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Short communication

Determination of ulifloxacin, the active metabolite of prulifloxacin, in human plasma by a 96-well format solid-phase extraction and capillary zone electrophoresis

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ABSTRACT

This paper described a method for quantification of ulifloxacin, the active metabolite of prulifloxacin in human plasma by capillary zone electrophoresis using lomefloxacin as the internal standard. The separation was carried out at 25 °C in a 60.2 cm × 75 μm fused-silica capillary with an applied voltage of 20 kV using 200 mM borate buffer (pH 10.5). The detection wavelength was 275 nm. Clean-up and preconcentration of the samples were developed by 96-well format solid-phase extraction. 0.25 ml of plasma sample and 0.25 ml of IS were loaded onto the preconditioned wells, and the wells were washed using 1 ml of 20% methanol in acid water (1% phosphoric acid), and the analytes were eluted using 1 ml of 95/5 methanol/ammonia water. The method was suitably validated with respect to stability, specificity, linearity, lower limit of quantitation, accuracy, precision, extraction recovery and robustness. The calibration graph was linear for ulifloxacin from 0.02 to 2 μg/ml. The lower limit of quantification was 0.02 μg/ml. The intra- and inter-day precisions were within 4.0 and 8.2%, respectively. The method developed was successfully applied to the evaluation of clinical pharmacokinetic study of prulifloxacin formulation product after oral administration to healthy volunteers.

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1. Introduction

Prulifloxacin, the lipophilic prodrug of ulifloxacin [1], is a new thiazeto-quinolone antibacterial agent with a broad-spectrum in vitro activity against various Gram-negative and Gram-positive bacteria and acts directly on bacterial DNA gyrase inhibiting cell reproduction that leads to cell death [2]. Following oral administration, prulifloxacin is absorbed from the intestinal tract and is immediately and quantitatively transformed by a paraoxonase into the active metabolite ulifloxacin by hydrolytic cleavage of the dioxolene ring (Fig. 1); no other known potentially active metabolites are founded [3–5]. Therefore, the in vitro antimicrobial activity studies were performed using ulifloxacin [5–8].

Common separation methods had been reported for the determination of the active metabolite ulifloxacin in various matrices based on HPLC determinations with UV [5,9], fluorescence [10] and mass spectrometric detection [11]. Some of these methods suffered from inadequate sensitivity, long analysis time and the use of toxic halogenated solvents. Capillary electrophoresis (CE) has also been used as an alternative to HPLC to separate quinolone antibiotics in biological samples over the last decade [12–16]. CE had become a powerful separation technique for high-throughput analysis because of its high efficiency, high resolution, rapid analysis and low cost of operation. Furthermore, it requires less consumption of samples and reagents. However, the use of CE in the bioanalysis of drugs and their metabolites is restricted due to the low concentration sensitivity of this technique. In order to increase preconcentration, the 96-well format solid-phase extraction (SPE) used in HPLC is recommended for the high-throughput analysis [17–20].

In this paper, we established a high-throughput Oasis 96-well SPE plate coupled with CE method for the determination of ulifloxacin in large numbers of samples generated from clinical studies. The method was evaluated in term of selectivity, sensitivity, linearity, accuracy, precision and stability in accordance to the recommendations published by the FDA [21], and applied to the analysis of ulifloxacin in clinical

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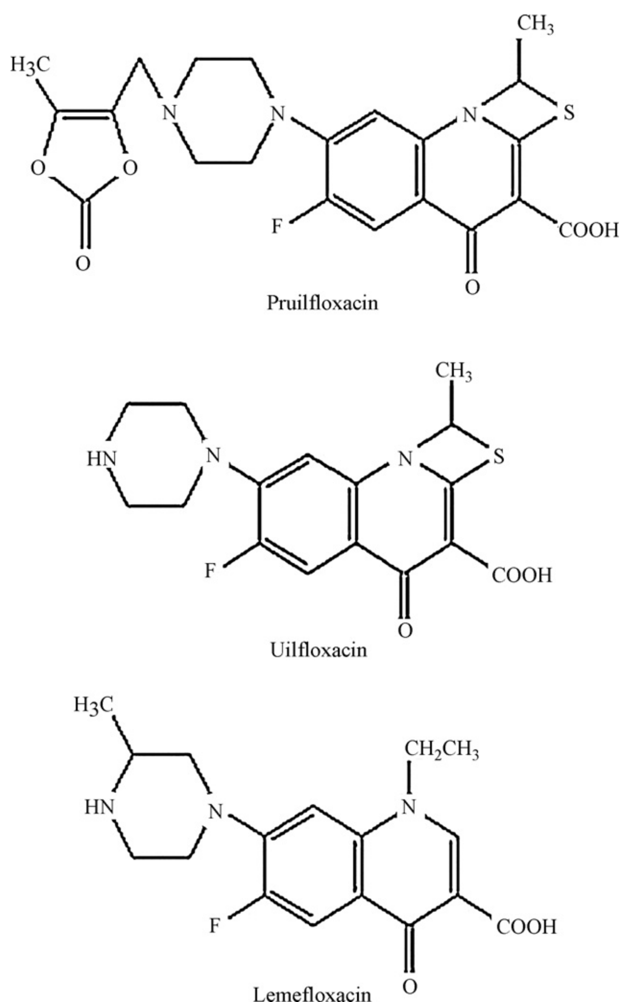

