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# A comparative study of capillary zone electrophoresis and pH-potentiometry for determination of dissociation constants

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**Abstract**

Acidity constants of six cephalosporin antibiotics, cefalexin, cefaclor, cefadroxil, cefotaxim, cefoperazon and cefoxitin are determined using capillary zone electrophoresis (CZE) and pH-potentiometric titrations. Since CZE is a separation method, it is not necessary for the samples to be of high purity and known concentration because only mobilities are measured. The effect on determination of dissociation constants of different matrices (serum, 0.9% NaCl, fermentation matrix) was examined. The advantages of CZE can be utilized in those fields where potentiometry has limitations (sample quantity, solubility, purity, simultaneous determinations), although p$K_a$ values that are close to each other can be determined by potentiometry with more accuracy.
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*Keywords:* p$K_a$ determination; CZE; pH-potentiometry; Cephalosporins

## 1. Introduction

The acid–base property of a drug molecule is a key parameter for drug development because it governs solubility, absorption, distribution, metabolism and elimination. Particularly, for developing new antibiotics, the p$K_a$ has become of great importance because the transport of drug into cells and cross other membranes is a function of physicochemical properties, and of the p$K_a$ of the drugs [1].

For the determination of p$K_a$ values the potentiometric titration is in general use because it is accurate, fast and automated instruments are commercially available, however, its shortcomings include the requirements to use milligram amount of pure compounds and a mixture of aqueous buffers [2].

An alternative to potentiometric titration is UV–vis spectrophotometric titration [3] because it can handle compounds with lower solubility and lower sample concentrations. But the compound must contain an UV active chromophore close enough to the site of the acid–base function in the molecule. The determinations are often interfered by impurities in the sample. NMR-pH titration is also an excellent technique in determining p$K_a$ values as protonation of a basic site leads to electronic deshielding effects on the adjacent NMR-active nuclei. Therefore the average chemical shifts of all measureable NMR-active nuclei, as a function of pH (or pD), are expected to reflect the fractional protonation of each basic group of the molecule. First, Beckers et al. [4] used capillary electrophoresis (CE) for determination of dissociation constants. Cai et al. [5] have proved that CZE is suitable for determination of the ionization constants of weak electrolytes and several fundamental equations relating the electrophoretic mobilities of ionized solutes were verified experimentally. Cleveland et al. [6] explored an automated CZE method for obtaining p$K_a$ values of acids and bases and described several advantages of it in determining p$K_a$ values over the two most common methods for p$K_a$ determination: potentiometric titration and ultraviolet spectroscopy. Ishihama et al. [7] determined dissociation constants of acidic, basic and ampholyte drugs. Gluck et al. studied the different aspects of p$K_a$ determination by CZE: problems of p$K_a$ calculation and its evaluation, the effect of experimental conditions on calculated p$K_a$ values [6,8,9]. The published works on determination of dissociation constants by CE have recently been summarized in a review [10].

The CE technique is expected to be especially useful if the amount of compound is very limited because using this technique only a few nanograms or less is commonly used for analysis. For CZE it is only necessary to determine the pH-dependent

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mobilities, which has several advantages: concentration is only limited by the LOD of the compound and the procedure does not require the measurement of solute concentration because the mobilities are averaged according to the molar fractions [6,11]. The p$K_a$ values may also be determined for compounds, which are impure or relatively unstable. These compounds are injected by short-end injection, for assuring the decomposition in a shorter path [12]. For the determination of p$K_a$ by CZE two factors such as ionic strength and temperature are important because effective mobility depends on the ionic strength of the background electrolytes and temperature [10]. The model equations for calculating the dissociation constants of multiprotic acids, bases and ampholytes are more complex. The p$K_a$ determination of multiprotic solutes is described in detail [7,8].

