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QUANTITATIVE ANALYSIS OF RAW MATERIAL OF TETRACYCLINE AND ITS RELATED SUBSTANCES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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An isocratic method for the analysis of tetracycline (TC) by high performance liquid chromatography (HPLC) using polystyrene-divinylbenzene copolymer packing material is described. The method allows the complete separation of TC, 4-epitetracycline, anhydrotetracycline (ATC), 4-epianhydrotetracycline (EATC). A fermentation impurity, 2-acetyl-2-decarboxamidetetracycline is also resolved from TC. The mobile phase combines tert.-butanol, water and phosphate buffer, tetrabutylammonium sulphate and sodium ethylenediaminetetraacetate at pH 9.0 for elution at a temperature of 60℃. Among the organic modifiers examined, only tetrahydrofuran and tert.-butanol gave good results. For practical reasons, tert.-butanol was retained as the final organic modifier. The preliminary experiments were carried out on polystyrene-divinylbenzene copolymer packing material (PRP-I and PLRP-S columns). The selectivity of the columns is comparable. PLRP-S column was chosen as it shows better separation of tetracycline – anhydrotetracycline. The method was used to analyse the commercial samples.

Keywords: *Tetracycline, High performance liquid chromatography, Polystyrene–divinylbenzene copolymer stationary phase*

INTRODUCTION

Tetracycline (TC) was first prepared by the catalytic reduction of chlortetracycline (Boothe *et al.* 1953; Conover *et al.* 1953). TC has also been reported to be produced from the Strains of *Streptomyces aureofaciens, Streptomyces avellanus, Streptomycs feofaciens, Streptomyces alboflavus* and many others (Weinstein and Wagman 1978). Among the TC group of antibiotics, TC is the most widely used in therapeutics.

TC has proved to be safe and effective in numerous commonly encountered infections and also in some specific cases of cholera, trachoma, atypical pneumonia, acne, conjunctivitis, plague and many other diseases. TC is used in the form of capsules, oral liquid

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and suppositories. Intramuscular injections are rather painful. TC is also used as an additive in agriculture and as a food additive in poultry and food-producing species (Ashworth 1985).

TC is the first member of this class of compounds whose structure was elucidated (Stephens *et al.* 1954). TC is a broad spectrum antibiotic having an identical 4-ring carboxyclic hydronaphthacene structure as a basic skeleton. The TC itself differs from other TC group of antibiotics chemically only by substituent variation (Mitscher 1978).

The molecule of TC retains its antimicrobial activity when substitution is made at position 5 to 9 (Mitscher 1978). Replacing the carboxamide group at position 2 by acetyl group, the therapeutic activity is decreased. Even by replacing one of the hydrogen of carboxamide by another group, the therapeutic activity is reduced. When any replacement is made at other positions, the therapeutic activity is completely lost (Mitscher 1978) as indicated below.

i. TC undergoes epimerization at position C-4, resulting in the formation of 4-epitetracycline (Mitscher 1978).
ii. Due to the presence of a hydroxyl group at C-6, acid degradation takes place resulting in the formation of anhydrotetracycline (ATC). This anhydroderivative also undergoes epimerization at C-4, resulting in the formation of 4-epianhydrotetracycline (EATC) (Mitscher 1978). This degradation product can also be formed by acid degradation of 4-epitetracycline (ETC) (Miller and Hochstein 1962).
iii. Another related compound, 2-acetyl-2-decarboxamidetetracycline was also described (Miller and Hochstein 1962). This compound was found to be a fermentation impurity of TC (Lancini and Sensi 1964; Keiner, Huttenrauch and Poethke 1967).
iv. The TC in neutral or slightly acidic conditions (pH 3 to 7) exits as zwitterions, and is amphoteric and has two acids and one basic functional groups (McCormick *et al.* 1957). The basicity of TC is due to the presence of the dimethylamine group at C-4. The acidity and basicity of TC is shown by pKa values (Stephens *et al.* 1956; Leeson, Kruger and Nash 1963; Benet and Goyanu 1965).

