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Investigation of *keto-enol* tautomerism and ionization of doxycycline in aqueous solutions

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Summary

The *keto* and *enol* tautomers of doxycycline were separated by liquid chromatography on poly(styrene-divinylbenzene). The *keto-enol* tautomerism occurred between C-11a and C-12. The chemical structure of the tautomers was elucidated by on-line and off-line UV spectrophotometry and by ^{13}C-NMR spectrometry. The kinetics of the equilibrium were investigated in the pH range 1–12. The equilibrium was subject to base catalysis. The tautomerism was enhanced by protonation of doxycycline. The assignment of the groups responsible for the micro-ionization of doxycycline was discussed. Activation parameters were determined at pH 4.0.

Introduction

Separation of *keto-enol* tautomers of chlortetracyline (CTC) and 4-epichlortetracycline (ECTC) by liquid chromatography (LC) on poly(styrene-divinylbenzene) (PSDVB) has been reported (Weng Naidong et al., 1990). The kinetics of the reaction were only investigated in acidic solutions due to the poor stability of CTC and ECTC in neutral and basic solutions. We report here the separation of *keto-enol* tautomers of doxycyline (DOX). *keto-enol* tautomerism occurs between C-11a and C-12 (Fig. 1). The kinetics of the equilibrium were investigated in the entire pH range. Based on pH-rate constant and pH-equilibrium constant profiles, the assignment of groups, responsible for the micro-ionization of doxycyline, is discussed.

Materials and Methods

Samples and reagents

Doxycycline hyclate (DOX · HCl) was obtained from Pfizer (Brussels, Belgium). 4-Epidoxycycline (4-EDOX) was prepared from DOX by storing a

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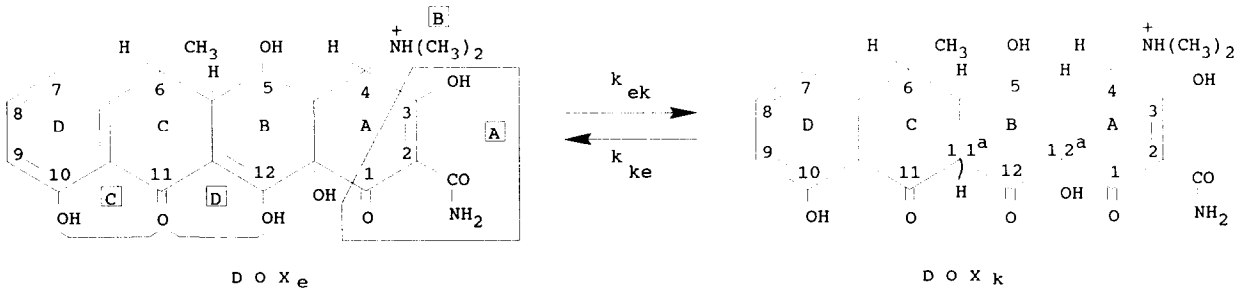

Fig. 1. Doxycycline structures of tautomers and position of dissociating groups.

DOX solution at pH 3. The 4-EDOX formed was isolated by using a previously described method (Weng Naidong et al., 1991).

Organic solvents were from Janssen Chimica (Beerse, Belgium). Double-distilled water was used. Other reagents were of pro-analysi quality (Merck, Darmstadt, Germany). Buffers for the calibration of pH measurements were prepared followed the instructions of the European Pharmacopoeia (1980).

Sample preparation and apparatus

DOX stock solution in methanol was prepared at 2 mg/ml. This stock solution was stable for 2 days at 5°C. 1 ml stock solution was added to 50.0 ml of the specified solvent and the time clock was simultaneously started. Sodium citrate-HCl, sodium citrate-NaOH and potassium phosphate buffers were used in the pH range 2–7. To examine the influence of ionic strength on the equilibrium, some experiments were performed with buffers containing varying potassium chloride concentration. In some experiments, the solvent was water with pH adjusted by HCl or NaOH. The solvents and the solutions were maintained at the temperature indicated.

