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Determination of the dissociation constants of the cephalosporins cefepime and cefpirome using UV spectrometry and pH potentiometry

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Abstract

UV spectrometry and pH potentiometry were used for the determination and direct characterization of the dissociation constants of cefepime (Cef) and cefpirome. The absorbance/pH profiles at two analytical wavelengths and different conditions were assessed and found to conform to those of diprotic acids. The titration curves indicated a triprotic acid profile with two overlapping dissociation constants. The comparison of the results of both techniques permitted the direct attribution of the three dissociation constants to the carboxylic group at position 4 of the Δ-3 cephem nucleus, the aminothiazole group and the amide group at position 7 of the Δ-3 cephem nucleus. Stability studies of Cef in alkaline solutions were also performed in order to evaluate the accuracy of the measurements carried out for the determination of the third pK_a value. The experimental pK_a values were compared to the corresponding predicted values derived by PALLAS/PKALC and Advanced Chemical Development (ACD) software packages.

Keywords: Cefepime; Cefpirome; Dissociation constants; UV Spectrometry; Potentiometry; pK_a prediction

1. Introduction

The important role of the degree of ionization in the biological behavior of chemical substances, as well as in their ability to passive transcellular diffusion and/or in their suitability as substrates for active transport is well established [1]. Several experimental approaches have been employed for the determination of the dissociation constants, including potentiometry, UV spectrometry, HPLC, phase solubility and recently capillary zone electrophoresis techniques [2–5]. In addition, relevant software have been developed for the rapid estimation of pK_a values based on the chemical structure [6,7], the reliability of which

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reflects the accuracy of the underlying experimental data. As inferred from MEDCHEM database, considerable differences are observed in the pK_a values measured in different laboratories [8], while the presence of several often overlapping dissociation constants renders difficulties in their correct attribution to the ionization centers.

Cefepime (Cef) and cefpirome (Cefp) are fourth generation cephalosporins with a broad spectrum of activity against many gram-positive and gram-negative bacteria. They contain one carboxylic group, an aminothiazole ring and positively charged quaternary nitrogen at position 3 of the cephem nucleus, responsible for the formation of zwitterionic species over a broad pH range. The chemical structures of Cef and Cefp are depicted in Fig. 1.

Two overlapping pK_a values of Cef (1.3/3.2 and 1.5/3.1) have previously been reported and indirectly attributed to the acidic (–COOH) and the basic (aminothiazole) center, respectively [9,10]. No details of the experimental protocol and the data treatment are given by the authors. Using capillary zone electrophoresis, one pK_a value has been reported for Cef (3.36) and Cefp (3.10) [5]. Both cephalosporins showed a zero electrophoretic mobility at pH above 4.5, whereas at lower pH they presented positive electrophoretic mobilities. Although this behavior is not commented on in the paper, it is our opinion that the determined pK_a relates to the protonated aminothiazole group, the carboxylic anion being masked by the formation of zwitterionic species with the permanently charged nitrogen group. The same authors reported the values 3.03 and 3.04 for Cef and Cefp measured by potentiometry. No literature data are found concerning the amide group in position 7 of the Δ-cephem-ring. One paper refers to the corresponding group of the structurally related cefotaxime and assigns a pK_a value of 10.9, indicating increased acidity, relatively to the common behavior of amides [11]. Cefotaxime, like Cef and Cefp,

