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Short communication

High-throughput determination of fudosteine in human plasma by liquid chromatography–tandem mass spectrometry, following protein precipitation in the 96-well plate format

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ABSTRACT

A 96-well protein precipitation, liquid chromatography–tandem mass spectrometry (LC–MS/MS) method has been developed and fully validated for the determination of fudosteine in human plasma. After protein precipitation of the plasma samples (50 μL) by the methanol (150 μL) containing the internal standard (IS), erdosteine, the 96-well plate was vortexed for 5 min and centrifuged for 15 min. The 100 μL supernatant and 100 μL mobile phase were added to another plate and mixed and then the mixture was directly injected into the LC–MS/MS system in the negative ionization mode. The separation was performed on a XB-CN column for 3.0 min per sample using an eluent of methanol–water (60:40, v/v) containing 0.005% formic acid. Multiple reaction monitoring (MRM) using the precursor-product ion transitions m/z 178 → 91 and m/z 284 → 91 was performed to quantify fudosteine and erdosteine, respectively. The method was sensitive with a lower limit of quantification (LLOQ) of 0.02 μg mL^{-1}, with good linearity (r > 0.999) over the linear range of 0.02–10 μg mL^{-1}. The within- and between-run precision was less than 5.5% and accuracy ranged from 94.2 to 106.7% for quality control (QC) samples at three concentrations of 0.05, 1 and 8 μg mL^{-1}. The method was employed in the clinical pharmacokinetic study of fudosteine formulation product after oral administration to healthy volunteers.

1. Introduction

Fudosteine, (−)-(*R*)-2-amino-3-(3-hydroxypopylthio) propionic acid, (for chemical structure, see Fig. 1), a new cysteine derivative, is a new mucoactive agent approved in Japan in 2001. It has a therapeutic effect against mucus hypersecretion caused by an increase in the number of goblet cells in chronic respiratory diseases such as bronchial asthma, chronic bronchitis, pulmonary emphysema, bronchiolectasia, pulmonary tuberculosis, pneumonoconiosis, atypical mycobacterial disease and diffuse panbronchiolitis [1,2].

Because fudosteine has little retention on the ODS column and lacks UV absorption and fluorescent functional groups, several methods require prior derivatization of fudosteine to achieve a suitable chromatographic behavior and detection sensitivity such as high performance liquid chromatography with fluorescence detection [3] and liquid chromatography–electrospray ionization mass spectrometry [4,5]. These two methods suffered from a complicated and time-consuming derivatization procedure and long chromatographic run time to elimination possible endogenous interference, which do not meet high-throughput analysis needs with respect to an efficient extraction procedure with small size plasma, reduced analysis time and high sensitivity when a large number of samples for quantification. Li et al. [6] have reported a LC–MS/MS method to determine fosfomycin in human plasma, and fudosteine was used as the IS. However, there was no detail method optimization and validation about fudosteine.

Several methods have been reported to determinations of the structure analogs of fudosteine such as carbocysteine and erdosteine in human plasma by LC–MS/MS following one-step protein precipitation [7] or solid-phase extraction automation in a

