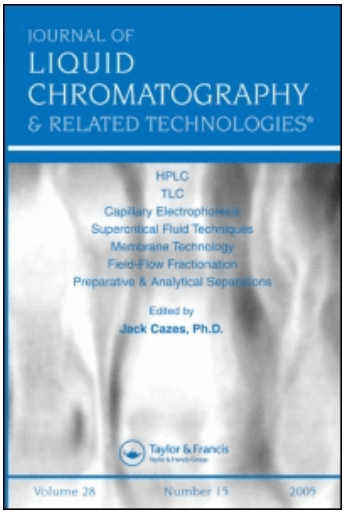

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Quantitative Analysis of Tetracycline by High Performance Liquid Chromatography on Polystyrene-Divinylbenzene Packing Materials

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QUANTITATIVE ANALYSIS OF TETRACYCLINE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ON POLYSTYRENE-DIVINYLBENZENE PACKING MATERIALS

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ABSTRACT

Isocratic high-performance liquid chromatography on a poly(styrene-divinylbenzene)copolymer column allows the complete separation and resolution of tetracycline, 4-epitetracycline, anhydrotetracycline and 4-epianhydrotetracycline. A fermentation impurity, 2-acetyl-2-decarboxamidotetracycline is also resolved from tetracycline. The mobile phase combines tert.-butanol, water and phosphate buffer, tetrabutylammonium sulphate and sodium ethylenediaminetetraacetate at a pH of 9.0 for elution at a temperature of 60 °C. The method was used to analyse official standards and commercial samples.

INTRODUCTION

Tetracycline (TC) epimerises at position C-4 to 4-epitetracycline (ETC) in weak acid conditions (pH 2-6). The

presence of a hydroxyl group at C-6 favors acid degradation resulting in the formation of anhydrotetracycline (ATC) which epimerises in weak acid conditions to 4-epianhydrotetracycline (EATC). Acid treatment of ETC also leads to the formation of EATC (1).

A comprehensive study on reversed-phase HPLC of TC and its degradation products using acid mobile phases was first published by Knox et al. (2). With acid mobile phases, complete separation of TC and ETC is obtained only with mobile phases which are too weak to elute the more strongly retained EATC and ATC within a reasonable time. Several modifications making use of acid mobile phases did not yield substantially better separations (3-8). Some improvement was achieved by the use of one step or continuous gradient elution (9-12). Methods using neutral mobile phases suffer from poor resolution between EATC and TC (13,14). Such a method is now prescribed by the United States Pharmacopeia (USP) and will be discussed in this paper (15). Major improvements were obtained by the use of mobile phases at pH 8-8.5 (16,17). A modified version of this method is prescribed by the USP for analysis of doxycycline (18). With this method, the epimers ETC-TC are well separated but EATC is eluted on the tail of the main peak (17). The instability of the packing material, attributed to the high pH of the mobile phase, is another disadvantage. Poly(styrene-divinylbenzene)copolymer (PSDVB) packing materials are known to be very stable in extreme pH conditions even though the use of acid mobile phase for analysis of tetracycline on these polymer materials offers no increased advantage over reversed-phase materials (19). However, with slightly alkaline mobile phases, very good separations were obtained with PSDVB materials in our laboratory for doxycycline (DOX) and for oxytetracycline (OTC) (20,21). In this paper an isocratic method for analysis of TC, using PSDVB copolymers, is described. The method is based upon those previously described for DOX and for OTC (20,21). Preliminary results obtained with a

short column have already been published (22). With a longer column, a fermentation impurity, 2-acetyl-2-decarboxamidotetracycline (ADTC), is also separated. This impurity has already been described but was never separated by HPLC (23-25). The HPLC method below has been used to compare official standards and to analyse a number of commercial samples.

EXPERIMENTAL

Reference Substances and Samples

The United States Pharmacopeia Reference Standard Lot I-1 (990 μ g/mg) (USP-RS), the European Pharmacopoeia Chemical Reference Substance (972 IU/mg) (Ph Eur-CRS) and the WHO Second International Standard (982 IU/mg) (WHO-IS) were compared. These reference substances are hydrochloride salts (TC.HCl). A reference substance of ADTC was donated by Pfizer (Groton, CT, USA). The TC.HCl house standard and the ETC.HCl, EATC.HCl and ATC.HCl reference substances are available from Janssen Chimica, Beerse, Belgium.

Several bulk samples were of known origin (Indonesia, Italy, USA or The Netherlands) and most obtained from wholesalers were of unknown origin. Bulk samples were available as bases, hydrochloric salts or laurylsulphate salts. Dosage forms containing TC were obtained from the Belgian market. The manufacturer of the formulated TC was mostly unknown.

