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Development of a capillary electrophoresis method for the simultaneous determination of cephalosporins

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Abstract: A rapid and simple capillary electrophoresis method has been developed for the simultaneous determination of six extensively used cephalosporin antibiotics (cefaclor, cefadroxil, cefalexin, cefuroxim, ceftazidim and ceftriaxon). The determination of cephalosporins was performed at pH 6.8, using a 25 mM phosphate/25 mM borate mixed buffer, and a voltage of 25 kV at a temperature of 25 °C. A baseline separation was achieved in approximately 10 min. The separation resolution was increased by the addition of an anionic surfactant, 50 mM sodium dodecyl sulfate, to the buffer solution. The proposed separation was evaluated based on the detection and quantification limits, effective electrophoretic mobility and relative standard deviation for the migration times and peak areas.

Keywords: cephalosporins; capillary zone electrophoresis; micellar electrokinetic chromatography.

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 and of their use for the treatment of infectious diseases.

Cephalosporins are semi-synthetic antibiotics, derived from cephalosporin C, found among the fermentation products of *Cephalosporium acremonium* over fifty years ago. These antibiotics, derived from 7-aminocephalosporanic acid, are composed of a *β*-lactam ring fused with a dihydrothiazine ring and differ in the nature of the substituents attached to the cephem ring.[1]

More than 60 cephalosporins in four generations are already available on the market, so it is important to develop new analysis methods for their quality control. The analysis of cephalosporins is not only limited to pharmaceutical

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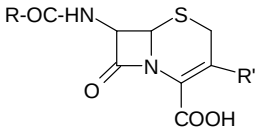
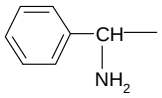
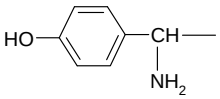
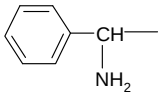
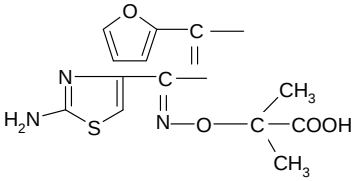
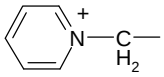
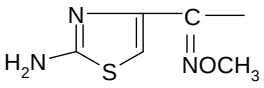
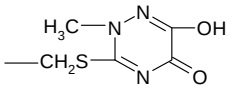
analysis, but also extends to food safety and environmental protection; all these analytical tasks require the development of high performance separation methods.

In this study, we analyzed six frequently used cephalosporin derivatives: cefalexin (CFL), cefadroxil (CFD) and cefaclor (CFC) – first generation oral cephalosporins; cefuroxim (CFR) – a second generation parenteral cephalosporin; and ceftazidim (CZI) and ceftriaxon (CTR) – third generation parenteral cephalosporins. Substitution of the various R and R′ groups results in cephalosporins with different pharmacological and pharmacokinetic properties. The chemical structures of the studied cephalosporins are presented in Table I.

TABLE I. The chemical structure of the studied cephalosporins[1]



Cephalosporin derivative	R	R′
Cefalexin – CFL		$-CH_3$
Cefadroxil – CFD		$-CH_3$
Cefaclor – CFC		–Cl
Cefuroxim – CFR	‼ EMBED ISISServer	$-CH_2-O-CO-NH_2$
Ceftazidim – CZI		
Ceftriaxon – CTR		

High performance liquid chromatography (HPLC) is usually the method of choice for the analysis of cephalosporins because of its specificity, rapidity and sensitivity. In recent years, capillary electrophoresis (CE), due to its short analysis time, high efficiency and low solvent and sample consumption; has proved to be a powerful analytical tool for the determination of pharmaceutical substances, being regarded as an alternative and also a complementary method to HPLC.[2,3]

In the last few years, some studies regarding the determination of cephalosporins by capillary zone electrophoresis (CZE),[4–9] micellar electrokinetic chromatography (MEKC)[10–12] and microemulsion electrokinetic capillary chromatography (MEEKC)[13] have been published.

