*Research Article*

# Study of the Electrophoretic Behavior of Cephalosporins by Capillary Zone Electrophoresis

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**Abstract**

*Purpose:* The aim of the study was the characterization of the electrophoretic behavior of cephalosporins from different generation having different structural characteristics in order to develop a rapid, simple and efficient capillary electrophoretic method for their identification and simultaneous separation from complex mixtures.

*Methods:* Ten cephalosporin derivatives (cefaclor, cefadroxil, cefalexin, cefazolin, cefoxitin, cefuroxime, cefoperazone, cefotaxime, ceftazidime, ceftriaxone) were analyzed by capillary zone electrophoresis using different background electrolyte solutions at different pH values. Electrophoretic mobilities of the analytes were calculated, the influence of the electrophoretic parameteres on the separation was established and the analytical conditions were optimized.

*Results:* Taking into consideration their structural and chemical properties cephalosporins can be detected over a pH range between 6 and 10. The best results were obtained using a buffer solution containing 25 mM disodium hydrogenophosphate - 25 mM sodium dihydrogenophosphate, at a pH – 7.00, + 25 kV voltage at a temperature of 25 °C, UV detection at 210 nm. Using the optimized analytical conditions we achieved the simultaneous baseline separation for seven cephalosporins in less then 10 minutes.

*Conclusion:* Using the described optimized electrophoretic procedures, capillary electrophoresis can be used for the identification and determination of cephalosporins in formulated pharmaceutical products and for their separation from complex mixtures.

## Introduction

Nowadays cephalosporins are one of the most important and probably the most frequently used antibiotics in the world, both in terms of the number of compounds currently on the market as well as regarding their use in the treatment of infectious diseases.

Cephalosporins are semisynthetic antibacterials derived from Cephalosporin C, a natural antibacterial substance, produced by the mould *Cephalosporium acremonium*. The cephalosporins are bactericidal and, like the penicillins, they act by inhibiting synthesis of the bacterial cell wall. The most widely used system of classification of cephalosporins is by generations and is based on the general features of their antibacterial activity, but it may depend to some extent on when they were introduced in therapy.[1]

The active nucleus, 7-aminocephalosporanic acid, is closely related to the penicillin nucleus, 6-aminopenicillanic acid, and consists of a beta-lactam ring fused with a 6-membered dihydrothiazine ring and having an acetoxymethyl group at position 3. Chemical modification of positions 3 and 7 has resulted in a series of drugs with different characteristics; substitution at the 7-amino affects generally the antibacterial action while substitution at position 3 have a more profound effect on pharmacokinetic properties.[2]
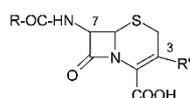
In this study 10 cephalosporins from different generations having different structural characteristics were analyzed by capillary zone electrophoresis (CZE): 1st generation cephalosporin derivatives (cefaclor, cefadroxil, cefalexin, cefazolin), 2nd generation cephalosporin derivatives (cefoxitin, cefuroxime), 3rd generation cephalosporin derivatives (cefoperazone, cefotaxime, ceftazidime, ceftriaxone). The cephalosporin derivatives were elected for this study taking in consideration their prevalence in therapy and also due to their particular structural and chemical characteristics.[3]

The chemical structures[3] and pKa values[4,5] of the studied cephalosporins are presented in Figure 1.

Although high performance liquid chromatography (HPLC) remains by far the most frequently used method in the analysis of cephalosporins, capillary electrophoresis (CE) is being increasingly employed due to its favorable characteristics (high separation efficiency, large flexibility, rapid method development and low consumption of samples and reagents).[6-8]

Separating simultaneously different types of cephalosporins from different generations remains a

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challenging task for HPLC, but the resolving power of CE can make this separation relatively fast and simple.[6] Cephalosporins are analyzed usually through two different electrophoretic techniques: capillary zone electrophoresis (CZE)[9-15] used for the separation of ionic or ionogenic cephalosporins when separation is based on the differences between the electrophoretic mobilities of the analytes; and micellar electrokinetic chromatography (MEKC)[16-19] used for the separation of both neutral and/or ionic or ionogenic cephalosporins when separation is based on the generation of a pseudostationary phase in which analyte partition takes place.