Solvents and Reagents

Organic solvents were purchased from Janssen Chimica (Beerse, Belgium). Tetrahydrofuran was distilled to assure the absence of peroxides. Tetrabutylammonium hydrogen sulphate (TBA) was

obtained from the same source. Other reagents were of pro analysi quality (Merck, Darmstadt, FRG). Water was freshly distilled in glass apparatus.

HPLC Apparatus and Operating Conditions

Isocratic elution was used throughout the study. For the development of the method, the solvent delivery system consisted of a Milton Roy minipump (Laboratory Data Control, Riviera Beach, FL, USA) equipped with a pulse dampener and a Bourdon manometer as described previously (22). A model LC3 UV variable-wavelength detector (Pye Unicam, Cambridge, UK) was used with this pumping device. For quantitative work, a SP 8700 XR pump (Spectra Physics, San Jose, CA, USA) and a Model 440 detector (Waters Assoc., Milford, MA, USA) were used. Some experiments were carried out using a Waters diode array detector Model 990. The Model CV-6-UHPa-N60 injector (Valco, Houston, TX, USA) was equipped with a 20 µ l loop. UV detection was performed at 254 nm. A Model 3390 A (Hewlett-Packard, Avondale, PA, USA) device was used for recording and integration. The columns (25 cm x 0.46 cm ID) were packed in the laboratory with different poly(styrene-divinylbenzene)copolymer (PSDVB) materials : PLRP-S 8 µ m, 100 Å (Polymer Labs., Church Stretton, Shropshire, UK); PRP-1 10 µ m (Hamilton, Reno, NV, USA) and RoGel 7-9 µ m (RSL-Alltech Europe, Eke, Belgium). The packing procedure was described previously (22). The column was immersed in a water-bath at 60 °C and the flow rate was 1.0 ml/min. Each evening the pump was rinsed with methanol-water (50:50), but the column was not. The back pressure never exceeded 1500 p.s.i.

Some analyses for tetracycline were performed according to the USP method with columns (25 cm x 0.46 cm ID) packed with different reversed-phase materials : Nucleosil C8 10 µ m or Polygosil C8 10 µ m (Macherey-Nagel, Düren, FRG), Zorbax C8 7 µ m

(Du Pont, Wilmington, DE, USA), Partisil ODS3 10 μ m (Whatman, Maidstone, UK), LiChrosorb RP18 10 μ m (E. Merck) and μ Bondapak C18 10 μ m (Waters).

Mobile Phases

The mobile phase finally used for analysis was prepared as follows. A quantity of tert.-butanol (6.0 - 8.5 % m/v, determined by the brand of packing material) was weighed and rinsed into a volumetric flask and 10 % v/v of 0.2 M potassium hydrogen phosphate buffer pH 9.0, 15 % v/v of 0.02 M tetrabutylammonium hydrogen sulphate (TBA) and 10 % v/v of 0.01 M sodium ethylenediaminetetraacetate (EDTA) was added to this mixture. During preparation of the latter two solutions, the pH was adjusted to 9.0 with sodium hydroxide solution. The mobile phases were diluted with water and degassed by ultrasonication.

Sample Preparation

All samples were weighed or pipetted to obtain the equivalent of about 20 mg of TC.HCl. Bulk samples of TC or TC.HCl were dissolved and diluted to 25.0 ml with 0.01 N hydrochloric acid. Bulk samples of TC.laurylsulphate were dissolved in 3 ml of 0.05 N sodium hydroxide and diluted to 25.0 ml with water. Samples of capsules and tablets were diluted to 25.0 ml with 0.01 N hydrochloric acid, ultrasonicated for 5 min at room temperature and then centrifuged at 2500 g for 5 min. The supernatant liquid was filtered through a membrane filter with 1.2 μ m pores. Samples in solution were diluted with 0.01 N hydrochloric acid so as to contain about 20 mg of TC.HCl in 25.0 ml. Samples were prepared immediately before analysis. Acidified solutions of TC were stable for 5 h at room temperature

in daylight and at least for 24 h at 6 °C in the dark. During this period the ETC content increased by no more than 0.4 %. Alkaline solutions were stable for 5 h at room temperature in the dark and for 2 h in daylight. At 6 °C in the dark these solutions were stable for at least 24 h. Products of unknown structure were formed by decomposition in alkaline medium.