The LC equipment consisted of a Waters M-25 solvent delivery system (Milford, MA, U.S.A.) a model CV-6-UHPa-N60 injector (Valco, Houston, TX, U.S.A.) equipped with a 50 μl loop, a Spectro Monitor model 3100 set at 249 nm (Milton-Roy, Riviera Beach, FL, U.S.A.) and an integrator model 3390 A (Hewlett-Packard, Avondale, PA, U.S.A.). A Waters model 990 photodiode-array detector was used to measure the on-line UV spectra. The 50 × 4.6 mm i.d. column

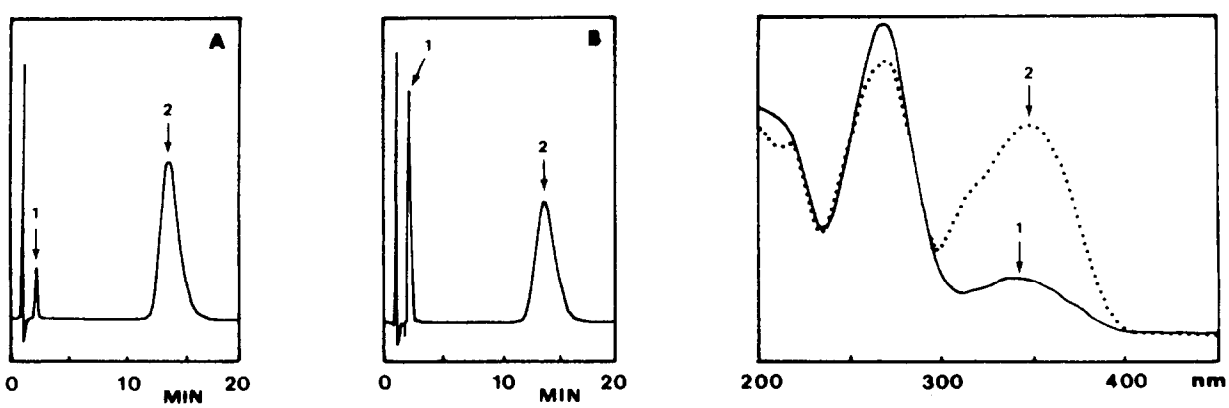

Fig. 2. LC chromatograms of doxycycline and the on-line UV spectra. Solutions examined: (A) 1 min after dissolution; (B) 30 min after dissolution (equilibrium); solvent, 0.01 N HCl; temperature, 10°C. LC conditions: column, PLRP-S 1000 Å (50 × 4.6 mm i.d.); mobile phase, 2-methyl-2-propanol-1 M perchloric acid-water (11 : 5 : 84 w/v/v); temperature, 10°C; detection at 249 nm; flow rate, 1.0 ml min^{-1}. The UV spectra were taken at the apex of the peaks and were normalized at 249 nm.

was packed in the laboratory with PLRP-S 1000 Å 8 μm (Polymer Labs, Curch Stretton, Shropshire, U.K.). The mobile phase was 2-methyl-2-propanol/1 M perchloric acid/water (11:5:84 w/v/v). The mobile phase was degassed by ultrasonication. The flow rate was 1.0 ml/min. The column was maintained at 10°C in a cooling system (Julabo, Seelbath, Germany).

Off-line UV spectra were recorded on a PU 8740 UV/Vis scanning spectrophotometer (Philips-Pye Unicam, Cambridge, U.K.) using a 10 mm cell. DOX · HCl (5.0 mg) was dissolved in 500 ml of 0.01 N HCl. The UV absorbance-time profile was followed. The time clock was started on solvent addition.

The stability of DOX in various solutions was determined by a quantitative LC method (Hoogmartens et al., 1989). No detectable degradation occurred throughout the study.

Results and Discussion

Structure analysis

Fig. 2 shows chromatograms of a fresh DOX solution (A) and a solution in 0.01 N HCl at equilibrium (B) at 10°C, 30 min after preparation. The first peak is much smaller in chromatogram A than in chromatogram B. Equilibrium was reached again upon collecting and reinjecting either of the two peaks. Fig. 3 shows off-line spectra of DOX obtained 1 min after the first contact with the solvent and at equilibrium (after 30 min). By subtraction of those spectra an isosbestic point was obtained at 249 nm. This also existed when the LC mobile phase was used as solvent. Based on the above observations and experience with the tautomerism of CTC and ECTC (Weng Naidong et al., 1990), DOX in solution may be a tautomeric mixture, as shown in Fig. 1.

