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Kinetics of Concomitant Degradation of Tetracycline to Epitetracycline, Anhydrotetracycline, and Epianhydrotetracycline in Acid Phosphate Solution

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Abstract □ The concentrations of tetracycline, epitetracycline, anhydrotetracycline, and epianhydrotetracycline in pH 1.5 phosphate solution were followed as a function of time at four temperatures. Separation and quantification of all four species were accomplished using high-pressure liquid chromatography. Through nonlinear regression analysis, rate constants for the reversible first-order epimerization of tetracycline and anhydrotetracycline and for the first-order dehydration of tetracycline and epitetracycline were obtained. Solutions to the differential equations obtained through Laplace transforms successfully predict concentrations found experimentally. The energy of activation for each reaction step was calculated and ranged from 15 to 27 kcal/mole. The rate constants for tetracycline and epitetracycline dehydration conform with those of earlier studies that used different experimental methods. The study shows that epimerization of tetracycline and anhydrotetracycline can take place at a low pH.

Keyphrases □ Tetracycline—kinetics of degradation at pH 1.5, effect of temperature □ Degradation kinetics—tetracycline at pH 1.5, effect of temperature □ Antibacterials—tetracycline, kinetics of degradation at pH 1.5, effect of temperature

Tetracycline degradation to toxic epianhydrotetracycline can take place through tetracycline epimerization to epitetracycline (1, 2) followed by dehydration to epianhydrotetracycline (3) or by dehydration of tetracycline to anhydrotetracycline (4) followed by epimerization to epianhydrotetracycline (5). The kinetics of each individual step have been studied separately under conditions where it was assumed that only the reaction of interest was operative. No reported studies followed all potential reactions simultaneously under the same experimental conditions. The use of high-pressure liquid chromatography (HPLC), which separates all four compounds (tetracycline, epitetracycline, anhydrotetracycline, and epianhydrotetracycline), permits such a study (5). This paper presents the results for the solution degradation of tetracycline at pH 1.5 together with the rate expressions defining these results.

EXPERIMENTAL

Materials—Tetracycline hydrochloride[1] (I), 4-epitetracycline ammonium salt[2] (II), anhydrotetracycline hydrochloride[3] (III), and 4-epianhydrotetracycline[4] (IV) were used as obtained. All other chemicals were reagent grade, and double-distilled deionized water was used to make all solutions.

Apparatus—A high-pressure liquid chromatograph[5] with a multiwavelength detector[6] was used with a 1-m × 2.1-mm strong cation-exchange column[7].

Separation and Quantification—The mobile phase employed in the HPLC separation consisted of 0.07 *M* phosphate–0.0075 *M* ethylenediaminetetraacetic acid adjusted to pH 7.0. To improve the separation for tetracycline and its degradation products, the operating procedure was a slight modification of a previously reported method (5). Specifically, the elution was carried out at a column temperature of 36° and a flow rate of 0.55 ml/min (575 psi). The eluent was monitored at 254 nm at 0.08 absorbance unit full scale (aufs).

Areas under the individual peaks were measured with a polar compensating planimeter[8]. Known injected amounts of I–IV in 0.03 *N* HCl were correlated with the areas under the chromatograms obtained. The slopes of the linear relationship between moles added and area were 2.625×10^{-10}, 2.681×10^{-10}, 1.154×10^{-10}, and 1.377×10^{-10} mole/cm^2 for I, II, III, and IV, respectively.

Kinetic Method—A phosphoric acid stock solution (1 *M*) was adjusted to pH 1.5 with a concentrated potassium hydroxide solution. The solution was scrubbed with nitrogen and allowed to equilibrate at the temperature desired. Appropriate amounts of I were weighed into volumetric flasks and dissolved in the phosphate solution. The pH did not change during the study. The reaction flasks, sealed with a rubber septum, were immediately placed in a water bath[9] that also protected the solution from light.

At appropriate time intervals, samples were withdrawn, placed in vials, and immersed in ice to stop the reaction. A fixed volume of this sample was assayed chromatographically by equating areas found with concentration through a standard curve. The reactions were followed until all I was lost.

