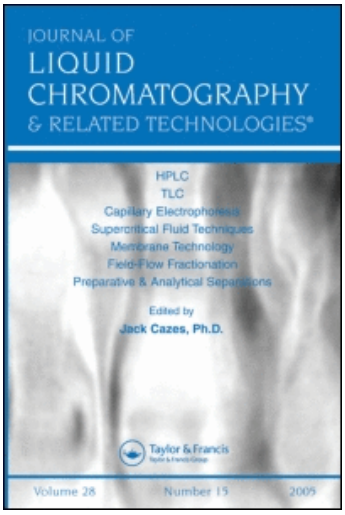

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Validated Method for Simultaneous Determination of Cefepime and L-Arginine in Cefepime for Injection by Capillary Zone Electrophoresis

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ABSTRACT

A rapid and accurate capillary zone electrophoresis (CZE) method is described for simultaneous determination of cefepime and L-arginine in cefepime for injection. Best results were achieved with the background electrolyte (BGE) prepared by titrating 40 mM sodium dihydrogen phosphate with phosphoric acid to pH 2.3 and an applied voltage of 30 kV in a bare fused-silica capillary. The capillary temperature was 30°C and detection was made at 195 nm. L-Histidine was used as internal standard (IS) to ensure acceptable precision. Separation was completed in less than

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12 min. The optimized method was validated for selectivity, linearity, accuracy, precision, ruggedness, repeatability, and detection limits. The linear range for cefepime and L-arginine was 57–571 and 39–394 $\mu g\,mL^{-1}$, respectively. Limit of quantitation (LOQ) was 6 $\mu g\,mL^{-1}$ for cefepime and 3 $\mu g\,mL^{-1}$ for L-arginine. Relative standard deviation (RSD) for peak area ratio (PAR, the area of analyte peak, divided by the area of the IS peak) was less than 1.0%.

Key Words: Capillary zone electrophoresis; Cefepime; L-Arginine.

INTRODUCTION

Cefepime is a fourth generation, semisynthetic cephalosporin. It has a *N*-methylpyrrolidinium at the 3-position, which renders it zwitterionic.[1,2] The effect of a zwitterion compound is a net neutral charge, which may better orientate the interior of bacterial cells and enhance outer-membrane penetration.[1] Like other fourth generation cephalosporins, cefepime demonstrates good activity against gram-negative organisms such as *Pseudomonas aeruginosa* and gram-positive organisms such as *Straphylococcus aureus*.[2] It also exhibits increased stability against β-lactamase-overproducing bacteria.

Cefepime for injection is a sterile, dry powder mixture of cefepime hydrochloride and L-arginine. The L-arginine is included to adjust the pH of freshly constituted solutions to 4–6. Cefepime hydrochloride and cefepime for injection were recently included in the United States Pharmacopeia (USP)[3] and will be included in the new edition (2005) of the Chinese Pharmacopeia (ChP). The purity of cefepime in cefepime hydrochloride is controlled by the USP.[3] According to the requirements of the ChP, and referring to aztreonam for injection in the USP,[4] a sterile, dry mixture of aztreonam and L-arginine, the purity of cefepime in cefepime for injection needs to be controlled and the limit will be 82.5–91.1% in the portion of cefepime hydrochloride, referring to the limit of cefepime in cefepime hydrochloride required by the USP.[3] For this reason, there is a clear analytical need to quantify L-arginine in cefepime for injection and the analytical technique employed should be capable of giving precise and accurate assay values.

A literature survey revealed that no references have been found for simultaneous determination of cefepime and L-arginine in pharmaceutical preparations. Utilizing an HPLC method provided by the USP,[4] which is used for the simultaneous determination of aztreonam and L-arginine in aztreonam for injection, cefepime was much less retained (the capacity factor was less than 1.0), which resulted in a poor resolution between cefepime and its

related substances. Moreover, the tailing factor of the L-arginine peak was usually more than 2.0, and the uncommonly used column that contains Diol packing is less stable in this mobile phase, so, a saturated column containing large particles of silica needs to be prepared.

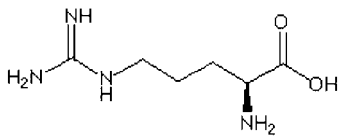
Capillary electrophoresis (CE) methods have been shown to be useful technologies in the separation of pharmaceuticals and pharmaceutical related products. Method precision, method ruggedness, limited dynamic range, and lack of experience in CE, as compared to HPLC, have impeded the widespread use of these methodologies in the pharmaceutical analytical laboratory. In the past several years, a great deal of work has been done to address these issues, particularly in the areas of method precision, sensitivity, and ruggedness, where data for CE methods have been reported to approach those of HPLC (Fig. 1).[5–20]

The present paper describes a rapid and reliable capillary zone electrophoresis (CZE) method for the simultaneous determination of cefepime and L-arginine in cefepime for injection. Development, optimization, and validation of this method are presented in this paper.

