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Determination of the active metabolite of prulifloxacin in human plasma by liquid chromatography–tandem mass spectrometry

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Abstract

A liquid chromatographic–tandem mass spectrometric method (LC–MS/MS) for the determination of ulifloxacin, the active metabolite of prulifloxacin, in human plasma is described. After sample preparation by protein precipitation with methanol, ulifloxacin and ofloxacin (internal standard) were chromatographically separated on a C_{18} column using a mobile phase consisting of methanol, water and formic acid (70:30:0.2, v/v/v) at a flow rate of 0.5 ml/min and then were detected using MS/MS by monitoring their precursor-to-product ion transitions, *m/z* 350 → *m/z* 248 for ulifloxacin and *m/z* 362 → *m/z* 261 for ofloxacin, in selected reaction monitoring (SRM) mode. Positive electrospray ionization was used for the ionization process. The linear range was 0.025–5.0 μg/ml for ulifloxacin with a lower limit of quantitation of 0.025 μg/ml. Within- and between-run precision was less than 6.6 and 7.8%, respectively, and accuracy was within 2.0%. The recovery ranged from 92.1 to 98.2% at the concentrations of 0.025, 0.50 and 5.0 μg/ml. Compared with the reported LC method, the present LC–MS/MS method can directly determine the ulifloxacin in human plasma without any need of derivatization. The present method has been successfully used for the pharmacokinetic studies of a prulifloxacin formulation product after oral administration to healthy volunteers.

Keywords: Prulifloxacin; Ulifloxacin; Liquid chromatography–tandem mass spectrometry; Human plasma

1. Introduction

Prulifloxacin (Fig. 1), the prodrug of ulifloxacin (AF3013, NM394), is a broad-spectrum oral fluoroquinolone antibacterial agent [1]. After oral administration and intestinal absorption, prulifloxacin is rapidly metabolized by paraoxonases [2] into ulifloxacin (Fig. 1), the active metabolite of prulifloxacin [2–7].

A rapid, selective and sensitive analytical method was required to determine the concentration of ulifloxacin in plasma for the pharmacokinetic study of a prulifloxacin formulation product in healthy volunteers. For the purpose, reversed-phase liquid chromatography (LC) is often preferred in common laboratories. As a result of a recent literature survey, LC with UV detection was available for the determination of ulifloxacin in biological samples [2,7]. On the basis of Fig. 1, it can be found that ulifloxacin exhibits a great polarity due to its amphiprotic property and probable formation of an internal salt. Taking into account its very short retention on a reversed-phase LC column, derivatization of the analyte prior to determination by LC is necessary to keep it resolved from polar endogenous compounds in biological samples. In the published method [2], ethylchloroformate was used for the derivatization of ulifloxacin into ethoxycarbonyl ulifloxacin, which had a reasonable retention on the column and acceptable resolution with polar endogenous compounds. Owing to the complexity and possible problems with a derivatization process, the determination of ulifloxacin without derivatization became our target in the present study. The need to quantify ulifloxacin in plasma for the pharmacokinetic study of prulifloxacin formulation products in clinical trials compelled us to develop a direct, rapid and sensitive analytical method. For the purpose, liquid chromatography–tandem mass spectrometry (LC–MS/MS) was adopted because of its high selectivity and sensitivity for analytes in biological samples. To our best knowledge, no reports are available for the direct determination of ulifloxacin in biological samples without any need of derivatization.

