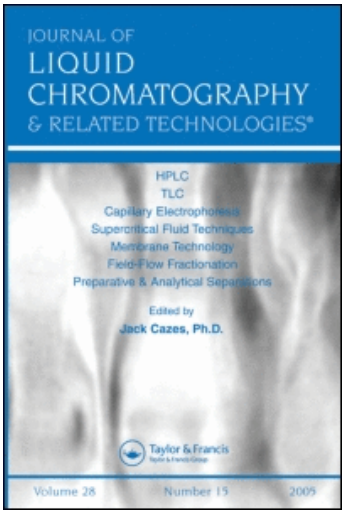

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COLLABORATIVE STUDY OF THE ANALYSIS OF TETRACYCLINE BY LIQUID CHROMATOGRAPHY ON POLY(STYRENE-DIVINYLBENZENE)

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ABSTRACT

A previously developed method for the analysis of tetracycline by liquid chromatography on poly(styrene-divinylbenzene) packing materials was examined in a collaborative study involving five laboratories and a total of seven columns. The main compound and the impurities of three tetracycline samples were determined. An analysis of variance, considering each column as a different laboratory, proved absence of consistent laboratory bias. The laboratory-sample interaction was significant. Estimates for the repeatability and the reproducibility of the method, expressed as relative standard deviations of the result of the determination of tetracycline, were calculated to be 0.9 and 1.5 % respectively.

INTRODUCTION

Tetracycline, an antibiotic produced by fermentation, is used as the base (TC), the hydrochloride (TC.HCl) or the laurylsulphate salt. From earlier LC work it is known that 4-epitetracyline (ETC), anhydrotetracycline (ATC), 4-epianhydrotetracycline (EATC) and 2-acetyl-2-decarboxamidotetracycline (ADTC) are the most important impurities present in commercial tetracycline samples [1]. A suitable LC method for the assay and purity control of TC samples should be able to separate the main compound TC from the potential impurities.

A previously described method for the analysis of TC on poly(styrene-divinylbenzene) (PSDVB) [1] has been examined by means of this multicentre study. This method was adopted for use in the monographs of tetracycline and tetracycline hydrochloride of the European Pharmacopoeia (Ph. Eur.) [2].

EXPERIMENTAL

Apparatus and Columns

The equipment consisted of a solvent delivery system 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 measured 25 x 0.46 cm i.d. Some of the columns were packed in the organizing laboratory, others were prepacked. Different brands of PSDVB stationary phases were used: PLRP-S 8 µm 1000 and 100 Å (Polymer Laboratories, Church Stretton, Shropshire, UK) and PRP-1 7-9 and 10 µm (Hamilton, Reno, NV, USA).

Mobile Phase

The required amount of 2-methyl-2-propanol was weighed and transferred quantitatively into a volumetric flask with water. Depending on the brand and age of the stationary phase, 7.7-8.5 % (m/v) was required to achieve satisfactory

separations. The mobile phase further contained 10 % (v/v) of a 3.5 % (m/v) dipotassium hydrogen phosphate solution adjusted to pH 9.0, 20 % (v/v) of a 1.0 % (m/v) tetrabutylammonium sulphate solution adjusted to pH 9.0, 1 % (v/v) of a 4.0 % (m/v) sodium edetate solution adjusted to pH 9.0 and the volume was made up to 100 % (v/v) with water. Dilute phosphoric acid (10 % m/m) or dilute sodium hydroxide solution (8.5 % m/m) was used to adjust the solutions to the required pH. The mobile phase was degassed by ultrasonication.

Samples, Chemicals and Solvents

The reference samples used were available from Janssen Chimica (Beerse, Belgium): tetracycline hydrochloride (TC.HCl-R), which was accepted to contain 90.7 % (m/m) TC base, 4-epitetracycline hydrochloride (ETC.HCl-R), 4-epi-anhydrotetracycline hydrochloride (EATC.HCl-R) and anhydrotetracycline hydrochloride (ATC.HCl-R). The latter three were accepted to contain 100 % of the hydrochloride salt. No official standards were used 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. The three samples to be examined were of commercial origin: two tetracycline hydrochloride samples (TC.HCl-S1, TC.HCl-S2) and one tetracycline base sample (TC-S1).

Chemicals complied with Ph. Eur. requirements [3]. Hydrochloric acid (0.01 N) was used as the solvent for the samples. For quantitative analysis, solutions containing 1.0 mg ml^{-1} were prepared. Sample solutions were found to be stable for 12 h at about 5 °C.