The effective mobility of a protonated compound is described by the following equation:

$$\mu_e = \frac{[H^+]/K_a}{1 + [H^+]/K_a}\mu_b \quad (1)$$

where $\mu_b$ is the electrophoretic mobility of the fully protonated compound and $K_a$ is the acid dissociation constant. This equation makes possible to determine the p$K_a$ values from the mobility data obtained from CZE measurements. The mobility is calculated from the migration time of the analyte and of the electroosmotic flow. The effective mobilities of the analytes were determined at different pH values using the following equation:

$$\mu_e = \frac{L_t L_{eff}}{V}\left(\frac{1}{t_m} - \frac{1}{t_0}\right) \quad (2)$$

where $\mu_e$ is the effective mobility, $V$ is the applied voltage, $L_t$ is the total capillary length, $L_{eff}$ is the effective capillary length (to the detector), $t_m$ is the migration time of the solute and $t_o$ is the migration time of the neutral marker.

The present work demonstrates the simultaneous determination of the dissociation constants of numerous analytes and performs analysis from complex matrices. Since there were several contradictions among literature data, the dissociation constants of four cephalosporins were reexamined and two others were determined, which p$K_a$ values are not yet determined previously. The p$K_a$ values of cephalosporins determined by both CE and potentiometry have been compared.

## 2. Experimental

### 2.1. Instrumentation

#### 2.1.1. Capillary electrophoresis determinations

The capillary electrophoresis instrument was a HP 3DCE model (Agilent, Waldborn, Germany). In all measurements hydrodynamic sample introduction was used for injecting samples. The sample solutions were introduced at the anionic end of the capillary with 50 mbar pressure for 2 s. Separations were performed using polyimide-coated fused-silica capillaries of 48 cm × 50 μm i.d. (effective length: 40 cm) (Polymicro Technology, Phoenix, AZ, USA). The applied voltage was +25 kV. The temperature of the capillary holder was kept constant at 25 °C. The detection was carried out by on-column photometric measurement at 270 and 200 nm. The diode array detection allowed to record the UV spectra in the points (in every 0.2 s) of the electropherogram. The electropherograms were recorded and processed by ChemStation computer program of 7.01 version (Agilent).

The obtained experimental data were plotted in the function of pH and the p$K_a$ values were calculated with the aid of a Matlab program (MathWorks, Inc., Natick, MA 01760, USA).

#### 2.1.2. Potentiometric titration

pH-potentiometric measurements were carried out at ionic strength of 0.2 M (KCl) and at 25.0 ± 0.1 °C. Carbonate-free KOH solutions of known concentrations (ca. 0.2 M) were used as titrant. HCl stock solutions were prepared from cc. HCl (both the acid and base were Merck products) and their concentrations were determined by potentiometric titrations using Gran's method [13]. A Radiometer pHM 93 instrument equipped with a Metrohm combined electrode (type 6.0234.100) and Metrohm 715 Dosimat burette were used for the pH-metric measurements. The electrode system was calibrated according to Irving et al., the pH-metric readings could therefore be converted into hydrogen ion concentration [14]. The water ionization constants, p$K_w$ is 13.76 ± 0.01 under the conditions employed. The titrations were performed in the pH range 2.0–11.0. In case of cefoperazon, due to its limited solubility back titrations were also carried out with this drug starting at pH ~ 11 and using HCl (ca. 0.2 M) as titrant until precipitation occurred. Initial volume of the samples was 10.00 ml. The ligand concentrations (cephalosporins) were varied in the range 0.002–0.004 M. The reproducibility of the titration points included in the evaluation was within 0.005 pH unit. About 150 titration points were used for each system. The samples were in all cases completely deoxygenated by bubbling purified argon for ca. 20 min before and during the measurements. The calculation of the acidity constants was performed with the aid of the SUPERQUAD program [15].