TABLE 1

^{13}C chemical shifts [a,b] and peak assignments for DOX and 4-EDOX hydrochloride tautomers in D_2O solution

Position	DOX		4-EDOX	
		Litt [d]	*enol* (major)	*keto* (minor)
1	194.2 (S)	192.4	190.8 (S)	187.8 (S)
2	97.0 (S)	96.2	97.3 (S)	97.7 (S)
3	187.3 (S)	187.5	188.4 (S)	188.6 (S)
4	69.4 (D)	69.5	70.1 (D)	69.3 (D)
4a	39.4 (D)	39.4	37.7 (D)	32.5 (D)
5	66.7 (D)	66.4	68.6 (D)	67.8 D)
5a	46.7 (D)	46.9	46.8 (D)	47.3 (D)
6	42.6 (D)	42.3	45.6 (D)	48.2 (D)
6a	148.2 (S)	148.4	147.8 (S)	148.0 (S)
7	117.2 (D)	116.7	117.0 (D)	121.6 (D)
8	138.6 (D)	137.3	138.0 (D)	139.6 (D)
9	117.1 (D)	116.3	116.6 (D)	116.7 (D)
10	161.4 (S)	162.7	161.4 (S)	161.8 (S)
10a	116.2 (S)	116.3	115.7 (S)	115.0 (S)
11	194.2 (S)	194.8	193.3 (S)	195.1 (S)
11a	108.4 (S)	107.8	107.3 (S)	57.4 [c] (D)
12	172.9 (S)	173.6	173.4 (S)	199.3 (S)
12a	74.1 (S)	74.0	75.3 (S)	82.1 (S)
CO-amide	170.4 (S)	172.4	171.9 (S)	172.2 (S)
4-NMe_2	43.0 (Q)	42.9	42.9 (Q)	47.9 (Q)
6-Me	16.1 (Q)	15.8	16.3 (Q)	22.0 (Q)

[a] Expressed in ppm downfield from tetramethylsilane (TMS); peak positions were measured relative to the centre of the multiplet of internal DMSO-d_6, set at 39.6 ppm vs TMS.

[b] The multiplicity of the signals in the OFR spectrum is indicated in parentheses: S, singlet for quaternary carbon: D, doublet for methine; T, triplet for methylene; Q, quadruplet for methyl carbon.

[c] Not visible in D_2O solution, but only in H_2O solution.

[d] In methanol (D_2O capillary).

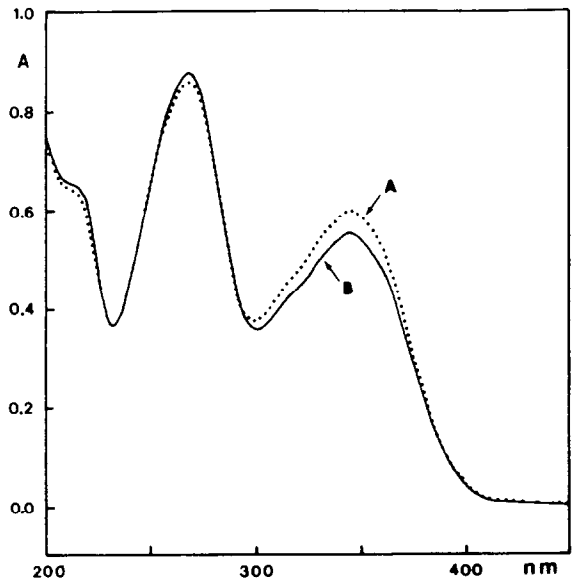

Fig. 3. UV spectra of doxycycline: (A) 1 min after dissolution; (B) 30 min after dissolution (equilibrium). Solution examined: 0.001% (w/v) in 0.01 N HCl at 10°C.

However, unequivocal proof of the structure of these tautomers by ^{13}C-NMR spectroscopy, as described for 4-ECTC (Weng Naidong et al., 1990), was not possible in this case. As before, the equilibrium position seems to be concentration-dependent, and under the normal conditions for good-quality ^{13}C spectra on the Jeol FX90Q apparatus (room temperature and at concentrations of 5–10%) only a single tautomer appears to be present. The ^{13}C shift values thus found (Table 1) were almost identical with the values published (Casy and Yasin, 1984) for DOX, and fully in accordance with a C-12 enolic structure which is the commonly accepted tautomeric form of ring B of these molecules. This is demonstrated mainly by the absence of the characteristic C-12 ketonic signal around 200 ppm and of a methine signal at about 60 ppm. Analogous LC and UV experiments were carried out with 4-EDOX. Solutions in 0.01 N HCl equilibrated for 120 min were analysed by the same LC method. More of the *keto* tautomer was found for 4-EDOX (82%) than for DOX (20%). This was confirmed by ^{13}C-NMR spectroscopy. The observed shifts of the minor component (Table 1) thus provided conclusive evidence for the proposed C-12 *keto* structure of this 4-EDOX tautomer.

