation of all the potential impurities and it gives comparable separations on different brands of PSDVB. In Table 1, it is observed that only small modifications of the 2-methyl-2-propanol content of the mobile phase are required. For one column (3a) the symmetry factor is higher than 1.25, whilst other

columns (1a, 2a, 4a) packed by the organizing laboratory with the same batch of stationary phase behaved much better. The theoretical plate number is low for all the columns. The good separation is due to favourable selectivity rather than good efficiency. The resolution between the small impurities MTC and 6-EDOX is better than 1.3 for all the columns. The resolution between 6-EDOX and DOX is always better than 2.0. There is no apparent difference in behaviour between the PRP-1 and PLRP-S materials or between home-packed and pre-packed columns. The repeatability of retention times in laboratory 3 is markedly inferior to those observed for the other collaborating laboratories. Close inspection of the chromatograms accompanying the results revealed that laboratory 3 also had some integration problems in so far that DOX peaks were terminated too early, giving lower figures and more variation. Incomplete integration of the DOX peak was also observed on some chromatograms of laboratory 4, especially for sample Ph. Eur. This emphasizes the importance of technical details and of the correct use of electronic integrators in the first place. The coefficient of correlation is sufficiently lower for the laboratories mentioned, for them to have some repeatability problems. It can be concluded, however, that the equipment was used in a linear range.

The results in Table 3 show that the RSD values for the mean of means are quite low. For three laboratories the RSD values are always smaller than 1.0, whilst the two other laboratories obtained values up to 2.9. This is probably owing to the above mentioned problems with stability and integration. The results for the related substances in Table 4 show that even very small amounts of about 0.1% of 4-EDOX, MTC or 6-EDOX can be determined without problems. Quantities of ADDOX smaller than 0.2% are difficult to determine with the system used. For levels above 0.2%, ADDOX, however, can be determined provided that the system is sufficiently stable and that the proper integration parameters are chosen.

In order to analyse further the results obtained for DOX, a number of statistical calculations were performed [6, 7]. In order to facilitate these calculations, each column was considered as a separate laboratory. The results were first examined for abnormally high intra-laboratory variation [7, p. 96]. The limit was exceeded for five means out of 12 in laboratory 3 and for two means out of four in laboratory 4. This confirms the stability and/or integration problems observed for these laboratories. In the next experiment the means were ranked to examine for outlying columns [7, p. 99]. No column had a total score of ranking outside the 7 and 41 limits. The lowest values were 8 for column 4a and 9 for column 3c, the highest 33 for column 2c. The lower values can be explained by incomplete integration as reported above. The ranked mean values were also examined for outlying mean values by using Dixon's criterion [7, p. 96]. For two means (columns 3c and 4a) the limit at the 5% level was exceeded but at the 1% level the limit was not exceeded. Therefore, all the means were used in further calculations.

An analysis of variance was carried out to search for consistent laboratory bias or significant laboratory sample interaction [6, p. 77]. The results which are shown in Table 5 reveal that there is no significant between laboratory variance at the 1% level, but there is at the 5% level. In other words, no consistent laboratory bias exists. The sample-interaction variance on the other hand is significant, even at the 1% level. To obtain a better idea of this variation, estimates of the repeatability of the analytical method (intra-laboratory variance) and of the reproducibility (inter-laboratory variance) were calculated [6, p. 80]. The RSDs thus obtained were 0.9 and 1.2%, respectively.

It can be concluded that the HPLC method described is suitable for control of related substances and for assay of DOX.

Table 5
Analysis of variance

Source of variation	Sum of squares	Degrees of freedom	Mean square	Variance ratio
11 Laboratories				
Between laboratories (L)	46.49	10	4.649	L/LS = 2.75 F 0.99(10,30) = 2.98 F 0.95(10,30) = 2.16
Laboratory-sample interaction (LS)	50.62	30	1.687	LS/S = 2.76 F 0.99(30,132) 1.7
Between replicates (S)	80.65	132	0.611	

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