### 2.2. Chemicals and samples

The sample solutions were prepared by dissolving the solid salts of cefalexin (Chinoin, Hungary), cefaclor (Lilly, Italy), cefadroxil (Bristol-Myers, Italy), cefotaxim (Lek, Slovenia), cefoperazon (Pfizer, Italy), cefoxitin (MSD, the Netherlands) in doubly deionised and ultrafiltered water obtained from a Milli-Q RG (Millipore) water purification system. The concentrations of the stock solutions of all cephalosporins were 0.1 mg/ml. The formulas and abbreviations of the analysed cephalosporins are summarized in Table 1. All other reagents (benzyl alcohol, NaOH, NaCl, $NaH_2PO_4$, $Na_2HPO_4$, $H_3PO_4$, $CH_3COOH$, $CH_3COONa$, $H_3BO_3$, HCl) for preparing buffer electrolytes were purchased from Spectrum-3D Ltd. (Hungary). Buffer solutions covering the pH range 2.0–9.7 at an ionic strength of 50 mM were prepared according to literature methods [10]. Sodium chloride was used to adjust the ionic strength and hydrochloric acid or sodium hydroxide to obtain the different pH values. pH of the buffer solutions was measured at 25 °C. The ionic strength was calculated using Peakmaster 5.1. Buffers were

Table 1
Structures of the studied cephalosporin antibiotics

| Name | Abbreviation | $R_1$ | $R_2$ |
|---|---|---|---|
| Cefoxitin | COX | | |
| Cefoperazon | CFP | | |
| Cefaclor | CFC | $—CH_2$-Cl | |
| Cefalexin | CFL | $—CH_3$ | |
| Cefadroxil | CFD | $—CH_3$ | |
| Cefotaxim | CTA | | |

filtered through a 0.45 μm syringe filter and stored in a refrigerator at +4 °C. A new capillary was washed for 20 min with 1.0 M NaOH. Before each injection the capillaries were preconditioned with the buffer electrolyte for 15 min and after electrophoresis the capillaries were postconditioned with the buffer electrolyte for 10 min. In the case of the analysis of biofluid samples the capillary was postconditioned after each run by flushing 1 M NaOH (5 min), 0.1 M SDS (5 min) and buffer (10 min) [16,17]. Benzyl alcohol (1%, v/v stock solution) was used as a marker for the determination of the electroosmotic flow (EOF). The concentration of the marker was 0.01% in the sample solutions.

## 3. Results and discussion

### 3.1. Application of CZE for determination of p$K_a$ values

The p$K_a$ value characterizes what kind of charge the ligand will have at a given pH. In the case of ampholytes, for example, at pH higher than p$K_a$2 the analyte is negatively charged and thus its peak appears after the EOF, while at pH lower than p$K_a$1 the analyte is positively charged, that is its migration precedes the EOF. At a pH higher than the p$K_a$ the analyte is usually negatively charged, and thus its peak appears after the EOF, while at pH lower than the p$K_a$, the analyte is positively charged, that is its migration precedes the EOF. The functional group(s) of the investigated cephalosporins may be protonated or deprotonated. The electric charge depends on the number of these functional groups of the compound, and on the pH of the electrolyte because the dissociation of these groups is controlled by pH.

In Fig. 1., the influence of pH of the running electrolyte on the migration times of cefalexin is shown. At higher pH cefalexin is in deprotonated form, therefore its migration velocity is smaller than that of the EOF. When decreasing the pH the effective mobility of cefalexin increases, the protonated form of it migrates before the EOF in the electropherogram. The calculated effective mobility data were plotted against the pH, and from the gained mobility curve of cefalexin the p$K_a$ values could be obtained (Fig. 2). The inflexion points of the curve specify