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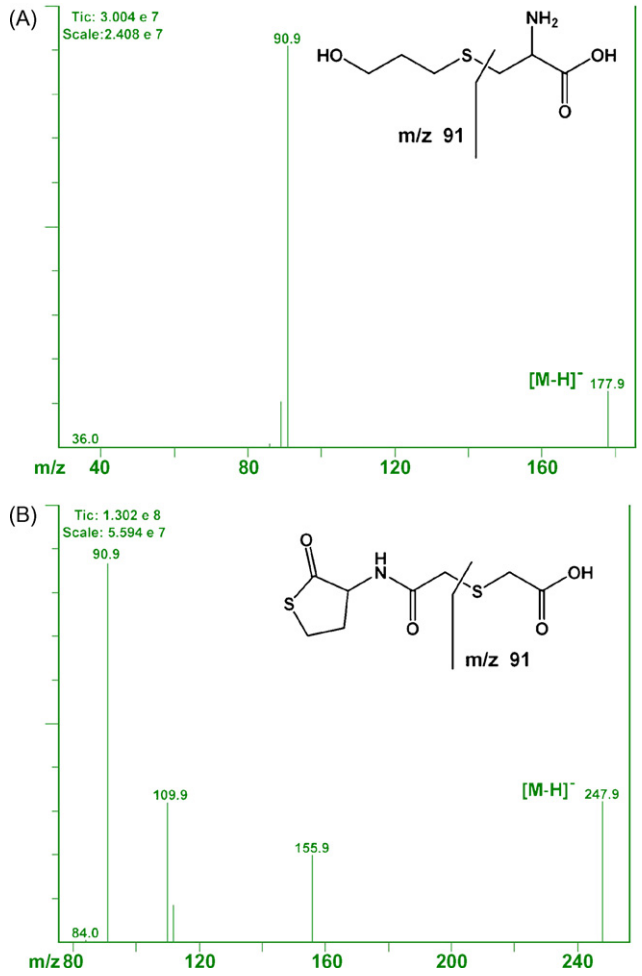

Fig. 1. Chemical structures and product ion spectra of $[M-H]^-$ of fudosteine (A) and erdosteine (B).

96-well format [8]. These method exhibited excellent performance in terms of selectivity, robustness, and high efficiency (running time of 2.0 min for carbocysteine and 4.0 min for erdosteine) with either simplicity or automation of sample preparation.

This paper presented a high-throughput analytical method to measure fudosteine in human plasma. Following 96-well protein precipitation, 50 μL of plasma sample was separated and monitored by an LC–MS/MS system for 3.0 min per sample. Results of the fully validation presented here demonstrate that the method is suitable for analyzing fudosteine in human plasma. It has been successfully applied to the clinical pharmacokinetic study of fudosteine tablets in healthy volunteers.

2. Experimental

2.1. Chemicals and reagents

Fudosteine reference (99.0% purity), erdosteine (IS, 99.0% purity) and tablet formulation of fudosteine 200 mg (Batch no. 030901C) were all from Shanghai Pharmaceutical (Group) Co., Ltd. Sine Pharmaceutical Laboratories (Shanghai, PR China). Methanol of HPLC grade was purchased from Merck KGaA (Darmstadt, Germany) and formic acid of HPLC grade was purchased from Tedia (Fairfield, USA). Ultra-pure water was generated in-housing using a Milli-Q system from Millipore (Bedford, MA, USA). Human blank plasma (sodium heparin as an anticoagulant) was obtained from Shanghai Changhai Hospital (Shanghai, PR China).

2.2. LC–MS/MS instrumentation

A Varian HPLC–MS/MS system (Varian, Inc., Palo Alto, CA, USA) consisted of a ProStar 430 autosampler, two ProStar 210 liquid chromatographic pumps, and a 1200 L triple quadrupole mass spectrometer equipped with an electrospray ionization source. Varian MS workstation Version 6.3 software was used for instrument control and data acquisition.

2.3. Liquid chromatographic conditions

The Separation was achieved with a Ultimate™ XB-CN column, 5 μm, 50 mm × 4.6 mm i.d. (Welch Materials, Inc., Ellicott, MD, USA) protected by a Security guard cartridge, 4.0 mm × 3.0 mm i.d. (Phenomenex, Macclesfield, Cheshire, UK) and a mobile phase of methanol–water (60:40, v/v) containing 0.005% formic acid, at a flow rate of 0.3 mL min^{-1}. The column and the autosampler temperature were kept at room temperature of 25 °C and the injection volume was 20 μL. The analysis time was 3.0 min per sample.

2.4. Mass spectrometer conditions

The mass spectrometer was operated in the negative ion mode. The electrospray capillary voltage was set to −25 V. Nitrogen was used as a drying gas set at 22 psi for solvent evaporation. The API housing and drying gas temperature was kept at 50 and 380 °C, respectively. Deprotonated analyte molecules were subjected to collision induced dissociation using argon as the collision gas at 1.8 mTorr to yield product ions. The optimized collision energy was 10 eV for the analyte and 8 eV for the IS. The detection voltage was set to 1650 V. MRM of the precursor-product ion transitions m/z 178 → 91 for fudosteine and m/z 284 → 91 for the IS were monitored for quantification. Product ion mass spectra for fudosteine and the IS were shown in Fig. 1.