RESULTS AND DISCUSSION

A modification of a previously reported HPLC assay for tetracycline and its degradation products (5) results in less overlap between I and its epimer (Fig. 1). The retention times for IV, III, II, and I were 5.5, 8.25, 22.75, and 30.25 min, respectively.

The relationship between the concentration of each of the four species in the reaction as a function of time gave results as shown in Fig. 2 (obtained at 75°). Four temperatures were used (60–80°), and duplicate kinetic experiments were run at each temperature. Studies made at higher temperatures yielded results involving large errors, making data analysis tenuous.

The concentration–time profile found at each temperature was assumed to be a consequence of the reaction illustrated in Scheme I.

[1] Lot 2K030-71 EA, Pfizer.
[2] Batch 430, British Pharmacopoeia Commission.
[3] Batch 428, British Pharmacopoeia Commission.
[4] Lot 3339-99-1, GS-6659, Pfizer.
[5] DuPont model 830.
[6] DuPont model 835.
[7] DuPont Zipax SCX.
[8] Model 62005, Keuffel and Esser Co.
[9] Haake model FS2.

$$\begin{array}{ccc} \text{tetracycline} & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & \text{epitetracycline} \\ \downarrow k_3 & & \downarrow k_4 \\ \text{anhydrotetracycline} & \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} & \text{epianhydrotetracycline} \end{array}$$

Scheme I

The rate constants for the individual reaction steps can be obtained from differential equations defined by the model. The equations are:

$$\frac{d[\mathrm{I}]}{dt} = k_{-1}[\mathrm{II}] - (k_1 + k_3)[\mathrm{I}] \quad \text{(Eq. 1)}$$

$$\frac{d[\mathrm{II}]}{dt} = k_1[\mathrm{I}] - (k_{-1} + k_4)[\mathrm{II}] \quad \text{(Eq. 2)}$$

$$\frac{d[\mathrm{III}]}{dt} = k_3[\mathrm{I}] + k_{-2}[\mathrm{IV}] - k_2[\mathrm{III}] \quad \text{(Eq. 3)}$$

$$\frac{d[\mathrm{IV}]}{dt} = k_2[\mathrm{III}] + k_4[\mathrm{II}] - k_{-2}[\mathrm{IV}] \quad \text{(Eq. 4)}$$

where the rate constants conform to the designation in Scheme I.

The differential equations for I and II (Eqs. 1 and 2) can be solved using Laplace transforms without considering the III and IV reactions since the dehydration process is irreversible first order for both I and II. The solutions to Eqs. 1 and 2 are:

$$[\mathrm{I}]_t = \frac{[\mathrm{I}]_0(k_{-1} + k_4 - a)}{(b - a)} e^{-at} + \frac{[\mathrm{I}]_0(k_{-1} + k_4 - b)}{(a - b)} e^{-bt} \quad \text{(Eq. 5)}$$

$$[\mathrm{II}]_t = \frac{[\mathrm{I}]_0 k_1}{(b - a)} e^{-at} + \frac{[\mathrm{I}]_0 k_1}{(a - b)} e^{-bt} \quad \text{(Eq. 6)}$$

where the subscripts to the concentration terms indicate any time, t, and initial, 0, concentrations and where a and b are complex constants used in the Laplace transforms. They are comprised of the reaction rate constants given in Scheme I and are partly defined in Eqs. 9–11.

When Eqs. 5 and 6 are substituted into Eqs. 3 and 4, the resulting differential equations for III and IV can be solved using Laplace transforms. The integrated equations resulting are:

$$[\mathrm{III}]_t = \frac{k_{-2}k_4k_1[\mathrm{I}]_0 + k_{-2}k_3(k_{-1} + k_4)[\mathrm{I}]_0}{abc} + \frac{k_{-2}k_4k_1[\mathrm{I}]_0 + (k_{-2} - a)(k_{-1} + k_4 - a)k_3[\mathrm{I}]_0}{(-a)(b - a)(c - a)} e^{-at} + \frac{k_{-2}k_4k_1[\mathrm{I}]_0 + (k_{-2} - b)(k_{-1} + k_4 - b)k_3[\mathrm{I}]_0}{(-b)(a - b)(c - b)} e^{-bt} + \frac{k_{-2}k_4k_1[\mathrm{I}]_0 + (k_{-2} - c)(k_{-1} + k_4 - c)k_3[\mathrm{I}]_0}{(-c)(a - c)(b - c)} e^{-ct} \quad \text{(Eq. 7)}$$

