J. Pharm. Pharmac., 1968, **20**, 539–546 Received December 11, 1967

Aspects of the epimerization of certain tetracycline derivatives

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The epimerization of several tetracycline derivatives was examined at several pH values using varying conditions of temperature and buffer strength. Rate coefficients for the epimerization of tetracycline and demethylchlortetracycline are reported and factors associated with the epimerization of chlortetracycline and oxytetracycline are discussed. Under the conditions used calcium had no effect on the rate of epimerization and copper promoted degradation other than epimerization.

THE C-4 epimerization of tetracycline and its analogues was first described by Doerschuk, Bitler & McCormick (1955). The epimer differed significantly from the analogue of normal configuration in several of its properties, the most important being its *in vitro* antibiotic activity. This was found to be less than 5% of the activity of the normal analogue.

Conditions promoting and inhibiting epimerization have been described by McCormick, Fox & others (1957). They found that epimerization occurs in a variety of solvent systems within the pH range of approximately 2–6 and that the rate of epimerization is increased by certain buffering agents. Remmers, Sieger & Doerschuk (1963) found that this C-4 epimerization follows first-order reversible reaction kinetics.

An understanding of the epimerization process and the factors that influence it is important in the preparation of liquid pharmaceutical dosage forms since a loss in potency can result from spontaneous epimerization. Also there have been reports of kidney damage following the ingestion of degraded tetracycline capsules (Frimpter, Timpanelli & others, 1963) and 4-epitetracycline and 4-epianhydrotetracycline were identified among the degradation products. 4-Epianhydrotetracycline has been specifically cited as the agent causing the kidney damage (Benitz & Diermeier, 1964; Lowe & Tapp, 1966). Thus, the possibility of epimerization must be considered when evaluating the stability and potential toxicity of formulations of the tetracycline drugs. We have expanded earlier epimerization studies and examined the influence of calcium and copper on epimerization.

Experimental

REAGENTS

Samples of tetracycline hydrochloride, chlortetracycline hydrochloride and demethylchlortetracycline hydrochloride were supplied by Lederle Laboratories and oxytetracycline hydrochloride was donated by Chas. Pfizer and Company. Analytical reagent grade sodium acetate was used to make approximately 0·1M and 1M buffer solutions at pH values 4·0, 5·0 and 6·0, the solutions being adjusted to the appropriate pH with

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concentrated hydrochloric acid. The solutions containing calcium or copper salts were prepared using analytical reagent grade copper chloride ($CuCl_2.2H_2O$) and calcium chloride ($CaCl_2.2H_2O$). Approximately 0·1N sulphuric acid solutions were used for the spectrophotometric assays; these were made with a Hitachi Perkin-Elmer spectrophotometer.

PROCEDURE

Solutions of the appropriate buffer were prepared and adjusted to the required pH. The buffers were allowed to equilibrate at the desired temperature in 50·0 ml volumetric flasks wrapped in aluminium foil to shield the solutions from light. The tetracycline derivative was added to the buffer to give solutions that were approximately 6×10^{-4}M with respect to the tetracycline derivative. At intervals, samples were withdrawn, diluted with 0·1N sulphuric acid, and assayed spectrophotometrically using an absorbancy ratio analysis to be described. These studies were made at pH values of 4·0, 5·0 and 6·0 in both 0·1M and M acetate buffers at temperatures of 30, 37 and 50° which were maintained by a regulated water bath. When the influence of calcium or copper on the epimerization was examined, the concentration of the metal was varied in the initial solution to give molar ratios of metal : tetracycline ranging from 1 : 5 to 5 : 1.

Results and discussion

A modification of the absorbancy ratio assay of McCormick & others (1957) was used to follow the epimerization. This assay makes use of significant differences in the spectral curves of tetracyclines and their epimers at 254 and 267 mμ when 0·1N sulphuric acid is used.

Remmers & others (1963) modified this assay to observe the kinetics of tetracycline epimerization. This assay is useful for kinetic studies because the time between sampling and completion of the assay is short. Also, since 0·1N sulphuric acid is used, any complex between a tetracycline and a metal would be broken and therefore the free tetracyclines could be assayed without interference from metal chelates.

