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High-throughput determination of faropenem in human plasma and urine by on-line solid-phase extraction coupled to high-performance liquid chromatography with UV detection and its application to the pharmacokinetic study

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ABSTRACT

An automated system using on-line solid-phase extraction and HPLC with UV detection was developed for the determination of faropenem in human plasma and urine. Analytical process was performed isocratically with two reversed-phase columns connected by a switching valve. After simple pretreatment for plasma and urine with acetonitrile, a volume of 100 μl upper layer of the plasma or urine samples was injected for on-line SPE column switching HPLC-UV analysis. The analytes were retained on the self-made trap column (Lichrospher C_{18}, 4.6 mm × 37 mm, 25 μm) with the loading solvent (20 mM NaH_2PO_4 adjusted pH 3.5) at flow rate of 2 ml min^{-1}, and most matrix materials were removed from the column to waste. After 0.5 min washing, the valve was switched to another position so that the target analytes could be eluted from trap column to analytical column in the back-flush mode by the mobile phase (acetonitrile–20 mM NaH_2PO_4 adjusted pH 3.5, 16:84, v/v) at flow rate of 1.5 ml min^{-1}, and then separated on the analytical column (Ultimate™ XB-C_{18}, 4.6 mm × 50 mm, 5 μm).The complete cycle of the on-line SPE preconcentration purification and HPLC separation of the analytes was 5 min. Calibration curves with good linearities (r = 0.9994 for plasma sample and r = 0.9988 for urine sample) were obtained in the range 0.02–5 μg ml^{-1} in plasma and 0.05–10 μg ml^{-1} in urine for faropenem. The optimized method showed good performance in terms of specificity, linearity, detection and quantification limits, precision and accuracy. The method was successfully utilized to quantify faropenem in human plasma and urine to support the clinical pharmacokinetic studies.

1. Introduction

The penem antibiotics, as a class, exhibit many positive properties such as a broad antimicrobial spectrum, a high level of resistance to hydrolysis by bacterial β-lactamases, and a high affinity for penicillin-binding proteins [1]. Faropenem (FAR), 4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid, 6-(1-hydroxyethyl) 7-oxo-3-(tetrahydro-2-furanyl)-, monosodium salt (shown in Fig. 1), is an orally available member of the penem class unique among carbapenems and other available beta-lactams, which has an unsaturated thiazole ring and is a structural hybrid between the penicillin and carbapenem nucleus. It is characterized by potent penicillin-binding protein activity and β-lactamase stability [2,3]. FAR presents a broad spectrum of activity against Gram-positive and -negative bacteria, and is also bactericidal. Preliminary reports indicate that FAR exhibited very good activity against the three major pathogens associated with community-acquired respiratory infections (Streptococcus pneumoniae, Moraxella catarrhalis and Haemophilus). The clinical use of FAR mainly includes four proposed indications: acute bacterial sinusitis, community-acquired pneumonia, acute exacerbations of chronic bronchitis, uncomplicated skin and skin structure infections [4–8].

Several different methods have been published for quantification of FAR in human plasma and urine by means of high-performance liquid chromatography with ultraviolet detection [9,10] and liquid chromatography-electrospray ionization mass spectrometry [11,12]. Nigori et al. [9] reported a HPLC-UV

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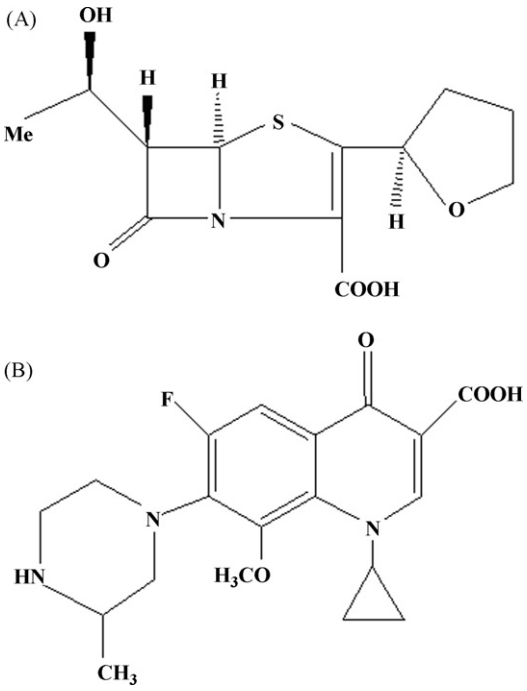

Fig. 1. Chemical structures of FAR (A) and the IS (B).

method for determination of FAR in human plasma. However, expensive solid-phase extraction cartridges were used in this assay for sample pretreatment and a lower limit of quantitation (LLOQ) was 200 ng ml^{-1}. Hu et al. [10] also developed a HPLC-ultraviolet method to quantitate FAR in human plasma. In their study, samples were divided into high concentration ones and low concentration ones which needed two calibration curves, thus it was time consuming and not quite suitable for high-throughput analysis. Gao et al. [11] and Wen et al. [12] established liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods to determine FAR in human biological samples. Although their methods can provide excellent sensitivity and selectivity, they are not available for some laboratories due to the costs associated with the acquisition and maintenance of this equipment. So a method meets high-throughput analysis together with economic, pragmatic, volant and convenient needs should be established in order to determine FAR in human biological samples.

As an alternative to existing methods, we developed a HPLC method with an automated on-line SPE system for the determination of FAR, which combined simplicity of self-made trap column with rapidity of short analytical column. The on-line SPE technique can reduce sample pretreatment steps, decrease the analysis time of bioanalytical methods and meet the increasing demand for automation and high-throughput analysis. Full validation was performed to assess the selectivity, sensitivity, linearity, accuracy, precision and LLOQ of the method, and the results presented here demonstrate that the method is suitable for analyzing FAR in human plasma and urine, and it has been successfully applied to the clinical pharmacokinetic studies of FAR tablets in healthy volunteers.