The high performance liquid chromatography (HPLC) of TC and its degradation products has been discussed extensively in the literature. A number of papers described the separation of TC in biological samples (Bruce, Cole and Ravenscroft 1981; Hermansson 1982; Onji, Uno and Tanigawa 1984; Krystyna and Arthur 1986; Moats 1986; Oka *et al.* 1987)

and in food (Ashworth 1985; Oka *et al.* 1987). Some of the early papers described the separation of TC and its degradation products on ion exchange materials (Butterfield *et al.* 1973; Sokoloski *et al.* 1977; Bagon 1979) and other methods using silica based reversed phase materials with acid mobile phase (Knox and Jurand 1975, 1979; Knox and Pryde 1975; Knox, Jurand and Pryde 1976; Tsuji and Goetz 1978; Steinbach and Strittmatter 1978; Oka *et al.* 1985).

Recently, some papers related to the use of polystyrene-divinylbenzene copolymer column with mobile phases at alkaline pH, were published for the high – performance liquid chromatographic separation of tetracyclines (Naeem *et al.* 1987, 1989a, 1989b, 1992; Hoogmartens *et al.* 1989).

The preliminary work was carried out by (Kabala *et al.* 1982) in developing the HPLC method for the purity control of TC, using reversed phase material as a stationary phase. But the 4-epimers of TC were not separated completely. This method needs improvement in order to achieve faster and better separation for the purity control and assay of TC by HPLC method. The present study is aimed to modify the above mentioned method which could separate the 4-epimers and anhydro derivative of TC in a resonable manner.

METHODS

HPLC of TC Solvent and Reagents

Organic solvents were from Janssen Chimica (Beerse, Belgium). Tertiary butanol (tert.-but.) and tetrabutylammonium hydrogen sulphate was obtained from the same manufacturer. Karl Fischer and other reagents were of pro analysis quality (Merck, Darmstadt, F.R.G.). Methanol of the HPLC grade was from Ruthburns, The U.K. Water was freshly distilled from glass apparatus in the laboratory.

Apparatus and HPLC Operating Conditions

Isocratic elution was used throughout the study. The solvent delivery system used for the experiments, consisted of SP 8700 XR pump from Spectra Physics, (San Jose, CA, U.S.A). The detector Model 440 was from Waters Assoc., (Milford, MA, U.S.A). The injector (for sample injection) was a Model of CV-6 UHPa-N60 from Valco (Houston, TX, U.S.A), which

was equipped with a 20 µl loop. The UV detection was performed at 254 nm. The recording and integration was carried out on an integratar Model 3390A (Hewlett-Packard, Avondale, PA, U.S.A).

The stainlessteel column 250mm x 4.6 prepacked with polystyrene-divinylbenzene copolymer material, (PLRP-S 8 µm, 100 Å) was purchased from Polymer Labs. (Church Stretton, Shropshire, U.K).

The column was immersed in a waterbath at 60°C and the flow rate was kept at 1.0 ml/min. Every evening the pump and the column was washed with methanol-water (50:50) for about 10 minutes in order to expel the impurities and other materials from the system. The back pressure of the system remained lower than 1500 psi during the experiments.

The pore size of filters used was 0.45 µm and were cleaned by sonication with methanol every third day. For water determination, Metrohm 633 Karl-Fischer Automat (Metrohm, Herisau, Switzerland), equipped with a 645 Multi-Dosimat and a multi-Burette E 485 was used. Glass fabricated sample weighing bottles with covers were purchased from the local market.

Mobile Phase

The mobile phase used for the analysis was prepared as follows.

Solution 1
0.2M solutions of dipotassium hydrogen phosphate and monopotassium dihydrogen phosphate were prepared separately in distilled water. The buffer solution of potassium hydrogen phosphate was brought to pH 9.0 by mixing sufficient quantities of dipotassium hydrogen phosphate into the monopotassium dihydrogen phosphate solution.

Solution 2
A solution of 0.02M tetrabutylammonium hydrogen sulphate in distilled water was prepared and made alkaline with sodium hydroxide solution to pH 9.0.

Solution 3
0.01M sodium ethylenediaminetetraacetate solution was prepared in distilled water and made alkaline with sodium hydroxide solution to pH 9.0.