Characteristic for the *enol*-to-*keto* conversion is the downfield shift of C-11, C-12 and C-12a, and the upfield shift of C-1 and especially of C-11a. The C-11a upfield shift is invisible in D_2O but visible in H_2O solution, and becomes a doublet signal in the OFR mode (Weng Naidong et al., 1990). Other less important shifts, not observed in previous experiments with 4-ECTC, are probably due to changes in conformational preferences accompanying the *enol-keto* conversion, and may be different for the various tetracycline members.

It can be concluded that DOX exists as *enol* in the solid state and as a mixture of the *keto-enol* tautomers in solution. As for CTC · HCl (Weng Naidong et al., 1990), it was not possible to isolate the *keto* tautomer in the solid state.

Kinetic Studies

Determination of rate constants and equilibrium constants

In methanol, DOX exists only as the *enol*, A DOX stock solution in methanol was used for kinetic studies. Stock solution (1.0 ml) was added to 50.0 ml solution used for the kinetic study. At this low concentration, the influence on kinetics from methanol was negligible. The LC method was validated to obtain quantitative results for the *keto-enol* tautomerism of DOX. The detector was set at 249 nm since this was the isosbestic point for the tautomers. Examination of linearity for DOX_{enol} (DOX_e) and DOX_{keto} (DOX_k) was carried out with solutions containing known amounts of DOX · HCl in 0.01 N HCl (up to 0.4 mg ml^{-1}). After equilibration the solutions were analysed and the mass corresponding to the *enol* or *keto* tautomers was calculated from the total mass injected, the area of each of the two peaks and the sum of the two peak areas. In all, four solutions were analysed three times. The coefficients of correlation (r) obtained were 0.9999 for DOX_k and 0.9996 for DOX_e. The limits of quantitation were 0.02 μg for DOX_e and 0.01 μg for

DOX_k. For an equilibrium reaction the following equations can be written (Martin et al., 1969):

$$\log \frac{DOX_e(0) - DOX_e(eq)}{DOX_e(t) - DOX_e(eq)} = \frac{k_{ek} + k_{ke}}{2.303} t$$

where $DOX_e(0)$ is the content (%) of DOX_e at time 0 = 100%, $DOX_e(eq)$, the content (%) of DOX_e at equilibrium and $DOX_e(t)$, the content (%) of DOX_e at time t. k_{ek} denotes the rate constant for transformation of DOX_e to DOX_k and k_{ke}, the rate constant for transformation of DOX_k to DOX_e.

$$K_{eq} = \frac{k_{ek}}{k_{ke}} = \frac{DOX_k(eq)}{DOX_e(eq)}$$

where K_{eq} is the equilibrium constant for transformation of DOX_e to DOX_k.

TABLE 2

Observed rate constants (min^{-1}) and equilibrium constants for the tautomerism of doxycycline at pH 2.0 as a function of ionic strength

Ionic strength (μ)	k_{ek} ($\times 10^2$)	k_{ke} ($\times 10^2$)	K_{eq} ($\times 10^2$)
0.10	5.83	22.17	26.31
0.17	5.83	22.17	26.31
0.37	5.73	22.0	26.03
0.61	6.33	22.14	28.59

Solvent: 3.06×10^{-2} M citrate buffer pH 2.0, with the ionic strength indicated and at 10°C.

The relative standard deviation (RSD) for the rate constants obtained was 3–10%. The lower RSD was obtained at lower pH. The RSD for the equilibrium constants was always less than 1%.

Values for K_{eq}, k_{ek} and k_{ke} obtained for pH 2.0 at different ionic strengths are reported in Table 2. The influence of the ionic strength is

TABLE 3

Observed rate constants (min^{-1}) and equilibrium constants for the tautomerism of doxycycline at different pH values as a function of the concentration of citrate buffer, at 10°C