CZE, also known as free solution capillary electrophoresis, is the simplest form of CE, the separation mechanism being based on differences in the charge-to-mass ratios of the analytes.[2]

MEKC is perhaps the most intriguing mode of CE, combining chromatographic and electrophoretic separation principles, the separation mechanism being based on the individual partition of the analytes between the micellar and aqueous phase.[2]

MEKC can separate both ionic and neutral substances, while CZE typically separates only ionic substances. Thus, MEKC has a great advantage over CZE for the separation of mixtures containing both ionic and neutral compounds.[2]

While MEKC proved itself to be especially useful for the determination of cephalosporins from biological samples (samples having high protein contents) as the disadvantageous matrix effects caused by organic materials are reduced, CZE proved itself to be a powerful analytical tool for determinations from pharmaceutical products.[6,7,8,14]

Structurally cephalosporins are a rather heterogeneous group; consequently, a large number of electrophoretic procedures using different analytical parameters could be used for their separation. Regarding previously published methods, several exemplify the fact that the simultaneous determination of structurally related cephalosporins is challenging because of their similar electrophoretic mobilities. For this reason, both structurally related cephalosporins from the same generation and cephalosporins from different generations and different structural characteristics were chosen as the subjects of the present study.[5,8,9,13]

The aim was to develop a rapid, simple and efficient method for the simultaneous separation of the studied cephalosporins and the optimization of the analytical conditions in order to obtain good separation resolution and a short analysis time.

EXPERIMENTAL

Cefalexin monohydrate, cefadroxil monohydrate, cefaclor monohydrate were obtained from Sandoz (Târgu Mureş, Romania); cefuroxim sodium from Medochemie (Cyprus), while ceftazidim pentahydrate, ceftriaxon sodium from Antibiotice (Iaşi, România). All the studied cephalosporins were of pharmaceutical grade.

Disodium hydrogen phosphate, methanol, phosphoric acid, sodium dodecyl sulfate (SDS), sodium tetraborate were purchased from Merck (Darmstadt, Germany). Sodium hydroxide 0.1 M was purchased from Agilent (Waldbronn, Germany). The water used in the study was purified with a Milli-Q water purification system (Millipore, Bedford, USA).

Capillary electrophoresis measurements were performed on an Agilent 6100 CE system with diode array detection. The electropherograms were recorded and processed by Chem-

station 7.01 (Agilent). The pH of the buffer solutions was determined with the Terminal 740 pH-meter (Inolab). The detection wavelengths were set at 210 and 270 nm. For the identification of the individual peaks, the UV spectrum of each cephaloasporin was recorded previously. Cephalosporins showed high UV absorption, and relatively similar spectra but small differences could be observed in the case of all components, which led to reliable and unambiguous identification.

The temperature was kept at 25 °C and a separation voltage of 25 kV was used. The samples were introduced into the system at the anodic end of the capillary by hydrodynamic injection applying a pressure of 50 mbar for 3 seconds. The separations were performed using polyimide-coated fused silica-capillaries of 56 cm (effective length: 48 cm)×50 μm I.D. (Agilent).

Stock solutions of 1 mg mL^{-1} for all cephalosporins were prepared in water and later diluted to the appropriate concentration. All the samples and buffers were degassed by ultrasound for 5 min before use and filtered through a 0.45 μm syringe membrane filter. The capillaries were preconditioned by washing with 0.1 M NaOH (2 min) and distilled water (3 min).

RESULTS

Preliminary study

The electrophoretic mobilities of cephalosporins depends not only on the number of ionizable carboxyl and amino groups of the analyte, but also on the pH of the buffer electrolyte because the dissociation of these groups is influenced by pH.[7,15]

In order to find suitable conditions for the separation of the studied cephalosporins, a series of preliminary experiments were conducted at different pH values and different compositions of the buffer (borate, citrate, phosphate) background electrolytes. In these preliminary experiments, satisfactory electrophoretic signals were obtained within the pH range 5–8, as the electrophoretic signals for some of the studied cephalosporins disappeared at pH values below 5. It was established that for a given type of background electrolyte, the magnitude of the total mobility of the analytes (electro-osmotic mobility plus electrophoretic mobility) depended mainly on the zeta potential, which decreased with decreasing buffer pH and/or increasing buffer concentration.[5] The migration behavior of the studied cephalosporins in different background electrolyte was significantly different, due to the different basicity on the side chain functional groups, but trends in the variation of the electrophoretic mobility were rather similar.