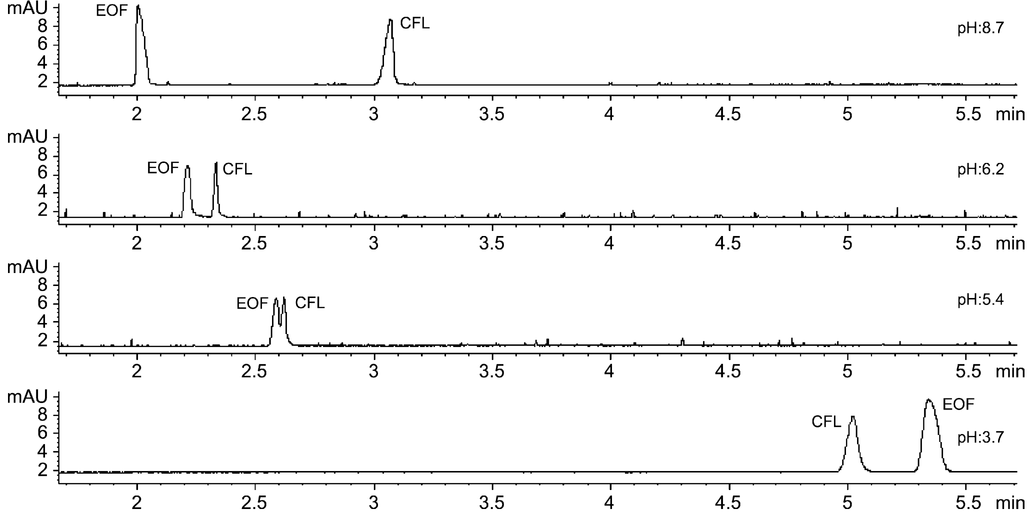


Fig. 1. The influence of pH of the buffer electrolyte on the migration times of cefalexin (conditions: capillary, 48 cm × 50 μm i.d.; ionic strength, 50 mM; applied voltage, +25 kV; injection, 50 mbar 2 s; temperature, 25 °C) detection: UV at 270 nm, the concentration of cefalexin was 0.1 mg/ml, EOF marker: benzyl alcohol, ionic strength of the electrolytes were constant, $I = 50$ mM).

the p$K_a$ values. The first of them (2.93) refers to the deprotonation of the carboxylic group, while the other (7.18) to that of the ammonium group. These values are in acceptable agreement with p$K_a$ data determined previously [18] and correspond well to the chemically expected acid–base character of these functional groups. Because the peaks of cephalosporins are relatively close to the EOF, the use of an additional reference material as marker was not used; (additional marker having permanent charge in some cases may be more advantageous).

### 3.2. *Determination of p$K_a$ values from solutions of complex matrices*

Taking advantage of the fact that CZE is an efficient separation technique, samples having more than one component can also be analyzed. The possible interfering effects of sample matrices including several inorganic and organic compounds on the p$K_a$ determination were studied. The determination of different matrix compounds has great importance because during practical analytical work the analytes are often solved not in pure distilled water, therefore matrices which are often used in pharmaceutical research and in pharmacokinetic measurements were chosen.

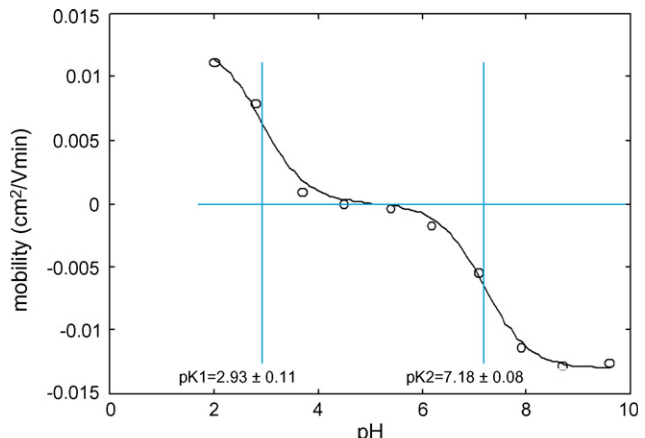


Fig. 2. Dependence of the effective mobilities of cefalexin on pH. Separation conditions were as for Fig. 1.

In our earlier works [16,17] for the analysis of biofluids the sodium dodecyl sulphate (SDS) content in the buffer electrolyte was found to be important to prevent the interferences from the high protein content. For p$K_a$ determination it is not possible to use electrolyte buffer containing SDS because in this case the mechanism of the separation would change (micellar elektrokinetic chromatographic separation (MEKC) instead of CZE). In MEKC the migration of the components does not depend exclusively on the charge-to-size ratio of the component, which is essential for p$K_a$ determinations. However, with a suitable three-step postconditioning the adsorption of the proteins onto the wall of the capillary could be prevented.

