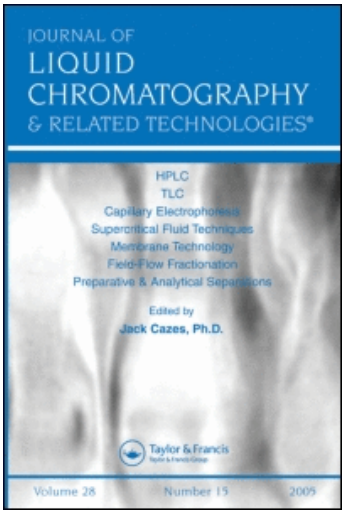

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Rapid Determination of Ciprofloxacin in Cerebrospinal Fluid by Micellar Electrokinetic Chromatography with Direct Sample Injection and its Application in Tuberculosis Meningitis

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Abstract: A micellar electrokinetic chromatography (MEKC) method with ultraviolet detection at 280 nm is described for analysis of ciprofloxacin in cerebrospinal fluid (CSF) by direct injection without sample pretreatment. The proposed method was applied in one tuberculosis meningitis patient monitoring the concentration of ciprofloxacin in CSF after oral administration of 500 mg of ciprofloxacin after 12 hr. The separation of ciprofloxacin from the CSF matrix was performed at 25°C using Tris buffer with sodium dodecyl sulfate (SDS) as the background electrolyte. Using moxifloxacin as an internal standard (IS), the linear range of the method for the determination of ciprofloxacin in CSF was between 0.1–5.0 μg/mL; the detection limit of the ciprofloxacin in CSF (signal to noise ratio = 3; injection 1.0 psi, 25 s) was 50 ng/mL.

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Keywords: Cerebrospinal fluid, Ciprofloxacin, Direct injection, Micellar electrokinetic capillary chromatography, Tuberculosis meningitis

INTRODUCTION

Tuberculosis (TB) is a chronic bacterial infection caused by the *Mycobacterium tuberculosis*. It is transmitted primarily via the respiratory route, mainly affecting the lungs in about 80–85% of cases. *Mycobacterium tuberculosis* can spread through the bloodstream and lymphatic system to the brain and may develop into severe tuberculosis meningitis. *Mycobacterium tuberculosis* readily develops resistance to single drug therapy. Three or four drugs consisting of isoniazid, rifampin, pyrazinamide, and ethambutol are usually used to reduce the bacterial population rapidly.[1] Fluoroquinolones, synthetic derivatives of nalidixic acid, display broad spectrum antimycobacterial activity. Fluoroquinolones therapy, such as ciprofloxacin for tuberculosis, is predominantly used in patients infected with multi-drug resistant *Mycobacterium tuberculosis*.[2] Their bactericidal effects involve binding to DNA-gyrase and DNA topoisomerase, inhibiting bacterial DNA replication and transcription.[3] It is very important that an adequate level of the drug is achieved in CSF for treatment bacterial meningitis. Therefore, the recommended dosage of ciprofloxacin in tuberculosis meningitis treatment is usually oral administration of 250–500 mg ciprofloxacin every 12 h with other antituberculosis drugs for adults. Ciprofloxacin is well absorbed from the gut and widely distributed among body tissues and fluids. However, the drug penetrates the intact blood brain poorly, resulting in low concentration in CSF in the absence of inflammation. Minimal inhibitory concentration for *Mycobacterium tuberculosisis* is $\leq 1.3\ \mu g/mL$ for these fluoroquinolones *in vitro*.[4]

Fluoroquinolones are used to treat a variety of infections in humans and also widely used to treat and prevent veterinary disease in food producing animals. Therefore, fluoroquinolones have been determined in many different samples such as meat, egg, plasma, or blood, by a variety of techniques. High performance liquid chromatographic (HPLC) techniques are the most frequently employed methods for the determination of one or simultaneous determinations[5–14] in plasma samples. These involve various pretreatment procedures prior to analysis. The time consuming pretreatment procedures include solid phase extraction, deproteination by organic solvents, etc., for measurement of the protein bound and unbound drug concentration. It is the free or unbound drug, in equilibrium with the receptor site, therefore, which is the pharmacologically active moiety.