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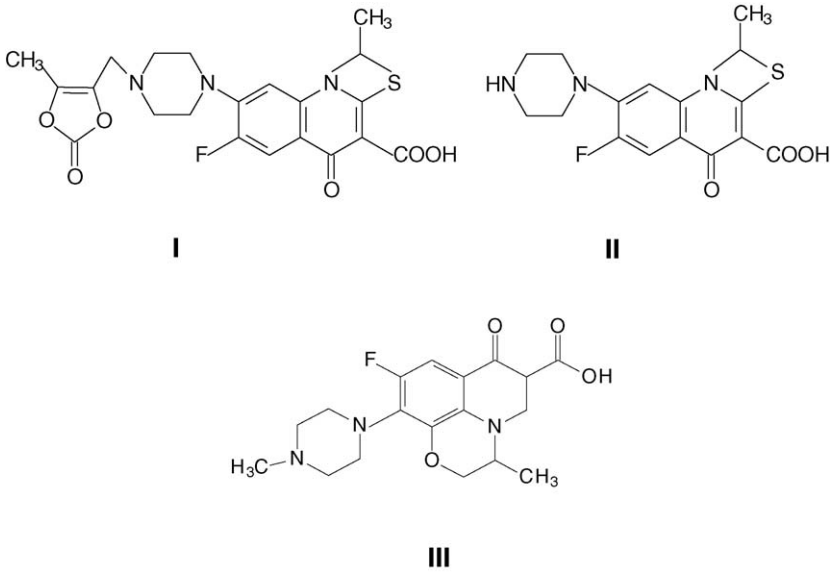

Fig. 1. Chemical structures of prulifloxacin (I), ulifloxacin (II) and ofloxacin (III).

The present paper describes a rapid, selective and sensitive LC–MS/MS method using an electrospray ionization source in selected reaction monitoring (SRM) mode for the determination of ulifloxacin in human plasma after a simple sample preparation. The described method was validated in terms of selectivity, sensitivity, linearity, accuracy, precision and stability, and successfully applied to the pharmacokinetic studies of prulifloxacin tablets in healthy volunteers.

2. Experimental

2.1. Chemicals and reagents

Ulifloxacin reference (98.0% purity) and prulifloxacin tablets (300 mg, batch no: 20010903) from Jingxin Pharmaceutical Company, Ltd. and Baiyunshan Pharmaceutical Company, Ltd. (Guangzhou, China), respectively. Ofloxacin (internal standard, I.S.) was from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Blank human plasma was from Shenyang General Military Hospital. HPLC-grade methanol was from Tianjin Concord Tech Reagent Company (Tianjin, China). Formic acid of analytical grade (88.0%) was from Tianjin Chemical Reagent Company (Tianjin, China). Purified water was used throughout this study.

2.2. Instrumentation

The LC–MS/MS system consisted of a Thermo Finnigan Surveyor liquid chromatograph and a Thermo Finnigan TSQ Quantum Ultra triple quadrupole mass spectrometer (San Jose, CA, USA) equipped with an electrospray ionization (ESI) source (Thermo Finnigan, USA). Data collection was performed with Xcalibur 1.1 software (Thermo Finnigan, USA). Peak integration and calibration were made with LCQuan software (Thermo Finnigan, USA).

2.3. LC–MS/MS conditions

Chromatographic separation was performed on a Diamonsil C_{18} column (200 mm × 4.6 mm I.D., 5 μm, Dikma, China) with a SecurityGuard C_{18} guard column (4 mm × 3.0 mm I.D., Phenomenex, USA). The mobile phase consisting of a mixture of methanol, water and formic acid (70:30:0.2, v/v/v) was delivered at a flow rate of 0.5 ml/min. The column temperature was maintained at 20 °C. The injection volume was 20 μl.

Positive ESI was used for the ionization. The optimized MS parameters were as follows. High-purity nitrogen served both as sheath gas with an operating pressure of 0.6 MPa and as auxiliary gas with a flow rate of 0.2 MPa. The capillary temperature was maintained at 350 °C. Collision induced dissociation (CID) was achieved using argon as the collision gas with a pressure of 1.2 mTorr and the collision energies for both ulifloxacin and ofloxacin were 28 eV. Quantification was performed by selected reaction monitoring (SRM) of the transitions of m/z 350 → m/z 248 for ulifloxacin and m/z 362 → m/z 261 for ofloxacin (I.S.), respectively, with a dwell time of 0.3 s per transition.

