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Determination of dissociation constants of cephalosporins by capillary zone electrophoresis

Yahya Mrestani[a,*], Reinhard Neubert[a], Angela Munk[b], Michael Wiese[b]

[a]*Institute of Pharmaceutics and Biopharmaceutics, Martin-Luther-University, Wolfgang-Langenbeck-Strasse 4, D-06120 Halle/S., Germany*

[b]*Institute of Pharmaceutical Chemistry, Martin-Luther-University, Wolfgang-Langenbeck-Strasse 4, D-06120 Halle/S., Germany*

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Abstract

Although the cephalosporins are important drugs in medical and in pharmaceutical research, the pK_a of these substances is frequently not known. Dissociation constants of cephalosporins were determined by capillary zone electrophoresis (CZE) as a new technique and potentiometric titration (PMT) at low solute concentrations. The CZE method relies on measuring the ionic mobility of the solute as a function of pH. Mobility and pH data are fitted to an equilibrium expression by using nonlinear regression. The total analysis time for the determination of the pK_a using CZE was 40 min. Potentiometric titration was performed as a comparative method to CZE for the determination of the dissociation constants. The total analysis time was 50 min and the sample concentration was between 200 and 300 μg/ml. © 1998 Elsevier Science B.V.

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1. Introduction

Cephalosporins have similar structures and are antibiotically active compounds (Fig. 1). However, only a small number of the cephalosporins can be orally administered. Therefore, there is a need to improve the oral bioavailability of these drugs. For this purpose the physicochemical properties of the cephalosporins must be determined. For characterizing these properties water solubility, partition coefficient, and dissociation constant are usually measured. Particularly, for developing new antibiotics, the negative log of the acid dissociation constant has become of great importance because the passage of many drugs into cells and across other membranes is a function of the internal environment, of the physicochemical properties and of the pK_a of the drugs [1]. Potentiometric, UV spectrophotometric [2,3], solubility and paper electrophoretic [4,5] methods have been employed for the determination of pK_a values. The determination of the dissociation constant using CZE has been reported previously [6,7]. The CZE technique has some advantages compared to other methods. It requires only small amounts of sample at low solute concentrations. The procedure does not require measurement of solute or titrant concentrations, only of migration times. Furthermore, the calculation of the pK_a is independent of solute purity. Potentiometric titration was used for pK_a determination of cephalosporins. It requires a small amount of pure sample mass. Insoluble compounds can be assayed in aqueous solutions or in

*Corresponding author.

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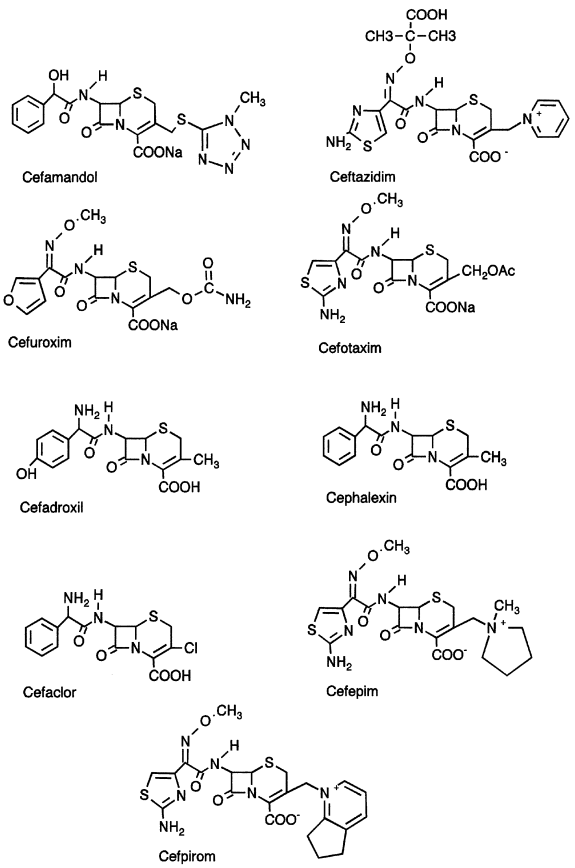

Fig. 1. Chemical structure of the investigated compounds.

water–solvent mixtures by taking very small masses. If not enough sample is available, the entire titration can be completed in 1 ml of solution with less than 0.1 ml of titrant added. The method in this report has several advantages compared to traditional half neutralisation or graphical methods. First, the models are based on the analytic solution of the charge balance equation, with no simplifying approximations. Second, the method can be used to determine dissociation constants in cases where no inflection point is present, such as for overlapping ionization processes.