2. Experimental

2.1. Chemicals and reagents

FAR tablets and standard reference material (95.4% purity) were supplied by Shanghai Shenjiu Medpharm Biotech Co. Ltd. (Shanghai, China). Gatifloxacin (internal standard, IS, 99.0% purity) (shown in Fig. 1) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol and acetonitrile of HPLC grade were obtained from Caledon Laboratories Ltd. (Georgetown, Ont., Canada). Perchloric acid of HPLC grade was purchased from Tedia Company (Tedia Fairfield, OH, USA). Other chemicals were all of analytical grade. Ultra-pure water was obtained by means of a Milli-Q apparatus from Millipore (Bedford, MA, USA). Human blank plasma was obtained from Shanghai Red Cross Blood Center (Shanghai, China).

2.2. Equipment

HPLC apparatus consisted of a Shimadzu system equipped with two LC-20AD pumps, an LC-10AD pump, an SIL-20A autosampler, a CTO-20AC column oven, an LV-306R automatic high-pressure switching valve, an SPD-20A UV–Vis detector for the second column and a DGU-20A$_3$ degasser. Shimadzu LC-solution software was used for data acquisition and mathematical calculations.

2.3. Preparation of stock and sample solutions

Stock solutions of FAR (1 mg ml^{-1}) and internal standard gatifloxacin (100 μg ml^{-1}) were prepared in methanol and stored at −20 °C. Primary stock solution of FAR was first diluted quantitatively with 20 mM NaH_2PO_4 (pH 3.5) to give working solutions with concentrations of 0.2, 0.5, 1, 2, 5, 10, 20, 40, 50, 80 and 100 μg ml^{-1} for the preparation of calibration and quality control (QC) samples. Internal standard gatifloxacin primary stock solution was diluted with 20 mM NaH_2PO_4 (pH 3.5) to give a working solution with concentration of 2.5 μg ml^{-1}. Calibration standards of FAR at concentrations of 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5 μg ml^{-1} and 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10 μg ml^{-1} were prepared by spiking appropriate amounts of the working solutions in blank plasma and urine, respectively. For each validation and assay run, the calibration curve standards were prepared fresh from the standard working solutions. Quality control (QC) samples which were used in the validation and during the pharmacokinetic studies were independently prepared at three level concentrations of 0.05 μg ml^{-1} (low QC), 0.5 μg ml^{-1} (medium QC) and 4 μg ml^{-1} (high QC) for plasma and 0.1 μg ml^{-1} (low QC), 1 μg ml^{-1} (medium QC) and 8 μg ml^{-1} (high QC) for urine. The QC samples were stored at −20 °C and brought to room temperature before processing together with the clinical samples.

2.4. Sample preparation

Protein precipitation was used for the preparation of plasma samples. 20 μl of IS (2.5 μg ml^{-1}) and 60 μl of 6% perchloric acid were added to a 100 μl plasma sample; the mixture was vortex-mixed for 1 min and centrifuged at 12,000 rpm for 10 min at 4 °C; 150 μl of the supernatant was transferred to another clean tube, then 25 μl of 1 M NaOH was spiked in order to adjust the pH to 4.0 approximately; after vortex-mixing, a volume of 100 μl upper layer was injected into HPLC system.

Urine samples were briefly vortex-mixed and centrifuged to remove large particles. A 50 μl urine sample was spiked with 100 μl of IS (2.5 μg ml^{-1}), oscillated for 1 min and centrifuged at 12,000 rpm for 5 min at 4 °C; then 100 μl of the upper layer was transferred to another clean tube and diluted with 400 μl of ultra-pure water; the mixture was vortex-mixed and then 100 μl of the supernatant was injected for HPLC analysis.

2.5. On-line SPE procedure

Fig. 2 shows a schematic diagram of the automated column switching HPLC system. The system was operated according to the

