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IONIZATION AND SOLUBILITY OF AN AMPHOTERIC β-LACTAM ANTIBIOTIC

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SUMMARY

Solution equilibria of an experimental antibiotic (code named CP-38,371) were investigated. In the pH range 2–4 the compound has two pK values. These overlapping macroconstants were determined by three methods: potentiometric titration, spectrometric titration, and curve-fitting of pH–solubility data. Potentiometric values of pK_1 = 2.86 and pK_2 = 3.81 at 25°C (μ = 0.2) were in agreement (±0.1 unit) with the constants determined by the other methods. CP-38,371 is amphoteric and pK_3 = 7.50 was determined by potentiometric and solubility methods. The complete microionization scheme for the compound was simplified by consideration of the UV spectral characteristics of the ionizing groups. Using absorbance–pH data, 4 microconstants for the important processes were calculated by the method of Edsall et al. (1958), and by a method developed by the authors. The two procedures gave identical results.

INTRODUCTION

The aminopenicillins, ampicillin and amoxicillin, are widely used in antibacterial therapy. The compound 6-[D-2-amino-2-(4-aminophenyl)-acetamido]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3,2,0]hept-2-yl-5-tetrazole, code named CP-38,371 [1], is a new antibiotic with a broad spectrum of activity in vitro. It differs from the aminopenicillins in the substitution of the penicillanic acid carboxyl group with a tetrazole and addition of a primary amino group at the 4-position of the aromatic ring. The antibacterial activity of its 4-hydroxy analog was reported by English et al. (1976). The acidity of tetrazoles is similar to that of carboxylic acids (Herbst, 1956). Unsubstituted tetrazole has pK_a = 4.9 and does not act as a base in dilute aqueous acid (Hansen et al., 1970).

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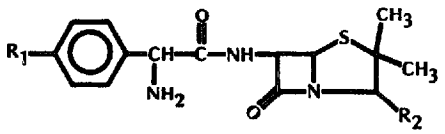
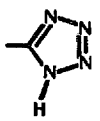


Name	R_1	R_2
Ampicillin	–H	–COOH
Amoxicillin	–OH	–COOH
CP-38,371	$–NH_2$	

Formula 1.

The purpose of this investigation was to study the ionization and solubility of CP-38,371 in aqueous solution. Three macroionization constants and 4 microconstants were determined. A new method for calculation of microconstants from spectrometric data is presented.

MATERIALS AND METHODS

CP-38,371 was synthesized in the Pfizer Central Research Laboratories. The amphoteric form was isolated by aqueous recrystallization as the trihydrate. Purity was approximately 97%, calculated on the anhydrous basis. The major impurity was the corresponding penicilloic acid.

Solubility

Suspensions of CP-38,371 in water were equilibrated at 25 ± 0.2°C in ampules using a vibratory mixer immersed in a water bath. The solution pH was adjusted using hydrochloric acid or sodium hydroxide. Preliminary experiments with periodic sampling indicated that 0.5 h was sufficient for equilibration. This short time was chosen to minimize the possible effect of degradation products on solubility. Degradation of CP-38,371 is insignificant under these conditions. Throughout the pH range 2–4 less than 5% degradation occurred during equilibration (unpublished data on the authors).

Assay

After equilibration the suspensions were filtered (1.2 μm), and drug concentrations in the filtrate were determined by high performance liquid chromatography using a Waters Microbondapak C-18 column. The mobile phase was 0.01 M phosphate buffer at pH 5.5 with 5% (v/v) acetonitrile. The drug was detected by ultraviolet absorption at 254 nm.

Spectrometry

UV spectra were obtained on a Beckman Acta III double beam instrument. For the spectrometric titration a single beam Gilford Model 240 was used. Aliquots of an aqueous

stock solution of CP-38,371 were diluted into various buffers containing hydrochloric acid, citric acid or sodium hydroxide. Ionic strength was adjusted to 0.20 with sodium chloride.

Potentiometric titration

The method of Albert and Serjeant (1971) was used. Drug at 5×10^{-3} M in 0.2 M NaCl was titrated with N HCl at 25 ± 0.2°C using a microburet. Volume change was negligible.

