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Collaborative study of the analysis of chlortetracycline hydrochloride by liquid chromatography on polystyrene–divinylbenzene packing materials

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Abstract: A previously established method for the analysis of chlortetracycline by liquid chromatography using polystyrene–divinylbenzene stationary phases was examined in a multicentre study involving four laboratories and a total of 12 columns. Three chlortetracycline hydrochloride samples were analysed. The main component and the impurities were determined. An analysis of variance, treating each column as a different laboratory, 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 of the result of the determination of chlortetracycline hydrochloride, were calculated to be 0.7 and 1.2%, respectively. When the analysis of variance was performed using only the results obtained on the wide pore (1000 Å) stationary phases, the laboratory–sample interaction strongly decreased. It is therefore proposed to use such materials for the analysis of chlortetracycline.

Keywords: *Chlortetracycline; liquid chromatography (LC); polystyrene–divinylbenzene stationary phase; collaborative analytical study.*

Introduction

Chlortetracycline (CTC) is a tetracycline antibiotic produced by fermentation which is used as the hydrochloride salt (CTC·HCl). During the fermentation process tetracycline (TC), some demeclocycline (DMCTC) and small amounts of demethyltetracycline (DMTC) are coproduced. Other impurities such as isochlortetracycline (ISOCTC) can be formed from CTC in alkaline medium, whilst in slightly acidic medium 4-epichlortetracycline (ECTC), 4-epitetracycline (ETC) and 4-epidemeclocycline (EDMCTC) can be present due to epimerization of the parent compounds CTC, TC, DMCTC. CTC is substantially stable in acid media and the formation of anhydro derivatives is minimal. Therefore anhydrochlortetracycline (ACTC) and epianhydrochlortetracycline (EACTC) are less likely to be formed and are considered to be minor impurities [1]. Other fermentation impurities 2-acetyl-2-decarboxamidochlortetracycline (ADCTC) and 2-acetyl-2-decarboxamidotetracycline (ADTC) were also detected in CTC·HCl samples [2, 3].

An acceptable LC method for the analysis of CTC·HCl samples should separate the therapeutically active components CTC and TC from all other potential components.

A previously described LC method using silica-based reversed-phases suffers from differences in selectivity between the different stationary phases and from reduced column lifetime due to the strong acidity of the mobile phase which contains 5% of 1 M perchloric acid. Moreover, ADCTC and ADTC are not separated from CTC or TC [1]. An improved LC method for the analysis of CTC on polystyrene–divinylbenzene (PSDVB) was introduced more recently [2]. This LC method has been examined by means of this multicentre

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study and the results obtained are shown to be satisfactory. Estimates for the repeatability and reproducibility of the method, expressed as relative standard deviation (RSD) of the result of the determination of CTC, were found to be 0.7 and 1.2%, respectively.

Experimental

The following laboratories participated in the study: (i) Laboratorium voor Farmaceutische Chemie K.U. Leuven (organizing laboratory) (Belgium); (ii) Dienst Geneesmiddelenanalyse, I.H.E. (Brussels, Belgium); (iii) Unité de Chimie Pharmaceutique, U.C. Louvain (Bruxelles, Belgium); (iv) European Pharmacopoeia Laboratory (Strasbourg, France). Attributed laboratory numbers used do not necessarily correspond to the order cited.

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 measured 25 × 0.46 cm i.d. All but one of the columns were packed in the organizing laboratory. Different brands of PSDVB stationary phases were used: PLRP-S 8 µm 1000, 300 and 100 Å (Polymer Laboratories, Church Stretton, Shropshire, UK); PRP-1 10 µm (Hamilton, Reno, NV, USA); RoGeL 7–9 µm, 7 nm (RSL-BioRad, Eke, Belgium); and TSK-gel 10 µm (Toyo Soda, Tokyo, Japan).

Mobile phase

The required amount of 2-methyl-2-propanol was weighed and transferred quantitatively into a volumetric flask with water. Depending upon the brand of stationary phase, 2.5–6.5% (m/v) of 2-methyl-2-propanol was required to achieve satisfactory separations. The mobile phase further contained 5% (v/v) of 1.0 M perchloric acid and the volume was made up to 100% (v/v) with water. The mobile phase was degassed by ultrasonication.