2.4. Preparation of calibration standards and quality control samples

A stock solution of 200 μg/ml for ulifloxacin was prepared in 88.0% formic acid and was serially diluted with water to give a series of standard solutions with concentrations of 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 μg/ml. A stock solution of 800 μg/ml for ofloxacin was prepared in methanol and then was further diluted with water to yield a working solution of 2.5 μg/ml.

Plasma calibration standards of ulifloxacin (0.025, 0.050, 0.10, 0.25, 0.50, 1.0, 2.5 and 5.0 μg/ml) were prepared by spiking appropriate amount of the standard solutions in blank plasma from healthy, non-smoking volunteers (total added volume less than 10% of the biological sample volume). Quality control (QC)

samples were prepared from a pool of six different sources of blank plasma at the concentrations of 0.025, 0.50 and 5.0 μg/ml and used in pre-study validation and during the pharmacokinetic studies.

2.5. Sample preparation

An aliquot of plasma (200 μl) was mixed with 100 μl of water, 100 μl of I.S. solution (2.5 μg/ml) and 400 μl of methanol and then the contents were briefly vortexed and centrifuged to separate the phases. The supernatant (400 μl) was transferred to another tube and evaporated to dryness at 35 °C under a gentle stream of nitrogen. The residue was reconstituted in 200 μl of the mobile phase and vortex mixed. A 20 μl aliquot of the reconstituted solution was injected onto the LC–MS/MS system for analysis.

2.6. Method validation

During pre-study validation, three validation runs were conducted on 3 separate days. Each validation run consisted of a set of the calibration standards at eight concentrations over the concentration range (each in triplicate), QC samples at three concentrations ($n = 6$ at each concentration), blank and stability samples. During routine analysis each analytical run included a set of calibration standards, QC samples in duplicate and unknowns.

The results from QC samples in three runs were used to evaluate the accuracy and within- and between-run precision of the method. The linearity was confirmed by plotting the peak area ratio of ulifloxacin to the internal standard versus ulifloxacin concentration. The unknown sample concentrations were calculated from the equation $y = a + bx$, as determined by a weighted ($1/x^2$) linear regression of the calibration standards.

The absolute recovery of ulifloxacin at low, medium and high concentrations (0.025, 0.50 and 5.0 μg/ml) was determined by comparing the responses from plasma samples spiked before extraction with the corresponding standard solutions without extraction.

The stability of ulifloxacin at low, middle and high concentrations in reconstituted solutions, the thawed plasma on the benchtop and the frozen plasma stored at −20 °C was determined.

2.7. Application of the LC–MS/MS method

The present LC–MS/MS method was successfully applied to the pharmacokinetic studies of prulifloxacin tablets in six healthy volunteers who were judged to be in good health through medical history, physical examination and routine laboratory tests. Written informed consent was obtained from each volunteer after detailed verbal and written information on the objective and the possible risks of the study. Volunteers were excluded if they had a history of smoking or drinking or if they were taking prescription medications within 14 days prior to the start of the study. The study protocol was reviewed and approved by China Drug Evaluation Center.

After an overnight fast (10 h), the volunteers were given single dose of one prulifloxacin tablet (300 mg) with 250 ml of warm water. Within 10 h after oral administration, the volunteers had a standard diet while water intake was free. Smoking and consumption of alcohol and beverages containing caffeine were not allowed during the study. About 2 ml of blood samples were collected into heparinized tubes before (0 h) and at 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 12.0, 24.0, 36.0, 48.0 and 72.0 h after oral administration. Plasma was separated by centrifugation at 2000 × *g* for 10 min and kept frozen at −20 °C until analysis.

3. Results and discussion

3.1. Method development

Since phosphate buffer is normally forbidden for an LC–MS/MS method, the chromatographic conditions for the present work, especially the composition of mobile phase, were optimized to achieve good resolution and symmetric peak shapes of analytes as well as short run time. It was found that a mixture of methanol, water and formic acid (70:30:0.2, v/v/v) could achieve our purpose and was finally adopted for the present work. Internal standard is necessary for the determination of analytes in biological samples. A stable-isotope labeled analyte would be preferable as the internal standard for the quantitation of the analyte in complex matrices by LC–MS/MS analysis. But because the stable isotope-labeled analyte was unavailable, ofloxacin, an analog of ulifloxacin, was finally used as the internal standard and found to be suitable for the present work.