8.5% m/v of tert.-but was weighed directly into a volumetric flask. A 10% v/v of (buffer solution) *Solution 1*, 15% v/v of *Solution 2*, and 10% v/v of *Solution 3* were then added and mixed by shaking. The volume was made up with distilled water. Mobile phase was degassed by sonication.

Reference Standards

The reference standard of ETC, EATC, ATC and TC secondary standard were obtained from Janssen Chimica (Beerse, Belgium).

In the following experiments PLRP-S column was used. Figure 1 shows the influence of the pH on the separation. pH 9.0 was retained, since at this pH, a better separation of TC/2-acetyl decarboxamide-tetracycline (ADTC) and EATC was obtained while the total analysis time did not increase considerably. The retention increased with increasing concentration of tetrabutylammonium hydrogen sulphate. A concentration of 5% v/v of 0.02M tetrabutylammonium hydrogen

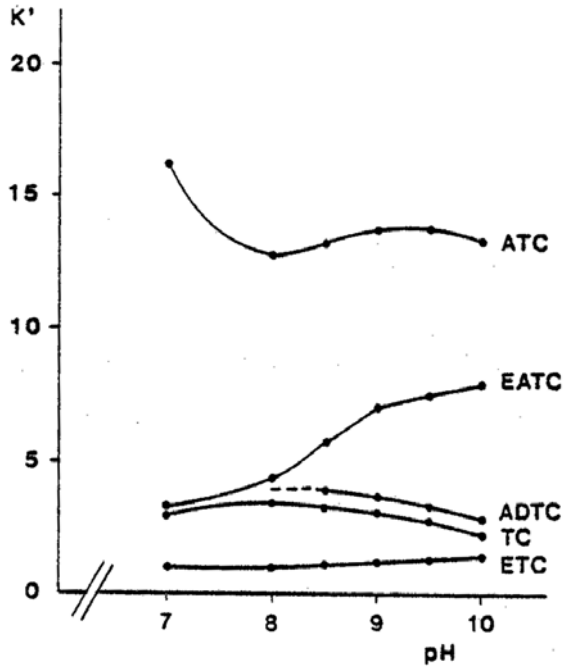

Fig. 1: Influence of the pH of the mobile phase on the separation of TC and related substances.

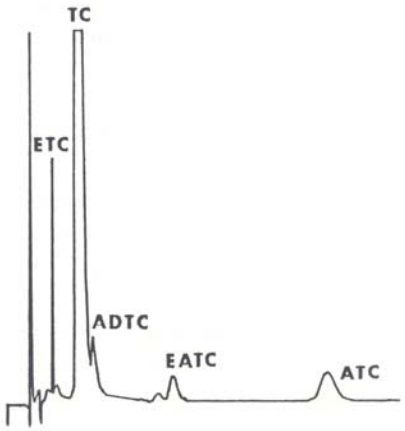

Fig. 2: Separation of TC and its related substances on PLRP-S column.

sulphate was originally chosen as suitable. In order to keep the total salt concentration at a minimum level, a content of 10% v/v of 0.2M phosphate buffer was used. The presence of sodium ethylenediaminetetraacetate (sodium edetate) in the mobile phase is necessary, otherwise the separation of TC-ADTC rapidly deteriorates. This was determined by comparing the separation without the use of sodium ethylenediaminetetraacetate. The amount of sodium edetate chosen was 10% v/v of 0.01M. Temperature of the column was kept at 60°C throughout the study. By decreasing the temperature to 50°C or increasing the temperature above 60°C, the separation obtained was not appreciable. No degradation of the product has been observed as the sample passes through the column at 60°C for a very short time.

RESULTS AND DISCUSSION

Figure 2 shows the complete separation of TC and its related substances on PLRP–S column. The analytical method for TC, using a mobile phase of pH 9.0 separates the potential impurities. The results are very satisfactory with the use of a stable polystyrene-divinylbenzene copolymer stationary phase. The analysis by the described method can be performed in a better way than classical silica based reversed phase materials. The silica based reversed phase materials did not give better separation of TC and in general the silica based reversed phase materials are not quite stable at elevated pH. This method has advantages for its selectivity, general applicability and stability.