Samples, chemicals and solvents

The reference samples used are available from Janssen Chimica (Beerse, Belgium): chlortetracycline hydrochloride (CTC·HCl-R), with an assigned content of 97.7% (m/m) CTC·HCl, 4-epichlortetracycline hydrochloride (ECTC·HCl-R, 90.9%, m/m), tetracycline hydrochloride (TC·HCl-R, 99.2%, m/m) and 4-epitetracycline hydrochloride (ETC·HCl-R, 98.1%, m/m). No official standards were used 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. The three samples to be examined were of commercial origin (CTC·HCl-S1, CTC·HCl-S2 and CTC·HCl-S3).

Chemicals complied with European Pharmacopoeia (Ph. Eur.) requirements [4]. Hydrochloric acid (0.01 M) was used as the solvent for the samples. For quantitative analysis, solutions were prepared containing 1.0 mg ml^{-1} of CTC·HCl. Sample solutions were found to be stable for 5 h at about 20°C [2].

Results and Discussion

In all, 12 columns were used in four laboratories. A typical chromatogram is shown in Fig. 1. Table 1 includes information regarding columns, conditions used and results of per-

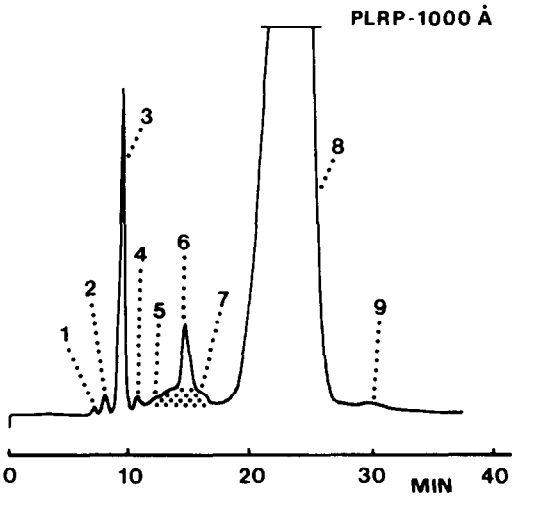

Figure 1
Chromatogram of CTC·HCl on PSDVB. Sample: CTC·HCl-S1. Mobile phase: 2-methyl-2-propanol (2.5 g/100 ml)–1.0 M perchloric acid (5.0 ml)–water (to 100.0 ml). Column 1a. Temperature: 60°C. Flow rate: 1 ml min^{-1}. Detection: UV at 254 nm. Peak identity and content (%, m/m): 1, ETC (0.06); 2, DMTC (0.2); 3, TC (3.7); 4, ADTC (0.4); 5, ISOCTC (<0.1); 6, DMCTC; 7, ECTC, dotted area (DMCTC + ECTC: 3.9); 8, CTC; and 9, ADCTC (0.5); all calculated as the hydrochloride.

Table 1
General information on columns and method performance

Laboratory	Column	Stationary phase	Particle size (μm)	2-Methyl-2-propanol (%, m/v in mobile phase)	*k'* CTC	*S* CTC	*n* CTC	*Rs* ECTC CTC	Repeatability (n = 5) Peak area RSD (%) TC	ECTC + DMCTC	CTC	Retention time CTC (min) Mean	RSD (%)	Linearity *r* CTC
1	a	PLRP-S 1000 Å	8	2.5	5.9	0.9	300	1.4	0.7	3.6	0.4	22.8	0.2	0.9995
	a'	PLRP-S 1000 Å	8	2.3	5.3	0.9	300	1.4	0.9	1.4	0.7	23.8	0.3	0.9999
	b	PLRP-S 300 Å	8	4.5	6.6	0.9	210	1.2	0.9	1.6	0.7	23.8	0.2	0.9995
	c	PLRP-S 100 Å	8	4.5	8.6	0.9	240	1.2	0.6	3.6	0.5	25.2	0.2	0.9996
	d	PRP-1	10	4.8	7.1	1.0	180	1.1	2.0	1.2	0.3	26.5	0.1	0.9995
	e	RoGeL	7–9	6.6	9.4	0.9	200	1.1	0.5	0.9	0.4	23.8	0.3	0.9999
	f	TSK-gel	10	3.7	7.5	0.9	280	1.4	0.3	1.3	0.3	25.0	0.1	0.9982
2	a	PLRP-S 1000 Å	8	2.5	5.5	0.9	370	1.4	1.0	2.0	0.9	17.8	0.1	0.9982
	d	PRP-1*	10	4.7	7.1	0.9	190	1.1	0.7	3.7	0.9	16.4	0.4	0.9949
3	a	PLRP-S 1000 Å	8	2.5	7.4	1.1	260	1.5	1.2	2.3	1.0	22.3	0.5	0.9980
	c	PLRP-S 100 Å	8	4.5	10.2	0.9	270	1.4	0.9	9.5	0.9	25.6	0.8	0.9992
4	a	PLRP-S 1000 Å	8	2.5	6.5	0.9	180	1.2	1.8	1.3	0.9	30.4	0.6	ND

