Note

Influence of temperature on the stability of solid tetracycline hydrochloride, measured by high-performance liquid chromatography

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The stability of tetracycline in aqueous solution has been examined extensively. Earlier analytical work was performed by microbiological and spectrophotometric methods[1-7]. Nuclear magnetic resonance spectroscopy and high-performance liquid chromatography (HPLC) have also been used[8,9]. The stability of tetracycline in methanol solution has also been examined, by paper chromatography[10] and recently by HPLC[11]. The stability of solid samples of tetracycline hydrochloride, tetracycline base and tetracycline phosphate. aged at room temperature for up to 8 years, has been followed[12,13]. No appreciable decomposition was observed. except for tetracycline phosphate. which showed a significant increase of 4-epitetracycline and anhydrotetracycline after a period of 4 years. No data are available on the stability at temperatures above room temperature.

In this paper we report on the stability of tetracycline hydrochloride during conservation in the solid state for about two years, at temperatures of 37°C, 50°C and 70°C. HPLC was chosen as the analytical method.

EXPERIMENTAL

Samples

Commercial tetracycline hydrochloride was kindly donated by Mr. G. Spaas, Kela Laboratorium. Hoogstraten. Belgium. Over a period of 27 months, and with intervals of about 2 months, small samples in vials with screw-caps were put in ovens at temperatures of 37°C, 50°C and 70°C. Meanwhile. the bulk of the tetracycline hydrochloride was kept at room temperature. in a well closed container. When tetracycline hydrochloride samples were kept at room temperature over a period of 8 years, no significant decomposition was observed[13]. The bulk sample was therefore considered to be stable at room temperature, over the period covered by our experiment.

Chemical reference substances (CRS) of the European Pharmacopoeia were used to prepare calibration curves for the hydrochlorides of tetracycline (TC), 4-epitetracycline (ETC), anhydrotetracycline (ATC) and 4-epianhydrotetracycline (EATC), whose chemical structures are shown in Fig. 1.
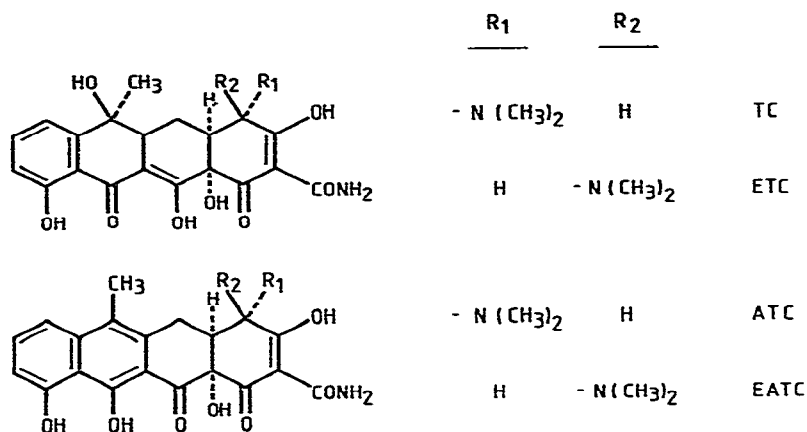
	R_1	R_2	
	$- N (CH_3)_2$	H	TC
	H	$- N (CH_3)_2$	ETC
	$- N (CH_3)_2$	H	ATC
	H	$- N (CH_3)_2$	EATC

Fig. 1. Structures. TC = Tetracycline; ETC = 4-epitetracycline; ATC = anhydrotetracycline; EATC = 4-epianhydrotetracycline.

Apparatus and reagents for HPLC

The HPLC apparatus consisted of a Varian Model LC 4200 chromatograph with gradient elution (Varian, Palo Alto, CA, U.S.A.), a Waters Model U6K injector (Waters Assoc., Milford, MA, U.S.A.), a Pye Unicam Model LC 3 UV detector (Pye Unicam, Cambridge, Great Britain), a Hewlett-Packard Model 3390 A integrator (Hewlett-Packard, Avondale, PA, U.S.A.) and a 25 cm × 4.6 mm I.D. column, packed with μBondapak phenyl (Waters).