EXPERIMENTAL

Reagents

Cefepime hydrochloride was from Shanghai Asia Pioneer Pharmaceuticals Co., Ltd. (Shanghai, P.R. China). L-Arginine was from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). Cefepime for injection (1 g) was from Bristol-Myers Squibb Australia Ptv Ltd. Cefepime hydrochloride reference standard, cefepime E-isomer hydrochloride reference standard, and L-arginine reference standard were kindly provided by Sino-American Shanghai Squibb Pharmaceuticals Ltd. (Shanghai, P.R. China). Sodium dihydrogen phosphate and phosphoric acid were purchased from SCR (Shanghai, P.R. China). L-Histidine was obtained from Sigma (St. Louis, MO). All other chemicals



L-arginine

Figure 1. Structures of cefepime, cefepime E-isomer, and L-arginine.

were atleast of analytical grade. Water used throughout the work was treated with a Millipore (Bedford, MA) Milli-Q water purification apparatus.

Solution Preparation

The internal standard (IS) was 0.4 mg mL^{-1} of L-histidine, dissolved in water. Calibration solutions were typically prepared by weighing 70 mg of cefepime hydrochloride reference standard and 40 mg L-arginine reference standard into a 200-mL volumetric flask and diluted to volume with IS solution. The sample solution was typically prepared by weighing a 280 mg sample into a 500 mL volumetric flask and diluted to volume with IS solution.

Capillary Electrophoresis

The capillary electrophoresis equipment was an $HP^{3D}CE$ system from Agilent, equipped with a diode array detector to confirm the purity of the analyte peaks from forced degradation. A 50 μm i.d. × 64.5 cm (56 cm detection length) bare fused-silica capillary was purchased from Agilent (Waldbronn, Germany), and was conditioned before initial use by rinsing with 0.1 M sodium hydroxide for 10 min.

The background electrolyte (BGE) was prepared by titrating 40 mM sodium dihydrogen phosphate with phosphoric acid to pH 2.3. Absorption was measured at 195 nm and the capillary temperature was 30°C. The BGE-filled capillary was electro-conditioned for 20 min at 30 kV before analysis. The flush procedure between runs was for 1 min with BGE, 0.5 min application of 30 kV between the inlet and outlet, and 2 min with BGE. The injection was carried out by pressure, 25 mbar for 6 sec, immediately followed by injection of BGE for 2 sec at the same pressure. The analyses were run in constant voltage mode at 30 kV after an initial ramp time of 0.5 min. The capillary was stored dry after rinsing with water for 5 min followed by air for 5 min.

RESULTS AND DISCUSSION

Method Development and Optimization

The pK_a value of cefepime is 1.12 (–COOH) and 3.1 ($–NH^+$).[21] As cefepime has a quaternary NMP^+ at the 3-position, it showed zero mobility at pH values above 4.5, whereas at lower pH the net charge of cefepime is positive, which results in positive mobility.[21] The pK_a values of L-arginine

are 2.18 (–COOH), 9.09 ($-NH_3^+$), and 13.2 ($=NH_3^+$),[22,23] respectively, hence, L-arginine presents positive mobility at lower pH. To achieve reasonable migration times with CZE for these two substances, the pH of the BGE should be less than 3, thus, phosphate buffer was chosen.

It is well known, that mobility mismatching between the analyte and the co-ion (component of BGE, which has the same charge with the analyte) can cause electro-migration dispersion and, as a result, the analyte peak will be fronting or tailing,[24] thus, the choice of the co-ion is very important during CZE method development.