**Figure 1.** The chemical structures and pKa values of the studied cephalosporins

CE has been successfully applied for the analysis of cephalosporins from different pharmaceutical forms,[15] environmental samples,[14] plasma,[9] blood serum,[20] urine and bile,[10] bronchial secretion,[13] but also in stability studies[12] and for the determination of dissociation constants.[4,5]

While MEKC proved to be especially useful for the determination of cephalosporins from biological samples reducing the disadvantageous matrix effects caused by organic materials, CZE proved to be a powerful analytical tool for determinations from pharmaceutical products.[10,13,15,19]

Structurally cephalosporins are a rather heterogeneous group;[2] consequently, a large number of electrophoretic procedures using different analytical parameters can be used for their separation. Previously published methods exemplify the fact that the simultaneous determination of

structurally related cephalosporins is challenging because of their similar electrophoretic mobilities.[9,12,14,15,18,19] For this reason, both structurally related cephalosporins from the same generation (CFL, CFD and CCL) and cephalosporins from different generations and different structural characteristics were chosen as the subject of the present study.

This paper presents a comprehensive study of the electrophoretic behavior of cephalosporins from different generations and also the development of a generic simulataneous separation method of several cephalosporins from complex mixtures.

## Materials and Methods
### Apparatus
CE measurements were performed on a Agilent 6100 CE system (Agilent, Waldbronn, Germany), with diode-array detection (DAD). CE Chemstation 7.01 (Agilent, Germany) software was used for instrument control, data acquisition and data analysis. Fused – silica capillaries (Agilent, Germany) with a total length of 48 cm, length to detector of 40 cm and internal diameter of 50 μm were used in the measurements.

The pH of the buffer solutions was determined with the Terminal 740 pH–meter (Inolab, Germany). The individual UV spectra of the studied cephalosporins were registered with UV-VIS Specord 210 spectrophotometer (Analytik Jena, Germany).

### Chemicals
Cefalexin monohydrate, Cefaclor monohydrate were obtained from Sandoz Târgu-Mureş (Romania); Cefadroxil monohydrate, Ceftriaxone sodium, Cefotaxime sodium, Cefazolin sodium, Cefoxitin sodium from Antibiotice Iaşi (Romania); Cefuroxime sodium from Medochemie (Cyprus); Ceftazidime pentahydrate, Cefoperazone sodium from Glaxo Wellcome (United Kingdom). All cephalosporins were of pharmaceutical grade.

Sodium tetraborate, disodium hydrogenophosphate, sodium dihydrogenophosphate, phosphoric acid, sodium hydroxide, methanol were obtained from Merck (Germany). All reagents were of analytical grade. Purified water was provided by a Milli-Q Plus water purification system (Millipore, USA).

### Sample and buffer preparation
The cephalosporins were dissolved in methanol, stock solution with a concentration of 100 μg/mL were prepared, further dilutions were made with the same solvent as required. Samples and buffers were filtered through a 0.45 μm syringe filter and injected immediately after preparation into the apparatus. Buffers were degassed by ultrasound for 5 minutes before use.

Electrophoretic runs were performed as quickly as possible, but no later than 4 hours after solution preparation, in order to avoid sample decomposition due to the instability of the beta-lactam ring.

## Analysis conditions

The capillary was preconditioned for 10 minutes with 1.0 M NaOH before the first run. Before each run, the capillary was flushed with 0.1 M NaOH for 1 minute and with the running buffer for 1 minute. In the preliminary analysis we applied some "standard" electrophoretic conditions: applied voltage + 25 kV, temperature 25 °C, injection pressure/time: 50 mbar/3 sec., injection at the anodic end of the capillary, UV detection at 210, 230 and 250 nm.