For the assay the mixture being analyzed must contain only a tetracycline and its corresponding epimer. Because of this, preliminary studies were made to determine its limitations. Solutions of tetracycline hydrochloride with pH values adjusted between the limits of pH 3·0 and 8·0 using sodium hydroxide or hydrochloric acid (buffers increase the rate of epimerization) were used. The absorbance of the solutions was measured at 254 and 267 mμ and curves made simultaneously, scanning from 380 to 220 mμ.

Appreciable degradation other than epimerization quickly took place at pH values above 6·0. At lower pH values, degradation other than epimerization was evident over a longer time, as seen by decreased absorbance in the 300–380 mμ region (Fig. 1).

To overcome this problem the original assay was modified. McCormick & others (1957) and Remmers & others (1963) observed epimerization to occur at a faster rate in the presence of buffers, the rate increasing with

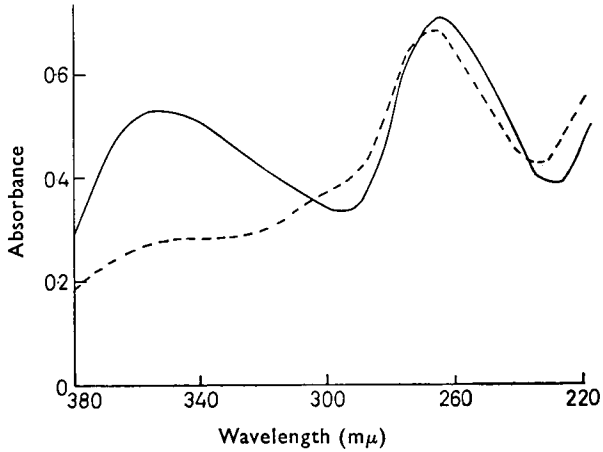


FIG. 1. Ultraviolet spectral curves showing the degradation of a solution of tetracycline hydrochloride at pH 7·0. Assayed in 0·1 N sulphuric acid after zero hr (———) and 1180 hr (– – – –).

increasing buffer strength. Since epimerization proceeds relatively slowly in water, we used acetate buffer systems. With the method described below, for most systems, the rate of epimerization was increased to such an extent that equilibrium mixtures of a tetracycline and its epimer could be obtained before other degradation became significant. This was established by determining the 254/267 and 355/298 ratios. The 254/267 ratio increased to an equilibrium level and remained relatively constant, whereas the 355/298 ratio increased to a point after which it began to decrease steadily, indicating that degradation other than epimerization was becoming significant. Thus, it can serve as a valuable corollary in indicating where the 254/267 ratio could be used validly to follow epimerization. The wavelengths used for the corollary assay varied with the tetracycline analogue examined whereas the 254/267 ratio was used for all the derivatives.

Remmers & others (1963) showed that the epimerization of tetracycline follows the kinetics of a first-order reversible reaction.

$$\text{Tetracycline} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Epitetracycline} \quad .. \quad .. \quad 1$$

The rate coefficients can be obtained using the equation describing the kinetics of such a reaction

$$\ln \frac{A_o - A_e}{A - A_e} = (k_1 + k_{-1})\, t \quad .. \quad .. \quad .. \quad 2$$

where A_o = % tetracycline hydrochloride at time 0 hr; A = % tetracycline hydrochloride at time t hr; A_e = % tetracycline hydrochloride at equilibrium; k = forward rate coefficient, hr^{-1}; k_{-1} = backward rate coefficient, hr^{-1}.

If log $(A - A_e)$ is plotted as a function of time, a straight line should

be obtained whose slope is equal to $-\frac{(k_1 + k_{-1})}{2{\cdot}303}$ (the method of least squares was used to estimate the slope). The individual rate coefficients can then be obtained knowing the equilibrium concentration of tetracycline using the expression:

$$\frac{100 - A_e}{A_e} = \frac{k_1}{k_{-1}} \qquad .. \quad .. \quad .. \quad 3$$

TETRACYCLINE AND DEMETHYLCHLORTETRACYCLINE

The values of the forward and backward rate coefficients for the epimerization of tetracycline hydrochloride and demethylchlortetracycline hydrochloride under various experimental conditions are summarized in Tables 1 and 2.