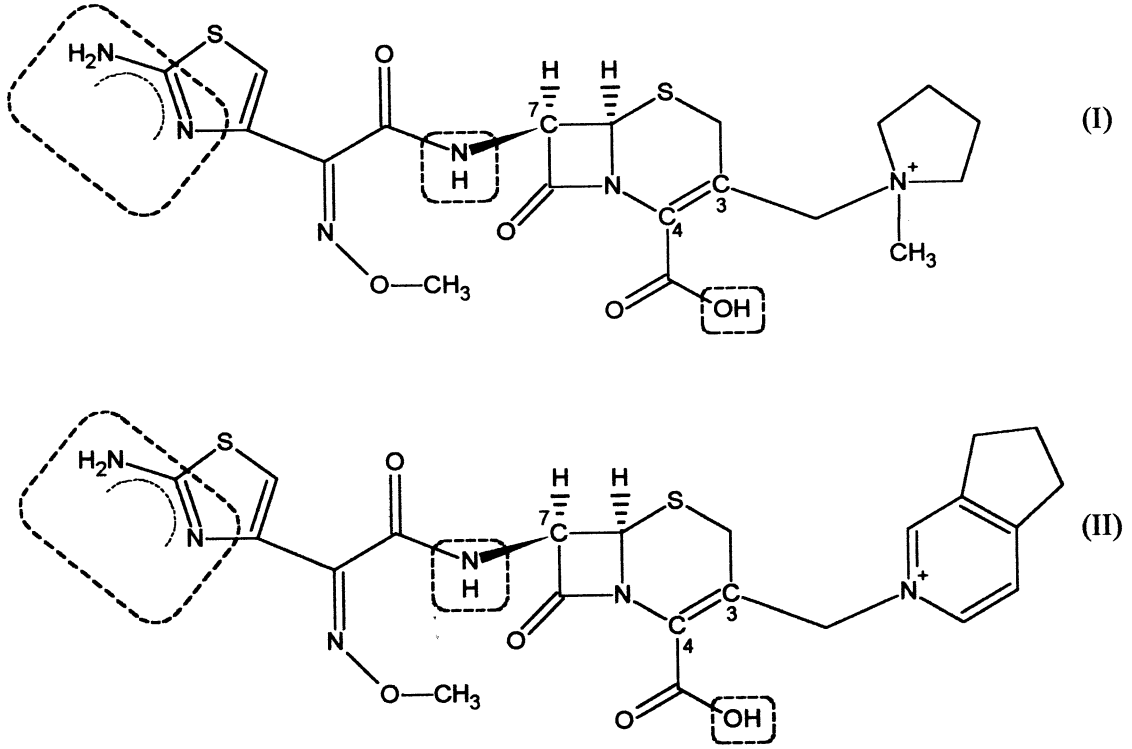

Fig. 1. Chemical structures of Cef I and Cefp II.

bears a methoxy group attached to the =N-group, capable to form internal hydrogen bond with the amide hydrogen.

In the present study UV spectrometry and potentiometry were used in order to determine and characterize the three dissociation constants of Cef and Cefp. Effort was given for the correct characterization and refinement of the two overlapping dissociation constants, since they are biologically more relevant and from analytical point of view more important. The combination of both analytical techniques permits the direct identification of the ionization centers, since the differentiation of the UV spectrum in the pH range 1–5 is due to the aminothiazole group [12]. Moreover, the stability of the Δ-cephem ring in alkaline solutions at ambient temperature was investigated in order to appreciate the reliability of measurements in the pH range above 9 for the determination of the third dissociation constant. Finally, the experimental pK_a values were compared to the predicted ones obtained by two different softwares.

2. Materials and methods

2.1. Materials

Cef dihydrochloride monohydrate was provided by Bristol-Myers Squibb. Cefp sulfate was provided by Aventis. All reagents were of analytical grade and were purchased from Fluka, Merck and Panreac. Fixanal® NaOH and HCl standard solutions, purchased from Riedel de Haen, were used as titrants.

2.2. Estimation of the dissociation constants from structure

Estimation of the three dissociation constants was performed using the PALLAS/PKALC, version 2.1 [6] and Advanced Chemical Development/pK_a predictor (ACD) [7] software packages. PKALC estimates the acidic and basic pK_a values using Hammet type equations for the relevant dissociation centers. In ACD/pK_a calculations, sets of equations are used, which parameterize a large number of experimental pK_a values using many variants of electronic substituent constants to cover combinations of the most common ionizable functional groups. In both softwares the input is the chemical structure drawn in a graphical mode.

2.3. UV spectrometry

UV spectrometry was used for the stability study of Cef in alkaline solutions and the pK_a determination. UV absorption spectra were recorded on a double beam Perkin–Elmer Spectrophotometer Lambda 7, using a 1-cm path length.

2.3.1. Cef stability in alkaline solution

The stability of Cef in alkaline solution using Universal buffer was studied at room temperature. Universal buffer was prepared as a mixture of equal volumes of 0.1 M H_3PO_4, 0.1 M H_3BO_3 and 0.1 M CH_3COOH. The resulting pH was ~ 2 and ionic strength 0.013 M, as calculated from the corresponding ion concentrations. pH was adjusted to the desired value by the addition of NaOH.