RESULTS AND DISCUSSION

pH–solubility profile

The effect of pH on solubility of CP-38,371 (Fig. 1) resembles the profiles reported for aminopenicillins (Hou and Poole, 1969; Tsuji et al., 1978). But since CP-38,371 has two primary amino groups, its solubility rises more sharply as pH decreases. The total solubility is expressed as the sum of the species:

$$S_T = C_O + C_M + C_D + C_A \quad (1)$$

where subscripts O, M, D and A denote the neutral, monoprotonated, diprotonated and anionic species, respectively. A general ionization scheme for CP-38,371 is shown below:

$$\text{Diprotonated} \underset{K_1}{\rightleftharpoons} H^+ + \text{Monoprotonated} \underset{K_2}{\rightleftharpoons} H^+ + \text{Neutral} \underset{K_3}{\rightleftharpoons} H^+ + \text{Anion}$$

Scheme 1

Substitution of the ionization constants defined in Scheme 1 into Eqn. 1 gives an expression for the pH–solubility profile:

$$S_T = C_O(1 + [H^+]/K_2 + [H^+]^2/K_1K_2 + K_3/[H^+]) \quad (2)$$

In this treatment it is assumed that the only solid phase is the neutral CP-38,371, i.e. the solubility of possible hydrochloride or sodium salts is not exceeded. The pK_a values were determined by visual curve-fitting of the data according to Eqn. 2. The theoretical line was in good agreement with the experimental points.

It is interesting to note that the measured S_T value for CP-38,371 in water (2.10 mg/ml) is slightly greater than the C_O value giving the best fit of the data (2.05 mg/ml). This is consistent with the amphoteric nature of CP-38,371 and nearness of pK_2 and pK_3 to isoelectric pH 5.6.

Potentiometric pK_a values

A modification of the graphical method of Speakman (1940) was used to determine pK_1 and pK_2 from potentiometric data. Since the original method involved titration of an

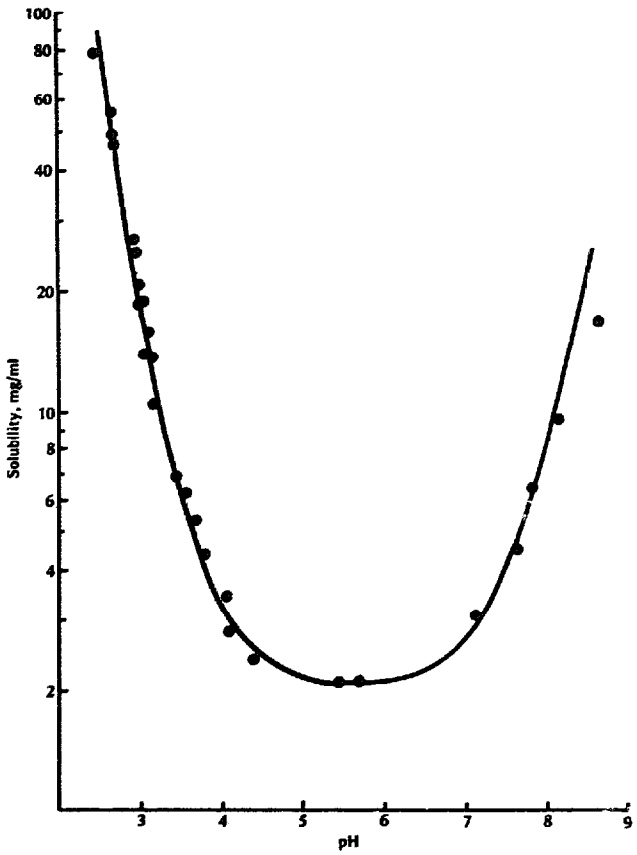


Fig. 1. Solubility of CP-38,371 at 25°C as a function of pH. The solid theoretical line was calculated from Eqn. 2 and the following constants: $pK_1 = 2.65$, $pK_2 = 3.75$, $pK_3 = 7.50$ and $C_O = 2.05$ mg/ml.

acid with hydroxide, the equations were redefined to apply to the present titration of a base with acid. The two ionization constants were calculated from the slope and intercept values obtained by linear regression ($r = 0.999$).