RESULTS AND DISCUSSION

In all, seven columns were used in five laboratories. A typical chromatogram is depicted in figure 1. Table 1 summarizes general information regarding columns, LC conditions used and results of performance checks carried out by each laboratory, using a mixture of TC.HCl-R, ETC.HCl-R and EATC.HCl-R. The columns were heated by either an oven or an immersion bath. The content of 2-methyl-2-propanol in the mobile phase required adaptation,

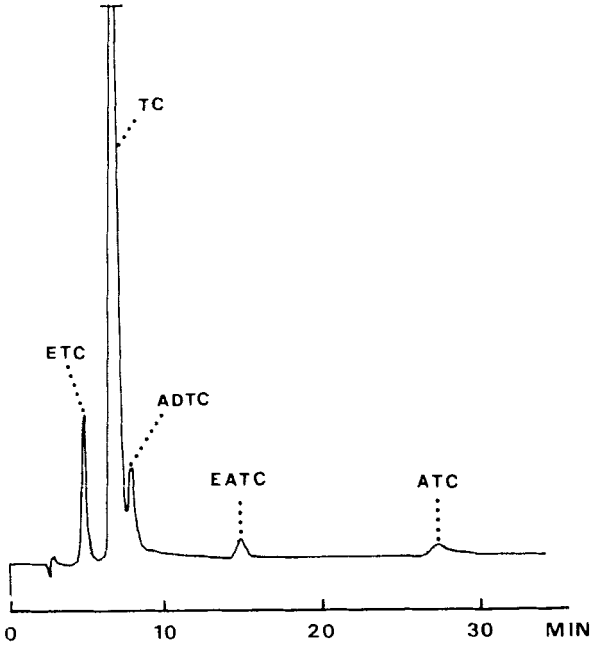

Figure 1. Typical chromatogram of TC.HCl-S1 obtained on column 1a. Mobile phase: 2-methyl-2-propanol (8.5 g/100 ml) - 3.5 % (m/v) dipotassium hydrogen phosphate pH 9.0 (10 ml) - 1.0 % (m/v) tetrabutylammonium sulphate pH 9.0 (20 ml) - 4.0 % (m/v) sodium edetate pH 9.0 (1 ml) - water (up to 100 ml). Temperature: 60 °C. Flow rate: 1 ml min^{-1}. Detection: UV at 254 nm.

ranging from 7.7 to 8.5 %, when using different columns. The calculations of the characteristics of the chromatographic parameters were carried out according to the Ph. Eur. [3]. The symmetry factor, *S*, and the theoretical plate number, *n*, were calculated for the TC peak. For all the columns the symmetry factor was between 1.0 and 1.2. The wide pore material (1000 Å), used by laboratory 5, gave better efficiency than the narrower pore materials. The resolution (*Rs*) was calculated for the pairs ETC-TC and TC-EATC. For ETC-TC the resolution was better than 3.0 on all the columns. For TC-EATC the resolution was 8.6 or better. Here also the 1000 Å column performed the best. The repeatability, expressed as the relative standard deviation (RSD, %), was calculated for five consecutive analyses of different solutions of sample TC.HCl-S1 and was found to be less than 1.0 % for

Table 1
General Information on Columns and Method Performance

L	C	Stationary phase	P	2-Methyl-2-propanol (% m/v in mobile phase)	k' TC	S TC	n TC	Rs ETC TC	Rs TC EATC	Repeatability (n = 5) Peak area RSD % ETC	TC	ADTC	EATC	ATC	Retention time TC (min) Mean	RSD (%)	Linearity r TC
1	a	PLRP-S 100 Å	8	8.5	2.7	1.0	2700	4.2	10.5	2.6	0.7	2.3	3.7	7.6	6.5	0.4	0.9935
	b	PRP-1	7-9	8.5	2.7	1.0	3200	4.1	13.1	1.7	0.3	1.1	1.9	1.8	6.1	1.9	0.9990
2	a	PLRP-S 100 Å	8	8.0	2.0	1.0	1500	3.7	8.6	7.6	0.6	1.0	4.0	9.7	5.5	0.1	0.9997
3	a	PLRP-S 100 Å *	8	8.5	1.5	1.2	3200	3.5	13.5	7.6	0.7	3.9	3.4	7.3	6.2	0.3	0.9930
4	a	PLRP-S 100 Å *	8	8.5	1.5	1.1	2800	4.3	11.3	2.6	0.6	2.4	1.6	3.4	5.9	0.1	0.9985
	b	PRP-1 *	10	8.3	1.9	1.1	1800	3.0	10.0	1.1	0.8	8.9	2.7	5.6	5.3	0.1	0.9937
5	a	PLRP-S 1000 Å *	8	7.7	0.5	1.0	4900	4.5	14.1	3.8	1.0	4.4	2.6	10.7	4.5	0.2	0.9990

L = laboratory, C = column, P = particle size (μm), k' = capacity factor, S = symmetry factor; n = theoretical plate number; Rs = resolution; RSD = relative standard deviation; r = coefficient of correlation for TC in the range 74-111 %, expressed as the base.
* Prepacked column, all other columns were laboratory-packed.