Owing to the unknown mobilities (μ) of cefepime and L-arginine in this type of CZE system, Na^+ was first selected as the co-ion. Using 5 mM NaH_2PO_4–10 mM H_3PO_4 buffer (pH 2.3) as BGE, the effective mobilities of cefepime and L-arginine, calculated with an equation previously reported,[25] were approximately 17×10^{-9} and $37 \times 10^{-9}\,m^2\,V^{-1}\,sec^{-1}$, respectively. As the mobility of Na^+ ($51.9 \times 10^{-9}\,m^2\,V^{-1}\,sec^{-1}$) is much higher than for the two drugs, several other co-ions such as histidine ($\mu = 29.2 \times 10^{-9}\,m^2\,V^{-1}\,sec^{-1}$),[26] creatinine ($\mu = 36.8 \times 10^{-9}\,m^2\,V^{-1}\,sec^{-1}$),[26] diethylammonium ($\mu = 34.1 \times 10^{-9}\,m^2\,V^{-1}\,sec^{-1}$),[26] triethylammonium ($\mu = 30.6 \times 10^{-9}\,m^2\,V^{-1}\,sec^{-1}$),[26] tetramethylammonium ($\mu = 42.6 \times 10^{-9}\,m^2\,V^{-1}\,sec^{-1}$),[26] tetra-*n*-butylammonium ($\mu = 18.5 \times 10^{-9}\,m^2\,V^{-1}\,sec^{-1}$),[26] and *tris*(hydroxymethyl)methylammonium ($Tris^+$, $\mu = 26.9 \times 10^{-9}\,m^2\,V^{-1}\,sec^{-1}$)[26] were evaluated instead of Na^+. It was found that the species of co-ion had a significant influence on the peak shape of L-arginine: the resultant peak of L-arginine might be fronting or tailing, and it was even broadened and deformed if $Tris^+$ was used. Peak shapes of the two drugs were not symmetrical simultaneously with any of the CZE systems examined above.

Due to the considerably large difference between the mobilities of the two substances in this type of CZE system, a stacking effect was utilized to improve the peak shape of the analytes. In electrophoretic processes, sample analytes are generally concentrated when they are introduced at a lower concentration than the BGE, i.e., the analytes can be concentrated in narrow bands by a stacking procedure.[13] Provided the generated Joule heating is not too high, which can yield lower peak efficiency, a higher buffer concentration can produce better peak shapes at high analyte concentrations and will also lead to improved peak reproducibility. Keeping the pH of BGE at 2.3, different concentrations of NaH_2PO_4 in BGE from 5 to 50 mM were examined. It was shown that the symmetry of peak shape improved significantly as the concentration of BGE increased. Considering the lower Joule heating generated and the higher peak efficiency yielded, 40 mM NaH_2PO_4 in BGE was chosen, and peak shapes of the two drugs were nearly symmetrical in this CZE system.

The influence of the BGE pH on migration time and resolution of analytes was investigated. With the pH of the BGE decreasing, migration times of all analytes decreased, meanwhile, the resolution between cefepime and its E-isomer also decreased. If the pH of the BGE was lower than 2.0, the resolution between cefepime and its E-isomer was less than 2.0, i.e., they cannot be baseline separated. At pH 2.2–2.4, the separation and analysis time were both satisfactory, and pH 2.3 was finally chosen since this BGE provided higher buffering capacity around the pK_a value of H_3PO_4 (pK_a 2.2). Advantages of this low pH were the lower absorption of the surface-analyte and a small EOF when using a bare fused-silica capillary. The sum concentration of NaH_2PO_4 and H_3PO_4 in this BGE was about 120 mM, calculated with a sophisticated simulation program Peakmaster (Charles University, Prague, Czech Republic). This optimal BGE had sufficient buffering capacity to provide consistent migration times and selectivities.

Calculation with Peakmaster showed there was an eigenzone with mobility of $62 \times 10^{-9}\,m^2\,V^{-1}\,sec^{-1}$, which originated from H^+ at this low pH (2.3), where H^+ considerably acts as the second co-ion.[24] Although the BGE does not have a pH in the safe region,[5–9] this system eigenzone (a migrating system zone) raised in the BGE with low pH does not disturb the separation, because its mobility is much higher than those of cefepime and L-arginine.

The pH of the sample solution (containing IS) dissolved in water, was determined to be higher than 4.5. For the sake of making cefepime possess a net positive charge, it seemed necessary to adjust the pH of the sample solution to less than 4.5. However, experimental results indicated that whether the pH of the sample solution was adjusted to 2.5 with 1 M H_3PO_4 or remained higher than 4.5, there was no significant difference with the peak shape and peak efficiency of cefepime. This phenomenon could be explained by the following reasoning:[27] to obtain good peak shapes, it is necessary for some species (might be buffering species) migrating into the sample zone to be able to dissociate to form hydrogen ions in order for the analyte in the sample to become positively charged, and an acid with more than two ionization constants (such as H_3PO_4) could be preferably used, provided that the difference between the constants of the acid and the analyte was not too large and the pH of the BGE was suitable. In this research, when a voltage was applied from the detector side, the ionized form of the acid ($H_2PO_4^-$, pK_a 2.2) migrates into the sample zone (with higher pH), this acidic ion will dissociate further and is able to donate hydrogen ions, hence, the conjugate acid of cefepime will be formed according to its equilibrium constant ($-NH^+$, pK_a 3.1). As a result, the net charge of cefepime will be positive.