The electrophoretic migration of cefalexin was compared in different matrices: 0.9 % NaCl solution, fermentation matrix and serum. The electropherograms in Fig. 3 show that the matrix composition has no remarkable effect on migration times of the cefalexin, only the distortion of peak shapes could be observed. In case of 0.9 % NaCl solution the inorganic component with high mobility (chloride ion) results in stronger leading peaks. In serum and fermentation matrix the interaction of the protein and other organic compounds with the analyte causes probably the peak broadening. The peak broadening could also be an effect of the adsorption of the proteins to the capillary wall.

The pH dependences of the electrophoretic mobility of cefalexin in different matrices are found to be very similar to each other and to that in pure water (Fig. 4) which means that the calculated p$K_a$ values did not differ from those determined in pure model solution. It may be concluded that the matrices studied have no significant effect on the quality of the determination of p$K_a$ values.

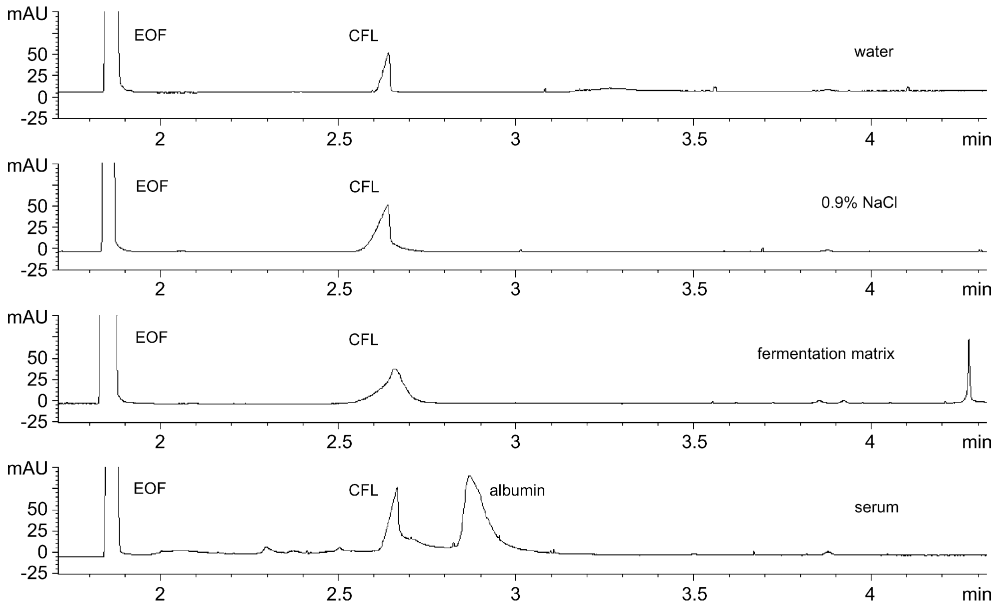


Fig. 3. The effect of different matrices on the migration time of cefalexin. Separation conditions were as for Fig. 1.

### 3.3. Simultaneous determination of p$K_a$ values of five analytes

All the cephalosporins consist of a ß-lactam ring fused with dihydrothiazine ring and side chains linked to carbon-7 position and to carbon-3 position, therefore they have similar structures. On the other hand, due to the different basicity of the side chain functional groups, they still have different behaviour at various pH values. Changing of pH results in different effect on the mobilities of the cephalosporins (since their p$K_a$ values are different), and even their migration order may be changed. The UV spectra of the cephalosporins could be used for identifying them [19]. With the help of the high separation resolving ability of CE the p$K_a$ values of several cephalosporins could be determined successfully at the same time (Fig. 5). With an increase in the pH the solutes migrate faster due to the stronger electroosmotic flow and the resolution was still sufficient to obtain the migration times. The separation was improved by decreasing the pH, however, the time of the measurement lengthened. Above pH 2 cefoperazon, cefotaxim and cefoxitin appeared after the EOF, however, cefadroxil and cefaclor have both positive and negative electrophoretic mobilities.