Table 2
Individual values (%, m/m) for the content of tetracycline, expressed as the base

Labo-ratory	Column	Samples											
		TC.HCl-S1				TC.HCl-S2				TC-S1			
1	a	88.89	90.29	89.05	89.31	89.08	90.25	89.09	90.46	83.56	85.57	84.46	84.62
	b	88.99	88.77	88.00	88.93	88.67	88.75	88.08	88.25	83.00	83.97	83.03	84.55
2	a	88.99	89.81	90.02	88.98	89.22	88.88	88.63	89.78	81.63	80.58	81.38	80.76
3	a	87.71	87.84	86.49	89.70	89.05	88.51	89.03	91.07	81.09	81.30	80.78	83.01
4	a	87.38	89.27	87.37	90.21	86.71	89.83	88.51	85.49	78.90	78.18	80.08	79.96
	b	91.04	90.20	88.95	90.36	90.42	90.47	91.07	90.90	80.79	81.98	80.54	81.26
5	a	87.62	90.23	88.91	88.70	90.52	90.37	88.11	89.06	80.99	82.21	80.87	83.25

Table 3
Mean Values (%, m/m) for TC

Laboratory	Column	Samples		
		TC.HCl-S1	TC.HCl-S2	TC-S1
1	a	89.39 (0.7)	89.72 (0.8)	84.56 (1.0)
	b	88.67 (0.5)	88.44 (0.4)	83.64 (0.9)
2	a	89.45 (0.6)	89.13 (0.6)	81.09 (0.6)
3	a	87.94 (1.5)	89.42 (1.3)	81.55 (1.2)
4	a	88.56 (1.6)	87.64 (2.2)	79.28 (1.1)
	b	90.14 (1.0)	90.71 (0.4)	81.14 (0.8)
5	a	88.86 (1.2)	89.51 (1.3)	81.82 (1.4)
Mean of means		89.00 (0.8)	89.22 (1.1)	81.87 (2.1)

RSD values (%) are given in parentheses.

Table 4
Mean of Mean Values (%, m/m) for Related Substances

	ETC	ADTC	EATC	ATC
TC.HCl-S1	1.50 (12.6)	0.48 (16.4)	0.31 (6.6)	0.28 (13.5)
TC.HCl-S2	1.53 (12.6)	0.24 (22.4)	0.25 (9.0)	0.25 (20.2)
TC-S1	8.51 (11.3)	1.05 (14.8)	0.07 (29.7)	0.63 (23.5)

For each column four results were obtained, leading to a mean value. The mean of these mean values, obtained on 7 columns, is shown. RSD values for reproducibility (%) are given in parentheses.

the main component. The repeatability for the related substances was satisfactory, the highest RSD value of 10.7 % being recorded for an impurity of 0.3 % m/m. The repeatability of the retention time is indicative for the quality of the pump system used while the linearity reflects the quality of the detector. The coefficient of correlation r was calculated for a calibration curve determined in the range 80-120 % of the prescribed amount to be analysed (74-111 %, expressed as the base). Some laboratories observed overloading of the detector when using the prescribed sample size. Laboratory 1 could not properly integrate more than 22 μg during the linearity tests on both columns examined and laboratory 5 had to decrease the sample size to 15 mg/50 ml instead of 25 mg/25 ml.

Table 5
Analysis of variance

Source of variation	Sum of squares	Degrees of freedom	Mean square	Variance ratio
Between laboratories (L)	52.98	6	8.83	L/LS = 1.91 *F* 0.995 (6,12) = 5.76 *F* 0.900 (6,12) = 2.33
Laboratory-sample interaction (LS)	55.50	12	4.63	LS/S = 5.16 *F* 0.995 (12,63) = 2.74
Between replicates (S)	56.46	63	0.90	

Samples were analysed four times, using independently prepared solutions. Individual results for the main compound , expressed as % (m/m) TC base, are listed in table 2. Mean and RSD values are given in table 3. Means of mean values and RSD on the mean values for the impurities are reported in table 4. These results were expressed in terms of the hydrochloride. ADTC.HCl was expressed as ETC.HCl. No difficulties were observed for the quantitation of the minor impurities ADTC, EATC or ATC. Some problems however were observed for the integration of the tetracycline base sample (TC.S1), which contained a high amount of ETC. The integration parameters had to be adapted in order to integrate properly both the ETC peak, eluted in front of the main TC peak, and the smaller ADTC peak, eluted on the descending part of the latter.

In order to analyse further the results obtained for the main compound, a number of statistical calculations were performed following described methods (4, 5). In order to simplify these calculations, each column was considered as a separate laboratory. The individual results were first examined for outliers. The means were ranked to examine for outlying columns and were also examined for outlying mean values by using Dixon's Criterion (4). Following the calculations of these statistical parameters, no columns or means were eliminated.

An analysis of variance was carried out in order to investigate consistent laboratory bias or significant laboratory-sample interaction (5). The results are listed in table 5. There is no significant between-laboratory variance even at the 10 % level. On the other hand, the laboratory (column)-sample interaction variance is significant, even at the 0.5 % level. These results mean that the analytical method

will show greater variation when carried out by different laboratories than within one laboratory, but evidently no consistent laboratory (column) bias exists. Estimates of the repeatability of the LC method [within laboratory (column) variance] and of the reproducibility [between laboratory (column) variance] were calculated (5). The RSD values thus obtained were 0.9 and 1.5 % respectively. Both values are satisfactory for a LC method.

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

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