A stock solution of 4.00×10^{-4} M Cef in water was 10-fold diluted with Universal buffer at pH 11.2 and immediately submitted to successive scanning, at time intervals 0, 13, 18, 21, 24 and 30 min. The successive UV spectra obtained are presented in Fig. 2. At $t = 0$ Cef presents two maxima at 230 and 264 nm. The maximum at 264 depends on the integrity of the β-lactam ring, as well as on the C3 and C4 double bond conjugation in the Δ^3 cephalosporin system [12]. The absorption at 264 nm decreases with time and the peak gradually disappears. At the same time the absorption at 230 nm increases and is shifted to lower wavelength. The wavelength at 264 nm was chosen for further stability studies. The stock solution of Cef was 10-fold diluted with Universal buffer previously adjusted at pH values 7.0, 8.0, 9.0 and 10.0 and the solution was immediately placed in a 1-cm cuvette. Absorbance was continuously recorded during a 30-min interval and plotted versus time (Fig. 3). Pseudofirst order degradation rate constants were calculated by linear regression analysis using Eq. (1).

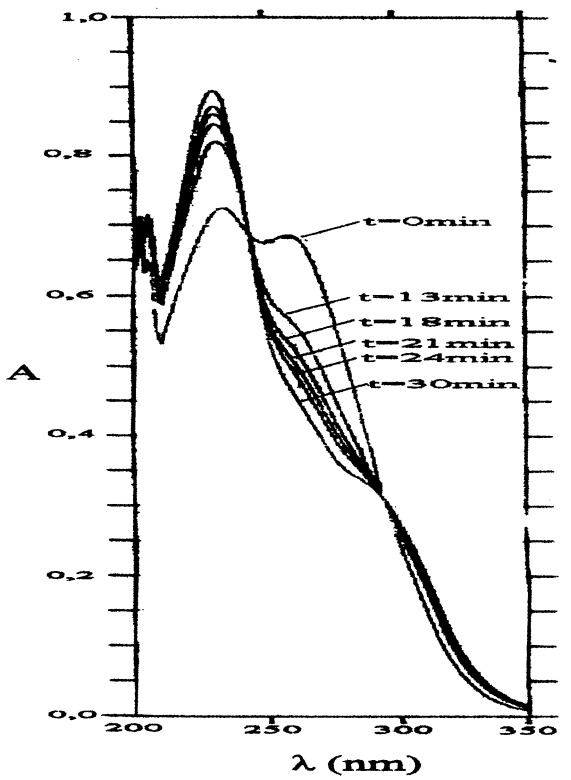

Fig. 2. UV absorption spectra of 4.00×10^{-5} M Cef in Universal buffer at pH 11.2, at different time intervals.

$$\ln A = \ln \mathrm{Ao} - kt \quad (1)$$

2.3.2. pK_a determination by UV spectrometry

A series of 21 buffer solutions were prepared in the pH range 1.8–10.9 using KCl/HCl (pH 1.8) or phosphate salts. Stock solutions of Cef and Cefp were prepared in water in concentration 4.00×10^{-4} M. Working solutions were prepared by 10-fold dilution of the stock solutions with the appropriate buffer. Experiments were performed also with Universal buffer in the pH range 1–6.5 using 12 solutions at final concentrations 2.00, 4.00 and 8.00×10^{-5} M for Cef and 4.00×10^{-5} M for Cefp. The whole procedure was repeated at fixed ionic strength 0.15 M, by the addition of the appropriate amount of KCl. Solutions were kept in dark. Measurements were carried out in successive days and within the same day using fresh working solutions.