The ionization constants of CP-38,371 are compared with those of ampicillin and amoxicillin in Table 1. The pK_as at 2.7 and 7.2 of the latter compounds are attributed to the carboxyl and α-amino groups, respectively (Hou and Poole, 1969). Although it is clear by structural analogy that $pK_3 = 7.5$ of CP-38,371 may be assigned to the α-amino function, the two ionization constants at lower pH cannot be unambiguously attributed to either the tetrazole or aromatic amino groups. The spectrometric data will show that these constants are not due to a single process but result from combination of micro-ionization equilibria.

Spectrometric titration

Ultraviolet scans for the diprotonated (pH 1.0), neutral (pH 6.1) and anionic (pH 10.0) forms of CP-38,371 are shown in Fig. 2. In the region of 250 nm absorbance

TABLE I

COMPARISON OF IONIZATION CONSTANTS OF CP-38,371 WITH AMPICILLIN AND AMOXICILLIN [a]

Compound	Temp. (°C)	μ	Ionization constants		
			pK_1	pK_2	pK_3
CP-38,371	25	0.2	3.00	3.57	7.50
		–	2.65 [b]	3.75 [b]	7.50 [b]
Ampicillin [c]	25	–	2.66	7.25	
Ampicillin [d]	37	0.5	2.67	6.95	
Amoxicillin [d]	37	0.5	2.67	7.11	9.55

[a] Potentiometric method unless otherwise noted.
[b] Solubility method.
[c] Hou and Poole (1969).
[d] Tsuji et al. (1978).

increases as pH increases from 1.0 to 6.1, but then decreases slightly from pH 6.1 to pH 10.0. Absorption at 246 nm was chosen as an analytical wavelength for determination of pK_1 and pK_2 due to its maximum absorptivity change with pH.

A spectrometric method for determination of closely overlapping pK_a values was described by Albert and Serjeant (1971). The procedure involves determination of three unknowns from absorbance–pH data: the two ionization constants, K_1 and K_2, and the absorptivity of the monoprotonated species (Scheme 1). The apparent absorptivity at some wavelength and constant pH is

$$\epsilon = \epsilon_D F_D + \epsilon_M F_M + \epsilon_O F_O \tag{3}$$

The subscripts are the same as described for Eqn. 1 and the fractions are defined as $F_D = [H^+]^2/T$, $F_M = K_1[H^+]/T$ and $F_O = K_1K_2/T$. The denominator $T = [H^+]^2 + K_1[H^+] + K_1K_2$. Substitution of the fractions into Eqn. 3 and rearrangement gives:

$$\frac{[H^+]^2}{K_1}\left(\frac{\epsilon - \epsilon_D}{\epsilon - \epsilon_O}\right) + K_2 = -[H^+]\left(\frac{\epsilon - \epsilon_M}{\epsilon - \epsilon_O}\right) \tag{4}$$

The absorptivities ϵ_D and ϵ_O were determined from the constant absorbance at low and high pH, respectively. Initial estimates of ϵ_M were obtained from Eqn. 3 by simplification and least squares analysis of the data segregated into groups at low and high pH. The average value of ϵ_M was then used to calculate preliminary estimates of K_1 and K_2 by least squares analysis of all the data according to Eqn. 4. A new value for ϵ_M was calculated using K_1 and K_2 and the process was repeated until the values become constant. Fig. 3 shows absorbance–pH data at 246 nm. The solid line was calculated using constants determined from the iteration procedure. Precision is estimated to be ±0.1 pK unit. Attempts to measure pK_3 (neutral → anionic species) at 246 nm were unsuccessful due to