Few capillary electrophoresis (CE) methods have been reported for the study or determination of fluoroquinolones in plasma matrices[15–18]

and in tissues.[19–21] These also involved sample pretreatment procedures prior to analysis. However, the use of CE with direct sample injection without sample pretreatment, in the bioanalysis of the drugs has contributed to clinical chemistry due to the speed, response to pharmacologically active moiety, and small sample size, especially in biological samples such as CSF. So far, no CE method with direct injection without sample cleanup enough for determination of ciprofloxacin in human CSF has been reported.

In this study, we demonstrate a simple and accurate MEKC with direct sample injection method for the quantification of ciprofloxacin, a pharmacologically active moiety, in one tuberculosis meningitis patient' CSF who is treated with ciprofloxacin (Ciproxin®) and three combined commercial tablets, Rifater®, for anti-tuberculosis.

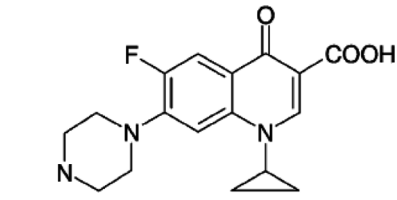
EXPERIMENTAL

Instrumentation

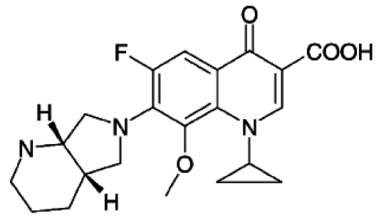
A Beckman P/ACE MDQ system (Fullerton, CA, USA) equipped with an ultraviolet (UV) detector and a liquid cooling device was used. MEKC was performed in an uncoated fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 31.2 cm (effective length 21 cm) × 50 mM I.D. The temperature of the separation was controlled at 25°C by immersion of the capillary in a cooling liquid circulating in the cartridge. The temperature of sample tray was at room temperature. Detection was carried out by the on-column measurement of UV absorption at 280 nm (cathode at the detection side). The CSF samples were in hydrodynamic mode at 1.0 psi for 25 s (1 psi = 69 mbar) keeping the separation voltage at 15 kV (anode at the injection end). A Beckman P/ACE MDQ Microsoft Software system was used for data processing.

Chemicals

Ciprofloxacin, (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl) 3-quinolone carboxylic acid (Figure 1), was purchased from ICN Biomedicals (Eschwege, Germany). Moxifloxacin and levofloxacin were kindly supplied by Bayer pharmaceuticals (Leverkusen, Germany) and Daiichi (Japan), respectively. Isoniazid, rifampicin, and pyrazinamide were purchased from Sigma, Fluka and MP Biomedicals, respectively. Sodium hydroxide (NaOH), tris(hydroxymethyl)-aminomethane (Tris), sodium dodecyl sulfate (SDS) and phosphoric acid (H_3PO_4, 85%) were supplied by Merck (Darmstadt, Germany). Other agents were of analytical reagent grade. Milli-Q (Millipore, Bedford, MA, USA) treated water was used for the preparation of buffer and related drugs.



Ciprofloxacin



Moxifloxacin

Figure 1. Chemical structures of ciprofloxacin and moxifloxacin.

CE Conditions

Solutions of various Tris buffers at different pH levels were prepared by neutralizing Tris solutions with H_3PO_4. Solutions of Tris-SDS buffer at various levels of SDS were obtained by dissolving different amounts of SDS in Tris followed by dilution with Tris buffer as a background electrolyte (BGE). The final electrolyte solutions containing Tris buffer (5 mM, pH 9.5) with 200 mM SDS were used for CSF samples analysis. The new capillary was conditioned with methanol for 15 min, 1 N HCl for 15 min, deionized water for 5 min, 1 N NaOH for 10 min, and deionized water for 2 min. The routine conditioning between runs every day was carried out using pressure with 1 N HCl (3.5 min), deionized water (2.5 min), 1 N NaOH (2 min), deionized water (2 min), and rinse buffer (3 min) under positive pressure applied at the injection end.