To obtain stacking effects, the sample is usually dissolved in water or in dilute buffer, which maximizes resolution and peak efficiency. Since cefepime is unstable in acidic aqueous solution, its degradation rate constants were

moderately increased by the addition of L-arginine, and its accelerated loss due to formate, acetate, phosphate, and borate buffer catalysis has also been quantitatively described,[28] therefore, choosing water as a sample solvent is appropriate for stabilizing the sample solution.

A system suitability test is proposed, although it is not required by the USP method used to assay cefepime in cefepime hydrochloride.[3] It was developed so that no sample of related substances is needed. The system suitability solution is prepared by dissolving 150 mg of sample in 50 mL of water and heating this solution at 40°C for 24 hr. Under these conditions, cefepime is partially converted into its E-isomer and some unknown degradation products. Resolution between cefepime and its E-isomer is established as a system suitability parameter and the limit is 2.0. The electropherograms in Figs. 2 and 3 show the separation of a system suitability solution and a real sample solution, respectively.

Since L-arginine in the BGE has no significant UV maximum but end absorption, to ensure the sensitivity of the method, a wavelength of 195 nm was employed for detection.

Method Validation

Selectivity

The method is selective for cefepime, its products of degradation, L-arginine and the IS, as shown by the electropherograms (Figs. 2 and 3). Injection, separately of the IS solution, 0.4 mg/mL of cefepime hydrochloride solution, 0.2 mg/mL of L-arginine solution and the stress-study sample solutions (all without IS included), water and BGE indicated that all these electrophero-

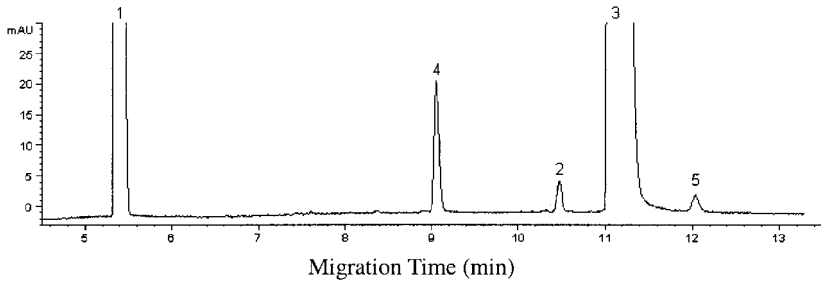


Figure 2. Electropherogram obtained from system suitability solution. Peak: 1, L-arginine; 2, cefepime E-isomer; 3, cefepime; 4 and 5, unknown degradation products. See operating conditions in Experimental section.

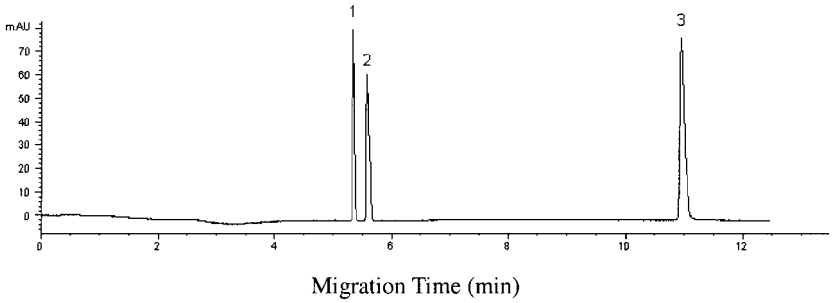


Figure 3. Electropherogram obtained from sample solution. Peak: 1, L-arginine; 2, L-histidine (IS); 3, cefepime. See operating conditions in Experimental section.

grams showed no interference in this method. Purity factors of the peaks of cefepime and L-arginine indicated the absence of coeluting impurities.

Linearity

Seven individual calibration curves (covering the range 57–571 $\mu g\,mL^{-1}$ of cefepime and 39–394 $\mu g/mL^{-1}$ of L-arginine) were prepared with IS solution. Each solution was analyzed in duplicate. The correlation coefficients for the data reported for peak area ratios (PARs, the area of analyte peak, divided by the area of the IS peak) of cefepime and L-arginine were 0.9998 and 0.9999, respectively.