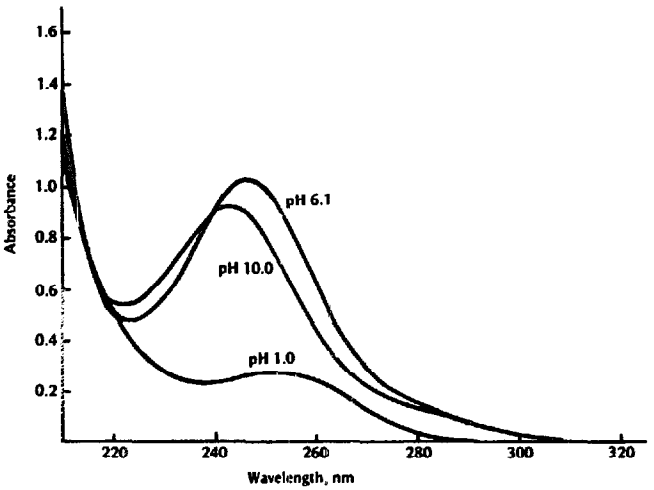


Fig. 2. Ultraviolet spectra of CP-38,371 at pH 1.0 (HCl), pH 6.1 (phosphate buffer) and pH 10.0 (phosphate buffer). Concentration: 1.0×10^{-4} M.

the small absorbance change involved. A pK_3 value of 7.5 was determined potentiometrically in water at 25°C; this was identical to the value obtained by the solubility method.

Microionization equilibria

The presence of 3 ionizing functional groups allows 8 possible molecular species to

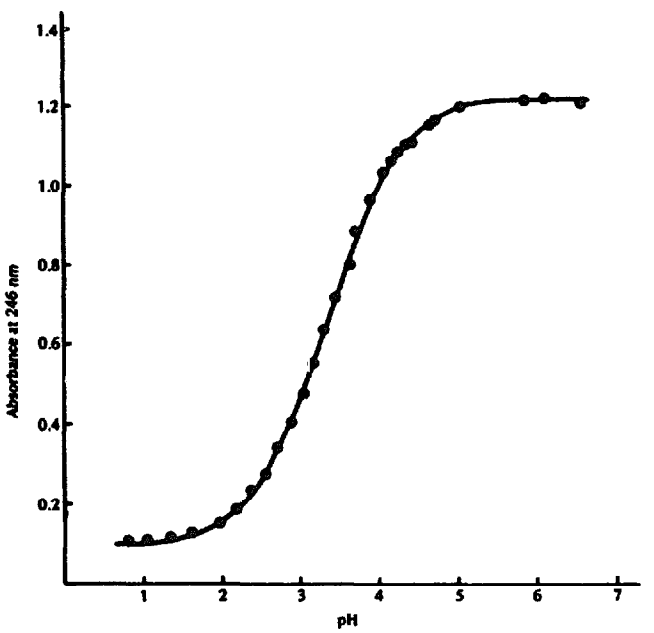
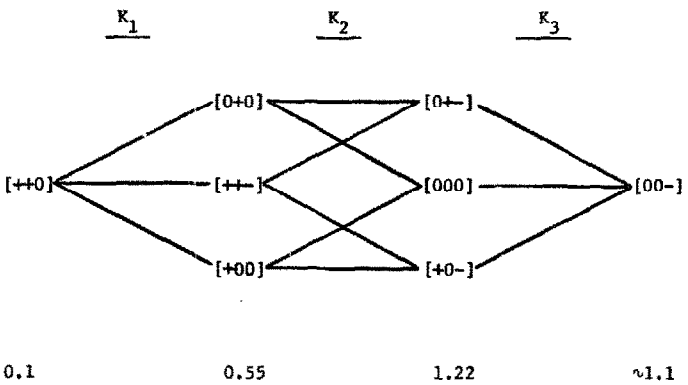


Fig. 3. Absorbance vs pH curve for 1.0×10^{-4} M CP-38,371 at 25°C. Citrate buffer was used at μ = 0.20 (NaCl) except below pH 2 where the buffer was hydrochloric acid (μ = 0.2). The theoretical curve (solid line) was calculated from Eqn. 3 using the following constants: 10^{-4} $\epsilon_D = 0.1$, 10^{-4} $\epsilon_M = 0.55$, 10^{-4} $\epsilon_O = 1.22\ M^{-1}\ cm^{-1}$, $pK_1 = 2.85$ and $pK_2 = 3.65$.

exist (Edsall and Wyman, 1958). The complete ionization equilibria of CP-38,371 are shown diagrammatically in Scheme 2, where the symbols refer to the charge on the aromatic amino, α-amino and tetrazole groups (left-to-right). Interconnecting lines indicate equilibria between the species.