Comparing the different systems, the best results were obtained using 100 mM phosphate buffer at a pH around 7. However, the current generated in the electrophoretic system exceeded 100 μA; therefore, experimental difficulties appeared due to excessive Joule heating. Moreover, the use of a simple phosphate buffer led to rather long migration times and low resolution for the separation of CFD, CFL and CFC.

In order to improve the separation resolution, a mixed buffer electrolyte containing both disodium hydrogen phosphate (3.55 g L^{-1}) and sodium tetraborate

(5.03 g L^{-1}) was chosen and the pH of the buffer solution was adjusted by the addition of sodium hydroxide, respectively phosphoric acid.

Although the stability of cephalosporins antibiotics in the solid state is usually satisfactory, they are slowly hydrolyzed on dissolution in water to different degradation products. Preliminary studies using an internal standard (ciprofloxacin hydrochloride) as reference showed that degradation (hydrolysis) was insignificant within 4 h of dissolution.[6,7] Electrophoretic runs were performed as quickly as possible, but no later than 4 h after solution preparation, in order to avoid sample decomposition due to the instability of the *β*-lactam ring.

Optimization of the separation conditions

Optimization of the separation was achieved by manipulation of several analytical parameters, such as buffer pH and concentration, applied voltage and system temperature, and by the addition different additives to the buffer solution.

The buffer pH plays an essential role in the separation of ionizable analytes as it determines the extent of ionization of the analytes. The influence of pH on the migration times of a mixture of the six cephalosporins in electrolyte solutions adjusted to pH 5–8. This pH range was chosen in order to minimize any possible degradation of the cephalosporins due to hydrolysis of the *β*-lactam ring. The migration times of all the studied cephalosporins increased with decreasing buffer pH, but in the pH range 6 to 7, the migration times did not change significantly and peak overlapping between CFL with CFC was avoided (Fig. 1).

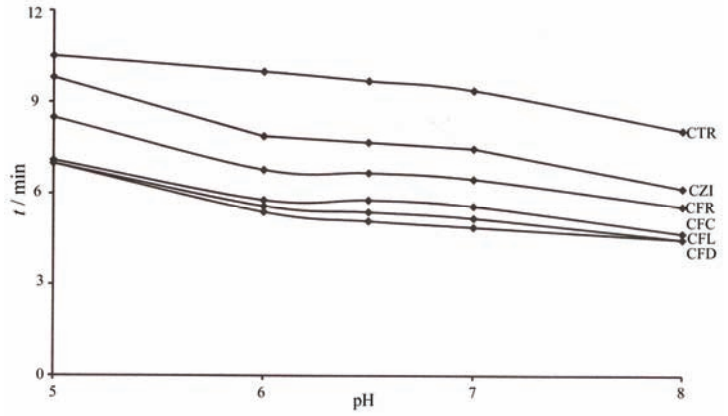


Fig. 1. Influence of pH on the separation of cephalosporins (separation conditions: capillary 56 cm×50 μm I.D., buffer electrolyte 25 mM phosphate – 25 mM borate, applied voltage: 25 kV, temperature: 25 C, detection: UV absorption at 270 nm).

At a given pH, increasing the buffer concentration resulted in a decrease in the electro-osmotic flow (EOF) and electrophoretic mobility and, consequently, an increase in the migration times of the analytes.

The separation of the six studied cephalosporins using the selected CZE parameters was achieved in approximately 10 min. The migration order was: CFD, CFL, CFC, CFR, CZI, CTR (Fig. 2).