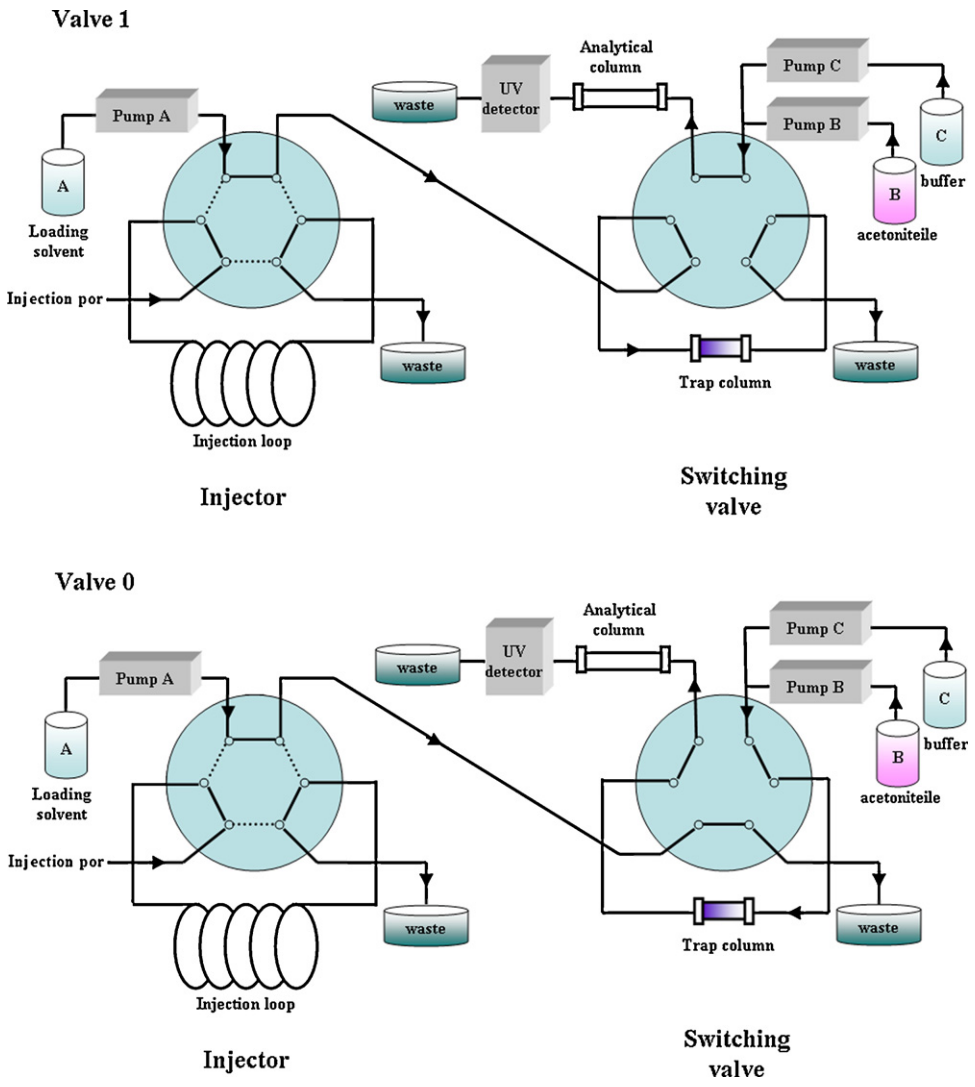

Fig. 2. Schematic diagram of the on-line SPE system using a six-port switching valve: step 1, valve 1; step 2, valve 0; step 3, valve 1.

following procedure where valve positions and switchover times are in parentheses. Step 1 (valve 1; 0–0.5 min): a plasma or urine sample was injected onto the trap column and the trap column was washed by loading solvent at a flow rate of 2 ml min^{-1} in order to remove endogenous interferences and enrich the interesting compounds. Data acquisition started in detector. Step 2 (valve 0; 0.5–2.5 min): the valve was switched from position 1 to 0 and the enriched compounds were eluted from trap column to analytical column in the back-flush mode for separating the analytes with the mobile phase. Step 3 (valve 1; 2.5–5 min): the valve was returned to the initial position, the trap column was equilibrated again with the loading solution for the next analysis and the analytical column was continuously eluted with the mobile phase until the end of this analytical procedure.

2.6. Chromatographic conditions

Chromatographic separation was achieved on a 50 mm × 4.6 mm, 5 μm, Ultimate™ XB-C_{18} column (Welch Materials, USA) with a 37 mm × 4.6 mm, 25 μm, Lichrospher C_{18} trap column (self-made). The column temperature of the trap column and the analytical column was maintained at 30 °C. The loading solvent (pump A) was 20 mM NaH_2PO_4 buffer (pH3.5) and the mobile phase (pumps B and C) was acetonitrile–20 mM NaH_2PO_4 (pH 3.5) (16:84, v/v). The flow rate of loading solvent was 2.0 ml min^{-1} and mobile phase was 1.5 ml min^{-1}. The autosampler was set with an injection volume of 100 μl and a run time of 5 min. The absorbance wavelength on the UV detector was set at 318 nm.

2.7. HPLC method validation

2.7.1. Specificity and selectivity

The chromatographic interference from endogenous compounds was assessed by comparing chromatograms of blank human plasma and urine, plasma and urine spiked with FAR and internal standard gatifloxacin, and plasma and urine samples obtained from FAR clinical pharmacokinetic studies.

2.7.2. Sensitivity

The lower limit of quantification (LLOQ) was determined during the evaluation of the linear range of calibration curve. LLOQ was defined as the lowest concentration yielding a precision with CV less than 20% and accuracy within 20% of the theoretical value (i.e. accuracy between 80 and 120%) for both within- and between-run analysis.

2.7.3. Linearity of calibration curve

Calibration curve was obtained by plotting the peak area ratios of FAR/IS to the spiked FAR theoretical concentrations in blank plasma and urine. Least-squares linear regression was used for curve fitting with $1/x$ as the weighting factor.

2.7.4. Precision and accuracy

The within- and between-run accuracy and precision were evaluated by repeated analyses of QCs at three levels (low, medium and high) on three sequential runs in five replicates. Accuracy was assessed by calculating the percentage deviation from the theoretical concentration. Precision was determined by calculating the coefficient of variation for within- and between-run replicates. The criteria for acceptability of data induced accuracy within ±15% deviation from the nominal values and a precision within ±15% relative standard deviation (RSD).

2.7.5. Recovery

The extraction recoveries of FAR were determined by comparing the peak area of the QCs with the peak area of the corresponding standard solution in 20 mM NaH_2PO_4 (pH 3.5) at equivalent concentrations and expressed in percentage. The recovery of IS was also determined similarly.

2.7.6. Stability

Analyte stability determinations comprised short-term temperature stability, long-term stability, autosampler stability and freeze–thaw cycles stability, which were evaluated by analyzing three QC levels in quintuple. The QC samples were analyzed after storage at room temperature for 2 h, at −20 °C for 1 month, in the autosampler at room temperature for 8 h after sample preparation and after three freeze–thaw cycles, which consisted of storage at −20 °C for a minimum of 12 h followed by thawing at room temperature.

2.8. Clinical pharmacokinetics design

The developed on-line SPE procedure was used to investigate the plasma profiles of FAR following oral administration of FAR tablets at single doses of 150, 300, and 600 mg and multiple dose of 300 mg. This method was also used to perform the determination of urine concentrations of FAR after oral administration of 150, 300, and 600 mg single doses to the healthy volunteers.