and:

$$[\mathrm{IV}]_t = \frac{k_2k_4k_1[\mathrm{I}]_0 + k_2k_3(k_{-1} + k_4)[\mathrm{I}]_0}{abc} + \frac{(k_2 - a)k_4k_1[\mathrm{I}]_0 + k_2k_3(k_{-1} + k_4 - a)[\mathrm{I}]_0}{(-a)(b - a)(c - a)} e^{-at} + \frac{(k_2 - b)k_4k_1[\mathrm{I}]_0 + k_2k_3(k_{-1} + k_4 - b)[\mathrm{I}]_0}{(-b)(a - b)(c - b)} e^{-bt} + \frac{(k_2 - c)k_4k_1[\mathrm{I}]_0 + k_2k_3(k_{-1} + k_4 - c)[\mathrm{I}]_0}{(-c)(a - c)(b - c)} e^{-ct} \quad \text{(Eq. 8)}$$

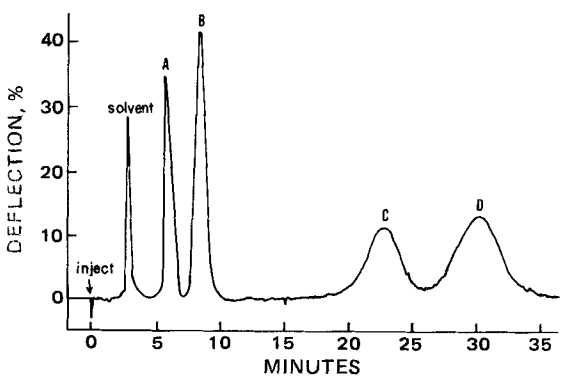

Figure 1—*Relationship between detector response and time in the HPLC separation of IV (A), III (B), II (C), and I (D).*

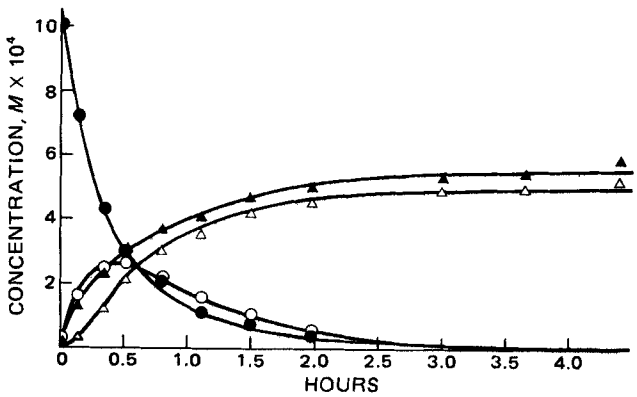

Figure 2—*Relationship between concentration and time for I (●), II (○), III (▲), and IV (△) in pH 1.5 phosphate solution and 75° The solid lines are the least-squares fit (NONLIN) of the experimental data shown.*

where:

$$a + b = k_1 + k_{-1} + k_3 + k_4 \quad \text{(Eq. 9)}$$

$$ab = k_1k_4 + k_{-1}k_3 + k_3k_4 \quad \text{(Eq. 10)}$$

$$c = k_{-2} + k_2 \quad \text{(Eq. 11)}$$

The experimental data, typified in Fig. 2, were fitted using both the differential (Eqs. 1–4) and integrated (Eqs. 5–8) forms of the equations through nonlinear regression analysis (NONLIN program[10]). To use this program, initial estimates of the six rate constants are required. The values of these initial estimates appear to be quite critical to the outcome of the NONLIN program when using the differential equations (Eqs. 1–4).