## Results and Discussion
### Preliminary study

In order to obtain the optimum capillary electrophoretic conditions for the determination of cephalosporins, the electrophoretic mobilities of the analytes were determined at different pH values using different background electrolytes: 25 mM phosphoric acid (pH – 2.50), 25 mM sodium didydrogenophosphate (pH – 5.00), 25 mM disodium hydrogenophosphate – sodium didydrogenophosphate (1:1) (pH – 7.00) and 25 mM sodium tetraborate (pH – 9.30); the pH of the buffers was adjusted to the desired pH by adding a 0.1M sodium hydroxide solution.

The pH of the buffer plays an important role in the separation of ionizable analytes such as cephalosporins since it determines the extent of the ionization of the analyte; moreover, the charge of the capillary wall surface and the zeta potential are also influenced by buffer pH. The electric charge of the cephalosporins depends on the number of carboxyl and amino groups of the analyte but also on the pH of the electrolyte because the dissociation of these groups is controlled by pH.[12,19]

The influence of the pH on the separation of cephalosporins from complex mixures was studied over a pH range from 2.5 to 11. The migration time of each component was recorded separately first then as a mixture. The cephalosporins exhibited different electrophoretic behavior at various pH values. The majority of the studied cephalosporins couldn't be detected in background electrolytes with an strong acidic pH (pH < 5), with the exception of CFL, CFD, CCL and CFP. All the studied cephalosporins were detected with quantificable electrophoretic signals over a pH range between 6 and 10, with the best results obtained using a buffer elecrolyte containing 25 mM disodium hydrogenophosphate – sodium didydrogenophosphate (1:1) at pH 7.00.

The effective electrophoretic mobilities of cephalosporins were determined at different pH values using the following equation:

$$\mu_{(cephalosporin)} = \frac{L_e \, L_t}{V \times (1/t_m - 1/t_{eof})}$$

where μ is the effective mobility of the analyte, V the applied voltage, $L_e$ the effective capillary length (to the detector), $L_t$ the total capillary length, $t_m$ the migration time of the analyte and $t_{eof}$ the migration time of the electroosmotic flow.

The variation of the electrophoretic mobilities of the studied cephalosporins at different pH values of the backgrund electrolyte is presented in Figure 2.
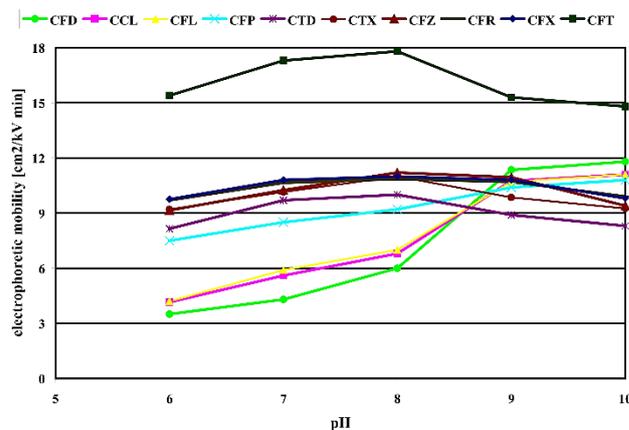


**Figure 2.** Variation of the effective electrophoretic mobilities of the studied cephalosporins at different pH values of the background electrolytes

Separation in CZE is based on differences between electrophoretic mobilities of the analytes, which result in different velocities of migration of ionic substances. The separation mechanism is mainly based on differences between solute size and charge at a given pH.

The beta-lactam ring of the cephalosporins can subsject of a possible degradation in acidic and basic environments, consequently it is advisable for their separation to use weak acid, neutral or weak basic medium (pH from 6 to 9).[11]

It is established that cephalosporins antibiotics are slowly hydrolyzed on dissolution to different degradation products due to the instability of their beta-lactam ring. Our preliminary studies using an internal standard (ciprofloxacin hydrochloride) as reference confirmed that degradation (hydrolysis) was insignificant within 4 hours of dissolution.[12]

All cephalosporins exhibited negative electrophoretic mobilities and moved in the direction of the electrophoretic flow (EOF).