Protein precipitation for the sample preparation was used in the present work. To obtain a clean chromatogram and achieve a sufficient extraction recovery, several solvents were tried and methanol was finally used as the precipitating solvent for the present work. The procedure for sample preparation was simple and rapid, and provided a clean chromatogram and satisfactory recovery for the analytes from the plasma.

The positive product ion mass spectra of ulifloxacin and the internal standard are shown in Fig. 2. The most intensive product ion was observed at *m/z* 248 for ulifloxacin and *m/z* 261 for the internal standard. Therefore, the precursor-to-product ion transitions *m/z* 350 → *m/z* 248 for ulifloxacin and *m/z* 362 → *m/z* 261 for the internal standard in the SRM mode were used for the quantitation of ulifloxacin and internal standard.

3.2. Selectivity

The selectivity of the method was investigated by analyzing blank human plasma extract (Fig. 3A) and an extract spiked with ulifloxacin (0.5 μg/ml) and ofloxacin (2.5 μg/ml) (Fig. 3B) and a volunteer plasma sample at 8 h after an oral dose of 300 mg prulifloxacin tablet (Fig. 3C). Ulifloxacin and internal standard exhibited retention times at 3.79 and 3.83 min, respectively. As shown in Fig. 3A, no significant interference from endogenous substances in drug-free human plasma at the retention time of the analytes was observed. In addition, by using LC–MS/MS,

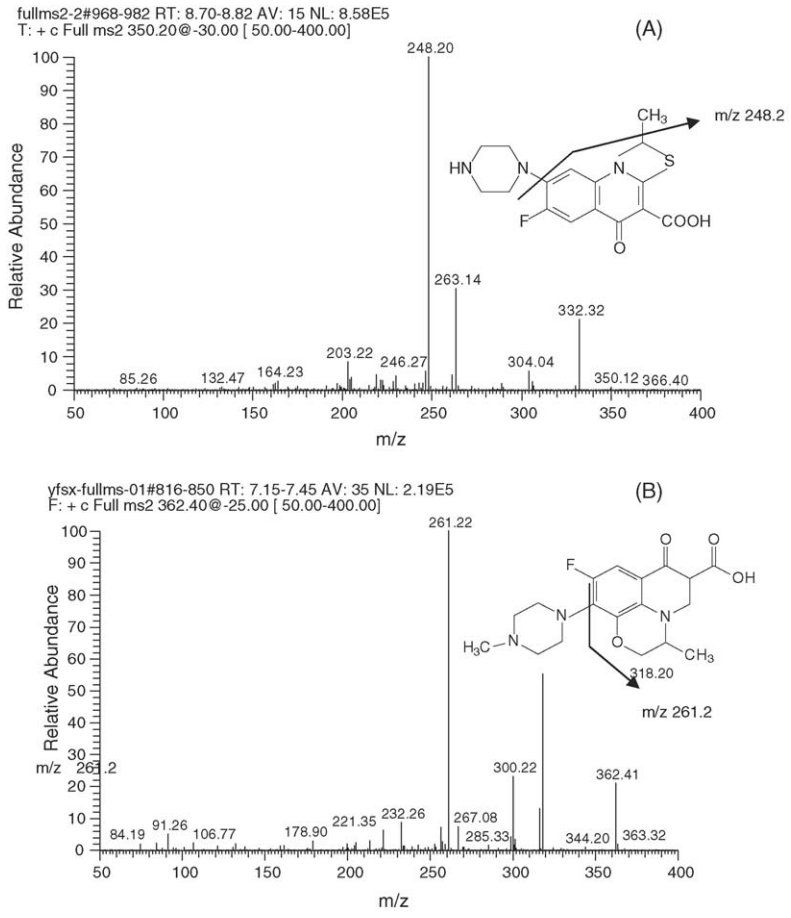

Fig. 2. Full scan MS/MS spectra of ulifloxacin (A) and ofloxacin (B).

the baseline resolution of the analytes was not required for the analysis due to the ability of the MS in SRM mode to deconvolute signals. The results demonstrated the satisfying selectivity of the present LC–MS/MS method for the determination of ulifloxacin in human plasma.