Fig. 4 shows the UV absorption spectra of Cefp solutions in Universal buffer in the pH range 1–6.5.

pK_a determination was accomplished by using absorbance data at two analytical wavelengths, 264 and 230 nm for Cef / 266 and 233 nm for Cefp. Absorbance data were converted to the observed molecular absorptivities $\varepsilon_{obs} = A/C$, where A is the absorbance and C the analyte concentration. Data of the whole absorbance—pH profile (diprotic acid behavior) were treated by non-linear fitting using model Eq. (2). Data in the pH range 1–6.5 were fitted in model Eq. (3) (monoprotic acid behavior) or its linear transformation, Eq. (4)[2].

$$\varepsilon_{obs} = \frac{\varepsilon_{H_2A}[H^+]^2 + \varepsilon_{HA^-}K_1[H^+] + \varepsilon_{A^{2-}}K_1K_2}{[H^+]^2 + K_1[H^+] + K_1K_2} \quad (2)$$

$$\varepsilon_{obs} = \frac{\varepsilon_{HA}[H^+] + \varepsilon_{A^-}K_1}{[H^+] + K_1} \quad (3)$$

$$\frac{\varepsilon_{obs} - \varepsilon_{HA}}{\varepsilon_{A^-} - \varepsilon_{obs}} = \frac{1}{[H^+]}K_1 \quad (4)$$

2.4. Potentiometric titration

The titration was performed with an automatic potentiometric titrator (Shanghai Rex, Shanghai) fitted with a glass-calomel conjugated electrode, a 10-ml automatic burette and a mechanical stirrer. The burette was calibrated by weighting the delivered doubly distilled and purified water and was found to have a delivery rate 0.0099 ± 0.0001 ml of titrant per count. The calibration of the electrode system was achieved with two standard buffers of pH either 1.68 (potassium tetraoxalate—Hanna) and 4.01 (hydrogen potassium phthalate—Shanghai Rex) or 4.01 and 6.86 (KH_2PO_4 0.025 M/Na_2HPO_4 0.025 M mixture) in order to obtain the standard e.m.f. of the cell (E^0) and the slope (s) of the glass electrode at 25 °C.

Fifty milliliter of 1.00×10^{-2} M solutions of Cef and Cefp were prepared in bi-distilled water, which had been degassed by boiling for 30 min. The

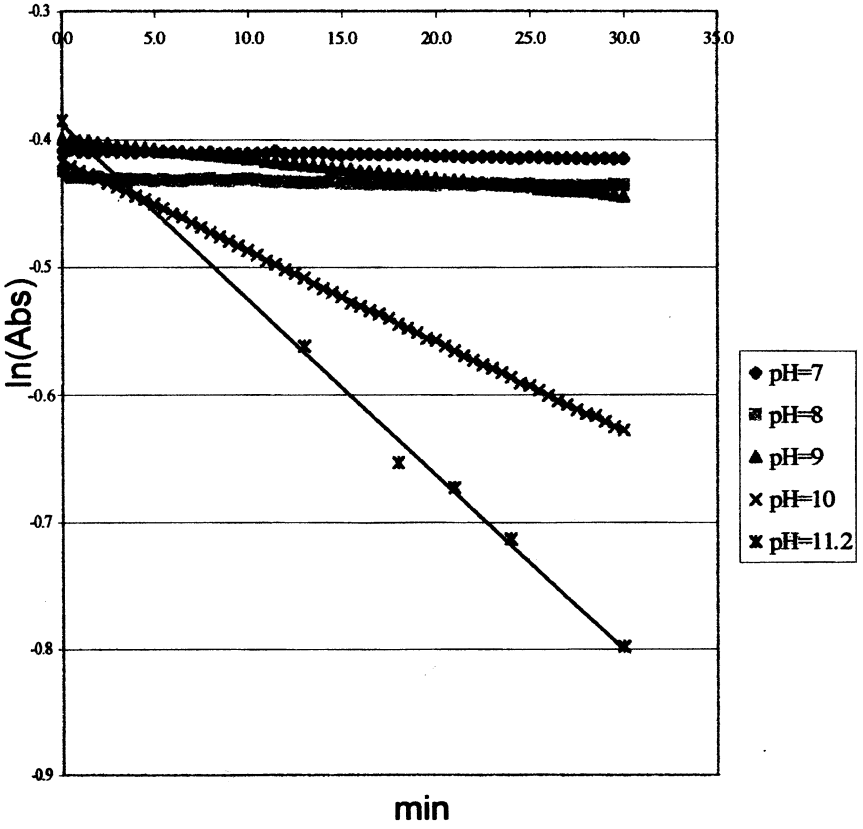

Fig. 3. Absorbance of 4.00×10^{-5} M Cef in Universal buffer at 264 nm versus time at different pH values.

solutions were titrated under nitrogen atmosphere using carbonate free KOH 0.999 M, standardized by titration with standard potassium hydrogen phthalate (Sigma). Each titration was performed in triplicate. For Cef the titration was repeated in the presence of exact excess (3.0 ml) of HCl 0.1 M, in order to have all the ionizable groups protonated.

