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Rapid and selective liquid chromatographic/tandem mass spectrometric method for the determination of fosfomycin in human plasma

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Abstract

A rapid and selective liquid chromatographic/tandem mass spectrometric method for determination of fosfomycin was developed and validated. Following protein-precipitation, the analyte and internal standard (fudosteine) were separated from human plasma using an isocratic mobile phase on an Ultimate™ XB-CN column. An API 4000 tandem mass spectrometer equipped with Turbo IonSpray ionization source was used as detector and was operated in the negative ion mode. Multiple reaction monitoring using the precursor to product ion combinations of *m/z* 137 → 79 and *m/z* 178 → 91 was performed to quantify fosfomycin and fudosteine, respectively. The method was linear in the concentration range of 0.10–12.0 μg/mL using 50 μL of plasma. The lower limit of quantification was 0.10 μg/mL. The intra- and inter-day relative standard deviation over the entire concentration range was less than 10.6%. Accuracy determined at three concentrations (0.25, 1.00 and 8.00 μg/mL for fosfomycin) ranged from −1.0% to −4.2% in terms of relative error. Each plasma sample was chromatographed within 5.0 min. The method was successfully used in a bioequivalence study of fosfomycin in human plasma after an oral administration of capsules containing 1.0 g fosfomycin (∼1.3 g calcium fosfomycin).

Keywords: Liquid chromatographic/tandem mass spectrometry; Fosfomycin; Plasma concentration

1. Introduction

Fosfomycin, (−)-(1*R*,2*S*)-(1,2-epoxypropyl)phosphonic acid (Fig. 1), is a broad-spectrum antibiotic with low molecular weight which inhibits the synthesis of bacterial cell walls by preventing the peptidoglycan synthesis. It appears to be little cross-resistant with other antibacterial agents, possibly because the chemical structure and action site are different from other agents [1]. Fosfomycin is well tolerated, with a low incidence of adverse events and has a rapid bactericide effect. In the clinic, it is widely used for the treatment of infections of the central nervous system, urinary tract infections and other infections caused by Gram-positive and certain Gram-negative bacteria [2].

Fosfomycin is very polar and lacks ultraviolet (UV) absorption. Although fosfomycin is extensively used as a bactericidal antibiotic in various infections [3,4], only a few methods have been reported so far for the determination of fosfomycin in serum or plasma, including gas chromatography (GC) [5,6], microbiological method [7], ion-exchange chromatography [8] and capillary electrophoresis (CE) [9-11]. In addition, several analytical methods including flow injection spectrophotometry [12], gas chromatography [13,14], microbiological method [15] and capillary electrophoresis (CE) [16] were also reported for the determination of fosfomycin in urine, muscle or pus. However, most of these methods have limitations such as low sensitivity [8,9–11,16], time-consuming derivatization steps [5,6,13,14] and long chromatographic run time [8].

An increase in sample throughput requires a reduced analysis time and simplified sample preparation, for which a simple, selective and rapid LC/MS/MS method was developed and validated in the present study. Following a simple protein precipitation, 50 μL of plasma sample was separated on an Ultimate™ XB-CN column and detected by mass spectrometry in the multiple reaction monitoring mode. The lower limit of quantification (LLOQ) was 0.10 μg/mL and this method has been successfully