It is interesting to notice that in every electrolyte CFT migrated much slower than the other cephalosporins, probably because of the characteristic highly acidic heteocyclic system on the 3-thiomethyl group attached to the 3 position of the cephem structure.[11]

In pH range 6 to 7, the structurally related CFD, CFL and CCL migrated closely to the EOF, with amost similar electrophoretic mobilities; also it is worth mentioning that the separation of CFL and CCL was resolved only in the pH range mentioned above.

## Method optimization

The individual UV spectra of the studied analytes were registered before the analysis for the reliable and unambigous identification of each cephalosporins from mixtures upon their characteristic UV spectrum. The UV spectra of the cephalosporins are relatively similar to

each one another, but definite, small differences can be observed in the case of all analytes.

Not all the studied cephalosporins could be resolved at a specific pH value, due to their very close electrophoretic mobilities. Migration behavior of the studied cephalosporins in a phospahte buffer was significantly different from that of cephalosporins in a borate buffer. However, the separability of cephalosporins using phosphate buffer proved to be better than that with borate buffer. This can be explained by the differences between the own electrophoretic mobilities of the analytes in different electrolyte solutions and also by the magnitude of the EOF at different pH which depends mainly on the zeta potential.

At pH – 7.00 we succeeded in the simultaneos baseline separation of seven cephalosporins, the order of migration being: CFD, CFL, CCL, CFP, CTD, CFZ, CTX, CFR, CFX, CFT (Figure 3).
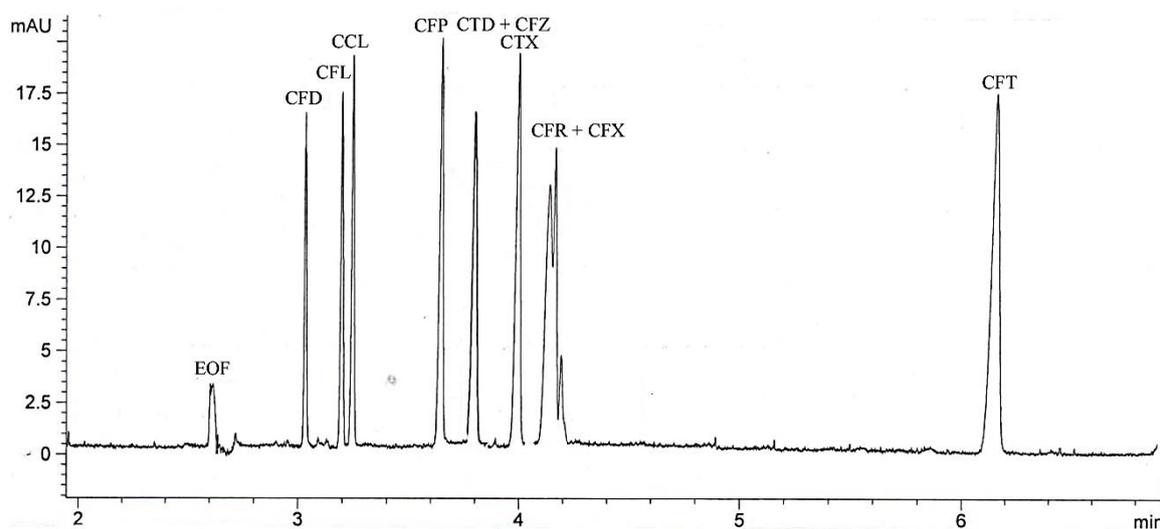


**Figure 3.** Electropherogram of the separation of the studied cephalosporins at pH – 7 using the standard analytical conditions

It is known that, for a given type of buffer electrolyte, the magnitude of the electroosmotic flow ($\mu_{eof}$) depends mainly on the zeta potential, which decreases with decreasing buffer pH and/or increasing buffer concentration. Hence, at a given buffer pH, an increase in the ionic strength resulted in a decrease in the zeta potential, thus leading to a decrease in the value of the electrophoretic mobility ($\mu$) of the analytes. An increase in the buffer concentration led to an increase in the migration times of the analytes, due to the slower EOF, but did not have a substantial effect on the separation.