A computerized Runge–Kutta method was used to obtain the six initial estimates (6). Trial and error values for the six rate constants defined in Scheme I were used until the Runge–Kutta estimation of the concentrations of I–IV at any time approximately agreed with the concentrations found experimentally. With these Runge–Kutta estimates as initial estimates, the rate constants generated by the NONLIN program were determined (Table I). The largest errors obtained were in the constants for the epimerization of III (k_2 and k_{-2}).

The use of the integrated forms of the equations in the NONLIN program yielded rate constants that were relatively insensitive to the initial estimates. The rate constants generated and their accompanying standard deviations were virtually identical to those found using the differential forms (Table I). For example, when the integrated equations (Eqs. 5–8) were used with the data obtained in Trial 1 at 80°, where the largest errors were found, the rate constants generated (standard deviations in parentheses) were 2.08 (0.153), 0.937 (0.279), 1.87 (0.654), 2.19 (0.729), 1.56 (0.0897), and 2.38 (0.172) for k_1, k_{-1}, k_2, k_{-2}, k_3, and k_4, respectively.

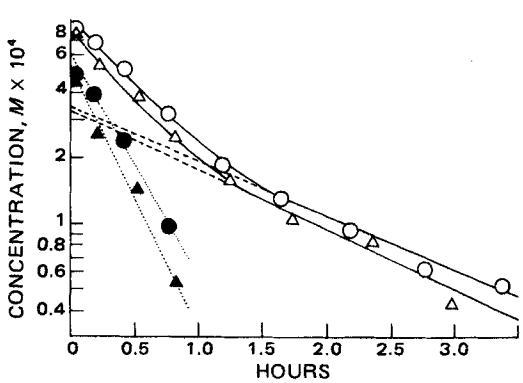

Figure 3—*Relationship between the logarithm of I concentration and time for two trials (○ and △) at 70°. Broken lines (- - -) indicate terminal slope and dotted lines (· · ·) indicate feathered initial data for Trial 1 (●) and Trial 2 (▲).*

[10] Unit 7292, The Upjohn Co.

Table I—Computer-Generated Rate Constants in Hours^{-1} for the Degradation of I in pH 1.5 Phosphate Solution at Several Temperatures

Temperature ± 0.5°	k_1[a]	k_{-1}	k_2	k_{-2}	k_3	k_4
60°[b]	0.414 (0.0274)[c]	0.373 (0.0531)	0.659 (0.162)	0.665 (0.166)	0.323 (0.0207)	0.206 (0.0344)
	0.392 (0.0192)	0.296 (0.0375)	0.585 (0.130)	0.607 (0.135)	0.302 (0.0156)	0.231 (0.0252)
70°	1.03 (0.0978)	0.661 (0.178)	1.31 (0.715)	1.46 (0.780)	0.687 (0.0714)	0.732 (0.118)
	0.982 (0.0504)	0.655 (0.0966)	1.33 (0.300)	1.46 (0.325)	0.743 (0.0369)	0.662 (0.0613)
75°	1.65 (0.0901)	1.43 (0.184)	1.88 (0.422)	2.07 (0.466)	1.28 (0.0677)	0.921 (0.123)
	1.53 (0.0800)	1.05 (0.159)	1.92 (0.436)	2.15 (0.483)	1.23 (0.0601)	1.11 (0.108)
80°	2.08 (0.153)	0.941 (0.282)	1.87 (0.673)	2.19 (0.750)	1.56 (0.0905)	2.37 (0.176)
	2.27 (0.208)	1.58 (0.405)	1.80 (0.909)	2.03 (0.996)	1.60 (0.128)	2.17 (0.251)

[a] Rate constant designation conforms to that of Scheme I. [b] The results of duplicate trials run at each temperature are listed. [c] Parentheses contain the NONLIN-generated standard deviations.