A clinical study was conducted on 30 Chinese volunteers (15 male and 15 female), aged from 20 to 28 years, who were judged to be in good health condition through medical history, physical examination and routing laboratory tests (hematology, blood biochemistry, and urine analysis). The volunteers were instructed to abstain from taking any medication including over the counter (OTC) drugs for at least 2 weeks prior to and during the study period and avoid any alcohol containing food and beverages 36 h prior to or during the course of the study. Informed consent was obtained from all the subjects after explaining the aim and risks of the study. The study protocol was approved by a local ethics committee. Healthy volunteers were assigned to 3 groups in random order based on computer-generated tables of random numbers and received single doses of 150, 300, and 600 mg of FAR under fasting conditions. Blood samples (2 ml) were collected into lithium heparinized tubes before administration (0 h) and at the time of 0.17, 0.33, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6 and 8 h after oral administration. Urine was collected over a period of 0–2, 2–4, 4–6 and 6–12 h post-dose. Plasma and urine samples were separated by centrifugation (2000 × *g*) for 10 min at 4 °C and frozen at −20 °C until analysis.

Volunteers who had been assigned to the FAR 300 mg group in the single-dose phase continued on to the multiple-dose phase and received FAR 300 mg three times daily for 5 days. This dose was chosen for the multiple-dose phase because it is likely to be a commonly used starting dose in clinical practice. Samples of venous blood (2 ml each) were drawn before drug administration on days 4, 5 and 6 to determine the Cssmin. On day 6, the volunteers received FAR 300 mg one time and blood samples were drawn at 0.17, 0.33, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6 and 8 h after drug administration. All other experimental conditions were the same as those in the single-dose phase.

3. Results and discussion

In the present study, on-line SPE with HPLC-UV method was considered to be a preferred technique due to its sensitivity, speed and selectivity. And a full validation was performed in accordance to the recommendations published by FDA [13].

3.1. Method development

3.1.1. On-line SPE procedure development

High-throughput analysis which is based on on-line SPE needs high speed of the loading solution to purify and preconcentrate target compounds. Because of the above reason, 2.0 ml min^{-1} was chosen as the speed of the loading solvent. In order to choose a suitable trap column, a number of trap columns were tested. It was found that there were two main problems: FAR had almost no retention in some tested columns, the limited retention might be due to the facts that the particle sizes of the filling materials were too large (e.g. BondElut C_{18}, 2.0 mm × 50 mm, 40–90 μm, Varian Inc.) or the lengths of these columns were not enough (e.g. Oasis® HLB, 2.1 mm × 10 mm, 25 μm, Waters Corp); but when we used long columns (e.g. YWG C_{18}, 2.0 mm × 100 mm, 20 μm), or columns with small filling materials (e.g. Capcell pak C_{18}, 4.0 mm × 20 mm, 5 μm, Shiseido), the high speed of the loading solution would lead to high pressure. After carefully consideration, a Lichrospher C_{18} column (4.6 mm × 37 mm, 25 μm) was packed in our laboratory. Because of the compatible length and particle size, this column gave satisfactory retention and was used for this study. Compared with other commercial trap columns [14–19], this self-made column had many advantages: it permitted large injection volume (100 μl), so that the analytes could not only be purified but also be preconcentrated; it permitted high speed of the loading solvent which was more effective to wash the endogenous compounds and reduce the analytical time; it could maintain low pressure and good reproducibility under high speed of the loading solution; it was much cheaper and more durable than most of commercial trap columns.

Loading solvents were also evaluated and optimized. For on-line SPE-HPLC method, the contents of organic solvent in mobile phase should be higher than loading solvent so that the enriched compounds retained in the trap column can be eluted to analytical column. As a result, we used pure water as loading solution, but almost no FAR and IS peaks were observed, which indicated that the analytes were ionized in water and were washed together with the endogenous components. Therefore, an acid solution should be used as loading solution to convert the analytes into neutral molecules that could be retained on the SPE column. Phosphate buffers (20 mM NaH_2PO_4) at different pHs were tested. The results indicated that the optimum apparent pH_{app} was 3.5, because this pH was close to pK_a value of the analyte and the IS, which could maximize the extraction efficiency for the target compounds. Meanwhile, the peaks of FAR and IS were symmetry and the endogenous compounds could be removed effectively in this pH.

When developing an on-line SPE method at first, the switching time must be determined. Switching time included two sections, transferring time and resetting time. The reduced transferring time

could not only limit the transfer of unwanted interfering compounds from the trap column to the analytical column, but also insure complete transfer of the drug and internal standard from the trap column to the analytical column. After careful comparison, it was found that 0.5 min was sufficient to remove most of the endogenous interferences in plasma and urine. Therefore, the SPE column was washed with the loading solvent (20 mM NaH_2PO_4 adjusted pH 3.5) for 0.5 min at 2.0 ml min^{-1} after the plasma or urine samples had been loaded. An adaptive resetting time could not only make FAR and the IS achieve satisfactory separation on the analytical column, but also give enough equilibration time to the next analytical cycle. On account of the above reasons, 2.5 min was chosen as the optimal resetting time.