S, symmetry factor; n, theoretical plate number; *Rs*, resolution; RSD, relative standard deviation; r, coefficient of correlation for CTC in the range 80–120%; ND, not determined.

*Prepacked column, all other columns were laboratory-packed.

formance checks carried out by each laboratory, using sample CTC·HCl-S1. It is shown that an adaptation of the 2-methyl-2-propanol content of the mobile phase, ranging from 2.5 to 6.6%, was required when the LC method was applied to different columns. The calculations of characteristics of the chromatography were carried out according to the monograph 'liquid chromatography' of the Ph.Eur. [5]. The symmetry factor, S, and the theoretical plate number, n, were calculated for the CTC peak. For all the columns the symmetry factor was between 0.9 and 1.1. The theoretical plate number for CTC was very low. Within the same laboratory the wide pore (1000 Å) materials gave better efficiencies than the narrower pore materials. This was not observed in previous experiments [2]. The 1000 Å material used in that study was not new, and this may explain the lower plate number. The resolution (Rs) was calculated for the pair ECTC–CTC. For all columns the resolution was better than 1.1. Based on this criterion also the 1000 Å columns performed somewhat better. The repeatability, expressed as the relative standard deviation (RSD, %), was calculated for five consecutive injections of different solutions of CTC on the same column and was found to be <1% for the main component. For the secondary component TC (3.7%, m/m), this repeatability was <2%. Summation of ECTC and the coeluted DMCTC impurities resulted in a repeatability which approached 10%. This can be explained by the difficulty of the integration of the sharp DMCTC peak and the broad ECTC peak. The broadness of the ECTC peak was explained by the occurrence of tautomerism [6]. This phenomenon may also explain the broadness of the CTC peak compared with that of TC. The repeatability of the retention time is a measure for the quality of the pump system used while the linearity is a measure of the quality of the detection system. The coefficient of correlation, r, was calculated for a calibration curve determined in the range 16–24 μg of CTC·HCl injected, corresponding to 80–120% of the prescribed amount to be analysed.

Samples were analysed four times, using independently prepared solutions. Individual results for the main compound, expressed as % (m/m) CTC·HCl, are reported in Table 2. Means and RSD values are given in Table 3. In two laboratories two integration modes were used for the same set of chromatograms. In the first mode, a horizontal baseline was drawn from the beginning of the chromatogram, starting before the ETC peak as described in the instructions of the collaborative study. In the second mode (1a*, 2a*, 2d*), this instruction was omitted and automated baseline construction was used. The means from both integration methods were compared using a student's t-test ($P = 0.05$) [7]. The test was not significant for results obtained on 1000 Å materials but for two out of three means (CTC·HCl-S1 and S3) obtained on a 100 Å column (2d) the test was significant. The results obtained by the second integration mode were not used for the calculation of the mean of the means and for the analysis of variance.