Sample Preparations and Method Validation

Drug free human control CSF samples were obtained from the Department of Neurology, Kaohsiung Medical University Hospital (Kaohsiung Taiwan). A 90 μL aliquot of human CSF was pipetted into a 1.5 mL Eppendorf vial, and 10 μL aqueous solution containing ciprofloxacin and 20 μL (10 μg/mL) of moxifloxacin were added to prepare the concentration of ciprofloxacin and moxifloxacin in CSF (2.5 μg/mL and 2.0 μg/mL, respectively) for evaluation of the optimal

separation conditions. To evaluate the quantitative applicability of the proposed method for ciprofloxacin determination, five different concentrations of ciprofloxacin (0.1, 0.2, 0.5, 1.0, 2.5, and 5.0 μg/mL) were analyzed using moxifloxacin (2.0 μg/mL) as an IS. The samples were mixed for 30 s. An aliquot of 30 μL of spiked drugs in CSF samples was transferred to a 0.2 mL minivial that could be placed into the sample tray (at room temperature) in a Beckman P/ACE MDQ system for CE analyses. The calibration graphs of ciprofloxacin in CSF matrix were established with the peak area ratio of ciprofloxacin to moxifloxacin (IS) as ordinate (y) versus the concentrations of the drug in μg/mL as abscissa (x). The LOQ is the minimum injected amount that gives precise measurements and is defined as the sample concentrations generating a peak height ten times the level of the baseline noise. The LOD was calculated on the basis of the baseline noise, which was defined as the sample concentration generating a peak height three times the level of the baseline noise (signal-to-noise ratio of 3).

Application

One female patient (49 years old) received oral administration of 500 mg of ciprofloxacin (Ciproxin®, Bayer) every 12 h and five tablets of three combined formulation, Rifater® (each tablet contains rifampin 120 mg, isoniazid 80 mg and pyrazinamide 250 mg) (Sanofi-Aventis, France) once daily for treatment of the suspicious tuberculosis meningitis at the Department of Neurology intensive care ward. Several days after, a CSF sample was withdrawn at 12 h after dosing to check the concentration of ciprofloxacin for therapy efficacy. The informed consent was obtained from the patient.

RESULTS AND DISCUSSION

In the analyses of drug molecules in biological samples, sample pretreatment is often needed to remove intrinsic compounds and isolate analytes from the protein matrix. The sample pretreatment suffered from time consuming cleanup procedures. There has been considerable interest in performing single step analyses with direct injection of human CSF on-column in CE without sample pretreatment. This is done by using SDS as a micelle forming agent to sweep the proteins. The protein SDS complexes are negatively charged and interaction with the negatively charged capillary wall is reduced. On the other hand, the larger protein SDS aggregates moved slower towards the detection window than drug SDS micelles, and hence the drug molecules can be eluted in front of the solubilized proteins.

Optimization of the Separation Conditions

MEKC is performed in buffer containing surfactants that are added above their critical micellar concentrations. Separation efficiency depends on the hydrophobicity of the solutes in MEKC. MEKC of the ciprofloxacin and moxifloxacin in human CSF under the 5 mM Tris buffer (pH 9.5) with 200 mM SDS can give a good peak response efficiency and resolution for ciprofloxacin and moxifloxacin from human proteins as shown in Figure 2b. After MEKC separation of ciprofloxacin and moxifloxacin in human CSF matrix using Tris buffer, the eluted compounds were monitored at 280 nm. Without adding SDS, severe interference of ciprofloxacin and moxifloxacin with plasma protein was observed.

Samples Injection

Two modes of sample injection, including electrokinetic and hydrodynamic injections, were commonly used to introduce the analytes into the capillary column in CE. Figure 3 shows electropherograms obtained after hydrodynamic and electrokinetic injections of ciprofloxacin and moxifloxacin spiked in human CSF, respectively. The hydrodynamic sample injection (1 psi, 25 s) (Figure 3a) can provide better detection efficiency than electrokinetic injection (10 kV, 25 s) (Figure 3b) in the determination of ciprofloxacin in human CSF. Therefore, hydrodynamic injection was used.