3.3. Linearity

The linearity was determined by plotting the peak-area ratio (y) of ulifloxacin to internal standard versus the nominal concentration (x) of ulifloxacin in plasma. The calibration curve was obtained by weighted ($1/x^2$) least-squares regression analysis. Representative regression equation for the calibration curve was $y = -5.19 \times 10^{-3} + 0.112x$ ($R^2 = 0.9949$, $n = 8$) over the concentration range of 0.025–5.0 μg/ml for ulifloxacin in human plasma.

3.4. Accuracy and precision

The accuracy was determined by calculating the percentage deviation observed in the analysis of QC samples and expressed as the relative error (R.E.). Within- and between-run precision was expressed as the relative standard deviation (R.S.D.). As shown in Table 1, for each QC level of ulifloxacin, the within- and between-run precision was less than 6.6 and 7.8%, respectively, and the accuracy was within 2.0%, indicating the acceptable accuracy and precision of the LC–MS/MS method for the determination of ulifloxacin in human plasma.

3.5. Lower limit of quantitation

The lower limit of quantitation (LLOQ) for the determination of ulifloxacin in human plasma was found to be 0.025 μg/ml

Table 1
Accuracy and precision for the determination of ulifloxacin in human plasma (3 days, six replicates each day)

Nominal C (μg/ml)	Found C (μg/ml)	Within-run R.S.D.[a] (%)	Between-run R.S.D. (%)	Relative error[b] (%)
0.025	0.025	3.6	5.8	0.6
0.50	0.503	4.4	7.3	0.7
5.00	5.102	6.6	7.8	2.0

[a] Relative standard deviation.
[b] R.E. (%) = 100 × (mean found concentration − nominal concentration)/nominal concentration.