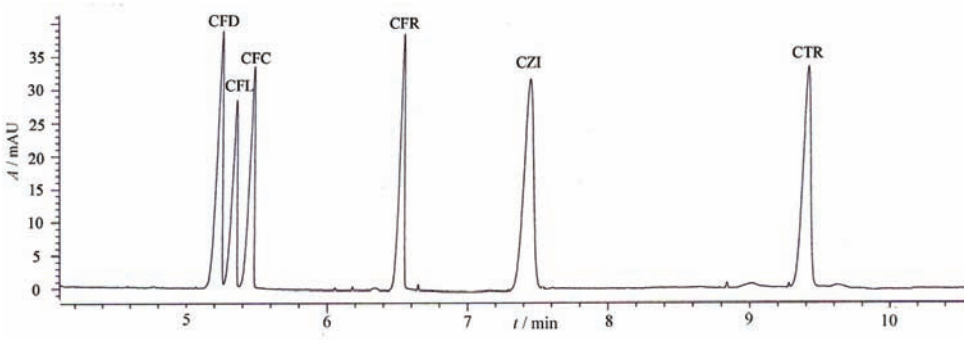


Fig. 2. Electropherogram of the separation of a mixture of the six cephalosporins (separation conditions: capillary 56 cm×50 μm I.D., buffer electrolyte 25 mM phosphate – 25 mM borate, pH – 6.8, applied voltage: 25 kV, temperature: 25 C, detection: UV absorption at 270 nm, concentration of each cephalosporin: 25 μg mL^{-1}).

Difficulties appeared in the separation of the three first generation oral cephalosporins (CFC, CFD and CFL), substances with very similar structural characteristics and, consequently, similar electrophoretic mobilities.

Cephalosporins are hydrophobic drugs that have similar polar groups but exhibit different partition behaviors between two phases (micellar and aqueous phase); consequently, MEKC is a well-suited method for their separation. Depending on the chemical structure of the cephalosporins, in addition to the hydrophobic interactions, various chemical interactions, such as dipolar interactions, may occur between them in the partitioning mechanism. Moreover, these analytes have carboxyl groups, and possible electrostatic repulsion between the analytes and the anionic SDS micelles might decrease, even if just partially, the micellar solubilization of the cephalosporins.[12,15]

Addition of SDS to the buffer solution led to a gradual increase in the migration times of the analytes, but improved resolution between the three structurally related cephalosporins (CFD, CFL and CFC). At neutral pH, a strong EOF moved in the direction of the cathode; SDS is an anionic surfactant consequently the electrophoretic migration of the anionic micelle was in the direction of the anode. As a result, the overall micellar migration velocity was slowed compared to the bulk flow of solvent. The separation was based on the differential partitioning of the analytes between the two-phase-system: the mobile aqueous phase and the pseudo-stationary micellar phase.

The migration times of the analytes increased with increasing SDS concentration as higher SDS concentration result in greater ionic strength and conse-

quently smaller EOF (Fig. 3). In MEKC, the migration times of the cephalosporins also increased with decreasing buffer pH.

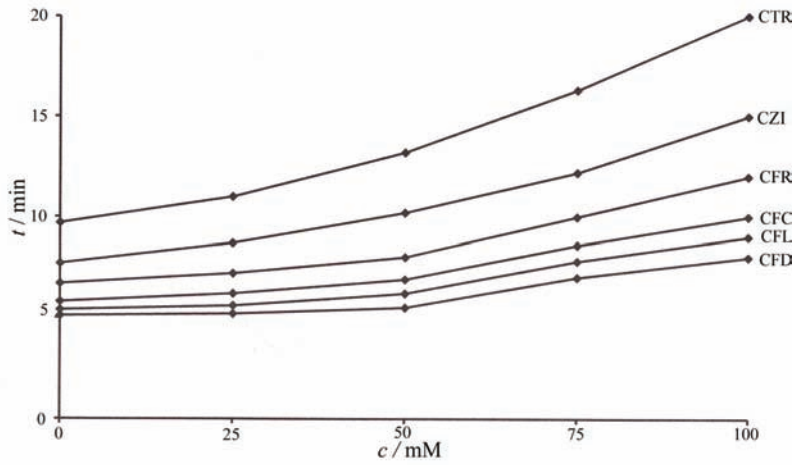


Fig. 3. Influence of SDS concentration on the separation of cephalosporins (separation conditions: capillary 56 cm×50 μm I.D., buffer electrolyte 25 mM phosphate – 25 mM borate, pH – 6.8, applied voltage: 25 kV, temperature: 25 °C, detection: UV absorption at 270 nm).