Effect of Concentration of Tris Buffer

Ionic strength or concentration of the running buffer has significant effects on solute mobilities, separation efficiency, and sensitivity. Hydrodynamic injection of the drugs in CSF using 200 mM SDS and Tris buffer (pH 9.5) at concentrations within the range of 3–30 mM was studied. MEKC of the ciprofloxacin and moxifloxacin in CSF under the 3–30 mM Tris buffer with 200 mM SDS can give good resolution from proteins. The peak shape of moxifloxacin did not obviously change at various Tris concentrations, but the peak width of ciprofloxacin was affected by buffer concentrations. Comparing concentrations of Tris from 3 mM to 30 mM on the effect of the peak height sensitivity for ciprofloxacin, 5 mM of Tris buffers gives a better response than any other and the sensitivity ratios are 0.43; 1.0; 0.6; 0.53; 0.53 at 3, 5, 10, 20, and 30 mM, respectively. The Tris buffer concentration for ciprofloxacin in CSF determination is set at 5 mM.

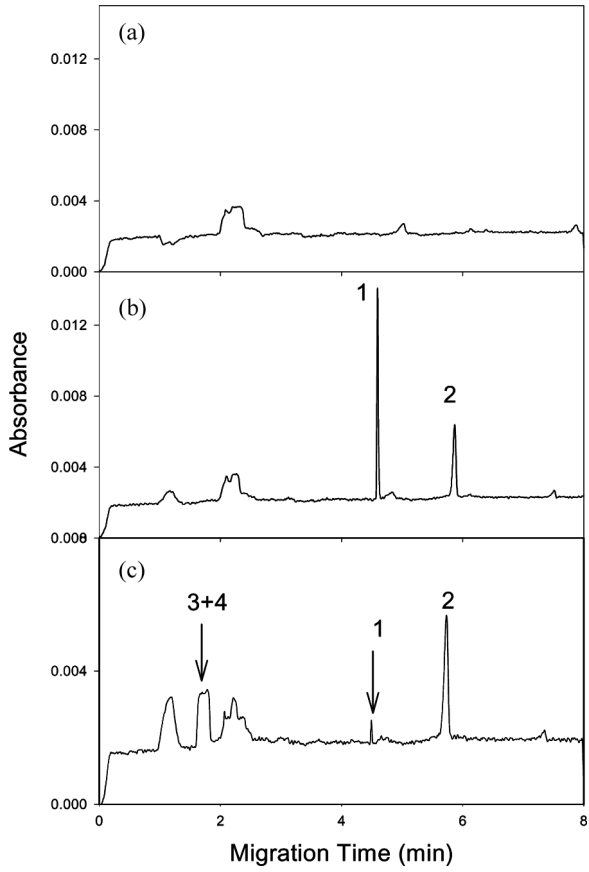


Figure 2. Electropherograms of ciprofloxacin and moxifloxacin in CSF samples determination. (a) human CSF blank; (b) human CSF spiked with ciprofloxacin and moxifloxacin 2.5 μg/mL and 2.0 μg/mL, respectively; (c) CSF sample from one patient with tuberculosis meningitis (49 year-old) oral dosing of 500 mg ciprofloxacin (Ciproxin®) after 12 h later (trough). He had received oral administration of 500 mg of ciprofloxacin every 12 h and Rifater® 5 tablets once daily for several days. Peaks: 1, ciprofloxacin; 2, moxifloxacin (as an IS); 3, isoniazid; 4, pyrazinamide. CE conditions: applied voltage, 15 kV (detector at cathode side); uncoated fused-silica capillary, 31.2 cm (effective length 21 cm) × 50 μm I.D.; sample size, 1 psi, 25 s; wavelength, 280 nm.