Scheme 2. Complete microionization equilibria.

Simplification of Scheme 2 is possible based on consideration of spectral properties of the functional groups. The ionizing functionalities shall be considered in left-to-right order.

Protonation of aromatic amines causes a large decrease in absorptivity due to the effect on the $\pi \rightarrow \pi^*$ transition of the aromatic ring (Silverstein and Bassler, 1967). Upon protonation the non-bonding electrons of the amine are no longer available for interaction with the π-electrons of the ring. Thus, the apparent absorptivity *increase* with increasing pH observed for macroionization process K_1 must be due in part to the equilibrium [++0] → [0+0].

The state of protonation of the α-amino group should have little effect on the UV spectrum of CP-38,371. Ivashkiv (1973) found that the absorptivity of ampicillin at 257 nm decreased 16% as pH was increased from 5.3 to 9.5. This is not surprising since the ring chromophore is separated from the amine by an aliphatic carbon atom. Thus, microionization equilibria of CP-38,371 involving deprotonation of the α-amino group, such as [++−] → [+0−] or [0+−] → [00−], would be expected to show a small *decrease* in absorptivity with increasing pH.

Tetrazole absorbs weakly in the UV region (Huisgen and Koch, 1955), showing only end absorption as the wavelength approaches 200 nm. The UV spectra of 6-aminopenicillanic acid (penicillin nucleus less the acyl side chain) and its tetrazole analog (a synthetic intermediate of CP-38,371) also do not exhibit absorbance maxima above 200 nm, and the spectra of the two compounds are nearly identical. Absorptivity at 246 nm is approximately 350 M^{-1} cm^{-1} which is much less than that of CP-38,371. The spectra of these compounds are not different at pH 1.1 and 5.5 indicating that pathways involving tetrazole ionization would not affect the UV spectrum of CP-38,371.

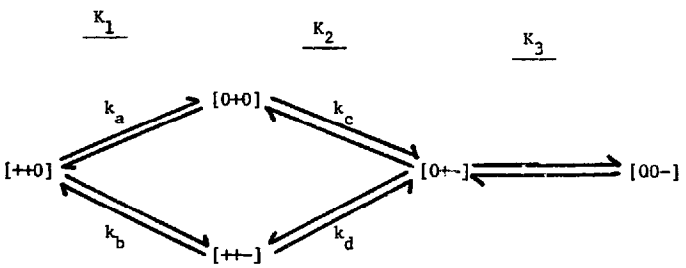
As shown in Scheme 2 the apparent absorptivity of CP-38,371 decreases approximately 10% due to ionization constant K_3. Based on the above discussion, pathway [000] → [00−] should show no detectable change in absorptivity. If the microprocess

[+0–] → [00–] were a significant part of K_3, ϵ should increase which is opposite to the observed change. Also, the prevalence of form [+0–] would cause the absorptivity of the neutral form to be more similar to that of the mono- or diprotonated forms. The [000] and [+0–] forms are thermodynamically unfavorable compared to the [0+–] zwitterion. Since the α-amine is approximately 10^4 times more basic than either the aromatic amine or the tetrazole, protonation should be favored on this site.

Therefore, it is concluded that the zwitterion [0+–] is the predominant electrical neutral form of CP-38,371 and K_3 is essentially [0+–] → [00–]. The low abundance of neutral molecule [000] is supported by the octanol/pH 5 buffer partition coefficient = 0.075 of CP-38,371 at 37°C. Ampicillin was also proposed to exist predominantly as zwitterion (Hou and Poole, 1969) which is consistent with its octanol/water partition coefficient of 0.007 at pH 5 (25°C) (Carney and Hurwitz, 1977).