Means of mean values and of RSD values for the impurities, separated on the different columns, are reported in Table 4. All results were expressed in terms of the hydrochloride salt. DMTC·HCl and ADTC·HCl were expressed as TC·HCl. ISOCTC·HCl and DMCTC·HCl were expressed as ECTC·HCl. ADCTC·HCl was expressed as CTC·HCl. The minor impurities ADTC and ISOCTC were separated on the wide pore materials but not on the narrow pore stationary phases except for RoGeL (le) on which the elution order was reversed. This is probably due to the higher content of 2-methyl-2-propanol which is necessary when using this material. The results in Table 4 also show that a small amount (approximately 0.5%) of ADCTC in samples CTC·HCl-S1 and S3 was detected on some 1000 Å columns. A concentration of 0.5% of ADCTC has been reported as the limit of quantitation for LC in these conditions [2]. No difficulties were observed for the quantitation of the well separated, minor impurities ETC and DMTC or for the quantitation of TC or of ECTC + DMCTC. The RSD values for the mean of the means for the latter were higher than those for TC, due to reasons explained previously.

In order to analyse further the results obtained for the main component, a number of statistical calculations were performed following described methods [8, 9]. To facilitate these calculations, each column was considered as a separate laboratory. The results were first examined for outliers. The means were ranked to examine for outlying columns [8]. The ranked mean values were also examined for

Table 2
Individual values (%, m/m) for CTC·HCl

Laboratory	Column	Samples											
		CTC·HCl-S1				CTC·HCl-S2				CTC·HCl-S3			
1	a	91.82	91.03	91.49	91.55	91.10	91.30	90.72	90.89	90.20	89.90	89.13	91.56
	a*	90.77	90.72	90.88	90.61	90.37	90.59	90.23	90.45	89.50	90.09	89.99	90.85
	a′	90.76	90.58	90.03	89.84	89.95	89.28	90.60	90.00	90.05	89.44	90.17	89.12
	b	90.71	90.98	90.97	91.70	90.99	90.62	91.29	90.52	90.02	90.49	90.15	89.77
	c	90.44	90.42	91.09	91.32	90.65	90.44	89.72	90.21	89.72	90.84	90.05	90.96
	d	91.39	90.86	91.25	91.10	90.60	90.38	91.06	89.64	89.78	90.23	91.20	90.45
	e	91.43	91.05	90.93	91.26	91.05	90.91	90.58	91.95	88.88	89.21	88.62	90.37
	f	88.30	90.11	89.11	90.45	91.36	90.57	90.59	90.99	90.34	89.78	90.07	90.08
2	a	91.16	91.21	90.19	90.19	90.62	90.47	92.32	92.25	90.68	90.35	89.99	89.58
	a*	91.73	91.58	90.38	90.34	90.83	90.70	92.39	92.38	90.95	90.66	90.07	89.78
	d	90.85	91.22	91.13	90.98	88.01	88.07	87.79	89.99	90.71	91.14	90.49	90.78
	d*	89.86	88.95	88.74	88.36	86.71	86.19	86.09	88.34	89.93	89.18	88.72	88.57
3	a	91.15	91.74	91.69	92.09	92.43	93.00	92.35	94.22	91.08	91.66	89.50	90.75
	c	89.20	89.41	91.14	89.81	90.34	88.09	88.66	88.49	86.36	86.95	87.97	86.67
4	a	91.87	91.06	89.84	89.63	89.33	89.68	89.56	90.61	90.49	89.59	89.05	91.23

*Using the same chromatograms, peak areas were integrated again without setting a horizontal baseline at the beginning of the chromatogram.

Table 3
Mean values (%, m/m) for CTC·HCl

Laboratory	Column	Samples		
		CTC·HCl-S1	CTC·HCl-S2	CTC·HCl-S3
1	a	91.47 (0.4)	91.00 (0.3)	90.20 (1.1)
	a*	90.77 (0.1)	90.41 (0.2)	90.11 (0.6)
	a′	90.30 (0.5)	89.96 (0.6)	89.70 (0.6)
	b	91.09 (0.5)	90.86 (0.4)	90.11 (0.3)
	c	90.82 (0.5)	90.26 (0.4)	90.39 (0.7)
	d	91.15 (0.3)	90.42 (0.7)	90.42 (0.7)
	e	91.17 (0.2)	91.12 (0.6)	89.27 (0.9)
	f	89.49 (1.1)	90.88 (0.4)	90.07 (0.3)
2	a	90.69 (0.6)	91.42 (1.1)	90.15 (0.5)
	a*	91.01 (0.8)	91.57 (1.0)	90.36 (0.6)
	d	91.05 (0.1)	88.47 (0.1)	90.78 (0.3)
	d*	88.98 (0.7)	86.83 (1.2)	89.10 (0.7)
3	a	91.67 (0.4)	93.00 (1.1)	90.75 (1.2)
	c	89.89 (1.0)	88.90 (1.1)	86.99 (0.8)
4	a	90.60 (1.2)	89.80 (0.6)	90.09 (1.1)
Mean of means		90.78	90.51	89.91
RSD (%)		0.7	1.3	1.1