TABLE 1. RATE COEFFICIENTS FOR THE EPIMERIZATION OF TETRACYCLINE HYDROCHLORIDE. (Assays in 0·1N sulphuric acid.)

Acetate buffer strength	pH		Rate coefficients (hr^{-1}) × 10^2		
			30°	37°	50°
0·1M	4·0	k_1	1·8	3·5	13
		k_{-1}	3·7	7·6	39
	5·0	k_1	0·8	1·9	6·9
		k_{-1}	2·3	5·7	33
M	4·0	k_1	14	33	88
		k_{-1}	25	62	170
	5·0	k_1	8·1	14	41
		k_{-1}	20	35	110
	6·0	k_1	1·1	2·7	5·8
		k_{-1}	3·3	9·4	19

TABLE 2. RATE COEFFICIENTS FOR THE EPIMERIZATION OF DEMETHYLCHLORTETRACYCLINE HYDROCHLORIDE. (Assays in 0·1N sulphuric acid.)

Acetate buffer strength	pH		Rate coefficients (hr^{-1}) × 10^2		
			30°	37°	50°
0·1M	4·0	k_1	2·2	4·5	12
		k_{-1}	4·6	10	28
	5·0	k_1	0·9	1·7	4·6
		k_{-1}	2·8	5·8	18
M	4·0	k_1	20	37	110
		k_{-1}	41	76	220
	5·0	k_1	7·9	15	44
		k_{-1}	21	42	120
	6·0	k_1	1·4	2·4	9·4
		k_{-1}	4·2	7·5	29

At pH 4·0 and 5·0 the epimerization progressed rapidly enough to allow an equilibrium to be reached before other degradation became a factor. However, at pH 6·0 in 0·1M buffer, the epimerization proceeded so slowly that it was apparent that other degradation was occurring in most instances before an equilibrium 254/267 ratio was reached.

The epimerization of tetracycline and demethylchlortetracycline proceeded fastest and to the greatest extent at pH 4·0, which was the lowest pH value studied. Remmers (1963) reported that epimerization took place most rapidly at a pH between 3·0 and 4·0. The equilibrium mixtures for both tetracycline and demethylchlortetracycline at pH 4·0 contained

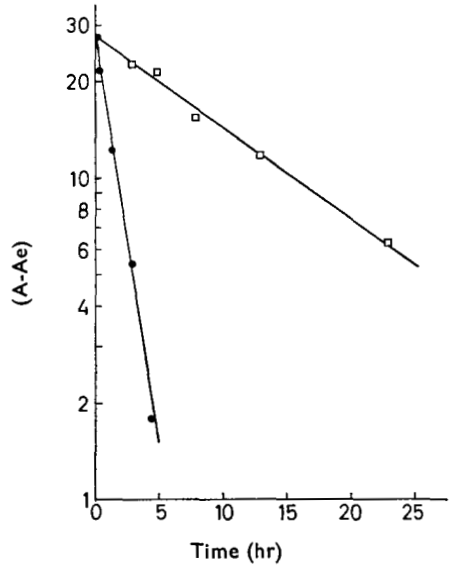


FIG. 2. The influence of buffer concentration on the epimerization of demethylchlortetracycline at pH 4·0 and 30° C. Assays were conducted in 0·1 N sulphuric acid. □ 0·1 M acetate buffer. ● M acetate buffer. See text p. 541 for definition of A–Ae.