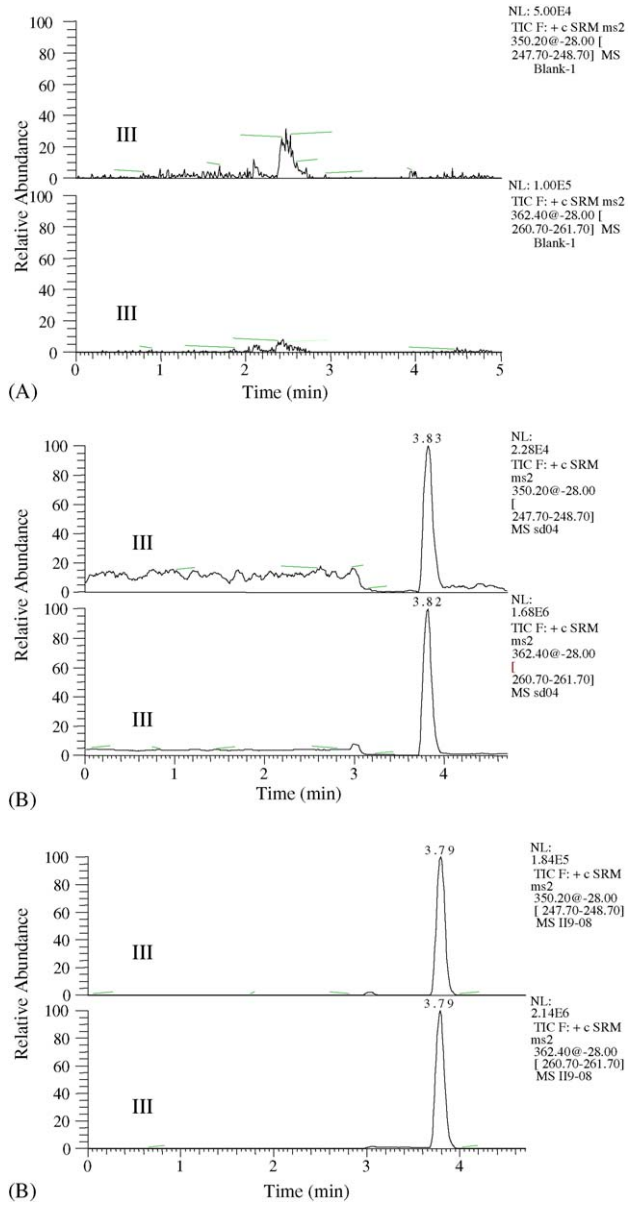

Fig. 3. Representative SRM chromatograms of: (A) blank human plasma sample; (B) blank plasma sample spiked with ulifloxacin (0.5 μg/ml) and ofloxacin (2.5 μg/ml) and (C) volunteer plasma sample with ulifloxacin of 0.48 μg/ml at 8 h after an oral dose of 300 mg prulifloxacin tablet. (II) Ulifloxacin and (III) ofloxacin.

with accuracy of 0.6% and within- and between-run precisions of 3.6 and 5.8%, respectively. The LLOQ is sufficient for pharmacokinetic studies of prulifloxacin formulation products in human.

3.6. Absolute recovery

The absolute recovery of ulifloxacin from human plasma was determined by comparing peak areas from plasma samples spiked before extraction with the corresponding standard solutions without extraction. The absolute recovery was found to be 92.1 ± 6.4%, 98.2 ± 5.9% and 93.8 ± 2.2% at the concentrations of 0.025, 0.50 and 5.0 μg/ml, respectively. The clean-up procedure by protein precipitation with methanol was found to be a reliable way of eliminating possibly interfering components from plasma with a satisfactory recovery.

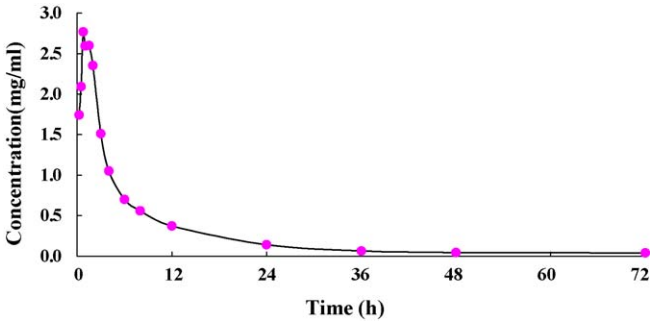

Fig. 4. Mean plasma concentration–time profile of ulifloxacin after oral administration of one prulifloxacin tablet (300 mg) to six healthy volunteers.

3.7. Stability

Ulifloxacin in reconstituted solutions was found to be stable for at least 12 h after sample preparation at room temperature with accuracy of less than 15% for three levels of QC samples. The analyte in the thawed plasma on the benchtop remained unchanged for at least 4 h before extraction and the analyte in the frozen plasma stored at −20 °C were stable for at least 2 weeks.

3.8. Application

The present LC–MS/MS method has been successfully used for the pharmacokinetic studies of prulifloxacin tablets in healthy volunteers over a period of 72 h after oral administration. The obtained mean plasma concentration–time profile is shown in Fig. 4. It can be found that prulifloxacin was immediately metabolized into ulifloxacin with the maximum concentration at about 0.75 h and ulifloxacin was detectable for at least 72 h (0.041 μg/ml) after oral administration.

4. Conclusions

A rapid and sensitive LC–MS/MS method for the direct determination of ulifloxacin in human plasma was developed. Compared with the reported LC method, the present LC–MS/MS method can directly determine ulifloxacin in human plasma without any need of derivatization prior to analysis with sufficient selectivity, acceptable accuracy and precision, and satisfactory recovery as well as short run time (less than 5 min). The present method is simple, sensitive and applicable for the pharmacokinetic studies of prulifloxacin formulation products.

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