Readings of the potential were recorded after stabilization of the electrode response (15 s) and transformed in the pH scale using the equation $\mathrm{pH} = E - E^0/s$.

The analysis of the titration curve data was based on the P method [12], P being the average number of the unbound protons, calculated according to Eq. (5).

$$P = \frac{[\mathrm{K}^+] + [\mathrm{H_3O}^+] - [\mathrm{OH}^-]}{C} \quad (5)$$

where $[\mathrm{K}^+]$ is the concentration of the potassium ion, calculated from the titrant volume added and C is the total concentration of the analyte.

If n is the number of dissociable protons, the P versus pH plot values at $P = n - 0.5$ correspond to the dissociation constants of the nth ionization center. For a diprotic acid, further refinement of the dissociation constants is achieved according to Eq. (6)

$$P = \frac{\mathrm{K_1}[\mathrm{H}^+] + 2\mathrm{K_1K_2}}{[\mathrm{H}^+]^2 + \mathrm{K_1}[\mathrm{H}^+] + \mathrm{K_1K_2}} \quad (6)$$

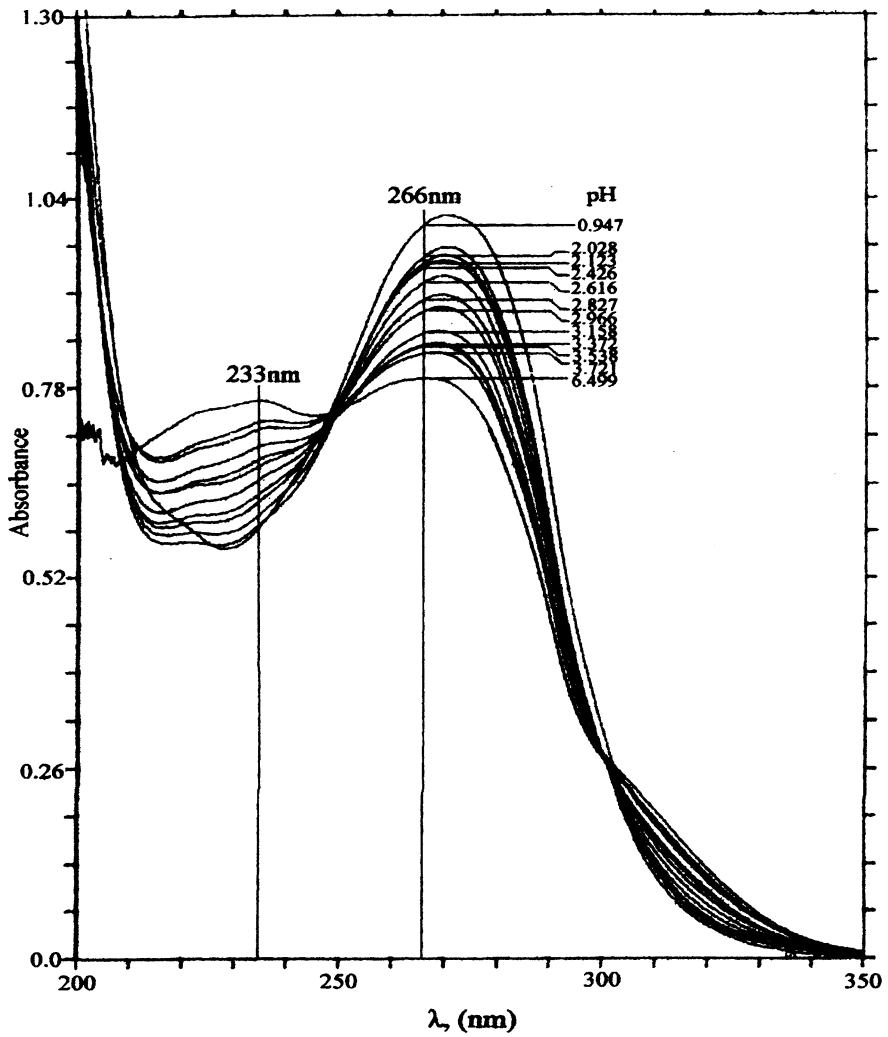

Fig. 4. UV absorption spectra of 4.00×10^{-5} M Cefp in Universal buffer adjusted at different pH values.