2. Theory

When a base is protonated, the effective mobility of the analyte, μ_e is given by the following equation [8,9]:

$$\mu_e = \frac{[BH^+]}{[BH^+] + [B]}\,\mu_b \tag{1}$$

where μ_b is the electrophoretic mobility of the fully protonated species. The equation can be rearranged to:

$$\mu_e = \frac{[H^+]/K_a}{1 + [H^+]/K_a}\,\mu_b \tag{2}$$

K_a is the acid dissociation constant.

The effective mobilities of the analytes were determined at different pH values using the following equation [10]:

$$\mu_e = \frac{L_g L_l}{V}\left(\frac{1}{t_m} - \frac{1}{t_0}\right) \tag{3}$$

where μ_e is the effective mobility, V the applied voltage, L_g the effective capillary length (to the detector), L_l the total capillary length, t_m the migration time of the solute, t_0 the migration time of neutral marker.For a weak acid, analogous equations are derived as follows:

$$\mu_e = \frac{K_a/[H^+]}{1 + K_a/[H^+]}\,\mu_a \tag{4}$$

where μ_a is the electrophoretic mobility of the fully deprotonated species.

The ionization constants of the ampholyte compounds were also determined using the method of Ishihama et al. [11].

3. Experimental

3.1. Apparatus

3.1.1. Capillary electrophoresis

Capillary electrophoretic experiments were performed using a Hewlett-Packard Model G1600A (Waldbronn, Germany) ^{3D}CE system with diode-array detector from 190 to 600 nm. CE CHEMSTATION equipped with a HP Vectra 486/66U workstation was used for instrument control, data acquisition, and data analysis. The system was controlled by win-

dows software, which was modified to the HP system. The detection wavelength was 270 nm. Fused-silica capillaries obtained from Hewlett-Packard [48.5 cm (length to detector 40 cm)×50 μm I.D.] were used for the determination and for the separation of cephalosporins.

3.1.2. Potentiometric titration

For automated pK_a determination a PCA101 analyser was used, consisting of a pH sensing circuit, a semi-micro combination pH electrode (Orion 8103SC), a temperature probe, an overhead stirrer, a precision dispenser, and a six-way valve. PTFE FEP tubing was used throughout. The instrument automatically moves the electrode/stirrer/dispenser-tip assembly in and out of sample, buffer and wash solutions. A weighed sample was supplied manually; the diluent and the titrants (0.5 *M* HCl and 0.5 *M* NaOH) were added automatically.

3.2. Chemicals

Cefotaxim, cefuroxim and cefpirom were obtained from Hoechst (Frankfurt/M., Germany). Cephalexin, cefadroxil, cefaclor, ceftazidim, cefamandol, cefepim and cephaloridin were obtained from Sigma-Aldrich (Deisenhofen, Germany). Acetone for chromatography, citric acid monohydrate, sodium hydrogenphosphate and sodium dihydrogenphosphate were obtained from Merck (Darmstadt, Germany).

3.3. Sample preparation

Standard solutions of cephalosporins were prepared at a concentration of 30 μg/ml in water. The samples were filtered through a 0.2-μm syringe filter and injected immediately into the apparatus. For titrations standard solutions of cephalosporins were prepared at concentrations of 200 to 300 μg/ml in water.

3.4. Buffer preparation

For capillary electrophoresis, buffer solutions were prepared by mixing two stock solutions (Table 1) and diluting to ionic strength $I=0.03$ [7]. The pH of the buffer was measured at 25°C using a HI 9321 Microprocessor pH meter (Hanna Instruments). The buffer solutions were filtered through a 0.2-μm syringe filter.

3.5. Analytical conditions

3.5.1. Capillary electrophoresis

A new capillary was washed for 15 min with 1.0 *M* NaOH at 40°C, followed by washing for 10 min with water at the same temperature and for 5 min with water at 25°C. Before each injection, the capillary was flushed with 0.1 *M* NaOH for 5 min and with the actual buffer solution for 5 min. The temperature was kept at 25°C, a separation potential of 30 kV was used. Acetone was used as a marker for the determination of the electroosmotic mobility. The samples [water–acetone (99:1, v/v)] were injected at a 50 mbar pressure for 9 s (hydrodynamic injection) with a sample volume of 18.8 nl. Detailed experimental conditions are listed in the figures.