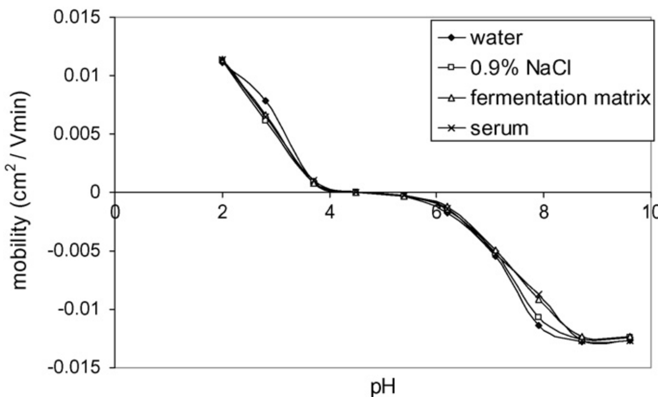


Fig. 4. Comparison of the mobility curves of cefalexin obtained in different matrices. Separation conditions were as for Fig. 1.

### 3.4. Comparative study of applicability of CZE and potentiometry for p$K_a$ determination

The p$K_a$ values of numerous cephalosporins have been determined by potentiometric titration, too. It should be highlighted that any comparison of the p$K_a$ values determined by different methods or instrumentation makes sense only if the experimental conditions are identical during measurements (temperature, ionic strength, concentration). In our CZE and potentiometric works all parameters were kept on the same level, only in the ionic strengths of the solutions were different. In case of CZE the 0.05 M ionic strength is a generally accepted compromise between the minimalization of the Joule-heating and the proper buffer capacity [10]. During potentiometric measurements the constant ionic strength is provided by a greater salt quantity (0.1 M or above), which is not considerably changed by other compounds using/forming during the titration.

The titration curve of cefadroxil as a representative example is shown in Fig. 6a. As it is seen, above pH 6 two deprotonation processes occur in the measurable pH range. Analyzing the pH-metric data with the SUPERQUAD program three p$K_a$ values can be obtained (Table 2). The first deprotonation constant (2.48) belongs to the carboxylic group. The deprotonation of this moiety takes places together with the neutralization of the strong acid content of the sample that is why the first deprotonation step is not visible in the titration curve. The second p$K_a$ value refers to the proton loss of the ammonium group of the ligand while the third one belongs to the deprotonation of the phenolic group. The determined values are in good agreement with data published previously [18], although the p$K_a$ value of the phenolic group was not published earlier. Comparison of the titration