Effect of pH of Tris Buffer

A 5 mM Tris buffer with SDS (200 mM) at a high pH (9.0, 9.5, 10.0, 10.5, and 11.0) was chosen, because high concentrations of proteins were present in the sample to be analyzed. At a pH above 9.0, a majority of

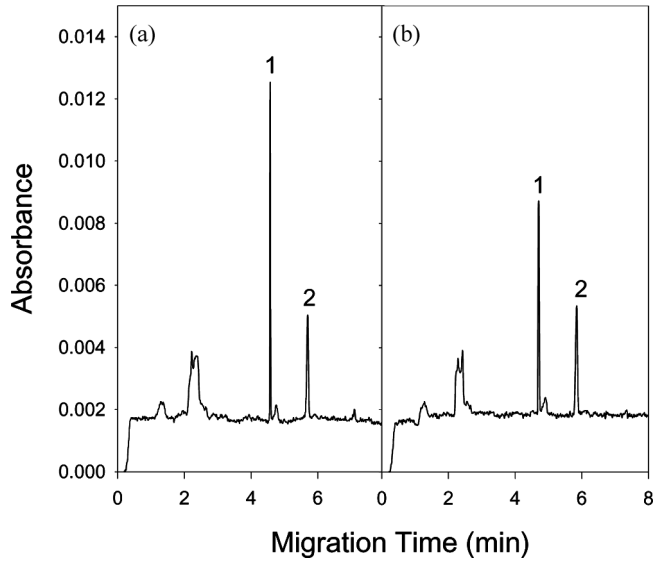


Figure 3. Electropherograms for the analysis of ciprofloxacin and moxifloxacin in human CSF using different injection modes. (a) hydrodynamic injection (1 psi, 69 mbar, 25 s); (b) electrokinetic injection (10 kV, 25 s). Peaks: 1, ciprofloxacin; 2, moxifloxacin. Other CE conditions as in Figure 2.

the silanol groups in the capillary is negatively charged. Ciprofloxacin and moxifloxacin have two relevant ionizable functional groups, the dissociation of the carboxylic group and the protonation of the nitrogen of the piperazine ring. Nearly all of the fluoroquinolones have two pK_a values, pK_1 of ciprofloxacin and moxification are 6.16 and 6.4, respectively, and pK_2 of ciprofloxacin and moxification are 8.62 and 9.5, respectively.[22] For these compounds, the anionic species dominates at the tested pH in aqueous solution, hence, interactions between analyte and the capillary wall would be reduced and a stable, reproducible separation can be obtained. The separation efficiency of the tested drug from CSF biological matrices had no obviously significant changes at various tested pH values.

Effect of Concentration of SDS

The mobility of the analytes in the MEKC system will depend on its own charge as well as the degree to which it partitions into the micelle. The effects of an SDS concentration range of 100–300 mM SDS in Tris buffer (5 mM; pH 9.5) for human CSF separation of ciprofloxacin were studied. The higher the concentration of SDS, the less was the interference for the tested drug determination in CSF matrix. The effect of the concentration

of SDS on migration of the drugs in human CSF is shown in Figure 4. Figure 4a-e represents 100, 150, 200, 250, and 300 mM of SDS in Tris buffer as a surfactant, respectively, affecting the separation efficiency of ciprofloxacin and moxifloxacin from the CSF matrix. Interference peak x overlapping with moxifloxacin existed at 100 mM SDS and partial overlapping with ciprofloxacin was observed at 150 mM SDS. When SDS concentration was increased, the peak of interference x slowed down. With the concentration of SDS $\geq$200 mM in Tris buffer, a baseline resolution of the drugs from CSF matrix was observed. The peak shape of the ciprofloxacin with a significantly broad peak width was obtained at the concentration of SDS $\geq$250 mM. This may be due to increased SDS concentration with increased conductivity of the BGE, and thus an increase in the current flow and Joule heating. The effect of SDS concentrations at 200 mM, 250 mM, and 300 mM on the plate number of ciprofloxacin is about 170000: 140000: 20000, respectively. Therefore, the 5 mM, pH 9.5 of Tris buffer with 200 mM SDS was the choice for the buffer concentration, pH, and surfactant concentration for determination of ciprofloxacin and moxifloxacin in CSF. Different applied voltages, including 10 kV, 12 kV, and 15 kV, were investigated regarding the effectiveness of the separation. A shorter migration time and higher plate number was observed at 15 kV; the resulting current was about 80 μA. Figures 2a and b present the typical electropherograms of MEKC separation of human CSF blank and ciprofloxacin and moxifloxacin spiked in CSF. Reproducibility of migration velocity of ciprofloxacin and moxifloxacin in human CSF was investigated, and the observed migration times were 4.60 ± 0.09 and 5.86 ± 0.17 min for ciprofloxacin and moxifloxacin in CSF, respectively. High accuracy in migration time