RESULTS AND DISCUSSION

Development of the Chromatographic Method

The development of the method was based upon experience previously obtained with the analysis of other tetracyclines (DOX, OTC) (20,21). Preliminary work was carried out on PRP-1. Among the organic modifiers examined, only tetrahydrofuran and tert.-butanol gave good separations. This was also observed for OTC (21). Mainly for practical reasons, tert.-butanol was finally retained as the organic modifier, as previously mentioned (22).

In the following experiments a PLRP-S column was used. Fig. 1 shows the influence of the pH on the separation. It is clear that at pH 9.0 a better separation of TC, ADTC and EATC is obtained. The small peak which was eluted right after the TC peak of commercial samples was identified as ADTC because it was coeluted with the main peak of the reference sample of ADTC and because the UV spectra of these peaks were identical as observed by diode array detection.

The retention increases directly with concentration of TBA in the mobile phase. The same behavior had been mentioned previously for DOX and OTC. As for these tetracyclines, a concentration of 5 % v/v of 0.02 M TBA was originally deemed suitable. In later experiments where 20-30 µ g amounts of TC

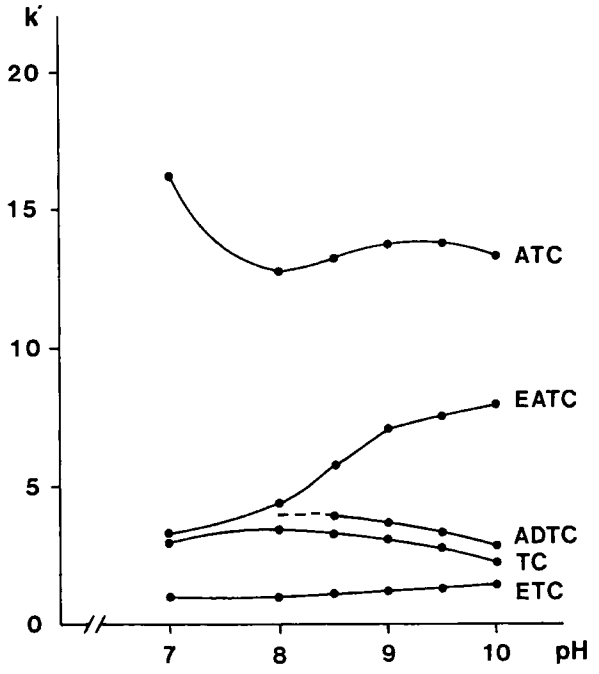

Figure 1.
Influence of pH of mobile phase on separation of tetracycline and related substances. Stationary phase : PLRP-S. Mobile phase : tert.-butanol (5.5 g) - 0.2 M phosphate buffer of indicated pH (10.0 ml) - 0.02 M tetrabutylammonium sulphate (5.0 ml) - 0.01 M sodium edetate (10.0 ml). The pH of latter two solutions adjusted to indicated pH with sodium hydroxide solution. Dilute to 100.0 ml with water. See experimental for other conditions.

were injected a splitting of the TC peak was observed whereby a secondary peak with a smaller k' value was formed. Peak splitting in reversed phase HPLC involving dynamic ion-exchange mechanisms has been discussed in the literature (26,27). No peak splitting occurred when 15 % v/v of 0.02 M TBA was added to the mobile phase. The effect of the phosphate buffer concentration has not been detailed. As in the case of DOX and OTC, retention decreases with increasing concentration to a level of 10 % v/v of 0.2 M phosphate buffer. At higher concentrations, retention does

not decrease further. In order to keep the total salt concentration at a minimum level, 10 % v/v of 0.2 M phosphate buffer was used hence. As in the analysis of DOX and OTC, the presence of EDTA in the mobile phase is necessary, otherwise the separation TC-ADTC deteriorates rapidly. The amount of EDTA chosen (10 % v/v 0.01 M) corresponds to that used for DOX (20). As previously mentioned for DOX and for OTC, a column temperature of 60 °C was used throughout the study. By decreasing the temperature to 50 °C, the column efficiency decreases from 2000 to 1400. By increasing the temperature above 60 °C, it does not increase significantly. The excellent repeatability mentioned in the calibration section below is an indication for the good stability of the sample during chromatography at 60 °C. Moreover, peak areas for TC obtained from 8 analyses at 50 °C, 60 °C and 70 °C respectively were not found to differ significantly.