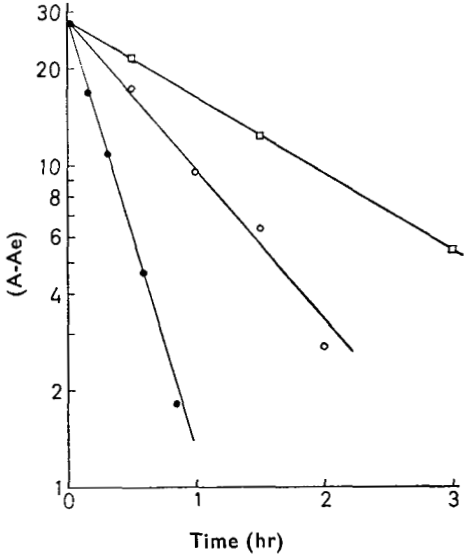


FIG. 3. The influence of temperature on the epimerization of demethylchlortetracycline in a pH 4·0 M acetate buffer. Assays were conducted in 0·1 N sulphuric acid. □ 30°. ○ 37°. ● 50° C.

approximately 34% of the corresponding epimer whereas, at pH 5·0, approximately 25% of the epimer was present at equilibrium. The epimerization proceeded slightly further when the M buffer was used.

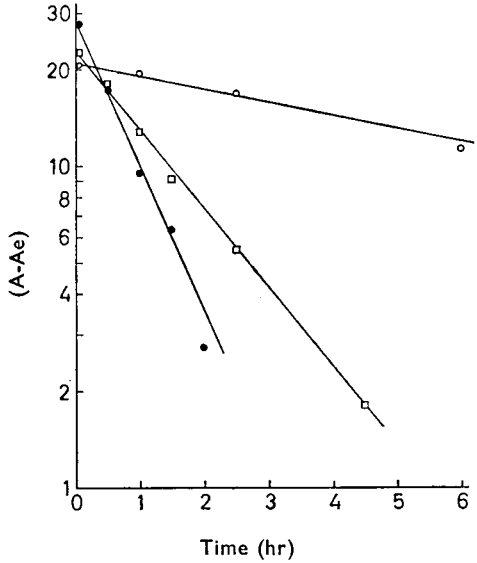


FIG. 4. The influence of pH on the epimerization of demethylchlortetracycline in M acetate buffer at 37°C. Assays were conducted in 0·1 N sulphuric acid. ● pH 4·0. □ pH 5·0. ○ pH 6·0.

Buffer concentration and temperature influenced the rate of epimerization (Tables 1 and 2). The influence of buffer concentration, temperature and pH have been illustrated in Figs 2–4.

TABLE 3. ENERGIES OF ACTIVATION FOR THE EPIMERIZATION OF TETRACYCLINE AND DEMETHYLCHLORTETRACYCLINE

Acetate buffer strength	pH	Reaction	Tetracycline E_a kcal/mole	Demethylchlortetracycline E_a kcal/mole
0·1M	4·0	Forward	19	16
		Backward	23	17
	5·0	Forward	21	16
		Backward	26	18
M	4·0	Forward	18	17
		Backward	18	17
	5·0	Forward	16	17
		Backward	17	17
	6·0	Forward	16	19
		Backward	16	19

The energies of activation for the epimerization of tetracycline and demethylchlortetracycline have been determined using the Arrhenius equation,

$$\log k = \frac{-E_a}{2{\cdot}303\ RT} + \log s \quad .. \quad .. \quad .. \quad 4$$

where k is the rate coefficient, E_a is the energy of activation, R is the gas constant, T is the absolute temperature and s is a constant referred to as the frequency factor. These values have been tabulated in Table 3. The energy of activation obtained for tetracycline when a pH 4·0, 0·1M

acetate buffer is used compares with the values obtained by Remmers & others (1963) using a phosphate buffer (20·4 kcal/mole for both the forward and backward reactions).