The optimum SDS concentration was set at 50 mM (14.42 g L^{-1}), as higher concentrations generated high currents (above 100 μA) and instability of the electrophoretic system.

The separation of the six studied cephalosporins using the selected MEKC parameters was achieved in approximately 15 min. The migration order was: CFD, CFL, CFC, CFR, CZI and CTR (Fig. 4).

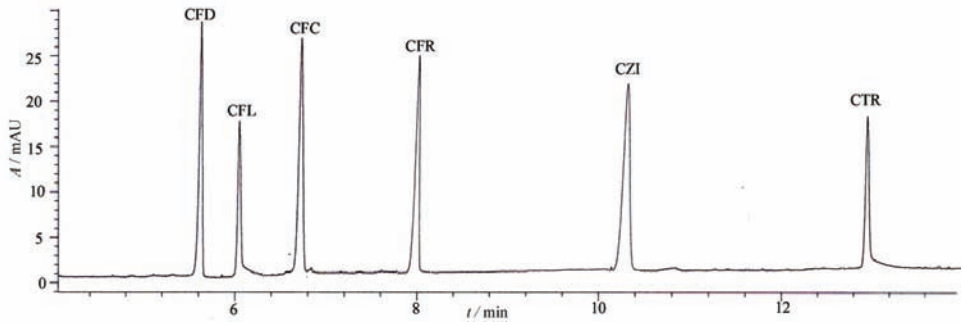


Fig. 4. Electropherogram of the separation of a mixture of the six cephalosporins (separation conditions: capillary 56 cm×50 μm I.D., buffer electrolyte 25 mM phosphate – 25 mM borate – 50 mM SDS, pH – 6.8, applied voltage: 25 kV, temperature: 25 °C, detection: UV absorption at 270 nm, concentration of each cephalosporin 25 μg mL^{-1}).

DISCUSSION

The migration order in CZE could be explained in terms of the electric charge and size of the analytes. In the CZE method, analytes with smaller molecular sizes migrate faster than the more bulky ones. At a pH above 5, the carboxylic group of the cephalosporins was fully dissociated, and the variation in the electrophoretic mobility of the analytes could be attributed to the deprotonation of either an amino group or a pyridinium group. There is also a possibility for the deprotonation of the benzyl position near to amino and pyridinium groups. It is also worth mentioning that at a pH between 5 and 7, CFL, CFD and CFC existed predominantly as zwitterions.[5]

In MEKC, analytes that have a greater affinity for the micelles have slower migration velocities compared to analytes that spend most of their time in the bulk phase. An analyte with charge opposite to that of the micelles will strongly interact with the micelle through electrostatic forces, while an analyte with the same charge as that of the micelle will interact weakly, due to electrostatic repulsion. The studied cephalosporins exhibited different hydrophobic–hydrophilic properties and contain various ring heteroatom, such as nitrogen, oxygen and sulfur, that form strong hydrogen bonds with water and micelles.[11]

Cephalosporins with more complex structures, *i.e.*, contain aromatic rings or long aliphatic side chains as substituents, are more strongly incorporated into the micelles and will migrate slower. CFD, CFL and CFC with simpler structures and obvious similarities will migrate first and the resolution between them will be poorer. CTR migrates much slower then the other cephalosporins, probably because of the characteristic highly acid heterocyclic system on the 3-thiomethyl group attached to the C-3 position of the cephem structure. The migration order of the three parenteral cephalosporins is in concordance with their pK_a values corresponding to the carboxylic group: CFR (pK_a 2.5), CZI (pK_a 1.9) and CTR (pK_a 1.7).[5,16]

Analytical performance

The analytical performance of the method was evaluated by measuring the detection (*LOD*) and quantification (*LOQ*) limits and relative standard deviations (*RSD*) for the migration times and peak areas. The optimized MEKC separation parameters were used for all these measurements.

All the cephalosporins exhibited negative electrophoretic mobilities and moved with the electrophoretic flow (Table II).

To evaluate repeatability of the peak areas and migration times, ten injections of mixture of cephalosporins were made and relative standard deviation was calculated (Table II).