Fig. 2 shows chromatograms obtained on the different PSDVB materials and using a mobile phase as described above. PRP-1 and PLRP-S materials behave in a very similar way. ADTC is somewhat better separated on PLRP-S, but this can be due partly to the smaller diameter of the PLRP-S particles. ADTC is distinctly better separated on the RoGel column where a larger concentration of tert.-butanol is needed to obtain the same retention time. On all the columns, the peaks from the main impurities ETC, EATC and ATC, for which TC samples are generally tested, are well separated. ADTC had never been mentioned before to be separated by HPLC. More extensively used columns tend to show somewhat shorter retention times. This can be adjusted by altering the concentration of the organic modifier but also, at least in part, by washing the column consecutively with water, 0.01 N phosphoric acid, methanol and dichloromethane. Filling in the small hollow space at the top of the column, as previously mentioned, was necessary in only a very few cases (22).

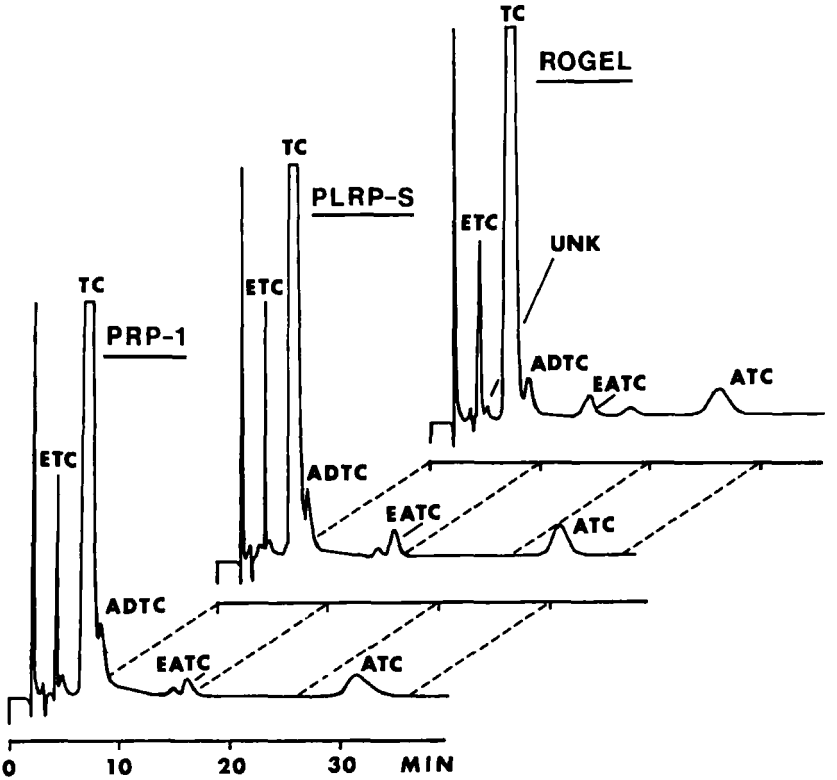

Figure 2.
Chromatograms of tetracycline sample on different poly(styrene-divinylbenzene)copolymer packing materials. Mobile phase : tert.-butanol (x g/100 ml) - 0.2 M phosphate buffer pH 9.0 (10.0 ml) - 0.02 M tetrabutylammonium sulphate pH 9.0 (15.0 ml) - 0.01 M sodium edetate pH 9.0 (10.0 ml) - water (up to 100.0 ml). Flow rate : 1.0 ml/min. Detection : UV at 254 nm. Temperature : 60 °C. Columns : 25 cm x 0.46 cm. PRP-1 10 µ m, x = 6.9; PLRP-S 8 µ m, x = 6.9; RoGel 7-9 µ m, x = 8.5. ETC = 2.5 % m/m, ADTC = 0.7 % m/m, EATC = 0.5 % m/m, ATC = 1.0 % m/m.
ETC = 4-epitetracycline, TC = tetracycline, ADTC = 2-acetyl-2-decarboxamidotetracycline, EATC = 4-epianhydrotetracycline, ATC = anhydrotetracycline.

Comparison with USP Methods

A HPLC method for ETC.HCl was proposed in Pharmacopeial Forum (28) for the analysis of tetracycline hydrochloride for topical solution, which is a mixture of TC.HCl and ETC.HCl. This method prescribes a mobile phase containing methanol-ammonium phosphate buffer pH 1.8 (20:80) and an ODS column comparable with the classical methods using acid mobile phases, referred to in the introduction. This isocratic method allows the separation of ETC and TC but EATC and ATC are not eluted. Therefore this method is not suitable for analysis of TC in general.