2.5. Preparation standards and quality control (QC) samples

The stock solution of fudosteine of 1 mg mL^{-1} was prepared in methanol. Working standard solutions at 0.2, 0.5, 1, 2, 5, 10, 20, 50, 80, 100 μg mL^{-1} were prepared from stock solution in methanol:water (1:1). A stock solution of 100 μg mL^{-1} for erdosteine was prepared in methanol and then was further diluted with methanol to yield a working solution of 1 μg mL^{-1}.

Calibration standards were prepared by spiking 45 μL human blank plasma with 5 μL of the standard working solutions of fudosteine to give nominal concentrations of 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5 and 10 μg mL^{-1}. For each validation and assay run, the calibration curve standards were prepared fresh from the standard working solutions. QC samples which were used in the validation and during the pharmacokinetic studied, were independently prepared at three level concentrations of 0.05, 1 and 8 μg mL^{-1}. The QC samples were stored at −20 °C and brought to room temperature before processed together with the clinical samples.

2.6. Sample preparation

Samples were prepared using protein precipitation in 96-well format plate (1 mL, Varian, Inc., Palo Alto, CA, USA). An eight-channel 10 μL pipetting tool and an eight-channel 100 μL pipetting tool (Eppendorf Research®, Eppendorf AG, Hamburg, Germany) were used for liquid transfer steps. Aliquots of 45 μL human blank plasma were transferred to 96-well plates and 5 μL of standard working solutions were added into 45 μL of human blank plasma to make the fresh calibration standards. 50 μL aliquots of clinical plasma samples and three levels QC samples, respectively were also

pipetted into 96-well plates. Using an eight-channel 300 μL pipetting tool, 150 μL aliquot of methanol containing 1 μg mL^{-1} of IS was added to each sample (standards, QCs and clinical samples) in order to precipitate the plasma protein. Plates were capped and mixed by vortex for 5 min and then subjected to centrifuge at 2500 × *g* for 15 min to remove any precipitated material. A 100 μL aliquot of the supernatant was transferred to another 96-well plate (350 μL) and 100 μL of mobile phase were added to each well. After mixing, the 96-well plate was covered to autosampler, and 20 μL of the mixture was injected into the LC–MS/MS system.

2.7. Validation of the analytical method

A full validation was performed to evaluate the performance of the method in accordance to the recommendations published by FDA [9]. A calibration curve ranging from 0.02 to 10 μg mL^{-1} of fudosteine was used in each run by plotting the peak area ratios of the analyte to IS against the nominal standard curve concentrations. The within- and between-run precision were evaluated by repeated analyses of QCs at three levels with five replicate samples analyzed each run. 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 matrix effects are generally due to the influence of coeluting, undetected matrix components reducing or enhancing the ion intensity of the analytes and affect the reproducibility and accuracy of the assay. To evaluate the matrix effects [10], five different lots of drug-free plasma were processed according to the protein precipitation described above and then spiked with the analyte at the final concentration after extraction and dilution of mobile phase. The absolute matrix effects of the plasma were expressed as the ratio of the mean peak area of analyte spiked postextraction to that of the neat standards at corresponding concentrations. The value of 100% indicates the response of the analyte in the plasma extracts was the same as that in the mobile phase and no absolute matrix effect was observed. The value of >100% indicates ionization enhancement, and the value of <100% illustrates ionization suppression. The relative matrix effects between five lots were measured by calculating the variability in the values. The same evaluation was performed for the IS. The recoveries of fudosteine and the IS were determined by calculating the ratios of the mean peak areas of five regularly prepared samples to that of spiked postextraction plasma samples.