3.1.2. Chromatography

The chromatographic conditions, especially the composition of mobile phase and types of column, were optimized through several trials to achieve good resolution and symmetric peak shapes for analyte and the IS, as well as shorter run time. Considering the high pressure when the trap column and analytical column were connected in series, a short column must be chosen as the analytical column. A number of C_{18} columns, such as Diamonsil C_{18}, Zorbax SB C_{18}, XB-C_{18}, AQ-C_{18} were tested. After carefully comparison, an Ultimate™ XB-C_{18} column (5 μm, 50 mm × 4.6 mm) was tried in the experiment and symmetric peak shapes could be achieved, the retention times for FAR and IS were suitable as well. Different compositions of acetonitrile/20 mM NaH_2PO_4 adjusted pH 3.5(15:85, 16:84 and 17:83) as mobile phase were also evaluated. It was found that the percentage of acetonitrile in the mobile phase had great influence. If the percentage was 15%, the retention time interval between the two peaks (FAR and IS) was large and the analytical time was long. When the percentage was adjusted to 17%, these two peaks could not be separated at baseline level. Eventually, a mixture of acetonitrile–20 mM NaH_2PO_4 (pH 3.5) (16:84, v/v) was adopted to achieve an efficient chromatographic separation of the analytes and no interference of endogenous peaks with FAR or the IS at their respective retention times was observed.

Gatifloxacin was used as internal standard for the method. The most important reason for selecting this compound was that it coeluted with FAR on the trap column, thereby minimizing the time interval required to insure complete transfer of the drug and internal standard from the trap column to the analytical column. This was an extremely important consideration, because selectivity of the on-line SPE technique diminishes when a greater volume of effluent from the clean-up column is introduced onto the analytical column. In addition, gatifloxacin has similar chemical and physical behavior to FAR, which was efficiently recovered from plasma and was stable in the solvent system used to reconstitute sample extracts.

3.2. Sample preparation

The instrumentation is not the bottleneck in high-throughput analysis but the sample preparation process. Unlike liquid–liquid extraction and off-line solid-phase extraction, on-line SPE has great advantages in permitting the direct injection of protein rich samples, such as plasma, after simple pretreatment or even no pretreatment. In this study, samples were simply purified before injecting onto the trap column. These procedures were very useful for extending the lifetime of the trap column and avoiding the time consuming process which was used to clean the trap column after every injection. For the protein precipitation 6% perchloric acid, 10% perchloric acid and acetonitrile were compared. 6% perchloric acid was selected because of its satisfactory efficiency compared to the other two. However, the pretreated samples were not stable enough and would degrade after 1–2 h. Eventually, it was found that adding 25 μl 1 M NaOH to 150 μl supernatant could adjust the pH to approximately 4.0 [20] and keep the samples stable for at least 8 h in the autosampler (shown in Table 1).

3.3. Method validation

3.3.1. Selectivity

The selectivity of the method was tested by comparing the chromatograms of six different batches of blank plasma and urine and the spiked plasma and urine. Fig. 3 showed the typical chromatograms of blank plasma and urine samples, blank plasma and urine samples spiked with FAR at LLOQ and the IS, and test plasma and urine samples after the oral dose of 150 mg FAR tablets to a volunteer. Under the optimized conditions the retention time of FAR and the IS was 3.3 and 4.2 min. All blank plasma and urine samples were found to be free of interferences with the compounds of interest.

3.3.2. Sensitivity

The LLOQs for FAR were 0.02 μg ml^{-1} in plasma and 0.05 μg ml^{-1} in urine with a precision of 7.7 and 11.1%, respectively, which were sufficient for clinical pharmacokinetic studies.

3.3.3. Linearity of calibration curve

A weighted $(1/x)$ linear regression was used to perform standard calibrations. The mean calibration equations were $y = 0.9794$(RSD = 1.2%, $n = 3)x + 0.002989$(RSD = 4.1%, $n = 3$) in plasma and $y = 0.7766$(RSD = 3.7%, $n = 3)x + 0.000851$(RSD = 9.1%,

Table 1
Comparison of stability at three QC levels for FAR in plasma with NaOH and without NaOH after protein precipitation.

Time	LQC (with NaOH) (0.05 μg ml^{-1})			MQC (with NaOH) (0.5 μg ml^{-1})			HQC (with NaOH) (4 μg ml^{-1})		
	Concentration (μg ml^{-1})	Accuracy (%)	RE (%)	Concentration (μg ml^{-1})	Accuracy (%)	RE (%)	Concentration (μg ml^{-1})	Accuracy (%)	RE (%)
0 h	0.0538	107.3		0.511	101.8		4.12	102.5	
2 h	0.0535	106.7	−0.6	0.487	97.0	−4.7	4.05	100.8	−1.7
4 h	0.0501	99.8	−7.1	0.496	98.8	−2.9	4.41	109.8	7.1
6 h	0.0495	98.7	−8.2	0.462	92.0	−9.6	4.18	104.0	1.5
8 h	0.0502	100.1	−6.8	0.477	95.0	−6.7	3.91	97.2	−5.2
Time	LQC (without NaOH) (0.05 μg ml^{-1})			MQC (without NaOH) (0.5 μg ml^{-1})			HQC (without NaOH) (4 μg ml^{-1})		
	Concentration (μg ml^{-1})	Accuracy (%)	RE (%)	Concentration (μg ml^{-1})	Accuracy (%)	RE (%)	Concentration (μg ml^{-1})	Accuracy (%)	RE (%)
0 h	0.0516	102.7		0.509	101.4		4.01	99.9	
2 h	0.0456	90.8	−11.9	0.455	90.6	−10.6	3.60	89.6	−10.4
4 h	0.0399	79.6	−22.9	0.413	82.3	−18.9	2.93	72.9	−27.0
6 h	0.0339	67.5	−34.9	0.322	64.1	−36.7	2.52	62.7	−37.3
8 h	0.0210	41.9	−60.3	0.279	55.6	−45.2	1.89	47.1	−52.9