The *LOD* and *LOQ* values were calculated within the concentration corresponding to signal-noise relation equal to 3 and 10, respectively (Table II).

TABLE II. Analytical parameters for the MEKC separation of the cephalosporin

Analyte	Migration time min	Electrophoretic mobility $cm^2\ kV^{-1}\ min^{-1}$	RSD migration time, %	RSD peak area, %	*LOD* $\mu g\ mL^{-1}$	*LOQ* $\mu g\ mL^{-1}$
CFD	5.52	–10.75	0.12	0.51	0.99	3.28
CFL	6.28	–12.98	0.15	0.75	2.59	8.63
CFC	6.87	–14.45	0.13	0.75	1.42	4.75
CFR	8.06	–16.76	0.09	0.42	1.96	6.55
CZI	10.16	–19.52	0.16	0.90	1.88	6.27
CTR	13.22	–21.97	0.16	1.03	2.73	9.09

To determine the linearity of the plots peak areas *vs.* concentration, six concentrations in a specific range and three replicates per concentration were used. The regression equations and correlation coefficients are presented in Table III. The correlation coefficients were higher than 0.99, which could be considered as evidence of good data fitting to a line regression.

TABLE III. Linearity regression data for the MEKC separation of the cephalosporin (concentration range: 5–100 $\mu g\ mL^{-1}$)

Analyte	Regression equation	Correlation coefficient
CFD	$y = 2.3594x + 4.5439$	0.999
CFL	$y = 1.6935x + 1.8458$	0.998
CFC	$y = 2.1355x + 3.0514$	0.999
CFR	$y = 2.0458x + 2.8607$	0.998
CZI	$y = 1.7939x + 5.0322$	0.992
CTR	$y = 1.668x + 3.4163$	0.997

CONCLUSIONS

This technique represents a suitable method for cephalosporin analysis, as it is easy to handle and exhibits good repeatability. It also increases knowledge about the electrophoretic behavior of cephalosporins, which could be advantageous in the qualitative and quantitative analysis of these frequently used β-lactam antibiotics.

The combined effects of buffer pH and buffer composition and concentration were taken into consideration to optimize separation of the examined cephalosporins. Correlations between the structural characteristics of the studied cephalosporins and their electrophoretic behavior were made in order to understand and explain the separation. Complete separation of the six studied cephalosporins was achievable under the optimized conditions. The use of a mixed borate–phosphate buffer proved to be superior to a simple phosphate or borate buffer in the CE separation of cephalosporins.

The method developed in this study, in comparison with others previously described in the literature, uses a mixed borate–phosphate buffer, which stabi-

lizes the electrophoretic system and presents a relatively short analysis time and a simple electrophoretic procedure.

Although in therapeutics, β-lactam antibiotics are not usually administered together, in this study, a mixture of six cephalosporins was analyzed in the same run to prove the suitability of the method for the determination of all six compounds in particular and for cephalosporins in general.

Using the described optimized procedures for CZE and MEKC, these techniques could be employed for the identification and determination of cephalosporins in formulated pharmaceutical products and for separations from complex mixtures.

И З В О Д

РАЗВОЈ МЕТОДЕ КАПИЛАРНЕ ЕЛЕКТРОФОРЕЗЕ ЗА ИСТОВРЕМЕНО ОДРЕЂИВАЊЕ ЦЕФАЛОСПОРИНА

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Развијена је брза и једноставна метода капиларне електрофорезе за истовремено одређивање шест широко коришћених цефалоспоринских антибиотика (цефаклор, цефадроксил, цефалексин, цефуроксим, цефтазидим и цефтриаксон). Одређивање цефалоспорина је изведено на pH 6,8, у пуферу 25 mM фосфат/25 mM борат, при напону од 25 kV и на температури од 25 °C. Постигнуто је раздвајање за око 10 min. Резолуција је побољшана додатком анјонског сурфактанта, 50 mM натријум-додецил-сулфата, у пуферски раствор. Предложена метода је евалуирана на основу граница детекције и квантификације, ефективне електрофоретске покретљивости и релативне стандардне девијације миграционих времена и површина пикова.

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