2.8. Application of the method to a pharmacokinetic study

The validated LC–MS/MS method was applied to a single oral dose study of fudosteine (400 mg tablet) in 10 healthy male volunteers who were signed the consent before clinical trial and the clinical trial was approved by a local ethics committee. About 1 mL of blood samples were collected into heparinized tubes before administration (0 h) and at 0.083, 0.17, 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 10 and 12 h after dosing. 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. LC–MS/MS optimization

Fudosteine gave a strong mass response in negative ESI mode. Although it was mentioned that erdosteine exhibit a fairly high sensitivity in positive ion detection mode rather than negative ion detection mode [8], high mass spectrometric response was also obtained in negative modes for its carboxyl group. By ESI, the analyte and IS formed predominately deprotonated molecular ions $[M-H]^-$ at *m*/*z* 178 and *m*/*z* 284 in full scan mass spectra, respectively. Parameters of MSD were tuned according to the MS signal response of the target compound. Automated MS/MS optimization for each analyte was accomplished using MS/MS Breakdown of Varian workstation. The dominant product ion for both fudosteine and the IS is *m*/*z* 91 using 10 and 8 eV collision energy, respectively (Fig. 1).

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. A number of C18 and C8 columns, such as Diamonsil C18, Zorbax SB C18, XB-C18, AQ-C18 Diamonsil C8, Zorbax SB C8 and XB-C8 were tested. It was found that fudosteine has almost no retention in these tested columns and strong ion suppression occurred when using 60% acetonitrile as mobile phase. Lower percentage of acetonitrile in the mobile phase could not increase the retention of fudosteine while it tended to reduce the efficiency of ionization. When using 60% methanol as mobile phase, only on Diamonsil C18 column (5 μm, 100 mm × 4.6 mm i.d., Dikma technologies) symmetric peak shapes could be achieved, but the retention time for fudosteine was long (4.1 min). After carefully comparison of many columns, an Ultimate™ XB-CN column adopted in Li's paper [6] to separate fosfomycin and fudosteine (IS) in human plasma, was tried in the experiment. But the peak broadening and unsymmetry were observed at mobile phase of 60% methanol. The acidic modifier, formic acid, in the mobile phase could improve peak shape, whereas a progressive decreasing response with higher formic acid concentration was observed. It was reported that the negative ESI response decreased at concentrations greater than 1 mM for all of the weakly acidic modifiers [11]. Among the mobile phase additives investigated (formic acid, ammonium formate, acetic acid and ammonium acetate), formic acid gave the best response under equivalent molar concentrations [12]. So the percentage of formic acid was optimized to maintain symmetric peak shape while being consistent with good ionization as far as possible in mass spectrometer. Eventually, a mixture of methanol–water (60:40, v/v) containing 0.005% formic acid was adopted to achieve an efficient chromatographic separation of the analyte and the endogenous plasma components for reducing the matrix effects and shorten running time on an XB-CN column.

It is necessary to use an IS to get high accuracy when using LC–MS/MS method. Erdosteine is a structurally similar analog to fudosteine and has similar chemical and physical behavior to fudosteine, which was adopted in the end.

3.2. Sample preparation

In order to increase sample throughput, the protein precipitation in 96-well format plates was used, which resulted in shorter sample preparation time. The selected protein precipitation was methanol because of its satisfactory efficiency and less extent ion suppression compared to acetonitrile. It was found that adding 100 μL mobile phase to 100 μL supernatant could yield symmetric peak shape and the least dilution for fudosteine.

3.3. Method validation

3.3.1. Selectivity

The selectivity of the method was tested by comparing the chromatograms of five different lots of blank plasma and the spiked plasma. Under the above conditions the retention time of fudosteine and the IS was 2.4 and 2.5 min, respectively. All plasma lots were found to be free of interferences with the compounds of