pH 2.0				pH 3.0				pH 4.0			
[Buffer] (M) ($\times 10^2$)	k_{ek} ($\times 10^2$)	k_{ke} ($\times 10^2$)	K_{eq} ($\times 10^2$)	[Buffer] (M) ($\times 10^2$)	k_{ek} ($\times 10^2$)	k_{ke} ($\times 10^2$)	K_{eq} ($\times 10^2$)	[Buffer] (M) ($\times 10^2$)	k_{ek} ($\times 10^2$)	k_{ke} ($\times 10^2$)	K_{eq} ($\times 10^2$)
0.765	5.09	20.11	25.30	1.008	6.11	24.35	25.10	1.400	5.22	21.15	24.68
1.530	5.42	21.55	25.13	2.015	7.27	29.38	24.75	2.800	9.37	38.32	24.45
2.295	5.37	21.65	24.82	3.023	8.78	35.37	24.83	4.200	12.87	52.89	24.33
3.06	5.83	22.17	26.31	4.030	9.44	37.25	25.33	5.600	16.40	66.34	24.72
Intercept ($k_0 \times 10^2$)	4.89	19.80			5.02	20.41			1.70	7.14	
Slope	0.28	0.82			1.14	4.44			2.65	10.72	
r	0.9182	0.9183			0.9900	0.9817			0.9991	0.9984	

pH 5.0				pH 6.0			
[Buffer] (M) ($\times 10^2$)	k_{ek} ($\times 10^2$)	k_{ke} ($\times 10^2$)	K_{eq} ($\times 10^2$)	[Buffer] (M) ($\times 10^2$)	k_{ek} ($\times 10^2$)	k_{ke} ($\times 10^2$)	K_{eq} ($\times 10^2$)
0.771	5.73	23.51	24.36	0.477	7.25	28.85	25.13
1.349	7.98	31.47	25.35	0.834	9.10	36.19	25.15
1.928	10.48	41.51	25.25	1.192	13.90	55.35	25.11
2.410	12.75	52.31	24.76	1.490	16.55	64.97	25.48
Intercept ($k_0 \times 10^2$)	2.32	8.98			2.10	8.88	
Slope	4.28	17.48			9.62	37.53	
r	0.9992	0.9945			0.9864	0.9860	

negligible in the range examined. The same was observed with solutions prepared with phosphate buffers or with non-buffered solutions.

The influence of the buffer concentration was investigated in the pH range 2–6 for citrate buffers and in the pH range 3–7 for phosphate buffers. The results are reported in Tables 3 and 4, respectively. The ionic strength of the buffer solutions was less than 0.3. From Tables 3 and 4, it is apparent that the buffer concentration has little influence on K_{eq}. A catalytic effect of the buffer on k_{ek} and k_{ke} was observed at pH 2–6 for citrate buffers and pH 3–7 for phosphate buffers. The slope increases exponentially with the pH values, indicating catalytic effect in decreasing order by citrate (Ci^{3-}), monohydrogen citrate (HCi^{2-}) and dihydrogen citrate (H_2Ci^{-}). The same order holds for phosphate (PO_4^{3-}), monohydrogen phosphate (HPO_4^{2-}) and dihydrogen phosphate $H_2PO_4^{-}$). That the reactions are susceptible to base catalysis can be explained by the fact that *keto-enol* tautomerism involves proton transfer from C-12 to C-11a, which is facilitated by bases. Linear relationships existed between the observed rate constants and the total buffer concentration. The intercept provided buffer-independent apparent first-order rate constants (k_0).

Experiments with citrate or phosphate buffers of higher pH values were discontinued because the reaction was too fast to be followed. Lower reaction rates could be achieved by decreasing the buffer concentration but then the buffer capacity was insufficient to compensate the shift of the pH values caused by adding DOX · HCl stock solution.

Influence of pH on tautomerism in relation with acid dissociation

The pH-rate constant profile is shown in Fig. 4. Since the ionic strength did not affect the rate constants, these experiments were carried out using non-buffered aqueous solutions, adjusted to the designated pH with HCl or NaOH. The k_0 values obtained as the intercepts in Tables 3 and 4 are also added to Fig. 4. The pH-equilibrium

TABLE 4

Observed rate constants (min^{-1}) and equilibrium constants for the tautomerism of doxycycline at different pH values as a function of the concentration of phosphate buffer, at 10°C