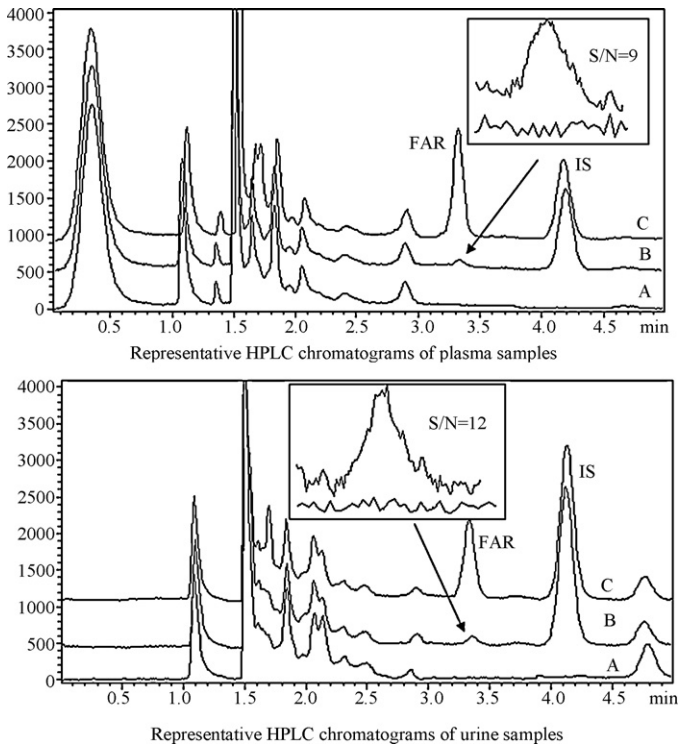

Fig. 3. Representative HPLC chromatograms of the method: (A) blank plasma and urine samples; (B) blank plasma and urine samples spiked with FAR at LLOQ and 2.5 μg ml^{-1} IS; (C) test plasma sample obtained at 1 h and test urine sample obtained at the period of 0–2 h after the oral dose of 150 mg FAR tablets to a volunteer.

$n = 3$) in urine, where y represented the peak area ratios of the analyte to the IS and x represented the plasma concentration of analyte in μg ml^{-1}. Calibration curves were linear in the range 0.02–5 μg ml^{-1} in plasma and 0.05–10 μg ml^{-1} in urine with the concentration coefficients consistently greater than 0.99.

3.3.4. Precision and accuracy

Table 2 shows a summary of within- and between-run precision and accuracy. In the range of 0.02–5 μg ml^{-1} in plasma, within- and between-run accuracy ranged from 99.0 to 105.2 and 101.4 to 102.4%, respectively. In the range of 0.05–10 μg ml^{-1} in urine, within- and between-run accuracy ranged from 96.2 to 100.3 and 98.4 to 99.6%, respectively. Therefore, the within- and between-run accuracies (% deviation) were within ±15% (85–115%) for all QC samples. The within- and between-run assay precision (CV) ranged from 2.9 to 9.4 and 4.4 to 7.4% for the plasma QC samples and 2.8 to 8.2 and 4.8 to 6.0% for the urine QC samples, respectively, which were also within the acceptable range of 15%. QC samples are a good representation of study samples and similar accuracy and precision of QC samples during clinical samples analysis were observed. The QC data indicate the accurate and reliability of this method in determination of FAR in human plasma and urine.

3.3.5. Recovery

The mean extraction recoveries of FAR were 89.9, 90.2 and 88.6% in plasma (n = 5) and 97.6, 99.5 and 98.3% in urine (n = 5) for low, medium and high QC samples, respectively. The mean extraction recovery of the IS was 95.4% ($n = 5$).

3.3.6. Stability

FAR primary stock solution (1 mg ml^{-1} in methanol) was stable for at least 2 months (data not shown) at −20 °C. The stability study results of FAR under various conditions were summarized in Table 3. FAR at three QC levels were stable in human plasma and urine for 2 h at ambient temperature, after three freeze–thaw cycles, as well as after storage at −20 °C for 1 month. FAR was also stable in the reconstituted buffer for 8 h in the autosampler at room temperature. The high stable property of FAR in human plasma and urine suggested that no special care was needed during sample preparation. The high stability of FAR in reconstituted buffer also suggested that a large batch of samples could be processed at one time within 8 h.

3.4. Application to clinical pharmacokinetic study

The method described above has successfully been applied to analyze plasma and urine samples obtained from 30 healthy volunteers who received single doses of 150, 300 and 600 mg FAR tablets. The mean plasma concentration-time profiles for FAR after oral administration of FAR at three single doses were presented in Fig. 4. FAR could be detected at all time points over the duration at all single doses. Pharmacokinetic parameters determined by non-compartment analysis method were listed in Table 4. The $AUC_{0\sim\infty}$ values of FAR were 3.9 ± 1.7 μg h ml^{-1}, 7.7 ± 1.8 μg h ml^{-1}

Table 2
Accuracy and precision for FAR in spiked plasma and urine samples.