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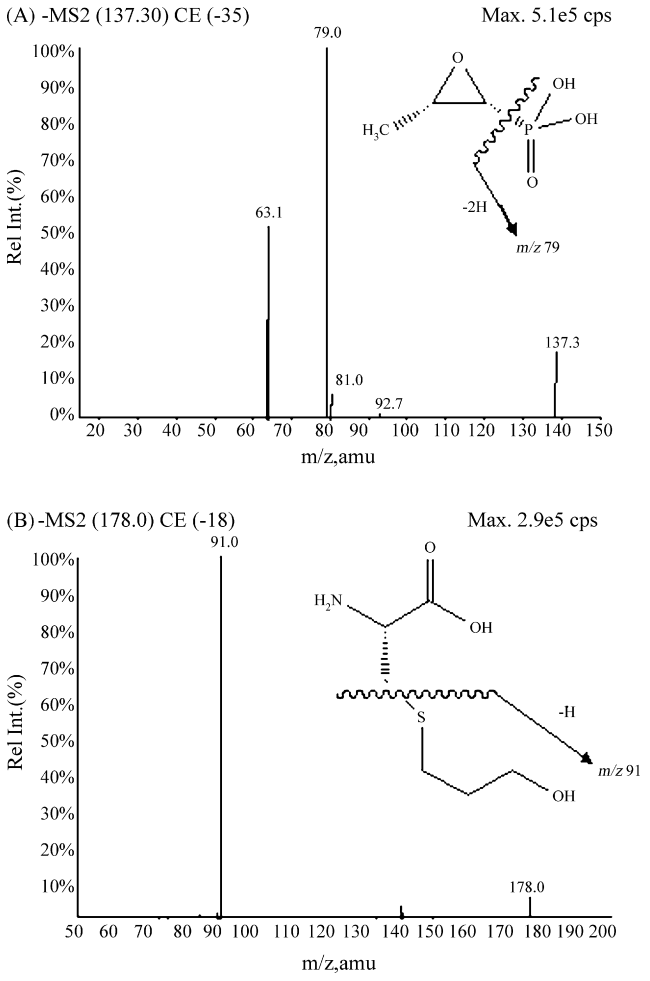

Fig. 1. Product ion spectra of $[M-H]^-$ of fosfomycin (A) and fudosteine (B).

applied to a bioequivalence study of fosfomycin after a single oral dose of 1 g fosfomycin (~1.3 g calcium fosfomycin).

2. Experimental

2.1. Materials

Fosfomycin (99.8% purity) and fudosteine (internal standard (IS), 99.0% purity) were both purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol (Sigma–Aldrich, Steinheim, Germany) and ammonium acetate (Tedia, Fairfield, OH, USA) were of HPLC grade, and other chemicals were of analytical grade. Distilled water, prepared from demineralized water, was used throughout the study. Drug-free plasma for the preparation of calibration standards was obtained from Shanghai Shuguang Hospital (Shanghai, China). The test and reference formulations were capsules containing calcium fosfomycin (fosfomycin 200 mg/capsule).

2.2. Instrument

A high-performance liquid chromatography system consisting of Shimadzu LC-20AD pump and Shimadzu SIL-HTA autosampler (Shimadzu Corporation, Kyoto, Japan) was used for sample delivery. An API 4000 triple-quadrupole mass spectrometer equipped with a Turbo IonSpray (ESI) source was used for mass analysis and detection (Applied Biosystems, Concord, Ontario, Canada). Data processing was performed on Analyst 1.4.1 software package.

2.3. Chromatographic conditions

Isocratic chromatographic separation was achieved on an Ultimate^{TM} XB-CN column, 150 mm × 4.6 i.d., 5 μm (Welch Materials, Ellicott, MD, USA) with a 4 mm × 3.0 mm i.d. SecurityGuard C_{18} guard column (Phenomenex, Torrance, CA, USA). The mobile phase consisted of methanol–5 mM ammonium acetate (10:90, v/v) at a flow rate of 0.7 mL/min. The column was maintained at room temperature (22 °C). The chromatographic run time for one sample was 5.0 min. For the first 2.5 min the eluent was diverted to waste.

2.4. Mass spectrometric conditions

The mass spectrometer was operated in negative ionization mode. The tuning parameters were optimized for fosfomycin and the IS by infusing a solution containing 0.80 μg/mL of both analytes at a flow rate of 30 μL/min into the mobile phase (0.3 mL/min) using a post-column 'T' connection. The nebulizer gas, heater gas and curtain gas (nitrogen) were set at 50, 50 and 20 p.s.i., respectively. The optimized Turbo IonSpray voltage and temperature were set at −3800 V and 400 °C, respectively. For collision-induced dissociation (CID), nitrogen was used as the collision gas at a back-pressure of approximately 4 p.s.i. Quantitation was performed using multiple reaction monitoring (MRM) of the transitions *m/z* 137 → 79 for fosfomycin and *m/z* 178 → 91 for the IS, respectively, with a dwell time of 200 ms per transition. The optimized collision energy (CE) of −35 eV was used for the analyte and −18 eV for the IS. The mass spectrometer was operated at unit mass resolution (peak width at half-height set at 0.7 Da) for both Q1 and Q3. The value of DP (declustering potential) was set at −40 V.