3. Results and discussion

3.1. Stability of Cef

Degradation kinetics for Cef has previously been reported to follow a U shaped rate-pH profile. That study however refers to elevated temperature in a pH range 1–8.6 [8]. Under the experimental conditions used for pK_a determination in the present study, Cef showed no stability problems in strong acidic solutions. In alkaline solutions however degradation was obvious. As illustrated in Fig. 3 the decrease in absorbance with time is significant at pH $\geqslant$10. Degradation apparent rate constants calculated according to Eq. (1), as well as the percentage of the intact substance after 30 min are presented in Table 1.

3.2. Determination of dissociation constants by UV spectrometry

The absorbance/pH profile of Cef at 230 nm over a pH range 1.8–11.2 corresponded to that of a diprotic acid (Fig. 5). Absorbance raised con-

Table 1
Degradation rate constants of Cef in alkaline solutions

pH	$k \times 10^4$ (min^{-1})	r^2	Percentage remaining after 30 min
7.0	2.03	0.916	99.3
8.0	2.41	0.812	98.9
9.0	14.94	0.997	95.6
10.0	70.73	0.9998	81.0
11.2	137.6	0.996	66.2

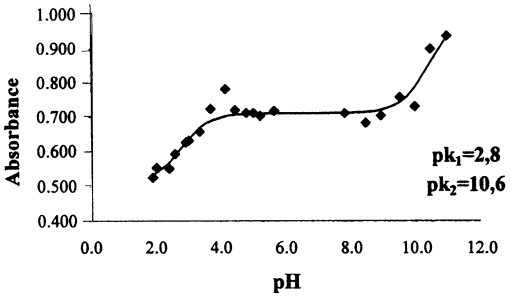

Fig. 5. Absorbance/pH profile of Cef at 230 nm.

stantly with increasing pH, it remained stable in a pH range 5–9 and it raised again at higher pH. The increase in absorbance at pH above 9 may be attributed to both the dissociation of the amide group and the degradation of the cephem ring, the latter however being not more than 5% during the time period of the experiment as deduced by the corresponding rate constants.

Fitting the data produced in the pH range 1.8–11.2 using Eq. (2), the following rough estimates were generated with $r^2 = 0.930$.

$$\varepsilon_{H_2A} = 1.26(\pm 0.08) \times 10^4 \ (mol^{-1}lcm^{-1})$$

$$\varepsilon_{HA^-} = 1.79(\pm 0.03) \times 10^4 \ (mol^{-1}lcm^{-1})$$

$$\varepsilon_{A^{2-}} = 2.61(\pm 0.24) \times 10^4 \ (mol^{-1}lcm^{-1})$$

$$pK_{a1} = 2.8(\pm 0.2)$$

$$pK_{a2} = 10.6(\pm 0.3)$$

where ε_{H_2A}, ε_{HA^-}, $\varepsilon_{A^{2-}}$ correspond to the molar absorptivities of the protonated aminothiazole, the deprotonated aminothiazole and the deprotonated amide group species, respectively. It should be noted that ε_{H_2A} and ε_{HA} coincide with experimentally observed molar absorptivities at pH 1.8 and >5, respectively. Due to a certain degree of degradation at pH >10, the value of pK_{a2} should be considered only indicative.

Refinement of pK_{a1} was achieved by the use of Universal buffer in order to keep buffer conditions stable over a pH range 1–6.5 and recording the spectra of 12 solutions within the same day at two analytical wavelengths. The robustness of the method was further validated by performing the same experiments at different concentrations of Cef, as well as in presence of 0.15 M KCl. No significant differentiation in the pK_{a1} values was observed by the above modifications of the experimental conditions. Data were analyzed by both non linear fitting and linear regression according to Eqs. (3) and (4) and the results are presented in Table 2.

Following the same experimental protocol the pK_a value of the aminothiazole group of Cefp was determined and refined. The obtained data are included also in Table 2.