More recently the USP introduced a HPLC method for TC (15). The use of a octylsilane C8 column is prescribed and the mobile phase is dimethylformamide-0.1 M ammonium oxalate-0.2 M dibasic ammonium phosphate (27:68:5), with the pH adjusted to 7.6-7.7 if necessary. The order of elution is ETC, EATC, TC and ATC. Using Zorbax C8, Nucleosil C8 or Polygosil C8, it was not possible in our laboratory to separate EATC from TC, not even when the dimethylformamide (DMF) content in the mobile phase was reduced from 27 % to 13.5 %. DMF was replaced with water and the flow rate was reduced to 1 ml/min. Better results were obtained with C18 columns. ETC, EATC and ATC were separated from TC on Partisil ODS-3, LiChrosorb C18 or μ Bondapak in about 25 min. The latter gave the best chromatographic separation as shown in fig. 3. By decreasing the DMF content to 13.5 %, a small impurity immediately before the TC peak was partly separated. This impurity was tentatively identified by chromatography as ADTC. With 13.5 % of DMF, ADTC was also separated on LiChrosorb RP18 but not on Partisil ODS3. On these two columns the total analysis time was about one hour. This method also allows chlortetracycline (CTC) to be separated from TC but the elution order depends upon the brand of the packing material used. For example on Nucleosil C8, CTC was eluted before ATC, on Partisil

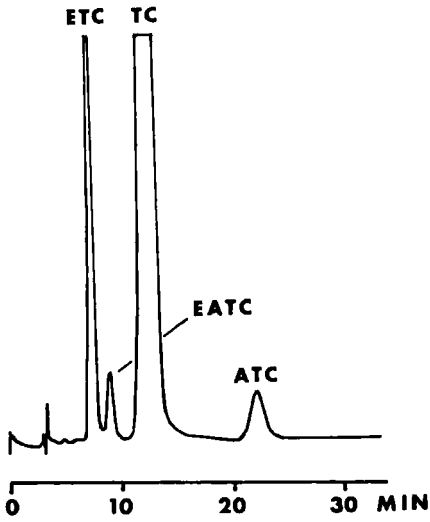

Figure 3.
Typical chromatogram of commercial tetracycline sample obtained by USP method (15). Stationary phase : μ Bondapak C18. Mobile phase : dimethylformamide - 0.1 M ammonium oxalate - 0.2 M diammonium hydrogen phosphate - water (21:68:5:6). Flow rate : 1.0 ml/min.

ODS-3 it ran together with ATC and on μ Bondapak it followed ATC. The major disadvantage of this method is the fact that it obviously is not applicable to all the columns generically described as octyl bonded. For C18 materials the results also depend upon the brand of packing material used. Another disadvantage is that ADTC is not separated, unless significantly longer analysis times of about one hour are accepted. As shown below, TC samples can contain up to 2 % of ADTC. Finally it must be emphasized that the analysis is performed at pH 7.7, a pH at which silica based reversed-phase materials lack stability. However, the use of a guard column provides some protection against decomposition of the column.

Advantages of the method using PSDVB materials are the applicability to the different polymer materials available on the

market, the stability of these materials under the operating conditions described above and the possibility to quantitate ADTC within a reasonable analysis time. One disadvantage of the method is that CTC can not be quantitated separately. Indeed, under the chromatographic conditions of pH and temperature described in this method, small amounts of CTC are completely transformed into isochlortetracycline (ISOCTC) (29). ISOCTC is eluted together with ADTC. The specific absorbance at 254 nm of ISOCTC in the mobile phase is about 1.1 times that of TC. However, it is better to have a combined result for ADTC and CTC than for CTC alone, obtainable with the USP method. Furthermore, CTC is an impurity which currently seems to occur rather rarely in TC samples. Tsuji et al. found levels of CTC below 0.05 % in TC.HCl bulk samples, except for some old samples (0.2 %) (30). In pharmaceutical preparations, Tsuji et al. always found less than the detection limit (0.3 %) (31). More recently Oka et al. found CTC (0.5 %) in only one pharmaceutical preparation, while for all other samples reported, the CTC level was below the detection limit (0.05 %) (32). The samples discussed in this paper and mentioned below in Tables 1 to 4 were also analysed with a method enabling the determination of CTC in TC. This method was developed for analysis of CTC samples and is discussed in detail elsewhere (33). The results confirm that in recent TC samples, CTC is present at a lower level than ADTC, but also indicate that CTC is not always a negligible impurity.

For reasons mentioned above, the method developed in this laboratory was adopted for subsequent use. All analyses were performed on a RoGel column.