2.5. Preparation of calibration standards and QC samples

A stock solution of fosfomycin with a concentration of 400 μg/mL was prepared by dissolving 10.0 mg of fosfomycin in 25 mL of water. The stock solution of fosfomycin was then serially diluted with drug-free plasma to obtain the desired concentrations. Effective concentrations in plasma samples were 0.10, 0.30, 0.80, 1.50, 4.00 and 12.0 μg/mL.

The quality control (QC) samples were prepared at concentrations of 0.25, 1.00 and 8.00 μg/mL with drug-free plasma in a similar way with the calibration standards, by a separate weighing of the pure standard. A 1.00 μg/mL solution of the internal standard (IS) was also prepared by diluting the 400 μg/mL stock solution of fudosteine with methanol.

These standards samples and QC samples were stored at −20 °C. Before processing of each analytical batch, the appro-

priate standards and QCs were brought to room temperature, and processed together with the clinical samples.

2.6. Sample preparation

A 50 μL aliquot of the IS solution (fudosteine, 1.00 μg/mL) was added to 50 μL of plasma samples. The sample mixture was deproteinized with 250 μL of methanol and the precipitate was removed by centrifugation at 11,300 × *g* for 7 min. Then, 30 μL of the supernatant were transferred to another clean test tube, and 100 μL of water were added. The mixture was vortexed for 10 s, and a 20 μL aliquot of mixture was injected for the LC/MS/MS analysis.

2.7. Method validation

Plasma samples were quantified using the ratio of the peak area of analyte to IS as the assay response. To evaluate linearity, plasma calibration curves were prepared and assayed in duplicate on three consecutive days over the range of 0.10–12.0 μg/mL. Least-squares linear regression was used for curve fitting with $1/x^2$ as the weighting factor.

The different validation parameters and the values for accepting the range of validation parameters were in accordance with international guidelines [17]. QC samples at three concentration levels (0.25, 1.00 and 8.00 μg/mL) were analyzed to assess the accuracy and precision of the method. Again, the assays were performed on three separate days, and on each day six replicates of QC samples at each concentration level were analyzed. The accuracy and precision were calculated using one-way ANOVA (analysis of variance). The accuracy was expressed by relative error (RE) and the precision by relative standard deviation (RSD). The intra- and inter-day precisions were required to be below 15%, and the accuracy to be within ±15%.

The LLOQ defined as the lowest concentration at which both precision and accuracy were less than or equal to 20%, was evaluated by analyzing samples which were prepared in six replicates.

The recovery of fosfomycin following the protein precipitation procedure was assessed by comparing the mean peak areas of the regularly prepared samples at three concentrations (0.25, 1.00 and 8.00 μg/mL) with the mean peak area of spike-after-extraction plasma samples, which represented the 100% recovery. To prepare the spike-after-extraction samples, blank human plasma was processed according to the sample preparation procedure as described above. All the supernatant was mixed with the appropriate standard solutions of fosfomycin at concentrations corresponding to the final concentration of the pretreated plasma samples. After vortexing, 100 μL of water was added to 30 μL of the mixture, vortexing for 10 s again. Similarly, recovery of IS was also evaluated by comparing the mean peak areas of six regularly prepared samples to mean peak areas of six standard solutions spiked in pretreated drug free plasma samples.

To evaluate the matrix effect, i.e., the potential ion suppression or enhancement due to co-eluting endogenous components, six different lots of blank plasma were extracted and then spiked with the analyte at 0.25 and 8.00 μg/mL. The corresponding peak areas of the analyte in spiked plasma post-extraction (A) were then compared to those of the solution standards in mobile phase (B) at equivalent concentrations. The ratio (A/B × 100) is defined as the absolute matrix effect (ME). A value of 100% indicates that the responses for fosfomycin in the mobile phase and in the plasma extracts were the same and that no absolute ME was observed. A value of >100% indicates ionization enhancement, and a value of <100% indicates ionization suppression. The assessment of the relative ME was made by a direct comparison of the analyte peak area values between different lots of plasma. The variability in the values, expressed as RSD (%), is a measure of the relative ME for the target analyte. The same evaluation was performed for the IS.