Level	Plasma			Urine		
	Low (0.05 μg ml^{-1})	Medium (0.5 μg ml^{-1})	High (4 μg ml^{-1})	Low (0.1 μg ml^{-1})	Medium (1 μg ml^{-1})	High (8 μg ml^{-1})
Between-run accuracy and precision ($n = 15$)						
Mean ± SD (μg ml^{-1})	0.051 ± 0.004	0.509 ±0.022	4.103 ± 0.246	0.100 ± 0.006	0.989 ± 0.059	7.905 ± 0.379
Accuracy (%)	102.4 ± 7.6	101.4 ± 4.5	102.2 ± 6.1	99.6 ± 5.9	98.5 ± 5.9	98.4 ± 4.7
RSD (%)	7.4	4.4	6.0	5.9	6.0	4.8
Within-run accuracy and precision						
Run I ($n = 5$)						
Mean ± SD (μg ml^{-1})	0.053 ± 0.003	0.517 ± 0.015	4.117 ± 0.225	0.099 ± 0.007	1.007 ± 0.051	7.904 ± 0.499
Accuracy (%)	105.2 ± 5.4	102.9 ± 3.0	102.5 ± 5.6	98.9 ± 7.4	100.3 ± 5.1	98.4 ± 6.2
RSD (%)	5.2	2.9	5.5	7.5	5.0	6.3
Run II ($n = 5$)						
Mean ± SD (μg ml^{-1})	0.050 ± 0.005	0.497 ± 0.029	4.074 ± 0.263	0.100 ± 0.006	0.995 ± 0.082	7.986 ± 0.222
Accuracy (%)	99.2 ± 9.3	99.0 ± 5.8	101.4 ± 6.5	99.9 ± 6.3	99.1 ± 8.2	99.4 ± 2.8
RSD (%)	9.4	5.8	6.4	6.3	8.2	2.8
Run III ($n = 5$)						
Mean ± SD (μg ml^{-1})	0.052 ± 0.004	0.513 ± 0.020	4.118 ± 0.300	0.100 ± 0.005	0.965 ± 0.044	7.825 ± 0.434
Accuracy (%)	102.8 ± 7.8	102.3 ± 4.1	102.5 ± 7.5	99.8 ± 5.2	96.2 ± 4.4	97.4 ± 5.4
RSD (%)	7.6	4.0	7.3	5.2	4.6	5.5

Table 3
Stability results of FAR in spiked plasma and urine samples ($n = 5$).

Stability	Plasma			Urine		
	Low (0.05 μg ml^{-1})	Medium (0.5 μg ml^{-1})	High (4 μg ml^{-1})	Low (0.1 μg ml^{-1})	Medium (1 μg ml^{-1})	High (8 μg ml^{-1})
Short-term stability (2 h at room temperature)						
Mean ± SD (μg ml^{-1})	0.049 ± 0.005	0.485 ± 0.011	3.841 ± 0.361	0.105 ± 0.009	1.012 ± 0.081	8.012 ± 0.609
RSD (%)	9.4	2.2	8.9	9.0	8.1	7.6
RE (%)	−2.4	−3.4	−4.4	4.6	0.8	−0.3
Long-term stability (1 month at −20 °C)						
Mean ± SD (μg ml^{-1})	0.048 ± 0.007	0.499 ± 0.029	3.829 ± 0.275	0.092 ± 0.007	0.989 ± 0.076	7.621 ± 0.825
RSD (%)	13.9	5.8	6.8	6.9	7.6	10.3
RE (%)	−4.4	−0.6	−4.7	−8.4	−1.5	−5.1
Autosampler stability (8 h at room temperature)						
Mean ± SD (μg ml^{-1})	0.052 ± 0.002	0.511 ± 0.023	4.103 ± 0.301	0.113 ± 0.005	1.038 ± 0.052	8.113 ± 0.755
RSD (%)	4.0	4.6	7.5	5.2	5.2	9.4
RE (%)	3.6	1.8	2.2	12.5	3.4	1.0
Freeze–thaw stability (3 cycles)						
Mean ± SD (μg ml^{-1})	0.053 ± 0.006	0.528 ± 0.031	3.767 ± 0.299	0.097 ± 0.004	1.102 ± 0.096	7.423 ± 0.512
RSD (%)	12.0	6.2	7.4	3.9	9.6	6.4
RE (%)	5.6	5.2	−6.2	−3.4	9.8	−7.6

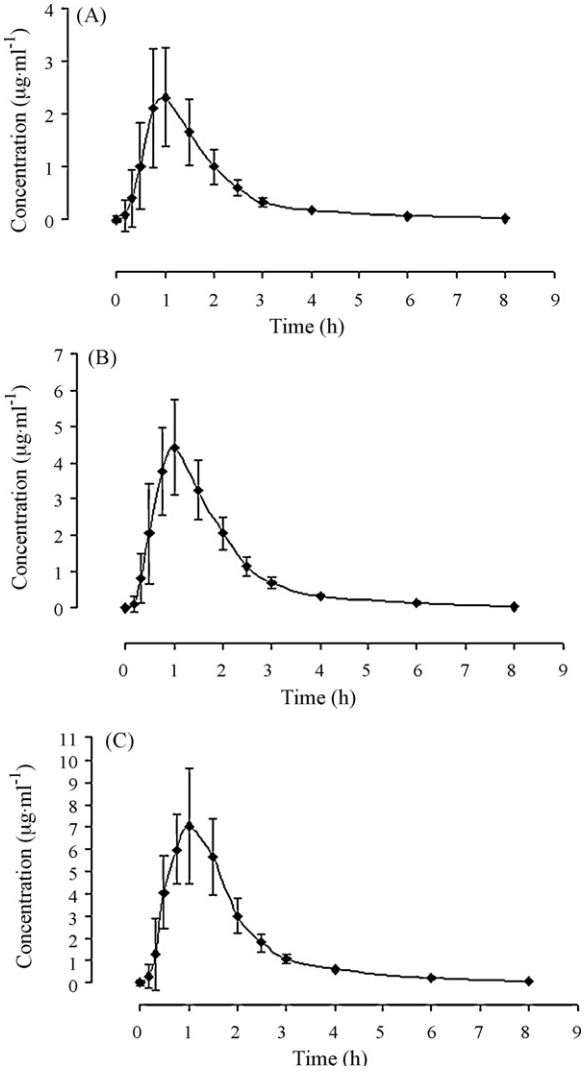

Fig. 4. Mean drug plasma concentration-time curves of FAR after single oral doses of 150 mg (A), 300 mg (B) and 600 mg (C).

Table 4
Pharmacokinetic parameters of FAR following oral administration of FAR tablets at different single doses (mean ± SD).