Table II—Values for Constants *a* and *b* Determined Experimentally at Four Temperatures and a Comparison of Their Sum with that Calculated from Rate Constants Generated by Nonlinear Regression Analysis

Temperature	Trial	a, hr^{-1}	b, hr^{-1}	$a+b$ Experimental	$a+b$ Calculated[a]	Ratio of Sums, Calc./Exp.
60°	1	0.91	0.22	1.13	1.32	1.17
	2	1.1	0.22	1.32	1.22	0.92
70°	1	2.4	0.59	2.99	3.11	1.04
	2	2.7	0.63	3.33	3.04	0.91
75°	1	3.8	0.99	4.79	4.81	1.00
	2	3.1	1.0	4.10	4.92	1.20
80°	1[b]	6.4[c]	2.1	8.5	7.8	0.92
	2[d]	6.4	2.1	8.5	7.6	0.89

[a] Using the data from Table I and Eq. 9. [b] Only six data points were available. [c] The 80° data for Trials 1 and 2 were used as one set in the semilogarithmic plots. [d] Only five data points were available.

The constants a and b in Eqs. 5 and 6 also can be obtained from semilogarithmic plots of concentration *versus* time using either I or II data, with the terminal slope reflecting b and the slope of the feathered initial data reflecting a. With the concentration–time data for I, semilogarithmic plots were constructed as represented by those in Fig. 3 for the two trials made at 70°. The a and b values obtained from such plots are given in Table II.

A comparison of the sum of the experimentally obtained a and b constants was made with the sum calculated according to Eq. 9 using the constants (Table I) generated by nonlinear regression analysis. These sums, together with their ratio (calculated to experimental), are given in Table II. The data at 80° are subject to considerable error since the number of data points available to estimate initial and terminal slopes was small due to the fast reaction rates. The agreement between the calculated and experimental sums of a and b is quite good. The average of the ratios (calculated to experimental) is 1.01.

Experimental confirmation of the constant c (Eq. 11) is difficult because it is necessary to construct a plot of the logarithm of the difference in equilibrium and time t concentrations as a function of time (Eq. 7 or 8). The difference in concentrations at long times is subject to large errors, making estimates of the terminal log-linear slope (constant c) highly inaccurate.

An Arrhenius plot of the average rate constant for each reaction step was made using the results obtained at four temperatures (Table I). Estimates of the activation energies for all six reactions are summarized in Table III. The epimerization reaction of the anhydro compounds appears to require less energy than the other steps, but the error involved in the determination of these energies is large, making any conclusion regarding energy effect tenuous.

In an earlier study (3), the rate constant for the dehydration of II, determined by following spectral changes, was 0.618 hr^{-1} at 71° and pH 1.53 (hydrochloric acid at 0.1 M ionic strength). The average value in the present study at 70° was 0.697 hr^{-1}, which is in reasonable agreement with the earlier value found under slightly different conditions. Schlecht and Frank (4) studied I dehydration at various temperatures and hydrogen-ion concentrations. By using their activation energy value (4) of 25.1 kcal/mole (ionic strength of 1 M), it is possible to calculate the rate constant at 60° if it is assumed that the hydrogen-ion concentration is reasonably close to the present hydrogen-ion activity (0.0361 M). Based on their rate constant at 50° (4), the rate constant calculated at 60° is about 0.44 hr^{-1}, which is of the same order of magnitude as the constants found in the present study, 0.323 and 0.302 hr^{-1}, again under different conditions.

In earlier studies on II (3) and I (4) dehydration, it was validly assumed that no epimerization was taking place at pH < 2. However, the present study shows that epimerization of both I and II can occur at pH 1.5, but the system used was different from that of both earlier studies. The present study used a 1 M phosphate solution adjusted to pH 1.5, which should contain significant amounts of monobasic phosphate, a species known to catalyze epimerization (1, 5). The significance of this result is that if a catalytic species is present in sufficient amounts, significant epimerization can occur even if the pH is less than 3.

Table III—Activation Energy for Each Step in I Degradation in pH 1.5 Phosphate Solution

Reaction as Indicated by Rate Constant	Activation Energy, kcal/mole
k_1	20.5 (19.0–21.7)[a]
k_{-1}	18.7 (10.2–27.0)
k_2	14.1 (3.84–24.4)
k_{-2}	15.3 (4.33–26.2)
k_3	19.4)12.8–25.8)
k_4	26.6 (17.3–36.0)

[a] Parentheses contain the upper and lower 95% confidence limits obtained from linear regression analysis.

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