The stabilities of fosfomycin in human plasma were evaluated by analyzing replicates ($n = 3$) of plasma samples at the concentrations of 0.25 and 8.00 μg/mL, which were exposed to different conditions (time and temperature). The spiked plasma samples were analyzed after storage at ambient temperature for 2 h, in auto-sampler for 24 h at room temperature after protein precipitation, at −20 °C for 30 days and after three freeze–thaw cycles from −20 °C to room temperature. These results were compared with those obtained for freshly prepared plasma samples. The analytes were considered stable in the biological matrix when 85–115% of the initial concentrations were found.

2.8. Application of the analytical method to a bioequivalence study

The LC/MS/MS procedure developed was used to determine fosfomycin concentrations in plasma samples 0–24 h after an oral administration of capsules containing 1.0 g fosfomycin (∼1.3 g calcium fosfomycin) to 20 healthy male volunteers in a bioequivalence study approved by the Ethics Committee. All volunteers gave written informed consent to participate in the study according to the principles of the Declaration of Helsinki. Blood (3 mL) was removed by venepuncture prior to dosage and at 0.5, 1, 1.5, 2, 3, 4, 5, 7, 9, 12, 15 and 24 h thereafter. Blood samples were collected into tubes containing sodium heparin as anticoagulant and centrifuged at 2000 × *g* for 15 min. The plasma was separated and stored at −20 °C until analysis.

3. Results and discussion

3.1. Optimization of the mass spectrometric condition

Fosfomycin is a polar compound, containing a phosphoric acid in its structure. It gave a strong mass response in negative ESI condition. For IS, fudosteine has a carboxyl group in its structure, high mass spectrometric response was obtained in negative mode as well.

Under (−) ESI mode, the analyte and IS formed predominantly deprotonated molecules $[M - H]^-$ at *m/z* 137 and *m/z* 178

in Q1 full scan mass spectra, respectively. No solvent adduct ions were detected. The most suitable CEs for the analyte and IS were determined by observing the response of the obtained fragment ion peaks. Fig. 1 displays the product ions spectra of $[M-H]^-$ ions from the analyte and IS. A predominant fragment ion at *m/z* 79 was formed when the collision energy was −35 eV and the intensity of $[M-H]^-$ ion for fosfomycin was reduced by more than 80%. As a result, the transition *m/z* 137 → 79 at CE −35 eV was used in MRM acquisition mode to obtain high specificity and low noise. Additional tuning of the ESI source parameters for the transition of *m/z* 137 → 79 further improved the sensitivity. For IS, the product ion spectrum of the $[M-H]^-$ ion showed a major fragment ion at *m/z* 91. The optimum collision energy (−18 eV) was determined by observing the maximum response obtained for *m/z* 91.

3.2. Optimization of the chromatographic condition

The high polarity of fosfomycin makes it difficult to be extracted from plasma with organic solvents. The protein precipitation is a common preparation method for polar compound, but the preparation procedure might lead to ion suppression when LC/MS/MS is applied. In addition, co-eluting endogenous materials are able to influence chromatograms and to contaminate the ion source, resulting in increased variation, particularly at the LLOQ level. Therefore, an appropriate chromatographic condition, including appropriate chromatographic column and suitable mobile phase, is needed for accurate quantification of fosfomycin in human plasma.

During method development, a number of reversed-phase C18 columns, such as Zorbax XDB C18, Atlantis dC18, Gemini C18 and Diamonsil C18 were tested. Strong ion suppression was observed on all tested reversed-phase columns, which was attributed to no retention for the polar compound. An Inertsil CN-3 normal-phase column was tried in the experiment, but broad peak shape (>2 min) was observed at all the tested mobile phases. In the further study, an Ultimate™ XB-CN was adopted to achieve symmetric and sharp peak shapes for both the analyte and IS.