Methanol, reinst (E. Merck, Darmstadt, G.F.R.), was purified by refluxing 1 l for 3 h in the presence of 10 g of silver nitrate and 20 g of potassium hydroxide, followed by distillation. Tap-water was distilled twice before use. Phosphoric acid was of pro analysi quality (E. Merck); 4-hydroxybenzoic acid, purissimum (Fluka, Buchs, Switzerland), was used as the internal standard (IS).

HPLC column packing procedure, mobile phases and operating conditions

Packing material (2.8 g) was suspended in 15 ml of carbon tetrachloride, the suspension was sonicated for 3 min and packed into the column, fixed to a 10-cm precolumn, using a Haskel pump DSTV-122 (Haskel, Burbank, CA, U.S.A.). The inlet pressure was 5 bar and methanol–water (80:20) was used as the pressurizing liquid. The solvents were of reagent grade and were distilled in glass before use.

The mobile phases in pump A and B both consisted of methanol–water–1 M phosphoric acid mixtures: A (5:90:5) and B (75:20:5). The gradient elution program was as follows: 20 % of B for 15 min, increased at a rate of 8 % of B per min during 5 min to a maximum of 50 % of B, held for 15 min, then decreased at a rate of 8 % of B per min during 5.0 min to a minimum of 20 % of B. Mobile phases were degassed by sonication. The flow-rate was set at 1.0 ml/min and 20-μl samples were injected. The column temperature was kept at 30°C by means of a water jacket. The detector was set at 280 nm and 0.08 a.u.f.s.

Sample preparation, calibration curves and reproducibility

Aliquots of 25.0 mg of tetracycline hydrochloride were dissolved in methanol,

5.0 ml of 0.025% (w/v) 4-hydroxybenzoic acid in methanol (IS) were added and the volume was made up to 25.0 ml. Using adequate amounts of the reference substances, calibration curves with a linear relationship between peak/area IS, y, and concentration in μg/ml, x, were obtained for TC, ETC, ATC and EATC. No corrections were made for small amounts of epimer, present in the reference substances. Linear regression and coefficients of correlation, r, were as follows: TC, $y = 0.02935\,x + 0.4337$, $r = 0.999$; ETC, $y = 0.01594\,x - 0.0033$, $r = 0.999$; ATC, $y = 0.064\,x - 0.0313$, $r = 0.996$; EATC, $y = 0.059\,x - 0.0583$, $r = 0.998$. The intercept found for TC is due to a gradient effect.

The commercial tetracycline hydrochloride sample was analysed on seven different days in the course of the experiment. Mean values, $\bar{X}$, for the content in % (w/w) and coeffients of variation, v, were: TC, $\bar{X} = 93.7$, $v = 2.5$; ETC, $\bar{X} = 6.0$, $v = 2.1$; ATC, $\bar{X} = 1.3$, $v = 9.5$; EATC, $\bar{X} = 0.7$, $v = 6.9$. When the sample was analysed six times on the same day the coefficients of variation were at least four times smaller. In order to reduce the amounts of chemical reference substances employed, the calibration curves were determined only once, and no compensation was made for small fluctuations in the course of the experiments.

RESULTS AND DISCUSSION

HPLC was used for the analysis of the samples because determination of the impurities is much more accurate than with a thin-layer chromatography. A system similar to that described here has already been discussed in detail[11]. Fig. 2 shows a typical chromatogram of the commercial tetracycline hydrochloride used. The four components are well separated.

The advantage of the phenyl column over the previously used LiChrosorb C8 column is that EATC and ATC are now separated with a mobile phase containing methanol; with the C8 column acetonitrile was necessary for this separation. The shape of the baseline, obtained during gradient elution with reagent grade methanol which had been purified by single distillation in the presence of silver nitrate and potassium hydroxide, is better than that obtained previously with expensive brands of acetonitrile. The silver used in this purification can be recovered. The choice of 280 nm as the wavelength for detection contributes also to the shape of the baseline, since
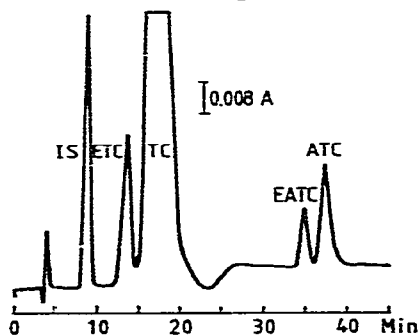