[Buffer] (M) ($\times 10^3$)	pH 3.0			pH 4.0			pH 5.0		
	k_{ek} ($\times 10^2$)	k_{ke} ($\times 10^2$)	K_{eq} ($\times 10^2$)	k_{ek} ($\times 10^2$)	k_{ke} ($\times 10^2$)	K_{eq} ($\times 10^2$)	k_{ek} ($\times 10^2$)	k_{ke} ($\times 10^2$)	k_{eq} ($\times 10^2$)
2.5	3.98	15.34	26.00	2.61	10.39	25.09	1.83	7.35	24.94
5.0	4.46	17.43	25.60	2.75	11.01	24.97	2.24	8.87	25.27
7.5	4.22	17.19	24.55	2.68	11.15	24.03	2.27	9.29	24.48
10.0	3.87	15.78	24.56	2.50	10.15	24.65	2.33	9.42	24.69
Intercept ($k_0 \times 10^2$)	4.28	16.17		2.74	10.82		1.79	7.08	
Slope	−0.02	0.04		−0.01	−0.02		0.06	0.27	
r	−0.2801	0.1353		−0.4844	−0.1556		0.8660	0.9000	

pH 6.0			pH 7.0		
k_{ek} ($\times 10^2$)	k_{ke} ($\times 10^2$)	K_{eq} ($\times 10^2$)	k_{ek} ($\times 10^2$)	k_{ke} ($\times 10^2$)	K_{eq} ($\times 10^2$)
2.69	10.47	25.72	5.85	21.97	26.65
3.55	14.19	25.05	10.26	37.72	27.20
3.86	15.34	25.18	15.00	57.66	26.10
5.21	20.70	25.15	17.84	70.86	25.18
1.86	7.22		2.06	5.40	
0.31	1.27		1.63	6.66	
0.9712	0.9719		0.9948	0.9973	

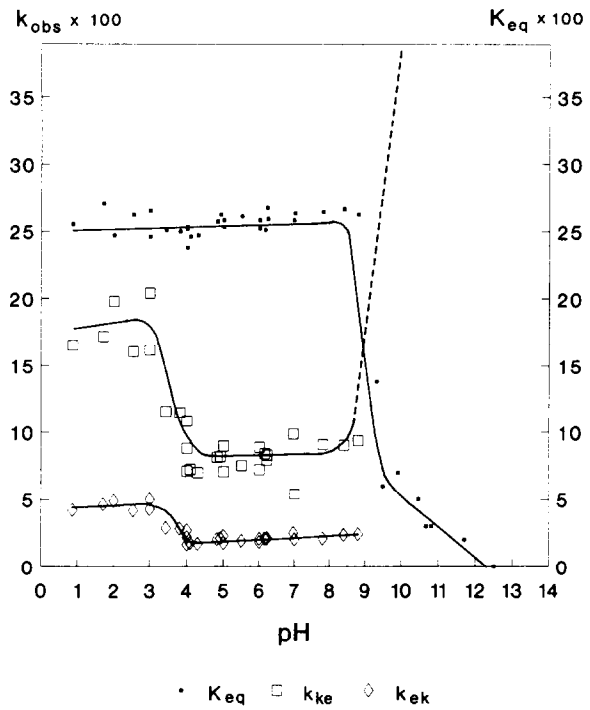

Fig. 4. pH-rate and pH-equilibrium constant profiles for the tautomerism of doxycyline.

constant profile is shown in the same figure. Between pH 1 and 8.5, a sigmoidal pH-rate constant profile was obtained. A similar curve was also obtained for CTC (Weng Naidong et al., 1990). For CTC, the tautomerism study was not performed at pH > 6 since CTC is unstable in neutral and alkaline medium. For both DOX and CTC, the reaction rate in both directions was higher at lower pH. Unlike CTC, for which the equilibrium slightly shifted towards the *keto* tautomer upon increasing pH, the K_{eq} for DOX remained unchanged from pH 1 to 8.5. In an alkaline medium at pH > 9, k_{ke} increased drastically and it was impossible to follow the transformation of the tautomers. Therefore, the rate constants were not measured at pH > 9. However, a large drop of K_{eq} between pH 8.5 and 9.5 was observed.

Although the acid dissociation constants for DOX have not been reported, the macroscopic pK_a values of tetracyclines in aqueous solution are approx 3, 7, 9 and 11 (Stephens et al., 1956; Leeson et al., 1963; Benet and Goyan, 1965; Ahmed and Jee, 1984). A tricarbonylmethane moiety at C-1–C-3 of tetracyclines was described as the group responsible for pK_{a1} (see Fig. 1) (Stephens et al., 1956; Leeson et al., 1963). Stephens et al. (1956) proposed that the dimethylammonium moiety at C-4 contributed to pK_{a2} and the phenolic moeity at C-10 to pK_{a3}. A reversal in assignment was proposed by other researchers (Garrett, 1963; Leeson et al., 1963). Later, it was found that assignment of the particular functional groups for pK_{a2} and pK_{a3} was dependent on the structure of each tetracycline and should be based on the determination of micro-ionization acid constants (Rigler et al., 1965; Kesselring and Benet, 1969; Bhatt and Jee, 1985). That the *enol* moeity at C-11a–C-12 responds for pK_{a4}, as proposed by Stephens et al. (1956), was confirmed by Rigler et al. (1965). The micro-ionization scheme is shown below, the superscripts refer to the charge on the acid-base sites:

$A^0B^+C^0D^0$

$$\xrightarrow{k_1} A^-B^+C^0D^0 \begin{cases} \xrightarrow{k_{12}} A^-B^0C^0D^0 \xrightarrow{k_{123}} A^-B^0C^-D^0 \\ \xrightarrow{k_{13}} A^-B^+C^-D^0 \xrightarrow{k_{132}} A^-B^0C^-D^0 \end{cases}$$

$$\xrightarrow{k_4} A^-B^0C^-D^-$$

The tautomerism reaction being influenced by acid dissociation of DOX is demonstrated by the results shown in Fig. 4. The sigmoid shape of the pH-rate profile is due to an acid/base dissociation of the drug molecule (Connors et al., 1986). The midpoint of the pH-rate curve of DOX is very close to the pK_{a1} value of tetracyclines. This was also observed for the pH-rate profile of CTC (Weng Naidong et al., 1990). The pH-rate profile demonstrates the dependence of the reaction rate on the pK_{a1} of DOX, and the greater reactivity of the protonated form compared with the zwitterion form. From Fig. 4, it is obvious that neither rate constants nor equilibrium constants are influenced at pH 5–8. A dominating pathway $A^-B^+C^0D^0 \xrightarrow{k_{12}} A^-B^0C^0D^0$ as described for

oxytetracycline (Bhatt and Jee, 1985) can probably be retained for DOX as well. The dimethylammonium moiety at C-4 is far away from the reaction centre of tautomerism and therefore deprotonation of it is assumed to have negligible influence. On the other hand, deprotonation of the phenolic moiety at C-10 should create a situation in favour of the *enol* form at C-11a–C-12 since the conjugated group at C-10–C-12 is more electron-rich, thus strengthening the intramolecular hydrogen bonding. The results in Fig. 4 show that k_{ke} is greatly increased. The dotted line indicates a trend for k_{ke}. Accurate determination was not possible due to the very fast transformation of the tautomers. K_{eq} is largely reduced at pH 8.5–9.5 with a midpoint at about 9 for K_{eq}. This midpoint is quite close to the pK_{a3} of tetracyclines. That small amounts of *keto* form still exist at pH 9.5–11.5 can be attributed to pK_{a4} of DOX. A completely deprotonated DOX would only exist in the *enol* tautomer, i.e., $K_{eq} = 0$, as obtained at pH 12.5 in Fig. 4.

That DOX_e was the predominant form was further confirmed by some rate measurements carried out in the presence of methanol. Upon addition of methanol, k_{ek} decreased and k_{ke} increased, resulting in a decrease in K_{eq} (see Table 5). Intramolecular hydrogen bonding is favoured by adding organic solvents such as methanol into aqueous solution (March, 1985) and leads to prevalence of DOX_e.

Arrhenius parameters

The influence of temperature was investigated in the range 0–30°C using an aqueous solution of DOX, adjusted to pH 4.0. Table 6 shows the obtained rate constants and equilibrium constants. The natural logarithms of the rate constants were plotted vs. $1/T$. From the slopes of the straight lines the apparent activation energies were calculated, i.e., 72.6 kJ mol^{-1} for *enol-keto* tautomerism and 59.8 kJ mol^{-1} for *keto-enol* tautomerism. The equilibrium constant is directly proportional to the temperature, which is consistent with an increase of temperature being unfavourable to the formation of intramolecular hydrogen bonding.

TABLE 5

Observed rate constants (min^{-1}) and equilibrium constants for the tautomerism of doxycycline in water at pH 4.0 as a function of the concentration of methanol, at 10°C

[Methanol] (% v/v)	k_{ek} ($\times 10^2$)	k_{ke} ($\times 10^2$)	K_{eq} ($\times 10^2$)
2	2.22	8.80	25.24
22	2.03	10.48	19.38
62	1.47	20.14	7.27
100			0

TABLE 6

Observed rate constants (min^{-1}), equilibrium constants and Arrhenius parameters for the tautomerism of doxycycline as function of temperature, in water at pH 4.0

Temperature (°C)	k_{ek} ($\times 10^2$)	k_{ke} ($\times 10^2$)	K_{eq} ($\times 10^2$)
0	0.50	2.41	20.74
10	2.22	8.80	25.24
20	5.11	16.40	31.18
30	12.55	34.99	35.89
r	−0.9932	−0.9895	
Slope ($-E_{obs}/R$)	−8741	−7199	
E_{obs} (kJ/mol)	72.6	59.8	

E_{obs}, apparent activation energy; R, molar gas constant.