The retention behavior of fosfomycin was improved by further optimization of the chromatographic condition, which was crucial to avoid the ion suppression. Lower proportion of organic phase in the mobile phase can increase the retention of fosfomycin on column and avoid matrix effect, but it tends to reduce the efficiency of ionization. In the present study, an investigation of the proportions of methanol and acetonitrile was performed. On an Ultimate™ XB-CN column, when the percentage of methanol in mobile phase was raised to 20% (flow rate set at 0.7 mL/min), the retention time of fosfomycin was short, but the mass response was decreased sharply due to ion suppression from plasma sample. When various percentages (equal to or more than 10%) of acetonitrile were employed instead of methanol, the serious ion suppression was still observed. Eventually, 10% of methanol was adopted in this experiment. Using the mobile phase consisting of methanol–5 mM ammonium acetate (10:90, v/v), the effects of matrix were minimal.

3.3. Sample preparation

In the present study, a one-step protein precipitation procedure was adopted to get a high recovery. The selected protein precipitant was methanol, due to its satisfactory efficiency in precipitation and extraction and less ion suppression compared to that observed with acetonitrile. The proportion of the water added to the supernatant was also evaluated in the early method development stage, it was found that adding 100 μL water to 30 μL supernatant could yield symmetric peak shape for fosfomycin rather than adding 50 μL water to 50 μL supernatant.

3.3.1. Method validation

3.3.1.1. Assay selectivity and matrix effect. Selectivity was assessed by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. Fig. 2 shows the typical chromatograms of a blank plasma sample, blank plasma sample spiked with fosfomycin at the LLOQ and IS, and a plasma sample obtained at 3.0 h after an oral administration of 1.3 g calcium fosfomycin to a volunteer. No interference from endogenous substances was observed at the retention times of the analyte and IS. Typical retention times for fosfomycin and fudosteine were 3.1 and 4.3 min, respectively.

The absolute matrix effects for fosfomycin at concentrations of 0.25 and 8.00 μg/mL were 108.1 ± 7.0% and 98.3 ± 14.7%, respectively. The relative matrix effect for fosfomycin at concentrations of 0.25 and 8.00 μg/mL were 8.0% and 5.4%, respectively. The absolute and relative matrix effects for IS (1.00 μg/mL) were 96.9 ± 5.5% and 96.9 ± 5.7%, respectively. As a result, ion suppression or enhancement from plasma matrix was negligible in the present condition.

3.3.1.2. Linearity and lower limit of quantification. The linear regression of the peak area ratios versus concentrations was fitted over the concentration range of 0.10–12.0 μg/mL in human plasma. A typical equation of the calibration curves was as follows: $y = 0.0230 + 0.253x$ ($r = 0.9963$), where y represents the peak area ratio of analyte to IS and x represents the plasma concentration of fosfomycin. Good linearity was obtained in this concentration range.

The lower limit of quantification was 0.10 μg/mL for determination of fosfomycin in plasma. The precision and accuracy at the concentration of LLOQ are shown in Table 1. Under the present LLOQ, the fosfomycin concentration could be deter-

Table 1
Precision and accuracy data for the analysis of fosfomycin in human plasma (in prestudy validation, 3 days, 6 replicates per day)

Concentration (μg/mL)		RSD (%)		Relative error (%)
Added	Found	Intra-day	Inter-day	
0.10	0.095	11.4	12.7	−5.1
0.25	0.24	7.2	7.9	−4.2
1.00	0.97	10.6	6.1	−2.9
8.00	7.92	1.9	5.1	−1.0