Fig. 1. Structure of prulifloxacin, ulifloxacin and lomefloxacin (internal standard).

pharmacokinetic studies of prulifloxacin tablets in healthy volunteers.

2. Experimental

2.1. Instrumentation

The employed CE system consisted of a Beckman P/ACE MDQ instrument (Beckman Instruments Inc., Fullerton, CA, USA) equipped with a photodiode array detection detector (PDA) and P/ACE System MDQ Software. Detection was performed at 275 nm, where ulifloxacin had the maximum absorption. Fused-silica capillaries (60.2 cm × 75 μm i.d., effective length 51 cm) were obtained from Hebei Yongnian Optical Fiber Factory (Hebei, China).

2.2. Materials and reagents

Ulifloxacin (NM394, 99.0% purity) and the tablet formulation of prulifloxacin (264 mg, lot 060157) were from Shanghai Modern Pharmaceutical Co. Ltd. (Shanghai, PR China). Lomefloxacin (99.0% purity) was obtained from National Institute for the Control of Pharmaceutical and Biological Product (Beijing, PR China). Sodium tetraborate of analytical grade were acquired from China Medicine (Group) Shanghai Chemical Reagent Corporation (Shanghai, PR China). Human control plasma (sodium heparin as an anticoagulant) was obtained from Shanghai Blood Center (Shanghai, PR China). Deionized (18 MΩ/cm) water was generated in-house using a Milli-Q System from Millipore (Bedford, MA, USA). All solutions were degrassed by ultrasonication (KQ-50DE, Kunshan Ultrasonic Instrument Co., Ltd., PR China).

2.3. Capillary electrophoretic conditions

The BGE used in this study was 200 mM borate buffer (pH 10.5). The capillary temperature was maintained at 25 °C and the separation voltage was 20 kV with the current of about 70 μA. The injection was hydrodynamic at a pressure of 2 psi for 5 s. Buffer solutions were prepared freshly every day and filtered through a 0.45 μm hydrophilic cellulose membrane filter prior to use. The capillary was conditioned by rinsing with 0.1 M NaOH, H_2O and separating buffer (2 min each) sequentially between the runs.

2.4. Preparation of standard solution and spiking control sample

The stock solution of 1 mg/ml of ulifloxacin was prepared by weighing 10 mg of ulifloxacin and made up to 10 ml with methanol. Working standard solutions of ulifloxacin were obtained in the concentration (0.1, 0.2, 0.5, 1, 2, 5 and 10 μg/ml) by further dilution of the stock solution with distilled water. A stock solution of 200 μg/ml of lomefloxacin (internal standard, IS) was prepared in methanol and diluted with distilled water to yield a working solution of 1 μg/ml.

The frozen human control plasma was thawed at room temperature, vortexed and centrifuged at 2124 × *g* for 10 min prior to use. Plasma calibration standards were prepared by spiking appropriate amount of the working solution in human control plasma. The concentration of ulifloxacin in plasma standards ranged from 0.02 to 2 μg/ml. Quality control (QC) samples (0.05, 0.2 and 1.6 μg/ml) were independently prepared by spiking appropriate amount of the working standard solution in human control plasma. The samples were submitted to SPE before CE analysis.