3.5.2. Potentiometric titration

The titration investigations were performed from pH 1.8 to 12.2 with 0.5 *M* HCl and 0.5 *M* KOH. The pH values were measured permanently through the Orion electrode.

Other experimental conditions were as follows: assay temperature, 25.1°C; average ionic strength, 0.158 *M*; salt type and strength, 0.15 *M* KCl, total volume of aqueous phase, 20 ml.

Table 1
Standard buffer

pH range	Constituent	Stock solution	Ionic strength
2–3.5	Citrate	1 *M* ($C_6H_8O_7 \cdot H_2O$ + NaOH), 0.1 *M* HCl	0.03
3.6–5.5	Acetate	1 *M* CH_3COOH, 1 *M* CH_3COONa	0.03
5.6–9	Phosphate	0.1 *M* NaH_2PO_4, 0.1 *M* Na_2HPO_4	0.03

4. Results and discussion

4.1. Choice of the electroosmotic marker

Acetone, mesityl oxide, benzene and dimethyl sulfoxide were tested as electroosmotic flow (EOF) marker substances. The best results (high absorbance and symmetrical peaks) were obtained by using acetone. In Table 2 the pH values and calculated μ_{EOF} are given for a mixture of 1% acetone in 99% water. The relative standard deviation of the calculated μ_{EOF} values was between 0.4% (neutral and alkaline conditions) and 3% (acidic region). In this method, the total analysis time depended on the electroosmotic velocity at the measured pH. In neutral and alkaline regions a very rapid determination of the solute was possible compared with the lower pH region. Long analysis times were required at lower pH values due to the slower EOF.

4.2. Effective mobility

The effective mobilities of cephalosporins were calculated from the measured apparent mobilities at different pH values using Eq. (3). Although the cephalosporins have similar structures they exhibited different behavior at various pH values. Cefepim, cefpirom and cephaloridin had a zero electrophoretic mobility at pH values above 4.5 whereas at lower pH these solutes had positive electrophoretic mobilities (Fig. 2).

Cefuroxim, cefotaxim and cefamandol had only negative electrophoretic mobilities and moved with the EOF (Figs. 3 and 4). Cefadroxil, cephalexin, cefaclor and ceftazidim showed zero electrophoretic mobilities (fully associated), negative electrophoretic mobilities (fully ionized) and positive electrophoretic mobilities (amino groups ionized) (Figs. 5 and 6) and migrated in the direction of the cathode. To control the reproducibility of the μ_e, three injections of the solutes were made from pH 2 to 9.5. The relative deviation of the effective mobilities was between 1% (neutral and alkaline conditions) and 3% (acidic region).

Table 2
Electroosmotic flow as function of electrolyte pH

pH	3	4	5	6	7.5	8.5	9.5
μ_{EOF}	8.5	9.2	12.5	28.2	53	62	66

μ_{EOF}: electroosmotic flow (10^{-5} cm^2 V^{-1} s^{-1}).

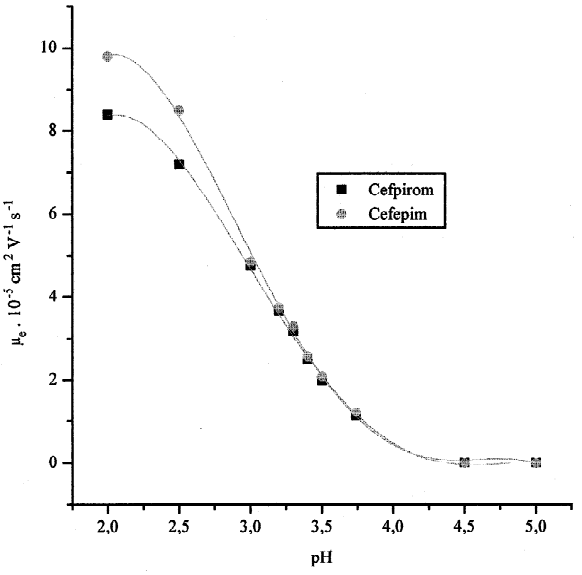

Fig. 2. Dependence of effective mobilities of cefpirom and cefepim. Capillary: 48.5 cm (40 cm to detector)×50 μm I.D.; field strength: 30 kV; temperature: 25°C; pressure injection: 9 s at 50 mbar; detection: 256 nm. The curve is interpolated calculation points.