The migration time increased with decreasing applied voltage, without any improvement in the resolution of the cephalosporins. An increase in temperature caused a slight decrease in migration times probably because of the decrease in the viscosity of the buffer. An applied voltage of + 25 kV and a temperature of 20 °C were therefore selected, in order to achieve the short analysis time and high resolution.

It could be observed that increasing of the injection time and pressure led to lowering of the detection limit without affecting the quality of peak shape and reproducibility.

On the basis of the results presented above the following analytical conditions were elected to standardize the separation procedure: 25 mM disodium hydrogenophosphate – 25 mM sodium didydrogenophosphate background electrolyte, pH – 7, + 25 kV voltage at a temperature of 20 °C, 50 mbar/1 second injection pressure/time, UV detection at 210 nm.

Using these conditions all peaks of the selected seven cephalosporin (CFD, CFL, CCL, CFP, CTX, CFR, CFT) mixture could be well resolved with good resolution and in a relatively short analysis time (Figure 4).

### Analytical performance
The analytical performance of the selected method for the determination of cephalosporins has been testeted in terms of reproductibility, linearity, limit of detection and quantification.

To verify the reproducibility of the peak areas, peak heights and of the migration times, six injections of the selected mixture of cephalosporins with a concentration of 10 μg/mL were made and relative standard deviation (RSD) was calculated (Table 1).

Statistical evaluation of the results revealed intra-day repeatability as RSD% between 0.08% and 0.2% for migration times, 0.76% and 1.15% for peak areas. Inter-day repeatability was calculated, from determination on three days, with RSD% between 0.25% and 0.47% for migration times, 0.96% and 1.74% for peak areas.

To determine the linearity, plots of peak area versus concentration over the range of 5 – 100 μg/mL. were made for the mixture of the seven cephalosporins. The regression equations and the correlation coefficients are presented in Table 2.

LOD and LOQ were estimated as: standard deviation of regression equation/slope of the regression equation multiplied by 3.3 and 10, respectively (Table 2).

The robustness of the method was examined by analyzing the selected mixture (n = 3) by making slight changes to the following parameters: buffer concentration (25-30 mM), applied voltage (22-25 kV), temperature (20-22 °C) and injection pressure (40-50 mbar/1 sec.), taking in consideration the variation of migration times. The slight variation of these parameter does not significantly modify the migration times (RSD <2 %).
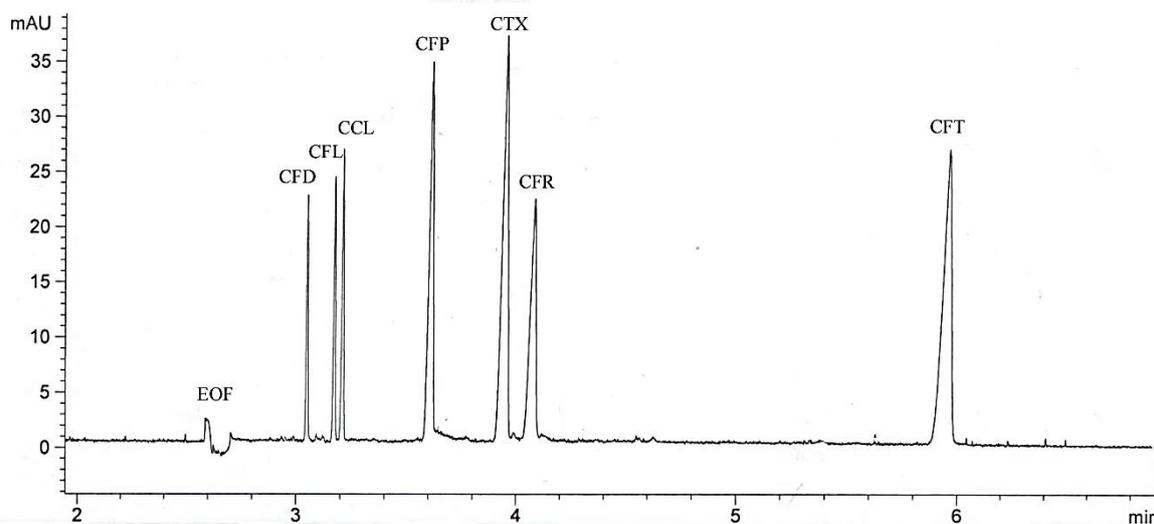