Calibration Curves and Reproducibility (Validation)

The calibration curves were obtained with the TC HCl house standard (purity 99.1%). The content of the reference substances ETC HCl , EATC HCl and ATC HCl was 98.1%, 95.8% and 95.7% respectively(expressed in hydrochloride salt). The following relationships were found, where y = peak area, x = amounts 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 = 16–20 µg ;
ETC , y = 10 269 x , r = 0.9999, S(y,x) = 55, CR = upto 2 µg ;
EATC , y = 23 890 x , r = 0.9995, S(y,x) = 175, CR = upto 0.5 µg ;
ATC , y = 18 052x , r = 0.9999 , S(y,x) = 233, CR = upto 2 µg ;

The detection limits were 0.01% for ETC, 0.05% for EATC and 0.1% for ATC. The house standard was analysed 38 times over a period of nine days. The relative standard deviation (RSD) for TC was 0.4%.

Analysis of Bulk Samples of TC Hydrochloride

The commercial bulk samples of TC were analyzed as described under the above conditions. Table 1 shows the results for TC hydrochloride bulk samples. The reproducibility of the TC assay is very good. The samples contain up to 2.4% of ETC and up to 1% of ADTC. EATC is present up to 0.4% and ATC up to 0.5%. The European Pharmacopoeia (1983) prescribes limit of 5% ETC and 0.5% for ATC and EATC. The United States Pharmacopeia XXI (1985) limits the amount of EATC to 2%. The unknown impurity (UNK) is also present in the TC hydrochloride commercial samples. The amount of impurities present in all samples examined is far below the levels prescribed, except for ATC. The water content was found to be within European Pharmacopoeia and United States Pharmacopeia limits (2%). The total content is close to 100% for all the samples. Here the UNK is expressed as ETC but is not included in the grand total.

It is observed that TC hydrochloride bulk samples are very stable. No significant difference in term of contents of epimer and acid degradation products is observed between the fresh and the old batches. For

Table 1: Composition of bulk samples of TC hydrochloride

Sample Number	Age of sample in *months	TC %	ETC	UNK	AD TC	EATC	ATC	Water K.F	Total %
1	NM	96.8	1.90	0.05	0.30	0.05	0.20	1.20	100.50
5	28	96.6	1.70	0.20	0.50	0.03	0.20	0.90	99.10
10	41	95.5	2.40	0.20	0.90	0.08	0.40	1.40	100.80
12	49	96.2	1.80	0.20	0.40	0.20	0.30	1.10	100.20
19	52	98.1	0.80	0.09	0.30	0.10	0.10	1.60	101.10
20	32	96.8	1.10	0.04	0.50	0.10	0.50	1.30	100.30
23	20	97.4	0.90	0.10	0.60	0.07	0.20	1.10	100.40
2	30	96.0	1.40	0.20	0.40	0.20	0.30	1.40	100.00
3	16	97.6	1.50	0.20	0.60	0.06	0.20	1.00	101.10
15	31	96.2	2.20	0.20	0.40	0.40	0.50	1.00	100.70
16	40	96.1	1.20	0.08	0.40	0.20	0.20	1.80	100.00
17	44	96.1	1.50	0.20	0.60	0.07	0.30	1.00	100.60
48	22	96.8	1.60	0.10	0.50	0.09	0.20	0.80	100.00
4	NM	96.6	1.80	0.20	0.50	0.05	0.30	0.90	100.30
18	NM	96.7	1.40	0.06	0.50	0.20	0.30	1.40	100.50
21	NM	96.4	1.50	0.20	1.00	0.09	0.30	0.90	101.40
50	9	97.6	1.20	0.08	0.60	0.05	0.20	0.90	100.60
51	6	97.1	1.30	0.10	0.70	0.10	0.30	0.60	100.20
49	28	97.7	1.10	0.09	0.40	0.05	0.10	0.80	100.20

Values in percent (m/m) expressed in terms of the hydrochloride; NM = not mentioned; UNK = unknown expressed in terms of ETC.HCl; * from the date of manufacture. TC = tetracycline; ETC = 4-epitetracycline; ADTC = 2-acetyl-2-decarboxamidotetracycline; ATC = anhydrotetracycline; EATC = 4-epianhydrotetracycline; K. F. = Karl Fischer ; RSD = Relative standard deviation.

example, sample 19 being 52 months old contains 0.8% of ETC and sample 51 being 6 months old contains 1.3% of ETC. The differences observed are probably due to the conditions of preparation rather than to the sample age.