Conclusion

The *keto* and *enol* tautomers of doxycycline were separated by LC on poly(styrene-divinylbenzene). By on-line and off-line UV spectrophotometry and ^{13}C-NMR spectrometry it was concluded that *keto-enol* tautomerism occurs between C-11a and C-12. The equilibrium reaction is subject to base catalysis. The assignment of functional groups responsible for micro-ionization of doxycycline was discussed.

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References

Ahmed, B.M. and Jee, R.D., The acidity and solubility constants of tetracyclines in aqueous solutions. *Anal. Chim. Acta,* 166 (1984) 329–333.

Benet, L.Z. and Goyan, J.E., Determination of the stability constants of tetracycline complexes, *J. Pharm. Sci.,* 54 (1965) 983–987.

Bhatt, V.K. and Jee, R.D., Micro-ionization acidity constants for tetracyclines from fluorescence measurements. *Anal. Chim. Acta,* 167 (1985) 233–240.

Casy, A.F. and Yasin, A., Application of ^{13}C nuclear magnetic resonance spectroscopy to the analysis and structural investigation of tetracycline antibiotics and their common impurities. *J. Pharm. Biomed. Anal.,* 2 (1984) 19–36.

Connors, K.A., Amidon, G.L. and Stella, V.J., *Chemical Stability of Pharmaceuticals; A Handbook for Pharmacists,* 2nd Edn, Wiley, New York, 1986, pp. 47–51.

European Pharmacopoeia, 2nd Edn, Maisonneuve, Sainte Ruffine, France, 1980.

Garrett, E.R., Variation of pK_a' values of tetracyclines in dimethylformamide-water solvents. *J. Pharm. Sci.,* 52 (1963) 797–799.

Hoogmartens, J., Naeem Hasan Khan, Vanderhaeghe, H., Van der Leeden, A.L., Oosterbaan, M., Veld-Tulp, G.L., Plugge, W., Van der Vlies, C., Mialanne, D., Melamed, R. and Miller, J.H.McB., A collaborative study of the analysis of doxycycline hyclate by high-performance liquid chromatography on polystyrene-divinylbenzene packing materials. *J. Pharm. Biomed. Anal.,* 7 (1989) 601–610.

Kesselring, U.W. and Benet, L.Z., Determination of a protonation scheme for isochlortetracycline using nuclear magnetic resonance. *Anal. Chem.,* 41 (1969) 1535–1539.

Leeson, L.J., Krueger, J.E. and Nash, R.A., Concerning the structural assignment of the second and third acidity constants of the tetracycline antibiotics. *Tetrahedron Lett.,* 18 (1963) 1155–1160.

March, J., *Advanced Organic Chemistry,* 3rd Edn, Wiley, New York, 1985, p. 67.

Martin, A.N., Swarbrick, J. and Cammarata, A., *Physical Pharmacy,* 2nd Edn, Lea & Febiger, Philadelphia, 1969, pp. 365–366.

Rigler, N.E., Bag, S.P., Leyden, D.E., Sudmeier, J.L. and Reilley, C.N., Determination of a protonation scheme of tetracycline using nuclear magnetic resonance. *Anal. Chem.,* 37 (1965) 872–875.

Stephens, C.R., Murai, K., Brunings, K.J. and Woodward, R.B., Acidity constants of the tetracycline antibiotics. *J. Am. Chem. Soc.,* 78 (1956) 4155–4158

Weng Naidong, Roets, E., Busson, R. and Hoogmartens, J., Separation of *keto-enol* tautomers of chlortetracycline and 4-epichlortetracycline by liquid chromatography on poly (styrene-divinylbenzene)copolymer. *J. Pharm. Biomed. Anal.,* 8 (1990) 881–889.

Weng Naidong, Verresen, K., Busson, R., Roets, E. and Hoogmartens, J., Isolation of doxycycline, 6-epidoxycycline and 2-acetyl-2-decarboxamidometacycline from commercial metacycline by preparative column liquid chromatography on silica gel. *J. Chromatogr.,* 586 (1991) 67–72.