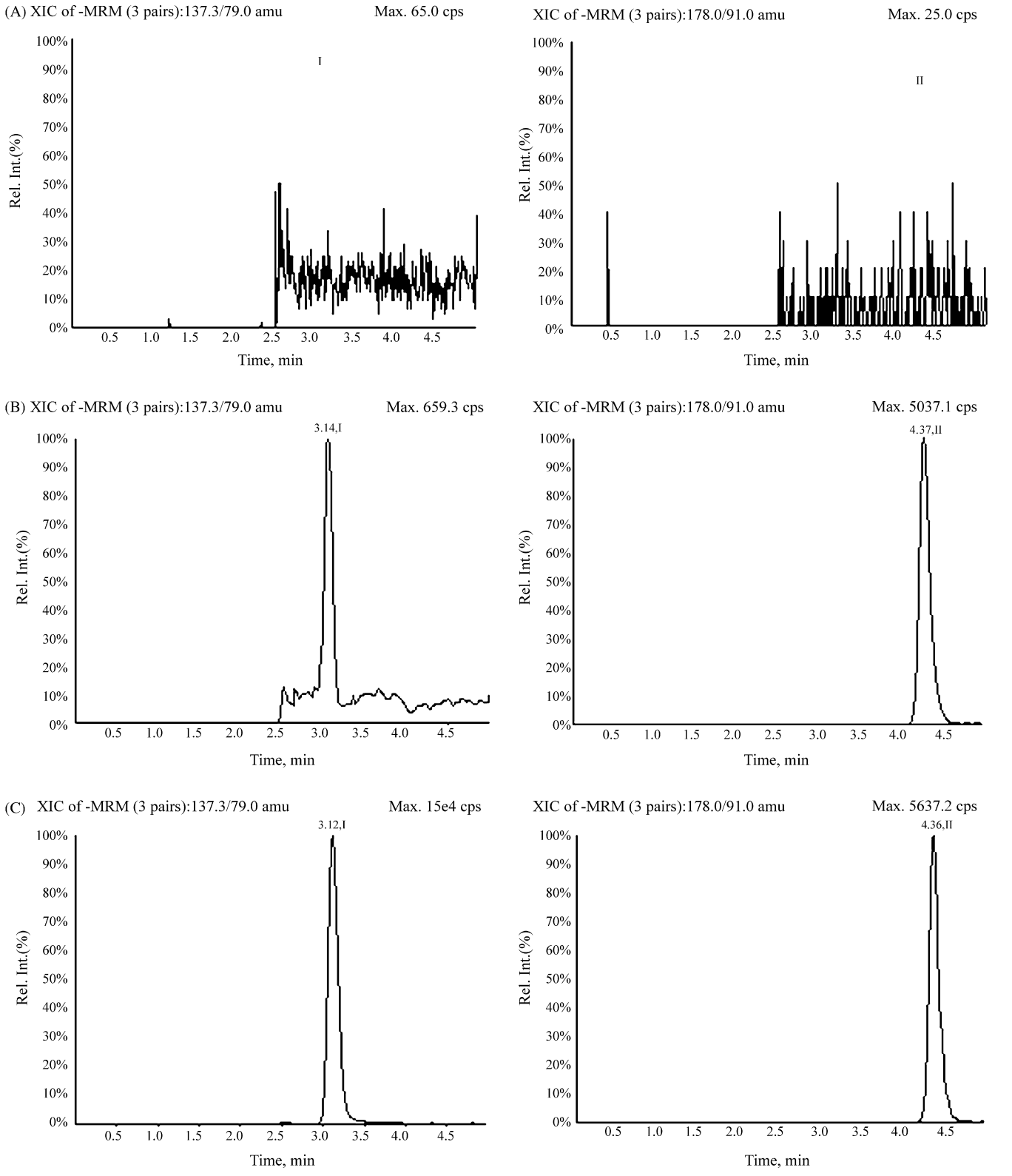

Fig. 2. Representative MRM chromatograms of fosfomycin (I) and IS (fudosteine, II) in human plasma: (A) Blank plasma sample; (B) plasma spiked with 0.10 μg/mL fosfomycin and 1.00 μg/mL IS; (C) plasma sample 3.0 h after an oral dose of 1 g fosfomycin (∼1.3 g calcium fosfomycin) to a volunteer (measured concentration 10.79 μg/mL).

Table 2
Summary of stability of fosfomycin in human plasma under various storage conditions ($n = 3$)

Storage conditions	Concentration (μg/mL)		RSD (%)	RE (%)
	Added	Found		
Short-term (2 h at 22 °C)	0.25	0.22	3.2	−11.8
	8.00	7.79	2.7	−2.6
Autosampler for 24 h (at 22 °C)	0.25	0.25	12.6	−1.8
	8.00	7.99	3.1	−0.2
Three freeze/thaw cycles	0.25	0.23	13.9	−6.9
	8.00	7.40	1.0	−7.4
Long-term (30 days at −20 °C)	0.25	0.26	5.1	4.6
	8.00	8.04	4.0	0.5

mined in plasma samples up to 24 h after a single oral dose of 1.3 g calcium fosfomycin, which is sensitive enough to investigate the pharmacokinetic behavior of the drug.