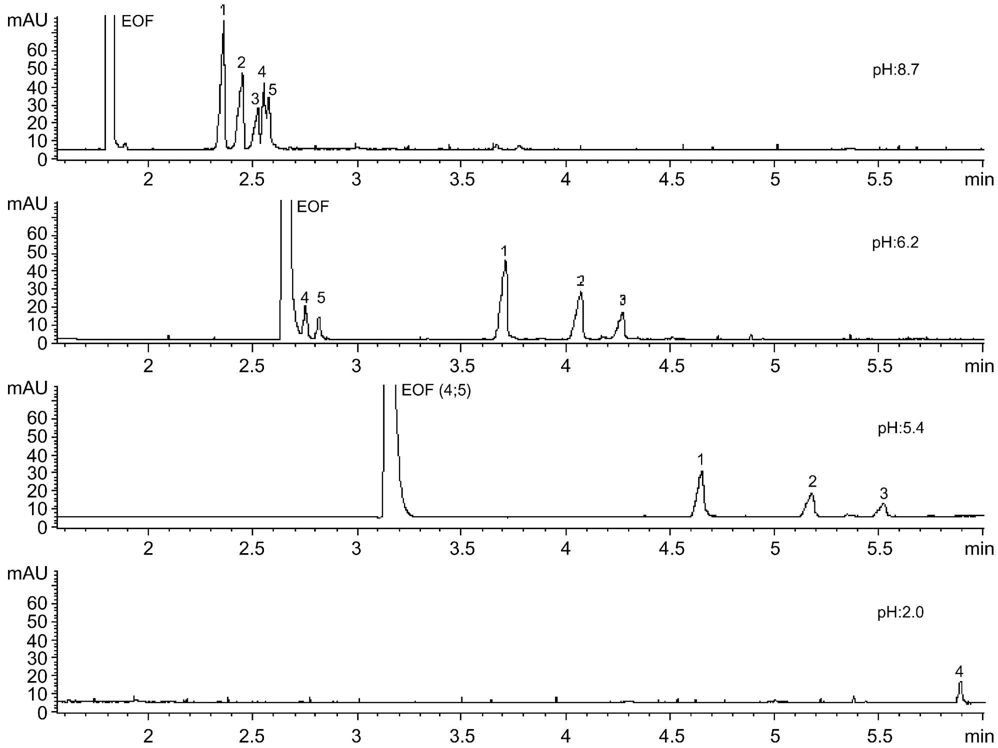


Fig. 5. Influence of pH on the separation of five cephalosporins. Separation conditions were as for Fig. 1. (1) cefoperazon, (2) cefotaxim, (3) cefoxitin, (4) cefadroxil, (5) cefaclor.

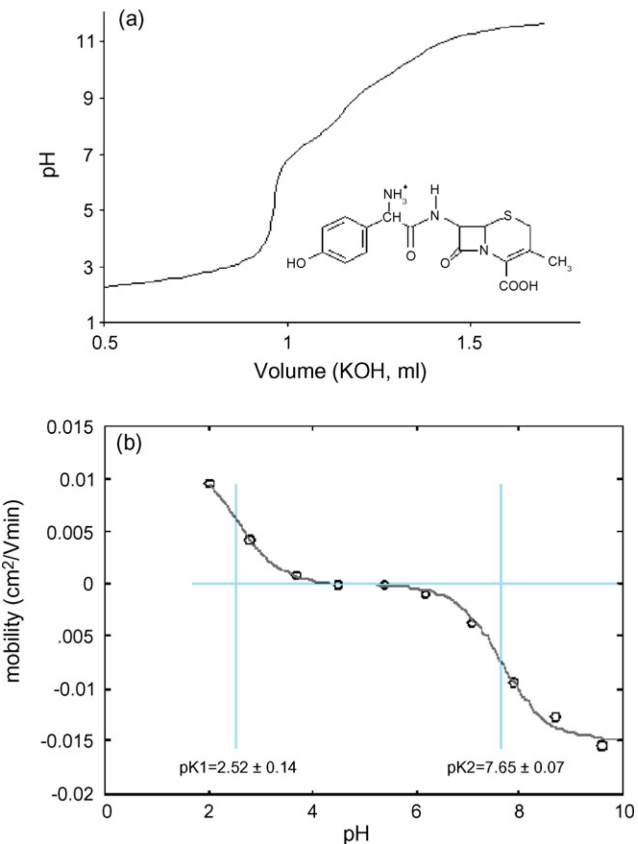


Fig. 6. Comparison of titration (a) and mobility (b) curves of cefadroxil. Separation conditions were as for Fig. 1. Potentiometric conditions: ionic strength, 0.2 M KCl; 25 °C.

curve and the electrophoretic mobility curve of cefadroxil in Fig. 6 shows that only two deprotonation steps can be observed in the mobility curve. The reason for this deviation can be that with CZE it is difficult to have as many measuring points for drawing a curve as with potentiometry, that is the “resolution” of the mobility curve is generally smaller than that of the potentiometric titration curve and thus small difference in p$K_a$ values can not be differentiated by CZE. In practice not more than 20 CZE runs (at 20 different pH values and buffers) are generally carried out for determination of a p$K_a$ value. Comparison of the p$K_a$ values obtained by the two methods indicates that those of the carboxylic group are in excellent agreement, while the difference in the second values is due to the limited power of CZE if the deprotonation processes are overlapped.

Analyzing of the titration curve of cefotaxim (not shown) resulted in two p$K_a$ values (Table 2). The value of 2.30 belongs to the carboxylic group while the other value to the aminothiazole group. In contrast to previous results [20] no further deprotonation process could be observed either with potentiometry or with CZE. Previously it has been assumed that the amide group of cefotaxim deprotonates having a p$K_a$ value of 10.87. Taking into account chemical considerations it is highly unlikely that an amide group would release a proton in the measurable pH range [21].