3.3. Determination of dissociation constants with potentiometric titration

The titration curves indicated a triprotic acid profile with two overlapping ionization constants—no inflection point between the two first equivalent volumes is observed. At higher pH values the time for the recording of the electrode response had to be compressed in order to avoid degradation. Longer time intervals between successive additions of the titrant led to a drift in the electrode response, while the appearance of the solution turned into colored. As a consequence, the titration curve at higher pH should be considered less accurate and it was used only for rough pK_a estimation according to the P plot.

As illustrated in Fig. 6 two dissociation constants could be derived according to the P plot.: At $P = 2.5$, pK_a 10.8 for both cephalosporins, corresponding to the amide group and at $P = 1.5$, pK_a 3.02 and 3.15 for Cef and Cefp respec-

Table 2
pK_a values of the aminothiazole group of Cef and Cefp produced by UV spectrometry under different conditions

Conditions	Model	230 nm	r^2	264 nm	r^2
Cefepime					
C: 2.00×10^{-5} M	N. L	2.98 ±0.10	0.972	2.85 (±0.07)	0.984
I: 0.013 M	L.	3.18 ±0.03	0.966	2.82 (±0.03)	0.972
C: 4.00×10^{-5} M	N. L	2.87 ±0.04	0.995	2.90 (±0.02)	0.998
I: 0.013 M	L.	3.09 ±0.02	0.993	2.94 (±0.03)	0.962
C: 8.00×10^{-5} M	N. L	2.95 ±0.05	0.992	2.87 (±0.05)	0.992
I: 0.013 M	L.	3.06 ±0.01	0.994	2.88 (±0.01)	0.993
C: 4.00×10^{-5} M	N. L	2.95 ±0.06	0.989	2.98 (±0.04)	0.995
I: 0.15 M	L.	3.03 ±0.01	0.995	3.02 (±0.02)	0.989
Cefpirome					
		233 nm		266 nm	
C: 4.00×10^{-5} M	N. L	3.03 ±0.04	0.994	2.83 (±0.06)	0.989
I: 0.013 M	L.	3.06 ±0.02	0.991	3.04 (±0.04)	0.943
C: 4.00×10^{-5} M	N. L	2.89 ±0.09	0.975	3.03 (±0.05)	0.992
I: 0.15 M	L.	3.12 ±0.03	0.993	2.97 (±0.03)	0.982

C, concentration; I, ionic strength. Model: N.L., non linear, Eq. (3), L, linear Eq. (4).

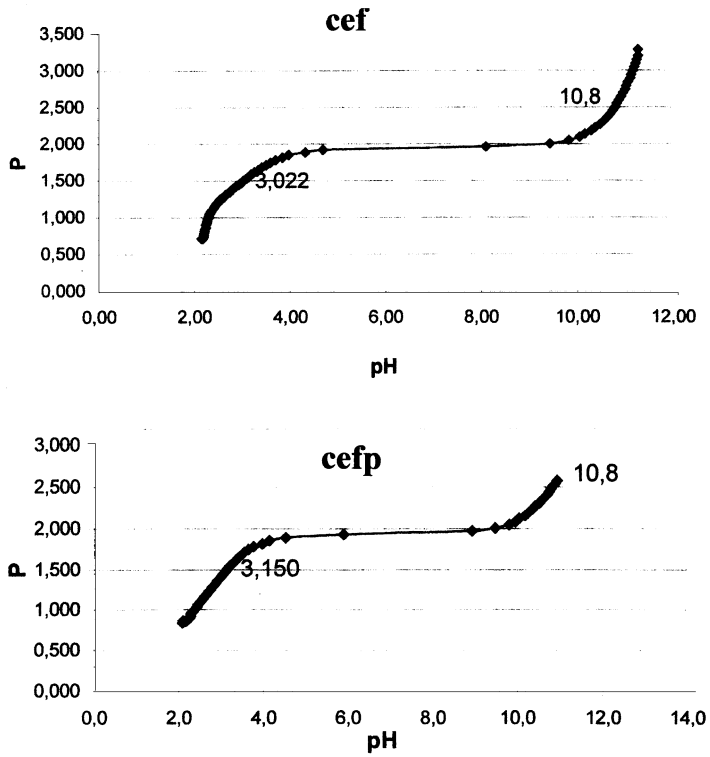

Fig. 6. P plots of Cef and Cefp.