3.3.1.3. Precision and accuracy. The precision and accuracy of the method were assessed by determining QC samples ($n = 6$) at three concentrations on the three consecutive days. Table 1 summarizes the intra- and inter-assay precision and accuracy for fosfomycin from QC samples. The intra- and inter-assay precisions were measured to be below 10.6% and 7.9%, respectively, with relative errors from −1.0% to −4.2%.

3.3.1.4. Extraction recovery and stability. Mean extraction recoveries of fosfomycin at 0.25, 1.00 and 8.00 μg/mL were 92.5 ± 2.9%, 93.4 ± 6.2% and 88.7 ± 10.1%, respectively ($n = 6$). Mean recovery of the internal standard (1.00 μg/mL) was 93.3 ± 2.2% ($n = 6$).

The stabilities of fosfomycin on bench top, in auto-sampler, after three freeze–thaw cycles and after long period of storage at −20 °C were investigated. The results are listed in Table 2, which indicated that the analyte was stable under the storage conditions described above, with the 88.2–104.6% accuracy. Besides, fosfomycin in solution and in plasma was proved stable in room light for 24 h. The stability of fosfomycin permitted to simplify the analytical procedures.

3.4. Application in bioequivalence study of two formulations

This validated analytical method has been successfully applied to determine the plasma concentration of fosfomycin after an oral administration of capsules containing 1.0 g fosfomycin (∼1.3 g calcium fosfomycin) to 20 healthy subjects in a bioequivalence study. Mean plasma concentration–time profiles of fosfomycin obtained from 20 healthy volunteers are presented in Fig. 3.

After an oral administration of the reference formulation, peak plasma concentrations were observed at about 2.4 h, and averaged 9.69 μg/mL. The plasma $t_{1/2}$ averaged 6.6 h. The mean $AUC_{0 \rightarrow 24\,h}$ and $AUC_{0 \rightarrow \infty}$ values were 68.9 and 75.4 μg h/mL, respectively. The test formulation showed similar results. These data demonstrated that the developed method could provide a satisfied sensitivity for bioequivalence studies at therapeutic doses.

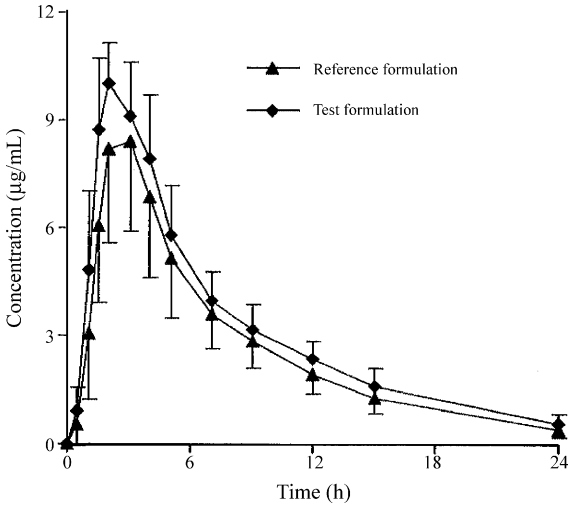

Fig. 3. Mean plasma concentration–time curves of fosfomycin after a single oral dose of 1 g fosfomycin (∼1.3 g calcium fosfomycin) to 20 healthy volunteers. Each point represents the mean ± SD.

4. Conclusions

An LC/MS/MS assay for fosfomycin in human plasma was developed and validated with respect to selectivity, linearity, precision and accuracy, and analysis of real samples was demonstrated. It was proved to be superior in sensitivity, selectivity and speed of analysis in comparison to the previously reported analytical methods. This method was successfully applied to a bioequivalence study for fosfomycin and was found to be reasonably sensitive and reliable.

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