Fig. 2. Chromatogram of commercial tetracycline hydrochloride. Column: μBondapak phenyl, 25 cm × 4.6 mm I.D. Mobile phases: methanol–water–1 M phosphoric acid. A (5:90:5) and B (75:20:5). Gradient elution: 20% B for 15 min, increased at 8% B per min for 5 min to 50% B, held for 15 min, then reset through a reverse gradient. Flow-rate: 1 ml/min. Detection at 280 nm and 0.08 a.u.f.s. IS = Internal standard; ETC = 4-epitetracycline; EATC = 4-epianhydrotetracycline; ATC = anhydrotetracycline.

the increase of the organic modifier during gradient elution is less visible at higher wavelengths. At the end of an analysis the mobile phase composition is reset to the original ratio through a rapid reverse gradient, by which procedure equilibrium is reached more rapidly than after a direct reset[14].

In Table I the results of the analysis of the heated samples are compiled. The content of TC, ETC, ATC and EATC was determined, the values reported being the means of two independent assays. At 37°C and at 70°C the stability was followed over a period of 27 months. At 50°C the experiment covers a period of only 16 months because a number of samples were lost due to overheating.

At 37°C and at 50°C no decomposition is observed for TC, nor for its related substances. The sum of the contents slightly exceeds the theoretical maximum of 100%, owing to the fact that the calibration curves were not corrected for small amounts of impurities, present in the reference substances. At 70°C a distinct decrease in TC is observed as well as a small increase in ATC. The ETC and EATC contents are practically unchanged. Since the total content, accounted for by TC and known related substances, decreases regularly, it is obvious that small amounts of one or more products of unknown identity formed which are not detected by our HPLC system. This was also observed during the decomposition of TC in methanolic solution[11]. The nature of this decomposition will be examined later.
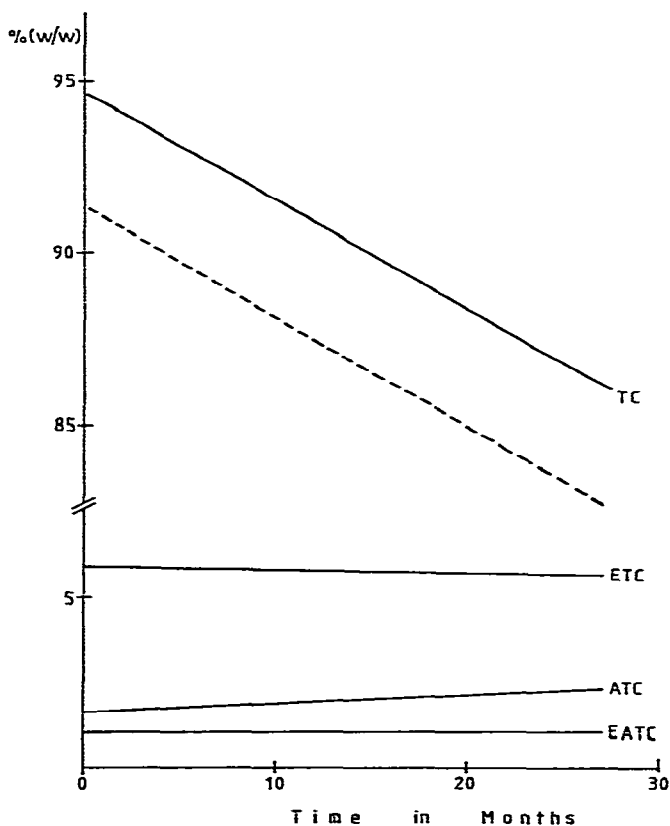
Fig. 3. Stability of solid tetracycline hydrochloride at 70°C. Least squares lines are shown. The broken line is the calculated line above which 95% of the results would fall.