2.5. Sample preparation

To a 2.0 ml 96-well plate, 0.25 ml aliquot of plasma sample were added to the designated wells followed by the addition of 0.25 ml aliquot of IS working solution (except the blanks to that 0.25 ml aliquot of water were added). The plate was capped with a 96-well mat, vortexed for 1 min at mid speed setting and then centrifuged at 2124 × *g* for 10 min. All wells in an Oasis HLB 96-well extraction plate (Waters, USA) were conditioned by sequential washes of 3 ml of methanol and conditioned with 3 ml of water. The entire volume of each sample (0.5 ml) was then transferred using an 8-channel pipetter (Eppendorf, Germany), into individual wells in the 96-well extraction plate and drawn through the plate using vacuum. The wells in the plate were then washed sequentially with 1.0 ml of water and 1.0 ml of a methanol–1% phosphoric acid (20:80, v/v) mixture. After drying the plate under vacuum for approximate 1 min, the retained analyte and IS in the wells were eluted to a 2.0 ml 96-well plate with 1.0 ml of a methanol–ammonia water (95:5, v/v) mixture. The extracts were evaporated to dryness under nitrogen (50 °C) using a self-constructed 96-well format plate evaporator, and then reconstituted using 50 μl of 10% BGE. The plate was capped with a 96-well mat, vortexed for 1 min at mid speed setting and centrifuged at 8276 × *g* for 10 min. 40 μl of the supernatant were transferred to the autosampler for injection onto the CE.

2.6. Clinical pharmacokinetic study

The method was applied to determine plasma concentrations of ulifloxacin from a clinical trial in which 10 healthy male volunteers

received a single oral dose of 264 mg of prulifloxacin tablet. About 1 ml of blood samples were collected in heparinized tubes before (0 h) and 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0 and 24.0 h postdosing and centrifuged at 2124 × g for 10 min to separate the plasma fraction. The obtained plasma samples were stored at −20 °C until analysis. The study was approved by a local ethics committee and performed after obtaining written consent from the subject.

3. Result and discussion

3.1. Method development

The fluoquinolones have two relevant ionizable functional groups, which mean that their acid–base chemistry involves two equilibria, the dissociation of carboxylic group and the deprotonation of the N_4 of the piperazine ring, placed at position 7. So we investigated the effect of pH by three different electrolytes: 200 mM borate (pH 9.5, 10.5 and 11.5, adjusted with 0.1 M NaOH/200 mM boric acid), 50 mM phosphate (pH 2.5, 3.5, 4.5 and 5.5, adjusted with 50 mM H_3PO_4) and the mixture of 50 mM borate and 50 mM phosphate (pH 6.5, 7.5 and 8.5, adjusted with 50 mM H_3PO_4). For the very low electroosmotic flow (EOF) at pH below 3.5, the analytes separated with a long migration time. As the analytes were mainly positively charged at pH 2.5–5.5, the migration times decreased significantly with the increase of buffer pH due to the increase of EOF. Meanwhile, the analytes were lack of adequate separation. In contrast, ulifloxacin and IS are predominantly in anioic form at pH above 6.5. The migration time increases slightly with the buffer pH, because of the electrophoretic flow was increased and moving in the opposite direction of EOF. With the pH above 9.5, ulifloxacin and IS had a baseline separation. Comparing three kinds of buffer, high concentration of borate produced a high ionic strength and resulted in a sharp peak. For the large mass of the borate ion, it would not produce a high current at high concentration. To weigh the merits and demerits, 200 mM borate buffer at the pH of 10.5 was chosen as the optimum allowing for the migration time and resolution.

Sample pre-treatment is a critical step in any biological sample analysis. To find out the most appropriate SPE washing and elution solution, Oasis HLB cartridges were used in the preliminary study. In order to optimize washing condition to achieve the best clean-up results without loss of analytes (ulifloxacin and IS), washing solvents with different percentage (5%, 10%, 20% and 30%) of methanol in acid water (1% phosphoric acid) were evaluated. Ulifloxacin was partially eluted at 30% of methanol. In order to achieve the best clean-up results without loss of analytes, 20% of methanol was chosen as the wash solvent. The recovery of ulifloxacin was low (<30%) when methanol was used as elution solvent, and an addition of 1% ammonia water to methanol showed a beneficial effect for the recovery of ulifloxacin. The addition of the base modifier made ulifloxacin in the ion form and reduced the retention of ulifloxacin in SPE columns. So the composition of the methanol elution solution was also investigated with different percentages (1%, 2% and 5%) of ammonia water. It was shown that the elution solution with 5% of ammonia water produced a maximum recovery for ulifloxacin and lomefloxacin. It achieved a similar recovery when it went to the 96-well extraction plate.

3.2. Method validation

Analytical method validation was carried out according to the recommendations published by the FDA.