Calibration Curves, Limits of Quantitation and Repeatability

The calibration curves were obtained with the TC.HCl house standard, found to contain 99.1 % of TC.HCl. The content of the

reference substances ETC.HCl, EATC.HCl and ATC.HCl was 98.1 %, 95.8 % and 95.7 % respectively, all expressed in terms of the hydrochloride salt. The determination of the purity of the house standard and the reference substances will be discussed elsewhere. The following relationships were found, where y = peak area, x = amount in micrograms of hydrochloride salt injected, r = correlation coefficient, $S_{y,x}$ = standard error of estimate, CR = range of injected mass examined. TC, y = 919 + 9128 x, r = 0.9992, $S_{y,x}$ = 625, CR = 12–18 μ g; ETC, y = 10 269 x, r = 0.9999, $S_{y,x}$ = 55, CR = up to 2 μ g; EATC, y = 23 890 x, r = 0.9995, $S_{y,x}$ = 175, CR = up to 0.5 μ g; ATC, y = 18 052 x, r = 0.9999, $S_{y,x}$ = 233, CR = up to 2 μ g.

The limits of quantitation were 0.01 % for ETC, 0.05 % for ADTC and EATC and 0.1 % for ATC, all calculated against the total amount injected (about 15 μ g of TC). Since the ADTC sample available was not chromatographically pure, the limit of quantitation for ADTC was determined by injecting mixtures of two solutions, one containing a TC.HCl sample with 0.5 % ADTC.HCl, the other containing the pure TC.HCl house standard. The house standard was analysed 38 times over a period of 6 days. The relative standard deviation (RSD) for TC was 0.4 %.

Comparison of Tetracycline Standards

The TC.HCl house standard was titrated with perchloric acid in non-aqueous medium. Three independent series of titrations gave a mean of 99.5 % TC.HCl (RSD = 0.4 %) for a total number of 18 titrations. Three independent series of Karl Fischer titrations gave a mean of 0.4 % water (RSD = 8.4 %) for a total number of 20 titrations. This result was confirmed by loss on drying (see Table 1). The total content of the TC.HCl house standard was therefore labelled nominally to be 99.5 % and this value was corrected for results obtained from HPLC by use of

calibration curves for the likely impurities. The total content of chromatographic impurities amounted to 0.4 %, and therefore the TC.HCl house standard was considered to contain 99.1 % of TC.HCl. ADTC was calculated in terms of TC.HCl, because no pure reference substance was available. For this purpose, the peak area ratio ADTC/TC in each chromatogram was used. The UV spectra of TC and ADTC were reported to be similar (24). The impurity of unknown identity (UNK), which eluted immediately after ETC, was calculated in terms of ETC.HCl. Because this method of expression was chosen arbitrarily, the result was not included in the subtotal. As verified by chromatographic retention, UNK is not oxytetracycline, a possible fermentation impurity which elutes just before ETC. Demethyltetracycline (DMTC) was coeluted with UNK but the identity of this minor impurity was not further investigated. To the authors knowledge DMTC was never mentioned as a possible impurity in the fermentation of tetracycline.

The content of the official standards was determined by HPLC by using the TC.HCl house standard as an external standard; table 1 summarizes the results obtained. The TC.HCl content was determined on several days by direct comparison with the chromatograms obtained the same day for the TC.HCl house standard. The impurities were determined as mentioned above for the TC.HCl house standard. The RSD values, given in parentheses below the assay values, are within acceptable limits for all determinations. The USP-RS contains 2.2 % of ETC.HCl and is less pure than the other standards. The unkown impurity (UNK) is present in all standards, and EATC.HCl is present in 0.1 % or less, indeed very low amounts in all standards. The USP-RS and WHO-IS contain somewhat more ATC.HCl than the other standards. The CTC.HCl content of all the standards did not exceed 0.05 %, as determined by another HPLC method (33). For all standards the subtotal is close to 100 %. Non-aqueous titration, Karl Fischer titration and loss on drying were not performed on the official standards, because of the amount of sample available was limited.

Table 1
Composition of Tetracycline Hydrochloride Standards

	House Standard	Ph Eur-CRS 972 IU/mg	USP-RS I-1 990 μ g/mg	WHO-IS (second) 982 IU/mg
Number of solutions	38	18	6	6
Number of analyses	38	18	12	12
Number of days	6	6	5	4
ETC	0.2	0.8	2.2	0.9
	(6.8)	(6.5)	(4.6)	(3.0)
UNK[a]	0.04	0.09	0.2	0.04
	(16.2)	(9.1)	(3.3)	(18.6)
ADTC	< 0.05	< 0.05	< 0.05	< 0.05
EATC	0.06	< 0.05	0.1	< 0.05
	(32.1)		(18.0)	
ATC	0.1	0.2	0.5	0.5
	(13.0)	(10.1)	(3.9)	(2.9)
TC	99.1	98.9	97.0	98.5
	(0.4)	(0.5)	(0.3)	(0.4)
Subtotal	99.5	99.9	99.8	99.9
Titration	99.5	ND	ND	ND
n (RSD)	18 (0.4)			
Water determined[b]	0.4	ND	ND	ND
n (RSD)	20 (8.4)			
Loss on drying determined	0.3[c]	ND	ND	ND
n (RSD)	3 (17.8)			
Loss on drying declared		0.3[c]	0.8[c]	0
		(34)	(35)	(36)