Consideration of the microionization processes of K_1 allows the pathway [++0] → [+00] to be eliminated. This step again involves the unfavorable proton loss by the more basic α-amino group rather than by the aromatic amine or the tetrazole. Also, since K_1 is the sum of the three processes, the [++0] → [+00] equilibrium constant should certainly be negligible compared to the sum of [++0] → [0+0] and [++0] → [++–].

Based on the above discussion, Scheme 2 may be simplified to Scheme 3. The micro-



Scheme 3. Simplified microionization equilibria.

constants and macroconstants in Scheme 3 are related as follows:

$$K_1 = k_a + k_b \tag{5}$$

$$K_2^{-1} = k_c^{-1} + k_d^{-1} \tag{6}$$

$$k_a k_c = k_b k_d \tag{7}$$

Consistent with the above discussion absorptivities for the microspecies [++–] and [0+0] may be estimated. Since [++–] and [++0] differ only by the proton on the non-UV absorbing tetrazole, then absorptivities at 246 nm may be considered equal, $\epsilon_{++-} = \epsilon_{++0} = 0.10 \times 10^4\ M^{-1}\ cm^{-1}$. Similarly, $\epsilon_{0+0} = \epsilon_{0+-} = 1.22 \times 10^4\ M^{-1}\ cm^{-1}$ is an equally valid assumption. The spectrometric method used to determine macroconstants pK_1 and pK_2 also provided a value for $\epsilon_M = 0.55 \times 10^4\ M^{-1}\ cm^{-1}$. For the monoprotonated form the apparent absorptivity (ϵ_M) and the microspecies absorptivities and fractions (F) are

related by:

$$\epsilon_M = F_{++-}\epsilon_{++-} + F_{0+0}\epsilon_{0+0} \tag{8}$$

$$F_{++-} + F_{0+0} = 1.0 = \frac{k_a}{k_a + k_b} + \frac{k_b}{k_a + k_b} \tag{9}$$

Solving these equations yields $F_{++-} = 0.60$ and $F_{0+0} = 0.40$. These fractions are related to the microconstants by:

$$\frac{F_{0+0}}{F_{++-}} = \frac{k_a}{k_b} = \frac{k_d}{k_c} = 0.667 \tag{10}$$

The 4 microconstants were calculated using Eqns. 5, 6, and 10 and the measured values of K_1 and K_2. Results are shown in Table 2. This method for calculation of microconstants has not been previously reported. It can be shown using Eqns. 3 and 8 that the spectrometric method of Albert and Serjeant (1971) determines the macroconstants and not microconstants. Edsall et al. (1958) described a different method for calculation of microconstants from spectrometric data. The same basic assumption is followed as in the present method, specifically, that the ionization of one of the functional groups (in CP-38,371, the tetrazole) does not change the apparent absorptivity. Adopting the terminology of Edsall et al. (1958) the fraction of species with the aromatic amine in the neutral form is:

$$\alpha_{NH} = \frac{\epsilon - \epsilon_D}{\epsilon_O - \epsilon_D} = \frac{k_a/[H^+] + k_a k_c/[H^+]^2}{1 + (k_a + k_b)/[H^+] + k_a k_c/[H^+]^2} \tag{11}$$

where ϵ is the apparent absorptivity at some pH. A function M is defined as:

$$M = \frac{[H^+]\alpha_{NH}}{1 - \alpha_{NH}} = \frac{k_a[H^+] + k_b k_d}{[H^+] + k_b} \tag{12}$$

The microconstants k_a and k_d are obtained from the intercepts of a plot of pM vs α_{NH}. Fig. 4 shows the data of Fig. 3 plotted in this manner. When H^+ is large, α_{NH} approaches zero and $pM = pk_a$. Similarly, for small values of H^+, α_{NH} approaches one and $pM = pk_d$. An estimate of pk_b was calculated and the final value was then obtained by curve-fitting of the data in Fig. 4 according to Eqn. 12. The curve was not a sensitive function of pk_b due to the similarity of pk_a and pk_d. The fourth constant pk_c was then calculated using Eqn. 7. This procedure was also used by Riegelman et al. (1962) to determine ionization constants of some phenylalkanolamines.