**Figure 4.** Electropherogram of the separation of the selected cephalosporins at pH – 7.00 using the optimized analytical conditions

**Table 1.** Analytical parameters of the studied cephalosporins separation (n = 6, sample concentration = 10 µg/mL)

| Cephalosporin | Migration time (min) | Electrophoretic mobility (cm²/kV min) | RSD (%) | | |
| --- | --- | --- | --- | --- | --- |
| | | | RSD (%) migration time | RSD (%) peak area | RSD (%) peak height |
| CFD | 3.08 | - 5.76 | 0.08 | 0.98 | 0.76 |
| CFL | 3.20 | - 6.72 | 0.13 | 0.84 | 0.79 |
| CCL | 3.30 | - 7.44 | 0.14 | 0.52 | 0.66 |
| CFP | 3.60 | - 9.38 | 0.12 | 1.06 | 0.80 |
| CTX | 4.00 | - 11.52 | 0.16 | 0.76 | 0.65 |
| CFR | 4.20 | - 12.43 | 0.17 | 1.01 | 0.82 |
| CFT | 6.10 | - 18.12 | 0.20 | 1.15 | 0.97 |

**Table 2.** Linearity regression data for the separation of the studied cephalosporins (n = 3, concentration range = 5 – 100 µg/mL)

| Cephalosporin | Regression equation | Correlation coefficient | LOD (µg/ml) | LOQ (µg/ml) |
| --- | --- | --- | --- | --- |
| CFD | Y = 1.0151X – 10.952 | 0.9905 | 2.40 | 8.00 |
| CFL | Y = 0.8693X – 11.396 | 0.9985 | 1.51 | 5.04 |
| CCL | Y = 0.5351X – 6.4519 | 0.9971 | 2.03 | 6.90 |
| CFP | Y = 1.1058X – 14.03 | 0.9983 | 1.62 | 5.40 |
| CTX | Y = 2.2811X – 28.448 | 0.9989 | 1.30 | 4.33 |
| CFR | Y = 1.9776X – 24.613 | 0.999 | 1.33 | 4.45 |
| CFT | Y = 0.9236X – 6.0564 | 0.9924 | 1.40 | 4.64 |

## Conclusion

In this study the electrophoretic behavior of ten frequently used cephalosporins from different generations having different structural characteristics was investigated using capillary zone electrophoresis. The separation of all studied cephalosporins can be succesfuly achieved using appropiate buffer electrolytes and variuos electrophoretic conditions.

Taking in consideration also the aspects presented in previously developed CE analysis methods already published in the literature, our study is a certainly needed thorough investigation of the buffer pH and composition

effects on the electrophoretic migration behavior of cephalosporins.

A simple, fast and reliable method for the simultaneous separation of seven derivatives was developed, demonstrating once more that this analytical procedure is well suitable for the analysis of cephalosporins.

The electrophoretic mobility of the studied analytes were shown to be highly dependent on the pH of the background electrolyte; as they possessed different ionic mobility at different pH values, with smaller electrophoretic migrations than the EOF and always migrated in the direction of the cathode.

The developed CZE method is easy to handle and shows good reproductiblity and linearity; the low amount of required sample and short analysis time appear to be the main advantages of the method. Using the described optimized conditions this technique can be used for the analysis and identification of cephalosporins in pharmaceutical products and also for resolving complex mixtures of drugs; other cephalosporins (not investigated in this study) can probably be likewise analyzed.

Performed analyses of selected cephalosporins in different separation conditions increases knowledge about behavior of these compounds, which could be helpful in identification and determination of cephalosporins by CE method.

## Ethical Issues
Not applicable.

## Conflict of Interest
Authors declare no conflict of interest.

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