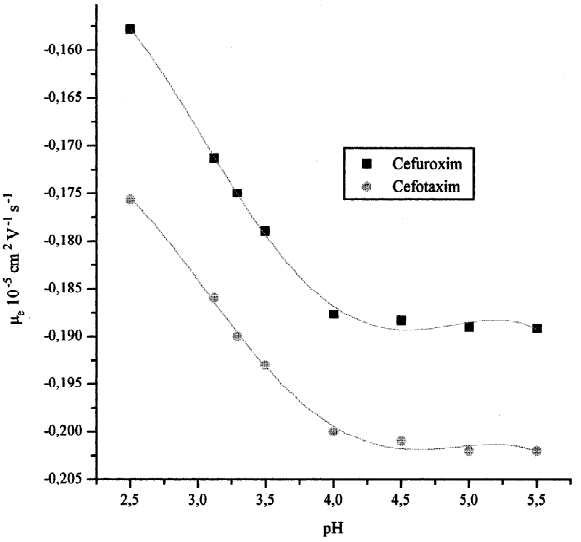

Fig. 3. Dependence of effective mobilities of cefuroxim and cefotaxin, other conditions as in Fig. 2. The curve is interpolated calculation points.

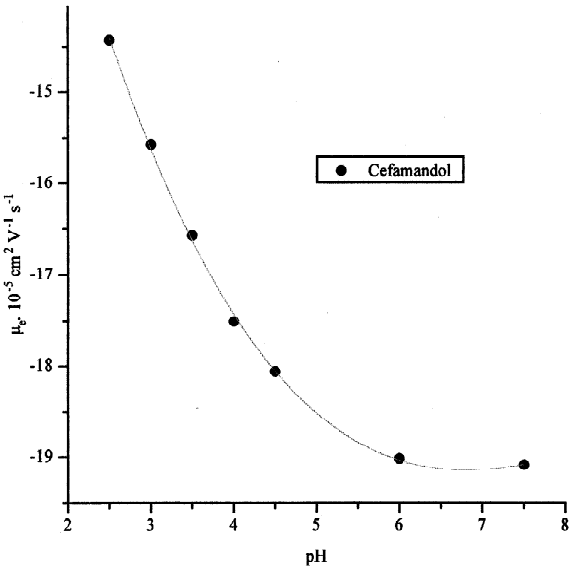

Fig. 4. Dependence of effective mobilities of cefamandol, other conditions as in Fig. 2. The curve is interpolated calculation points.

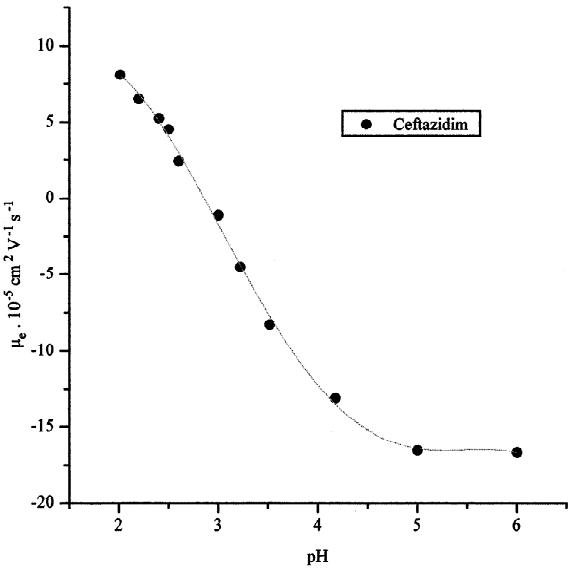

Fig. 6. Dependence of effective mobilities of ceftazidim, other conditions as in Fig. 2. The curve is interpolated calculation points.