For cefoperazon two p$K_a$ values were obtained by CZE, one in acidic and one in alkaline range, which could be attributed to the carboxylic and the phenolic hydroxyl group, respectively. In this system potentiometry had limitations due to the poor solubility of the ligand in the acidic pH range therefore it was not possible to determine the p$K_a$ value of the carboxylic group by

Table 2
Comparison of p$K_a$ values of cephalosporins determined by CZE and potentiometry

| | Literature data | | Own measurements | |
|---|---|---|---|---|
| | CZE[a] | Potentiometry[a] | CZE | Potentiometry |
| Cefalexin | 3.11 ± 0.16[b] | 2.34 ± 0.09 | 2.93 ± 0.11 | 2.53 ± 0.01 |
| | 6.79 ± 0.27[b] | 7.08 ± 0.06 | 7.18 ± 0.07 | 7.13 ± 0.01 |
| Cefaclor | 2.69 ± 0.09[b] | – | 1.74 ± 0.38 | <1.5 |
| | 7.38 ± 0.66[b] | 7.19 ± 0.06 | 7.19 ± 0.03 | 7.07 ± 0.02 |
| Cefadroxil | 2.86 ± 0.18[b] | 2.65 ± 0.05 | 2.52 ± 0.14 | 2.48 ± 0.03 |
| | 7.14 ± 0.20[b] | 7.59 ± 0.18 | 7.65 ± 0.07 | 7.37 ± 0.02 |
| | | – | – | 9.64 ± 0.01 |
| Cefotaxim | 2.09 ± 0.21 | 2.09 ± 0.21[a], 2.93[c] | – | 2.30 ± 0.01 |
| | | 3.07[c] | 3.20 ± 0.19 | 3.37 ± 0.03 |
| | | 10.87[c] | – | – |
| Cefoperazon | – | – | 3.13 ± 0.27 | – |
| | – | – | 8.99 ± 0.44 | 9.15 ± 0.02 |
| Cefoxitin | – | – | 3.15 ± 0.24 | 2.75 ± 0.04 |

[a] Mrestani et al. [18].
[b] Ishihama et al. [7].
[c] Aleksic et al. [20].

potentiometry. Due to the precipitation appearing in the system, potentiometric data could only be treated above pH 5. With this ligand, therefore CZE, which is suitable to handle very diluted samples appeared more successful in p$K_a$ determination over potentiometry.

Cefoxitin exhibits one deprotonation process in the measurable pH range. The obtained p$K_a$ value belongs to the carboxylic group of the molecule.

For both cefalexin and cefaclor two p$K_a$ values could be determined either with potentiometry or with CZE. Due to the electron withdrawing effect of the chlorine substituent in the case of cefaclor the carboxylic p$K_a$ is decreased compared to that of cefalexin, therefore it was not possible to give an accurate value for it with the aid of potentiometry only with aid of CZE. For both ligands the higher p$K_a$ values belong to the ammonium groups.

## 4. Conclusion

In this work CZE was performed as a comparative method to the potentiometric titration and its advantages and limitations are presented in determining the p$K_a$ values. According to the results the CZE method is capable of determining the p$K_a$ values from real samples with complicated matrices. The direct analysis from real samples has great importance because it is not necessary to extract and purify the analyzed compound so accurate p$K_a$ values are easy to obtain during drug discovery, development and pharmacokinetic measurements. CZE can be used for the determination of physicochemical properties at physiological conditions. CZE does not require high purity substances because it enables separation of impurities and decomposition products from the main component. With the help of the high separation resolving ability of CZE the p$K_a$ values of several compounds could be determined at the same time. The advantages of CZE can be used in those fields where potentiometry has limitations (sample quantity, solubility, purity, sample with several components) although with potentiometry there is a chance to determine p$K_a$ values, which are close to each other. However, modern CE instruments (mainly those which incorporate parallel capillaries) enable to improve the resolution of the obtained mobility curve. Although the CZE was found to be particularly advantageous for the determination of acidity/basicity constants when the analyte is not isolated or its concentration is low; the largely undefined factors that influence mobility introduce uncertainty into the p$K_a$ determination as shown in several papers [12,22,23].

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