Analysis of Bulk Samples of TC Base

Table 2 shows the results for bulk samples of TC base, represented in the same way as in Table 1. For some samples (27, 57, 61), the ETC limit is exceeded but EATC results are well within the limits.

Table 2: Composition of bulk samples of TC base

Sample Number	TC %	ETC	UNK	ADTC	EATC	ATC	Water (K.F.) %	Total %
27	77.1	5.8	0.4	2.0	0.2	0.7	10.3	96.5
57	76.5	5.6	0.2	0.5	0.1	1.1	6.1	90.1
58	93.7	0.9	0.2	0.3	<0.05	0.3	3.2	98.6
59	85.8	4.3	0.2	1.1	0.07	0.9	5.2	97.6
60	83.4	4.5	0.2	1.3	0.06	0.9	6.2	96.5
61	79.5	6.3	0.2	1.5	0.2	1.2	8.2	97.1
62	89.2	4.0	0.3	1.3	0.1	0.6	3.7	99.2
63	82.8	4.7	0.2	0.7	0.07	0.7	7.3	96.0
64	82.6	4.5	0.2	1.3	0.1	0.6	8.1	97.4

Values in percent (m/m) expressed in terms of the base; UNK = unknown expressed in terms of ETC; TC = tetracycline; ETC = 4-epitetracycline; ADTC = 2-acetyl-2-decarboxamidotetracycline; ATC = anhydrotetracycline; EATC = 4-epianhydrotetracyclin; K.F. = Karl Fischer; RSD = Relative standard deviation

Almost all samples exceeded the European Pharmacopoeia limit prescribed for ATC since these contain up to 1.2% of ATC. The European Pharmacopoeia and United States Pharmacopeia limit the water content up to 13%. Water is found to be within limits for all samples. It was observed that several samples took up moisture upon storage in the laboratory. Repeated HPLC and Karl Fischer determinations confirmed the total contents (Table 2).

In most of the samples the total content is distinctly less than 100%, for sample 57 it is only 89.1%. Non-aqueous titration of the base content gave results comparable with HPLC except for sample 57.

In order to examine this phenomenon of low total content, a TC base sample was prepared by neutralizing a solution of commercial TC hydrochloride to pH 5.5 with aqueous sodium hydroxide solution. The precipitate was washed and then kept in the laboratory atmosphere untill constant weight. For this sample, the total base content obtained by HPLC (78.7%) corresponds well with the base content obtained by non-aqueous titration in acetic acid with perchloric acid (78.7%). The sample contained 20.5% water and the total content (99.2%) was close to the theoretical value.

Semi-micro determination of water content was carried out following the European Pharmacopoeia (1983). The weighing bottles were washed with distilled water, rinsed with ethanol and dried in an oven at 120°C for 2 hours. Then these bottles were cooled down in a dessicator over

diphosphorus pentaoxide. The sample was weighed in a weighing bottle and closed immediately with the cover to avoid penetration of moisture into the weighed sample. The sample was then transferred into the automatic titration flask through a special chamber and titration was carried out following the European Pharmacopoeia (1983).

The samples of hydrochloride salt contain less anhydroderivatives as compared to the base samples. The reason for this may be that, base samples contain more water content (up to 13% limit according to The United States Pharmacopiea XXI (1985)) as compared to the hydrochloride salts. The anhydroderivatives are well separated from the other components. The separation of 4-epimer is important because it decreases the biological activity which is also completely separated from the tetracycline.

CONCLUSION

The HPLC method for the analysis of tetracycline enables accurate analysis for purity control of raw material and its preparations. The results obtained have shown that the HPLC method described is suitable for quantitative analysis of tetracycline in bulk, pharmaceutical preparation and biological samples.

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