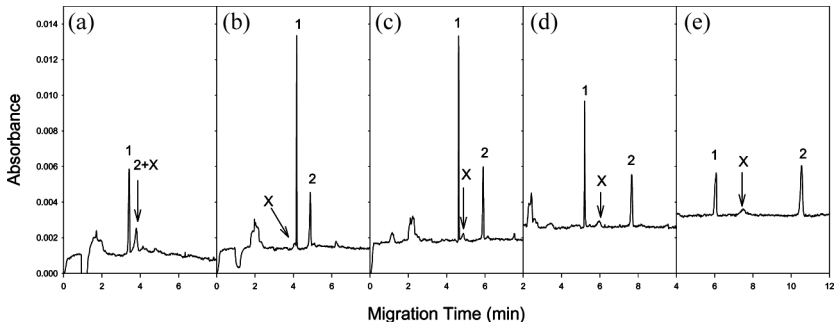


Figure 4. Effect of SDS concentrations (100–300 mM) with Tris 5 mM (pH 9.5) on the migration of ciprofloxacin and moxifloxacin. Peaks: X, unknown peak; 1, ciprofloxacin and 2, moxifloxacin in human CSF, at 2.5 μg/mL and 2.0 μg/mL, respectively. (a) 100 mM; (b) 150 mM; (c) 200 mM; (d) 250 mM; (e) 300 mM. Other CE conditions as in Figure 2.

for ciprofloxacin and moxifloxacin ($n = 20$) was found with RSDs of 1.9% and 2.9%, respectively. Separation was achieved within 7 min. Compared with sample pretreatment needed for bioassay, the proposed method developed for ciprofloxacin determination in CSF with sample direct injection is very rapid. The apparent mobility (μA) was calculated according to the equation: $\mu A = \mu E + \mu EOF = (lL/tV)$, where l = length along the capillary (cm) to the detector, V = voltage, t = migration time (s), and L = total length (cm) of the capillary;[23] the electrophoretic mobility values (μE) of ciprofloxacin and moxifloxacin in human CSF are $-4.63 \times 10^{-4}\,cm^2\,V^{-1}\,s^{-1}$ and $-4.98 \times 10^{-4}\,cm^2\,V^{-1}\,s^{-1}$, respectively, under this MEKC condition.

Validation of Ciprofloxacin Spiked in CSF

The concentration of ciprofloxacin in human CSF over the range of 0.1–5.0 μg/mL and fixed concentration of moxifloxacin at 2.0 μg/mL, was used as an IS to establish the calibration curve for ciprofloxacin determination in CSF. The linear regression equations in CSF for ciprofloxacin was obtained as follows: $Y = (0.4349 \pm 0.0016)X + (0.0138 \pm 0.0053)$ and $Y = (0.4375 \pm 0.0022)X + (0.0036 \pm 0.0115)$ for intra-day and inter-day assays, respectively. The straight lines obtained from several separate experiments had good correlation coefficients of 0.999. The data indicate a high linearity of ciprofloxacin for the intra-day and inter-day assays. Repeatability was determined by RSD of the slope of the linear regression equations. The RSDs of intra-day and inter-day average slopes of the equations were all less than 0.5%. The relative recoveries of ciprofloxacin were obtained from the calibration graph constructed from CSF spiked with different amounts of ciprofloxacin at low, medium, and high concentration levels. Table 1 shows all of relative recoveries were >95%. The LOQ is the minimum injected amount that gives precise measurements. The LOQ in CSF was 0.1 μg/mL and the LOD of the proposed method for ciprofloxacin in human CSF (1.0 psi, 25 s) was found to be 50 ng/mL. The selectivity of the proposed method was briefly tested on the separation of fluoroquinolone antibiotics including ciprofloxacin, moxifloxacin, and levofloxacin with other antituberculosis drugs including isoniazid, pyrazinamide, and rifampin. Under present MEKC conditions, a complete separation of ciprofloxacin and moxifloxacin with other antituberculosis drugs was obtained as shown in Figure 5.