As shown in Table 2, the two methods give identical results. The method of the authors is somewhat simpler since the microconstants are calculated directly from K_1, K_2 and ϵ_M. The method is highly dependent on ϵ_M since this value leads directly to the ratio of the microconstants. Using Eqn. 4 it was found, however, that variation of ϵ_M could be easily detected in the fit of the theoretical curve (Fig. 3). This method also appears to be

TABLE II

MACRO- AND MICROIONIZATION CONSTANTS OF CP-38,371 AT 25°C (μ = 0.2)

Macro-constant	Micro-constant	Spectrometric method [a]	Spectrometric method [b]
pK_1		2.85	2.80
	pk_a	3.25	3.22
	pk_b	3.07	3.0
pK_2		3.65	3.61
	pk_c	3.25	3.19
	pk_d	3.43	3.41
	k_a/k_b	0.67	0.60

[a] Described in the text.
[b] According to Edsall et al. (1958).

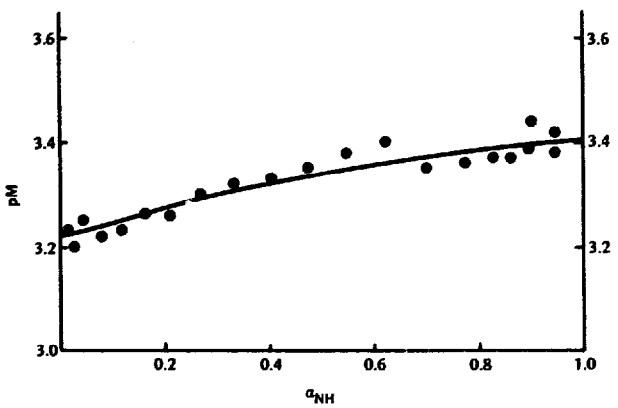


Fig. 4. Plot of the spectrometric data according to Eqn. 12.

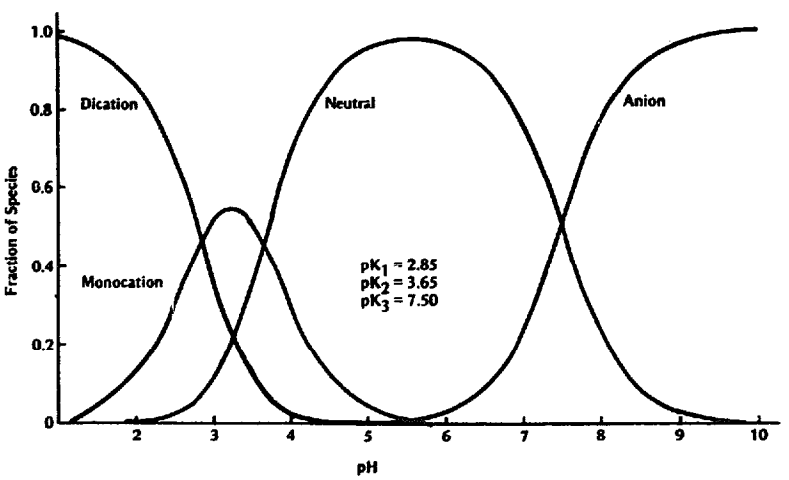


Fig. 5. Distribution diagram for CP-38,371.

less complicated than that of Streng et al. (1976), which required both spectrometric and potentiometric data to calculate the microconstants.

Distribution of species of CP-38,371 as a function of pH is shown in Fig. 5. Fractions were calculated from:

$$\text{Dication} = \frac{[H^+]^2}{[H^+]^2 + K_1[H^+] + K_1K_2 + K_1K_2K_3/[H^+]}$$

$$\text{Monocation} = K_1[H^+]/\text{denominator}$$

$$\text{Neutral} = K_1K_2/\text{denominator}$$

$$\text{Anion} = K_1K_2K_3[H^+]^{-1}/\text{denominator}$$

According to Table 2 the curve for the monocation can be further divided into species [0+0] : [++–] in a 2 : 3 pH-independent ratio.

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