Accuracy

Accuracy was determined by applying the described method to synthetic mixtures containing known amounts of each drug corresponding to 80%, 100%, and 120% of label claim. The accuracy was then calculated as the

Table 1. Accuracy of the CZE method for simultaneous determination of cefepime and L-arginine.

	Mean recovery (%)		
Drugs	80% of label claim ($n = 3$)	100% of label claim ($n = 3$)	120% of label claim ($n = 3$)
Cefepime	100.6	100.5	100.3
L-arginine	100.3	99.9	100.1

percentage of analyte recovery by the assay. Mean recoveries for cefepime and L-arginine from the formulations are shown in Table 1, which indicate good accuracy of the method for simultaneous determination of the two drugs.

The contents of cefepime and L-arginine in a batch sample determined using this method were 52.1% and 35.6% ($n = 6$), respectively, which agreed with the result obtained with the USP method used for assay of cefepime in cefepime hydrochloride[3] and for the assay of L-arginine in aztreonam for Injection:[4] 51.9% and 35.8% (both $n = 4$), respectively. Using the described method, the purity of cefepime in this batch sample was 84.9%, which also agreed with that obtained with the USP method:[3,4] 84.8%, calculated on the anhydrous (3.0% water contained) and L-arginine-free basis.

Precision

The intraday relative standard deviations (RSDs) of migration times of L-arginine, IS, and cefepime were 1.2%, 1.3%, and 1.8% ($n = 30$), respectively, while using the same inlet and outlet BGE vials, the RSDs were 0.1%, 0.2%, and 0.4% ($n = 10$), respectively. As a result, there was no need to correct the peak areas for their corresponding migration times. The RSDs of the PAR values of 10 consecutive injections of the standard solution were 0.3% (L-arginine) and 0.9% (cefepime), respectively.

Limits of Detection and Quantitation

The limits of detection (LOD) of cefepime and L-arginine were estimated at 2 and 1 $\mu g\,mL^{-1}$ (signal-to-noise of 3), respectively. The limits of quantitation (LOQ) of cefepime and L-arginine were estimated at 6 and 3 $\mu g\,mL^{-1}$ (signal-to-noise of 10), respectively.

Ruggedness

The method proved to be robust with respect to small changes in BGE composition as was derived during method development. The migration time ranges (min) of L-arginine, IS, and cefepime during four consecutive days were 5.21–5.46, 5.44–5.74, and 10.27–11.48, respectively.

Utilizing another bare fused-silica capillary (SGE, Melboume, Australia, 50 μm i.d. $\times$ 32.5 cm, 24 cm detection length) under the same conditions except substituting 10 kV for 30 kV, the injection time was decreased from 6 to 3 sec for the reason of lower load with this shorter column, the separation and peak shapes were also excellent.

Applying this method with another Agilent instrument (Agilent ^{3D}CE system) and another bare fused-silica capillary (Yongnian, Hebei, P.R.

China, 75 μm i.d. × 60.5 cm, 52 cm detection length) under the same conditions, the retention times (min) of L-arginine, IS, cefepime E-isomer, and cefepime were 5.03, 5.21, 8.88, and 9.46, respectively. It was, therefore, expected that routine use of this method on these two types of CE instruments should not give rise to problems.

Repeatability

Six individual weighing samples of the same batch were taken and analyzed. The RSDs of results were 0.3% (L-arginine) and 0.4% (cefepime), respectively, which confirmed the acceptable repeatability of sample preparation. Ten individual calibration solutions were prepared and analyzed in duplicate. The calculated response factors produced precisions of 0.9% RSD (L-arginine) and 1.1% RSD (cefepime) for PAR.

Sample Solution Stability

Three sample solutions (stored at ambient temperature: 25°C) were separately injected at 0, 1, 2, and 4 hr. The results remained almost unchanged and no significant degradation was observed within the given period, indicating that the sample solutions were stable for atleast 4 hr.

BGE Stability

It was found that the BGE had a shelf life of more than 1 week when stored at room temperature and protected from light.

BGE Depletion Effects

Because of the higher buffering capacity of the BGE, up to 30 injections using the same inlet and outlet BGE vials were carried out with no significant shift in migration time or peak area, even utilizing the smaller volume vials (1 mL) in the HP^{3D} CE system used.

CONCLUSIONS

The CZE method developed for simultaneous determination of cefepime and L-arginine in cefepime for injection has sufficient selectivity, linearity, accuracy, precision, ruggedness, and repeatability. The developed method can be used for the assay of the two components in cefepime for injection.

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