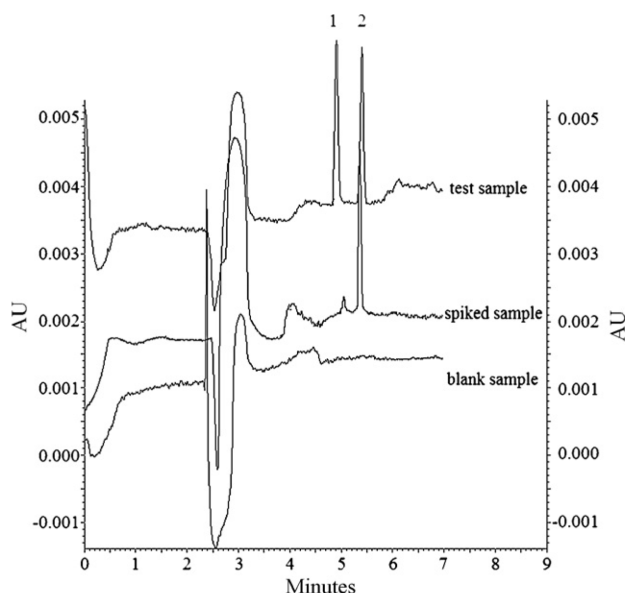

Fig. 2. Representative chromatograms of extracted blank plasma, plasma spiked with 0.02 μg/ml (LLOQ) of ulifloxacin and 0.5 μg/ml of IS, test plasma spiked with 0.5 μg/ml of IS at 4 h after the dose of 264 mg. Peaks: (1) ulifloxacin and (2) lomefloxacin. Running buffer 200 mM borate (10.5); capillary 60.2 cm × 75 μm i.d., effective length 51 cm, uncoated; hydrostatic injection 2 psi × 5 s; applied voltage 20 kV (+) → (−); column temperature 25 °C; detection 275 nm.

3.2.1. Specificity

Specificity is described as the ability of a method to discriminate the analyte from all potentially interfering substance. Specificity of the method was investigated by both peak purity and spiking experiments with pure standard compounds. Peak purity was evaluated by means of the P/ACE System MDQ Software. The total peak purity values of ulifloxacin and the IS were 1.0000. There appeared to be no interference from the endogenous substance, which were analyzed under the same optimized condition (Fig. 2).

3.2.2. Linearity of calibration curves and lower limits of quantification (LLOQ)

The calibration graph of ulifloxacin in human plasma with seven different concentrations of ulifloxacin in the range of 0.02, 0.05, 0.1, 0.2, 0.5, 1 and 2 μg/ml was established with the peak area ratio of ulifloxacin to IS as ordinate (y) versus the concentration of ulifloxacin in μg/ml as abscissa (x). The calibration curves were defined by the following equations: $y = 2.5267x + 0.2166$, $r = 0.9992$. The LLOQ, which is determined as the lowest concentration on the standard curve, of ulifloxacin in human plasma was found to be 0.02 μg/ml with accuracy of 110.6% and intra- and inter-day precision of 5.8% and 9.3%, respectively.

3.2.3. Accuracy, precision and extraction recovery

The inter-day precision and accuracy were calculated from repeated analyses of identical samples on 5 consecutive days for these concentrations of ulifloxacin. The results in Table 1 show that the intra- and inter-day variances at three QC levels were all below 8.2%. It was shown that the accuracy were from 97.3% to 99.7%. The extraction recovery was found to be 79.1 ± 6.7%, 82.7 ± 2.4% and 91.0 ± 1.7% at the concentration of 0.05, 0.20 and 1.60 μg/ml, respectively. The extraction recovery of IS was 84.2 ± 1.2%.

3.2.4. Stability

Analyte stability determinations comprised short-term temperature stability, long-term stability, autosampler stability and freeze-thaw cycles stability, which were evaluated by analyzing

Table 1
Intra- and inter-precision of the determination of ulifloxacin in human plasma ($n = 5$)

	Concentration (μg/ml)		Accuracy (%)	R.S.D. (%)
	Added	Found		
Intra-day	0.05	0.049 ± 0.002	97.3 ± 3.9	4.0
	0.20	0.196 ± 0.004	97.8 ± 2.1	2.1
	1.60	1.594 ± 0.032	99.7 ± 2.0	2.0
Inter-day	0.05	0.049 ± 0.004	97.4 ± 8.0	8.2
	0.20	0.194 ± 0.009	96.9 ± 4.5	4.6
	1.60	1.569 ± 0.063	98.1 ± 3.9	4.0

three QC levels in quintuple. The mean values and standard deviations of the ratios between the concentrations found and initial concentration were used for stability evaluation. Ulifloxacin had an acceptable stability at room temperature for 2 h, at −20 °C for 1 month, in the autosampler at room temperature for 8 h after solid-phase extraction and after three freeze-thaw cycles with the values 99.8–101.7%, 98.1–99.1%, 99.6–103.1% and 98.7–101.2%, respectively, at the three concentrations studied.