4.3. Calculation of pK_a values

In the present paper we studied the determination of the dissociation constants of cephalosporins by CZE as a new technique as well as by potentiometric titration as comparison method. For the determination of pK_a by CZE some factors are important, such as ionic strength and temperature. All ionic strengths were set to 0.03 [9], because K_a, μ_a and μ_b depend on the ionic strength of the background electrolytes. Another important factor in the pK_a determination was the temperature. An effective control of capillary temperature seemed to be important for reproducible operation. The capillary was thermostated in a high velocity air stream. Therefore, these pK_a values may contain some errors because the temperature of the solution inside the capillary was not exactly 25°C. Under these conditions, the pK_a values of cephalosporins were determined. Eqs. (2) and (4) were used in nonlinear regression to determine the pK_a values. Fig. 2 shows plots of the electrophoretic mobilities of cefepim, cefpirom and cephaloridin against pH. The interpolated curves were obtained from Eq. (2) using weak bases. The pK_a of weak acids (cefuroxim, cefotaxim, cefamandol) were determined using Eq. (4) in nonlinear regression (Figs. 3 and 4). For ampholyte compounds, such as ceforoxil, cephalexin, cefaclor,

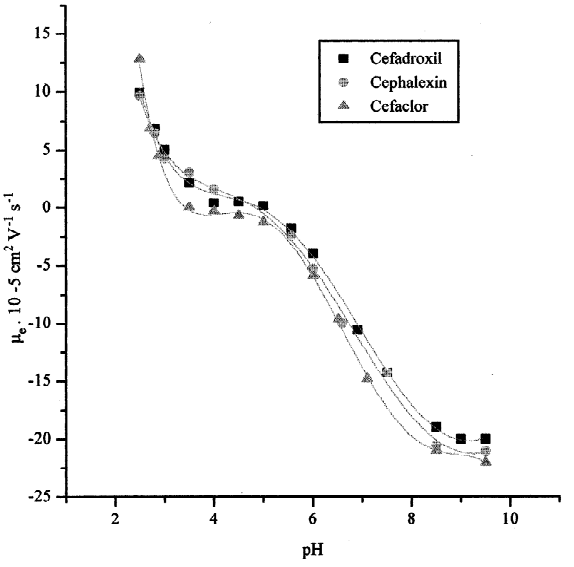

Fig. 5. Dependence of effective mobilities of cefadroxil, cepalexin and cefaclor, other conditions as in Fig. 2. The curve is interpolated calculation points.

Table 3
Dissociation constants of cephalosporins

Samples	CZE	pH metric
Cefamandol	2.46±0.24[a]	2.60±0.07
Ceftazidim	2.19±0.01[c]	2.91±0.06
	3.98±0.35[c]	3.81±0.07
Cefuroxim	2.04±0.13	2.17±0.11
Cefotaxim	2.09±0.21	2.9±0.18
Cefadroxil	2.86±0.18[c]	2.65±0.05
	7.14±0.20[c]	7.59±0.18
Cephalexim	3.11±0.16[c,b]	2.34±0.09
	6.79±0.27[c]	7.08±0.06
Cefaclor	2.69±0.09[c]	
	7.38±0.66[c]	7.19±0.06
Cefepim	3.36±0.40	3.03±0.05
Cefpirom	3.10±0.13	3.04±0.10

[a] 2.6 Molecular Design, San Leandro, CA, personal communication.
[b] 3.2 Molecular Design, San Leandro, CA, personal communication.
[c] Calculated according to Ishihama et al. [11].

ceftazidim the pK_a values were obtained by non-linear regression analysis using the method derived by Ishihama et al. [11].

All measurements were made at the same ionic strength and temperature.

Table 3 summarizes the pK_a values of cephalosporins in aqueous solution using CZE compared to the potentiometric titration method.

5. Conclusion

In this investigation, the determination of dissociation constants of cephalosporins by CZE and potentiometric titration has been studied. The results of these studies showed that CZE is suitable for the determination of pK_a values. In general, this technique has some advantages for the determination of the pK_a of compounds with low water solubility. For example, compounds of limited water solubility need not be dissolved in a cosolvent and it is not necessary to accurately know the concentration of a titrate or solute. Therefore, highly pure and stable samples are not necessary, because CZE is a highly efficient separation technique. Potentiometric titration could also be used for the pK_a determination. The cephalosporins studied showed different effective mobilities at various pH values. All anions had smaller electrophoretic migrations than the EOF and migrated in the direction of the cathode.

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