Values in percent (m/m) expressed in terms of the hydrochloride; n = number of analyses; RSD values are given in parentheses; ND = not determined owing to the limited amount of sample; UNK = unknown; NA = not available; [a]expressed in terms of ETC.HCl, not included in the subtotal; [b]Karl Fischer; [c]at 60 °C for 3 hours over P_2O_5 under vacuum.

The amount of water and volatile substances contributes little to the total mass of the standards. The accumulated total mass obtained from the analytical values available is always close to 100 %. The declared content for the USP-RS of 990 μ g/mg (expressed as the hydrochloride) is an overestimation because it contains only 97.0 % of TC.HCl. In comparison, other standards would therefore contain more than 100 μ g/mg, which is not theoretically possible if the micrograms are actual mass units. In fact the micrograms reported by the USP have to be interpreted as micrograms of activity. This can be a source of confusion (37). The Ph Eur-CRS (972 IU/mg) and the WHO-IS (982 IU/mg), which are of about the same declared activity as determined by microbiological assay, have indeed an equivalent content.

Analysis of Commercial Samples

Commercial samples were analyzed by the method described above for the standards. Table 2 shows some of the results obtained for TC.HCl bulk samples. For simplicity, RSD values for the impurities are not mentioned. A repeatability of less than 0.5 % for the TC assay is considered very good. The samples contain up to 2.4 % of ETC and up to 1 % of ADTC. EATC is present at a level up to 0.4 % and ATC up to 0.5 %. The Ph.Eur. prescribes limits of 5 % for ETC and 0.5 % for EATC and ATC (38). The USP limits the amount of EATC to 2 % (15). The impurity UNK mentioned in Table 1 is also present in commercial samples of TC.HCl. In most samples, the content of CTC is lower than that of ADTC. These results indicate that CTC is not always a negligible impurity. The amount of impurities present in all samples examined is well below the levels allowed, except for ATC. The water content is always found to be below the Ph.Eur. and USP limits (2 %). Owing to the rather limited amount of

Table 2
Composition of Bulk Samples of Tetracycline Hydrochloride

Sample origin	TC	ETC	UNK[a,b]	ADTC	EATC	ATC	CTC[b,c]	H_2O	Total %
Manufacturer									
B	96.8 (0.2)	1.9	0.05	0.3	0.05	0.2	0.02	1.2 (0.9)	100.4
Wholesalers									
1	95.5 (0.3)	2.4	0.2	0.9	0.08	0.4	0.02	1.4 (2.6)	100.7
2	96.0 (0.4)	1.4	0.2	0.4	0.2	0.3	0.08	1.4 (0.8)	99.7
3	96.2 (0.4)	2.2	0.2	0.4	0.4	0.5	0.05	1.0 (0.6)	100.7
4	96.1 (0.5)	1.5	0.2	0.6	0.07	0.3	0.15	1.0 (7.2)	99.6
5	96.6 (0.3)	1.8	0.2	0.5	0.05	0.3	0.5	0.9 (1.8)	100.1
6	96.7 (0.4)	1.4	0.06	0.5	0.2	0.3	0.08	1.4 (2.9)	100.5
7	96.4 (0.5)	1.5	0.2	1.0	0.09	0.3	0.09	0.9 (2.7)	100.2
8	97.6 (0.5)	1.2	0.08	0.6	0.05	0.2	0.08	0.9 (1.7)	100.5
9	97.7 (0.5)	1.1	0.09	0.4	0.05	0.1	0.13	0.8 (3.3)	100.1

Values in percent (m/m), expressed in terms of the hydrochloride, are the mean of 3 analyses; KF = Karl Fischer; UNK = unknown; [a]expressed in terms of ETC.HCl; [b]not included in the grand total; [c]determined by another HPLC method (33); RSD values are given in parentheses.