*Using the same chromatogram, the peak area was integrated again without setting a horizontal baseline; these results are not included in the mean of means.

outlying mean values by using Dixon's Criterion [8]. Following the calculations of these statistical parameters, no columns or means were eliminated.

An analysis of variance was carried out to search for consistent laboratory bias or significant laboratory–sample interaction [9]. The results are shown in Table 5. Using the results from all 12 columns, there is no significant between-laboratory variance at the 1% level, but there is at the 5% level, i.e., no consistent laboratory (column) bias exists. On the other hand the laboratory (column)–sample interaction variance is significant, even at the 1% level. This means, as expected, that more variation will occur when the method is performed by different laboratories (columns). To obtain a better idea of this variation, estimates of the repeatability of the analytical method (within laboratory (column) variance) and of the reproducibility (between laboratory (column) variance) were calculated [9]. The RSD values thus obtained were 0.7 and 1.2%, respectively. Both the repeatability and the

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

	ETC	DMTC	TC	ADTC	ISOCTC	ECTC + DMCTC	ADCTC
CTC·HCl-S1	0.6	0.17	3.66	0.36	ND	3.91	0.47*
	(22)	(13)	(2.1)	(20)		(7.3)	(21)
CTC·HCl-S2	0.6	0.14	3.28	0.28†	ND	4.30	ND
	(16)	(19)	(4)	(33)		(11)	
CTC·HCl-S3	0.09	ND	5.85	0.67‡§	0.43§	2.33	0.49*
	(14)		(2)	(30)	(8)	(14)	(23)

Normally, for each column four results were obtained, leading to a mean value. The mean of these mean values, obtained on 12 columns, is shown. ND, not detected.
*Detected on two columns (1a and 2a).
†Not detected on two columns (1d and 4a).
‡Coeluted with ISOCTC on six columns (1b, 1c, 1d, 1f, 2d, 3c).
§Mean of the results from PLRP-S 1000 Å columns.
RSD (%) are given in parentheses.

Table 5
Analysis of variance

Source of variation	Sum of squares	Degrees of freedom	Mean square	Variance ratio
12 Laboratories (columns)				
Between laboratories (L)	74.02	11	6.73	L/LS = 2.91 F 0.99 (11,22) = 3.19 F 0.95 (11,22) = 2.27
Laboratory–sample interaction (LS)	50.74	22	2.31	LS/S = 5.50 F 0.99 (22,108) < 2.03
Between replicates (S)	44.96	108	0.42	
5 Laboratories (columns)				
Between laboratories (L)	24.71	4	6.18	L/LS = 4.94 F 0.99 (4,8) = 7.01 F 0.95 (4,8) = 3.84
Laboratory–sample interaction (LS)	9.97	8	1.25	LS/S = 2.45 F 0.99 (8,45) > 2.82 F 0.95 (8,45) < 2.18
Between replicates (S)	22.77	45	0.51	

reproducibility are satisfactory for a chromatographic method. When the analysis of variance was repeated using only the results obtained on the 1000 Å materials (five laboratories) the between-laboratory variance is still significant at the 5% level whilst the laboratory (column)–sample interaction is not significant at the 1% level but still is significant at the 5% level. This is in accordance with the better resolution obtained on the 1000 Å columns. However, using only the results obtained on the 1000 Å columns, estimates for repeatability or reproducibility did not change.

It can be concluded that the LC method described is suitable for control of related substances and for the assay of CTC·HCl. Preference should be given to the 1000 Å stationary phase.

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