tively, in accordance with the dissociation constants derived by the UV spectrometry. The pK_a value corresponding to $P = 0.5$ could not be derived graphically, since titration starts at higher pH value, due to the strong acidic function of the carboxylic group. Titration was repeated after calibration of the electrode for lower pH and pK_a values were refined by non-linear fitting using Eq. (5). The pK_a values obtained for Cefp are pK_{a1} 1.62 ($\pm$0.02), pK_{a2} 3.11 ($\pm$0.01) with $r^2 = 0.995$. For Cef Eq. (5) led to pK_{a2} 3.07 ($\pm$0.01) with $r^2 = 0.994$. However no reliable pK_{a1} value could be obtained by the same procedure, since critical data points at lower pH values are missing due to the higher acidity of the Cef carboxylic group. To overcome this problem titration was repeated in presence of an exact excess of HCl. Fitting the data in Eq. (5) resulted to $r^2 = 0.998$ and the following values were determined: pK_{a1} 1.12 ($\pm$0.01) and pK_{a2} 3.21 ($\pm$0.01). pK_{a1} reflects the strong acidic character of the carboxylic group of Cef but it should be considered cautiously since it is not within the concentration rule [2]. Moreover the excess of HCl may be considered as a source of error leading to a small deviation in the value of pK_{a2}.

3.4. Comparison between experimental and predicted pK_a values

Predicted and mean experimental pK_a values are presented in Table 3. pK_a values from ACD are accompanied by standard errors of about 0.5 log units. PKALC does not provide standard errors upon calculation, however a previously published validation equation for various drugs reported a standard deviation $s = 0.60$, reflecting a rather large tolerance in predictions [13]. Under these considerations, PKALC predicted correctly the strong acidic character of the carboxylic group of Cef, while it failed in the case of Cefp. This is due to the fact that the quaternary pyridinium substituent of Cefp is not available in the library of the program and a phenyl group was used instead for the calculation. ACD underestimated the acidity of the carboxylic group of both cephalosporins, predicting pK_a values higher than 1.5 log units.

The prediction of the pK_a value of the aminothiazole group is satisfactory by both methods. Particularly ACD provided accurate estimates of this pK_a. A very weak acidic character was assigned to the amide group by PKALC, while for the same group ACD predicted higher acidity than experimentally testified.

4. Conclusions

There is satisfactory agreement between the dissociation constants of Cef and Cefp determined by UV spectrometry and potentiometry. The combination of the two methods permits the direct assignment of the pK_a values to the ionization centers. The acidity of the carboxylic center is highly influenced by the quaternary nitrogen substituent which promotes the acidic character, more evidently in the case of Cef. The relatively high acidity found for the amide group is in

Table 3
Predicted and experimental pK_a values of Cef and Cefp

Ionization center	Cefepime				Cefpirome			
	PKALC	ACD	pK_a (UV)[a]	pK_a (POT)[b]	PKALC	ACD	pK_a (UV)[a]	pK_a (POT)[b]
$COOH \rightarrow COO^-$	0.78	2.46 $\pm$ 0.50		1.12 $\pm$ 0.01	2.54	2.97 $\pm$ 0.50		1.62 $\pm$ 0.02
$NH^+ \rightarrow N$ (aminothiazole)	2.49	2.87 $\pm$ 0.50	3.00 $\pm$ 0.04[c]	3.07 $\pm$ 0.04	2.49	2.87 $\pm$ 0.50	3.04 $\pm$ 0.04[c]	3.11 $\pm$ 0.01
$NH \rightarrow N^-$ (amide)	16.27	7.82 $\pm$ 0.40	10.6 $\pm$ 0.3 (n.r.)	10.8[d] (n.r.)	16.27	7.90 $\pm$ 0.40	n.m.	10.8[d] (n.r.)

n.m., non measured; n.r., non refined.
[a] measured by UV spectrophotometry.
[b] measured by potentiometry.
[c] mean value of the data presented in Table 2, derived by the linear model (Eq. (4)).
[d] derived graphically.

accordance with the corresponding pK_a value reported for cefotaxime [10]. Calculation systems agree in the correct prediction of aminothiazole basicity, give a rough estimate of the acidity of the carboxylic group and fail to provide reliable results for the amide group.

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