Table 3
Composition of Bulk Samples of Tetracycline Base

Manufacturer	TC	ETC	UNK[a]	ADTC	EATC	ATC	H_2O	Total %
A	77.1 (0.3)	5.8	0.4	2.0	0.2	0.7	10.3 (0.3)	96.1
B	76.5 (0.4)	5.6	0.2	0.5	0.1	1.1	6.1 (1.4)	89.9
C	79.5 (0.2)	6.3	0.2	1.5	0.2	1.2	8.2 (0.4)	96.9
D	82.8 (0.3)	4.7	0.2	0.7	0.07	0.7	7.3 (0.4)	96.3

Values in percent (m/m), expressed in terms of the base, are the mean of 3 analyses; KF = Karl Fischer; UNK = unknown;
[a]expressed in terms of ETC, not included in the total base content; RSD values are given in parentheses.

sample available, Karl Fischer titrations were performed instead of loss on drying, which is prescribed by the official compendia. The total content is nearly 100 % for all samples. The impurity UNK is expressed as ETC.HCl but is not included in the grand total as was the case for the standards.

Table 3 shows some of the results obtained for bulk samples of tetracycline base (TC), represented like Table 2. The Ph Eur provides for a 5 % limit for ETC and a 0.5 % limit for EATC and ATC (39). The USP limits the amount of EATC to 2 % (15). The ETC limit is exceeded in some samples, but EATC results are well within limits. All samples exceed the Ph Eur limit prescribed for ATC. Figures for the CTC content were not obtained for the TC base samples because the CTC peak was not clearly resolved from other small impurities of unknown identity which are present in all the commercial TC samples. The Ph Eur and USP limit the

water content to 13 %. As for the hydrochloride salts Karl Fischer titration was used instead of the official method which is loss on drying. Water is found to be within limits for all samples. It was observed that several samples in screw cap containers took up moisture upon storage in the laboratory. In most samples the total content is distinctly less than 100 %. This indicates that not all the impurities are detected by the HPCL system.

Table 4 shows some of the results obtained from the analysis of bulk samples of TC laurylsulphate. ETC is found to be present at a level up to 3 % in most of the samples. Some samples contain more than 10 % of ETC; ADTC is present up to 0.5 % and the concentration of impurity UNK is calculated as noted above. Most samples contain less than 0.5 % of EATC which is substantially below the 2 % limit cited in the USP (15). In one sample this EATC limit is exceeded, because about half of this sample was transformed to ATC. In TC laurylsulphate ATC is an important impurity. In most samples, the CTC content is also found to be smaller than the ADTC content. The content totals nearly 100 % for all samples which indicates that the HPLC method is capable of detecting all degradation products, in this case.

Tablets, capsules, veterinary oral powders, human and veterinary gynaecological tablets and veterinary solutions were also analysed. The USP limits the amount of EATC in pharmaceutical dosage forms to 3 % (15). Even the USP limit of 2 % EATC for bulk samples was never exceeded in the specialties examined. Solid samples contained up to 4.4 % of ETC, up to 2.3 % of ADTC and up to 2.2 % of ATC. Most of the solid samples had a TC content between 95 % and 110 %. The USP limits for formulated preparations are 90 % to 125 % (15). The content of solutions containing TC base was found to be distinctly low. Only 8 months after preparation, liquid samples kept at pH 4.2 contained up to 63.5 % of ETC and up to 3.5 % of ATC. Seven

Table 4
Composition of Bulk Samples of Tetracycline Laurylsulphate

Sample origin Wholesalers	TC	ETC	UNK[a,b]	ADTC	EATC	ATC	CTC[b,c]	H_2O	Total
1	95.0 (0.2)	2.1	0.08	0.3	0.06	1.0	0.02	1.5 (3.7)	100.0
3	86.5 (0.5)	2.1	0.1	0.2	0.3	9.3	0.08	2.7 (0)	101.2
4	83.9 (0.4)	11.2	0.05	0.2	0.4	2.6	0.04	3.0 (0)	101.3
7	40.8 (0.9)	1.8	0.1	0.1	2.4	51.7	0.03	4.0 (2.0)	100.8
9	89.0 (0.4)	1.8	0.02	0.5	0.3	7.1	0.02	2.5 (5.6)	101.2
10	76.0 (0.1)	14.1	0.06	0.2	0.07	7.7	0.1	2.5 (0.2)	100.7

Values in percent (m/m), expressed in terms of the laurylsulphate, [a]are the mean of 3 analyses; [b]KF = Karl Fischer; UNK = unknown; [a]expressed in terms of ETC; [b]not included in the grand total; [c]determined by another HPLC method (33); RSD values are given in parentheses.

months after preparation a liquid sample of pH 2.2 contained 14.6 % of ETC and 5.4 % of ATC. TC distinctly is not the tetracycline of choice for the preparation of solutions.

The results obtained have shown that the HPLC method described above is most appropriate for the analysis of tetracycline in bulk samples and in pharmaceutical preparations.

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