Parameter	150 mg ($n = 10$) Single dose	300 mg ($n = 10$) Single dose	600 mg ($n = 10$) Single dose
C_{max} (μg ml^{-1})	2.7 ± 0.8	5.2 ± 1.2	8.1 ± 1.3
T_{max} (h)	0.9 ± 0.1	1.1 ± 0.3	1.1 ± 0.3
$AUC_{0\sim t}$ (μg h ml^{-1})	3.9 ± 1.7	7.6 ± 1.8	12.6 ± 3.2
$AUC_{0\sim\infty}$ (μg h ml^{-1})	3.9 ± 1.7	7.7 ± 1.8	12.7 ± 3.2
$t_{1/2}$ (h)	1.2 ± 0.2	1.2 ± 0.1	1.2 ± 0.1
MRT (h)	1.8 ± 0.2	1.8 ± 0.2	1.8 ± 0.2

and 12.7 ± 3.2 μg h ml^{-1} for 150, 300 and 600 mg, respectively, which increased with the dose, showing apparent dose-dependent relationship ($r = 0.9902$). Accumulative excretion amount of FAR in urine were 9.1 ± 4.9 mg, 18.5 ± 10.9 mg and 36.5 ± 20.7 mg within 12 h after oral administration (Fig. 5).

The 10 subjects who received FAR at a dose of 300 mg in the single-dose phase of the PK study continued on to the multiple-dose phase and received 300 mg of FAR three times daily for 5 days and one time on day 6. The mean plasma concentration–time profile for FAR after multiple oral doses was shown in Fig. 6, and the PK parameters were summarized in Table 5. Steady state was achieved after administration of FAR for 3 consecutive days. Mean (±SD) plasma concentrations on the 4th, 5th and 6th days before dosing were 0.039 ± 0.012 μg ml^{-1}, 0.046 ± 0.011 μg ml^{-1} and 0.040 ± 0.010 μg ml^{-1}, respectively. No significant differences

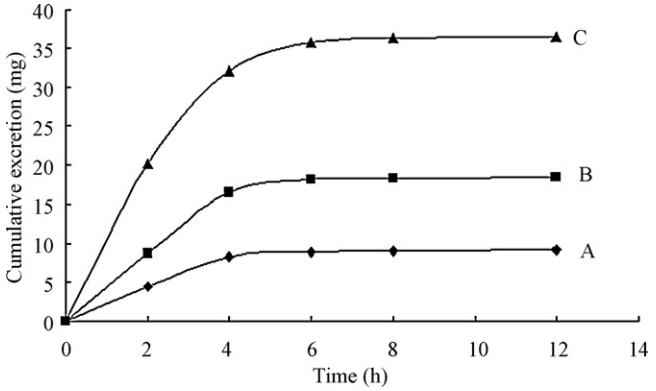

Fig. 5. Cumulative excretion of FAR in healthy volunteers after single oral doses of 150 mg (A), 300 mg (B), 600 mg (C).

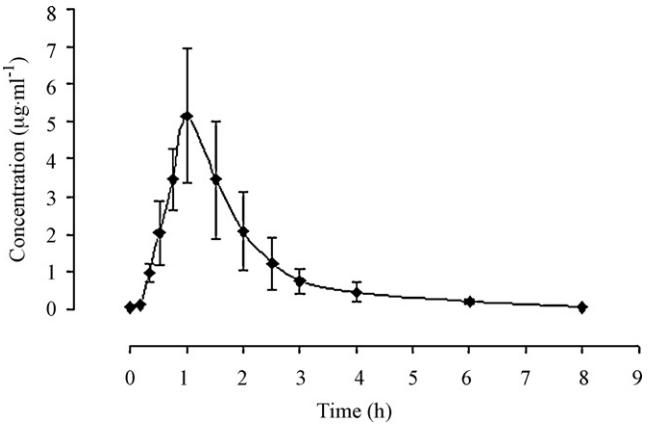

Fig. 6. Mean drug plasma concentration–time curve of FAR after multiple oral doses of FAR (300 mg three times daily for 5 days).

Table 5
Comparison of pharmacokinetic parameters for FAR after administration of a single dose (300 mg) and multiple doses (300 mg three times daily for 5 days) in healthy Chinese subjects (mean ± SD).

Parameter	300 mg (n = 10) Single dose	300 mg (n = 10) Multiple doses
C_{max} (μg ml^{-1})	5.2 ± 1.2	5.7 ± 1.4
T_{max} (h)	1.1 ± 0.3	1.0 ± 0.2
$AUC_{0\sim t}$ (μg h ml^{-1})	7.6 ± 1.8	8.4 ± 2.9
$AUC_{0\sim\infty}$ (μg h ml^{-1})	7.7 ± 1.8	8.5 ± 2.9
$t_{1/2}$ (h)	1.2 ± 0.1	1.3 ± 0.3
MRT (h)	1.8 ± 0.2	2.0 ± 0.2

in PK parameters were observed between the subjects who received a single dose and those who received multiple doses of FAR. As expected, the AUC and Cmax were higher after administration of multiple doses than after a single dose.

4. Conclusion

The present method for the determination of FAR in human plasma and urine has proved to be rapid, sensitive and selective, and it requires relatively small volumes of samples. It is thus suitable for the pharmacokinetic studies in humans. To date, no application of on-line SPE for analysis of FAR in human plasma and urine has been reported. Sample pretreatment procedures for plasma and urine were characterized by easy-to-use methods and speed. They provided sufficient clean-up of the biological samples prior to analysis and showed no significant loss of the analytes during sample handling. In conclusion, this paper describes a sensitive and accurate on-line SPE method for the quantification of FAR suitable to monitor plasma concentrations during clinical pharmacokinetic studies in humans. This method is also potentially helpful for the determination of other antimicrobial agents.

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