Application

Administration of antimicrobial agents that are known to reach effective concentrations in the CSF is a very important mainstay in the therapy of

Table 1. Precision and accuracy for the analysis of ciprofloxacin in human CSF

Concentration known (μg/mL)	Concentration found (μg/mL)	RSD (%)	RE (%)
Intra-day (n = 4)*			
0.2	0.19±0.01	5.3	−5.0
1.0	1.00±0.02	2.0	0.0
5.0	5.04±0.04	0.8	0.8
Inter-day (n = 5)*			
0.2	0.20±0.01	5.0	0.0
1.0	1.01±0.04	3.9	1.0
5.0	5.03±0.10	2.0	0.6

*Intra-day data were based on four replicate analyses and inter-day were from five consecutive days.

severe brain infections. This issue becomes a particularly important link to the development of bacterial resistance under subinhibitory antibiotic concentrations at the target site. One patient with tuberculosis meningitis consecutively received oral administration of 500 mg of ciprofloxacin (ciproxin®, Bayer) every 12 h and five tablets of the three combined formulation, Rifater® (Sanofi-Aventis, France) once daily. Several days later, a CSF sample was withdrawn at 12 h after dosing for checking the concentration of ciprofloxacin in the brain. The electropherogram of CSF after oral administration of the above drugs is shown in Figure 2c.

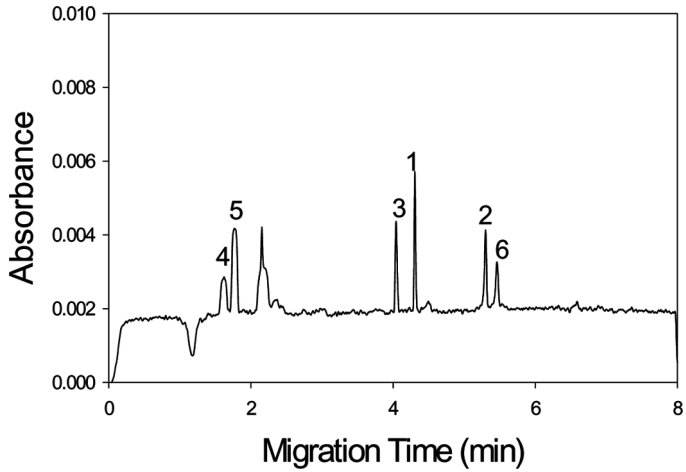


Figure 5. Electropherogram of selectivity study in human CSF. Peaks: 1, ciprofloxacin; 2, moxifloxacin; 3, levofloxacin; 4, isoniazid; 5, pyrazinamide; 6, rifampin.

The concentration of ciprofloxacin in the patient's CSF at 12 h was 0.154 μg/mL. It is lower than MIC (1.3 μg/mL). Exceeding the MIC over a dosing interval (t > MIC) was considered for favorable a clinical and bacteriological outcome. The antituberculosis components, isoniazid and pyrazinamide (peak 3 and peak 4), were overlapped with each other and rifampin did not appear in Figure 2c. This indicates that ciprofloxacin and rifampin penetrates the intact blood brain barrier poorly, even in the presence of inflammation. Therefore, physicians should take the concentration of medicine into consideration when they are making a decision regarding treatment responses. The method is suitable for the analysis of ciprofloxacin and moxifloxacin in CSF for pharmacokinetic and pharmacodynamic investigations in humans. The characteristics of the small amount sample volume needed and the lack of the necessity for sample pretreatment of this MEKC analytical method for quantitation of ciprofloxacin and moxifloxacin in CSF make it very useful in clinical use.

CONCLUSION

MEKC, with sample direct injection without sample pretreatment, for determination of ciprofloxacin in CSF described here, represents a rapid, sensitive, and efficient analytical method. The analytical characteristic of the proposed method is satisfactory for pharmacokinetic and clinical use. Validation of the method for determination of ciprofloxacin in CSF showed that the method has a high accuracy.

ACKNOWLEDGMENTS

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