CHLORTETRACYCLINE

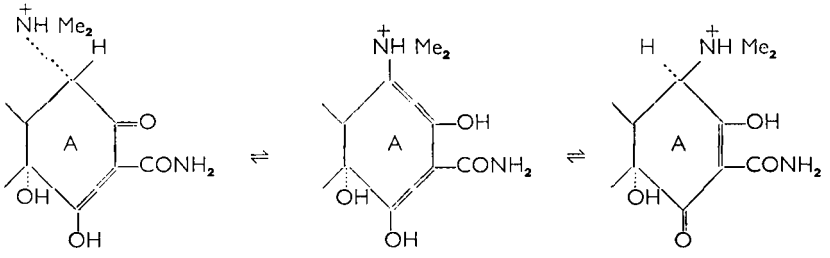
Chlortetracycline is unstable in aqueous solutions; we found that degradation other than epimerization occurs rapidly, even at pH 4·0.

OXYTETRACYCLINE

Epimerization of oxytetracycline occurred very slightly, or not at all. The 254/267 ratio remained relatively constant, whereas the 353/298 ratio eventually decreased, indicating other degradation was taking place.

Jarowski (1963) stated that tetracycline will epimerize much more easily than oxytetracycline. He suggested that the hydroxyl group at position 5 of oxytetracycline could hydrogen bond with the dimethylamino-group and thus inhibit the tendency to epimerize. By this reasoning, tetracycline and demethylchlortetracycline, which lack this hydroxyl group, would show a greater tendency to epimerize. This would satisfactorily explain our findings.

Recently, Huettenrauch & Keiner (1966) investigated the influence of structural modification at C-2 on the epimerization of several tetracycline derivatives. They described the epimerization of *N*-(pyrrolidinomethyl)-tetracycline and *N*-(pyrrolidinomethyl)oxytetracycline and reported that, after cleavage to form tetracycline and oxytetracycline, respectively, the oxytetracycline epimerized more readily. This conflicts with our results although acetic acid was used and, by acting as a stronger protonating solvent than water, it should promote the rearrangement of ring A (on which the dimethylamino-group is a substituent) so as to favour the epimerization process. For example, the following scheme could be suggested for the epimerization process in acid:



I II III

Rigler, Bag & others (1965) have suggested the above structures for tetracycline (I) and its epimer (III). These authors also noted striking differences in the basicities of the nitrogen atoms (of the dimethylamino-group) of the two epimers and indicated that the difference in conformation of ring A may be partly responsible for this.

As well as promoting the rearrangement of ring A, the acetic acid may interact with the hydroxyl group at position 5 of the oxytetracycline, thus competing with the bonding of this hydroxyl group with the dimethyl-ammonium group. This would result in at least partial removal of this

inhibition of the epimerization process and therefore, in acetic acid, the 5-hydroxy group may not interfere with the epimerization.

THE INFLUENCE OF CALCIUM ON EPIMERIZATION

Above pH 6·0 epimerization of tetracycline occurs only slightly and at a very slow rate. Kaplan, Granatek & Buckwalter (1957) reported that epimerization is markedly inhibited by calcium or magnesium above a pH of 6.

We found that in the presence of calcium the rate of epimerization was unchanged at pH values below 6·0 even when a 5 : 1 calcium : tetracycline concentration ratio was used. These results are not surprising in the light of potentiometric studies previously made in these laboratories in which the formation of calcium–tetracycline complexes was not observed below a pH of 6·0. Thus, for calcium to inhibit the epimerization process, it may be necessary for it to complex with tetracycline.

THE INFLUENCE OF COPPER ON EPIMERIZATION

Since calcium had no influence on the rate of epimerization of tetracycline over the pH range examined, the effects of a metal having different complexation characteristics at these pH values was studied. Copper–tetracycline molar ratios ranging from 1 : 5 to 2 : 1 were used. It was found that degradation other than epimerization occurred at a faster rate than it did when copper was not present in the system. Thus, it would appear that copper promotes the degradation of the tetracyclines.

Kaplan, Lannon & Buckwalter (1965) reported the inactivation of tetracycline with a cupric–morpholine complex but stated that cupric ion as cupric acetate, or copper tyrosinase did not inactivate tetracycline. Although the biological activity of the solutions used by us was not measured, it was apparent that degradation was occurring rapidly in the presence of copper.

Since it was the purpose of this study to investigate the epimerization process, the other routes of degradation were not analyzed.

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