TABLE I

COMPOSITION OF HEATED TETRACYCLINE HYDROCHLORIDE SAMPLES, IN % (w/w)

Time (months)	37°C					50°C					70°C				
	TC	ETC	ATC	EATC	Total	TC	ETC	ATC	EATC	Total	TC	ETC	ATC	EATC	Total
2	94.2	6.1	1.0	0.7	102	92.3	6.0	1.1	0.7	100.1	93.8	5.9	1.5	1.0	102.2
2.5	93.8	6.0	1.2	0.7	101.7	93.5	6.0	1.3	0.7	101.5	94.5	5.9	1.7	0.9	103.0
4.5	93.2	6.1	1.2	0.7	101.2	93.3	5.9	1.3	0.8	101.3	94.5	5.9	1.7	0.9	103.0
6	93.9	6.1	1.2	0.7	101.9	92.8	5.9	1.1	0.7	100.5	92.8	5.9	1.6	0.9	101.2
8	93.1	6.0	1.4	0.7	101.2	93.2	5.9	1.2	0.8	101.1	91.9	5.7	2.0	1.1	100.7
10	94.5	6.0	1.4	0.8	102.7	92.6	5.8	1.5	0.9	100.8	91.1	5.6	2.7	1.6	101.0
13	92.9	6.1	1.1	0.7	100.8	93.8	6.0	1.2	0.7	101.7	90.5	5.8	1.5	0.8	98.6
15	94.1	6.1	0.9	0.6	101.7						88.3	5.9	1.7	0.9	96.8
16	93.5	6.0	1.2	0.6	101.3	92.9	6.0	1.2	0.6	100.7	90.2	5.6	1.8	0.7	98.3
18	93.9	6.1	0.9	0.6	101.5						88.6	5.7	1.6	0.8	96.7
21	92.7	6.1	1.2	0.7	100.7						87.9	5.7	2.2	1.1	96.9
23	93.6	6.1	1.2	0.7	101.6						87.3	5.7	2.1	1.1	96.2
25	93.1	6.0	1.2	0.7	101.0						88.1	5.7	2.0	0.9	96.7
27	93.1	6.1	1.3	0.7	101.2						86.3	5.7	2.7	1.2	95.9
Regression															
Intercept	93.9	6.05	1.2	0.7		92.9	5.9	1.2	0.8		94.7	5.9	1.6	1.0	
Slope	−0.027	0.0007	0.0002	−0.0013		0.019	0.0001	0.0033	−0.0038		−0.316	−0.0082	0.0237	0.0018	

The least squares lines, expressing the stability results at 70°C, are shown in Fig. 3. As far as it was followed, the decomposition of TC shows a zero-order pattern. The broken line in Fig. 3, parallel with the least squares line for TC, is the calculated line above which 95 % of the results would fall. It is drawn at a vertical distance of 2 $s_{y,x} = 3.4\%$ of the calculated line, $s_{y,x}$ being the standard error of estimate. On this line, t_{90}, the point at which the content reaches 90 % of the original value, is measured to be 19 months. Activation energy calculations cannot be performed since decomposition was only observed at one temperature. Shelf-live calculations were therefore carried out by an approximate method, discussed by Connors et al.[15]. Here

$$t_{90}(T_2) = t_{90}(T_1)/Q_{10}^{(\Delta T/10)}$$

with temperature $T_2 = T_1 + \Delta T$ and $Q_{10} = 2, 3$ or 4. The value $Q_{10} = 3$ gives the most likely estimate, while for $Q_{10} = 2$ or 4 the upper and lower limits are obtained. The t_{90} values, calculated in years for 50°C and 20°C, are reported in Table II.

TABLE II

CALCULATED t_{90} VALUES (YEARS) OF TETRACYCLINE HYDROCHLORIDE

Q_{10} value	Temperature (°C)	
	50	20
2	6.3	50.7
3	14.2	349
4	25.2	1621

These values show that, at 20°C and under conditions of low humidity, tetracycline hydrochloride is a very stable product, having a t_{90} of at least 50 years, but probably of about 350 years. The calculations a͟ o explain why in the course of our experiment no decomposition was observed at 50°C nor at 37°C.

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