3.2.5. Robustness

The robustness of the method was evaluated by deliberate variation of the method parameters, such as pH, borate buffer concentration and voltage. The change in the capillary zone electrophoresis (CZE) results of the same sample was monitored by varying these parameters, and it was found that there was little change in the efficiency and resolution of the anlaytes (Table 2). Hence, the method was considered robust and reliable.

3.3. Application to clinical pharmacokinetic study

The developed and validated CE method was used to analyze pharmacokinetic profiles of ulifloxacin in healthy volunteers after a single oral administration of a tablet formulation containing 264 mg of prulifloxacin. Profiles of the mean plasma concentration of ulifloxacin versus time were shown in Fig. 3. The pharmacokinetic parameters of ulifloxacin were calculated from the curve. The mean value of T_{max} and C_{max} were 1.08 ± 0.50 h and 1.47 ± 0.42 μg/ml, respectively. The elimination half-life of ulifloxacin was 6.98 ± 1.35 h. The $AUC_{0\sim24}$ and $AUC_{0\sim\infty}$ values were 5.98 ± 1.48 μg h/ml and 6.44 ± 1.56 μg h/ml, respectively. The LC method in a previous publication [10] was used as a comparison method to evaluate the validity of the method developed. The correlation graph of ulifloxacin concentration determined by both methods was established with the concentration of ulifloxacin by CZE as ordinate (y) versus the concentration of ulifloxacin by HPLC as abscissa (x). The results show the correlation ($r = 0.9880$)

Table 2
Robustness of the analytical method upon variation of CZE separation condition

Parameter	Experimental electrophoretic plate (N)		Resolution
	Ulifloxacin	Lomefloxacin	
Voltage			
18 kV	275,364	269,701	2.9
20 kV	326,218	329,450	2.4
22 kV	306,731	357,203	2.2
Buffer pH			
10.0	309,704	285,607	1.8
10.5	326,218	329,450	2.4
11.0	339,768	314,762	3.8
Buffer concentration			
180 mM borate	346,370	337,010	2.0
200 mM borate	326,218	329,450	2.4
220 mM borate	268,405	296,403	2.8

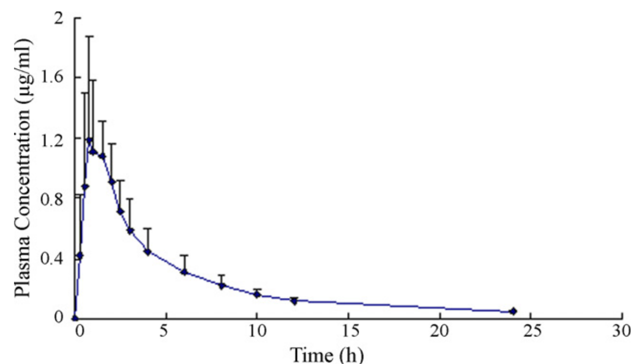

Fig. 3. Mean plasma concentration-time curve in 10 male, healthy Chinese subjects when administered oral dose of a tablet formulation containing 264 mg of prulifloxacin.

between the methods. A comparison of pharmacokinetic parameters obtained by both methods carried out using the Wilcoxon's test. It was indicated that there were no significant differences between the results obtained by both methods ($p > 0.05$). Even though the dose of administration was different, our results of pharmacokinetic parameters were similar to Nakashima's et al. [5] and Picollo's et al. [9] research.

4. Conclusion

A rapid, sensitive and selective 96-well format solid-phase extraction capillary zone electrophoresis method has been developed and validated for the determination of ulifloxacin in healthy volunteers after a single oral administration of prulifloxacin. The combination of the CE with 96-well format SPE greatly simplified the preparation process and decreased the time required for sample preparation. While reviewing the earlier analytical methods for the quantitation of NM394, the LLOQ (0.02 μg/ml) and sample consumption (0.25 ml) of our method were low compared to previously reported method [5,8,11]. Furthermore, it saved a great amount of payment of solvent for the determination of large numbers of samples. The analysis of pharmacokinetic parameters confirmed that the CZE method was an alternative method for routine analysis of ulifloxacin in plasma comparing with the reference method. The method possessed good characteristics of specificity, sensitivity, precision and accuracy and proved to be reliable. It is expected that this approach can be applied to the extraction and analysis of numerous samples in clinical pharmacokinetic studies of prulifloxacin prodrugs.

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