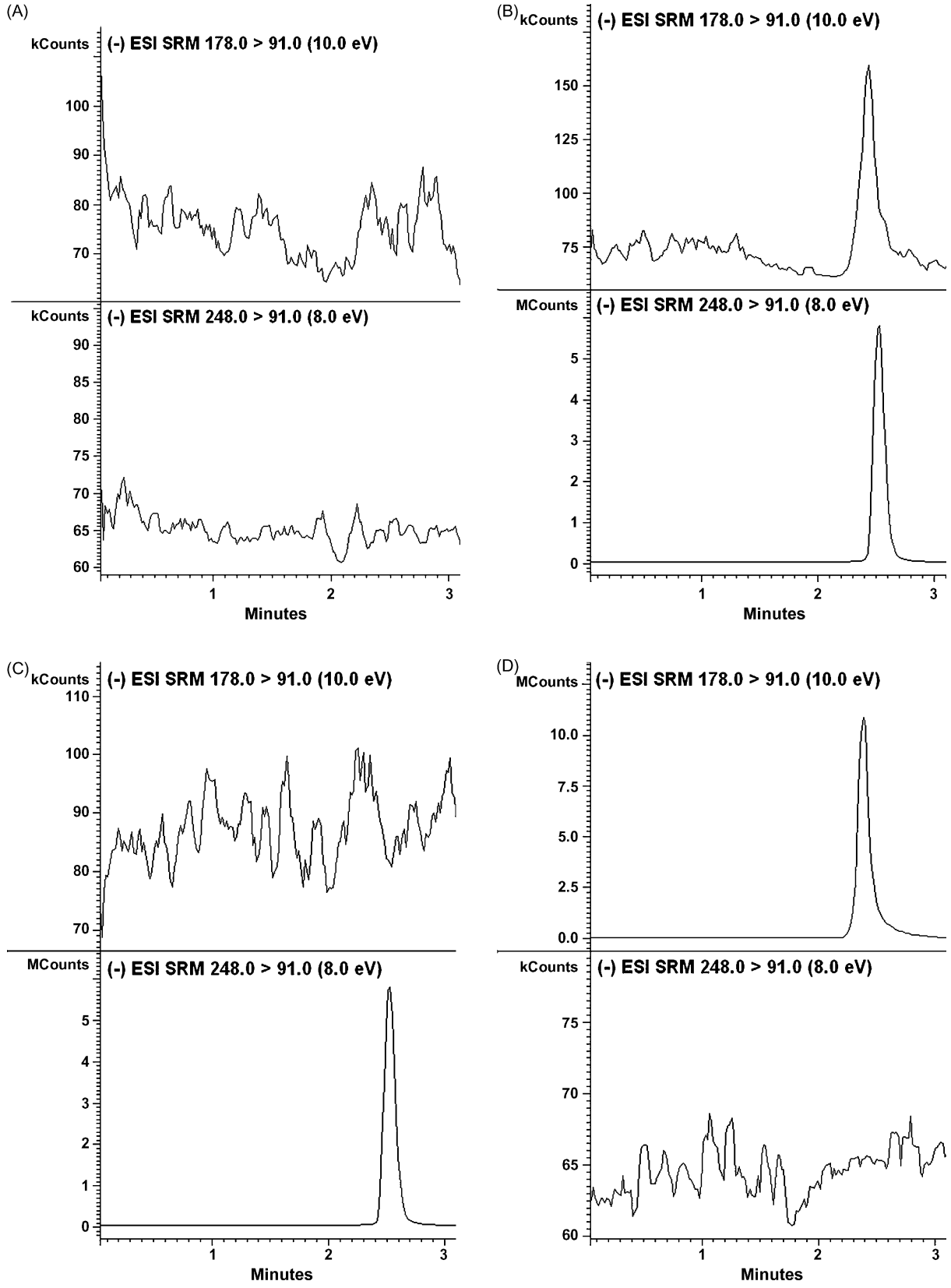

Fig. 2. Representative MRM chromatograms: (A) blank plasma sample; (B) blank plasma spiked with 0.02 μg mL^{-1} fudosteine and 1 μg mL^{-1} IS; (C) blank plasma spiked with 1 μg mL^{-1} erdosteine; (D) blank plasma spiked with 10 μg mL^{-1} fudosteine; (E) plasma sample collected from a subject 0.5 h after receiving a 400 mg oral dose of fudosteine (concentration 7.92 μg mL^{-1}).

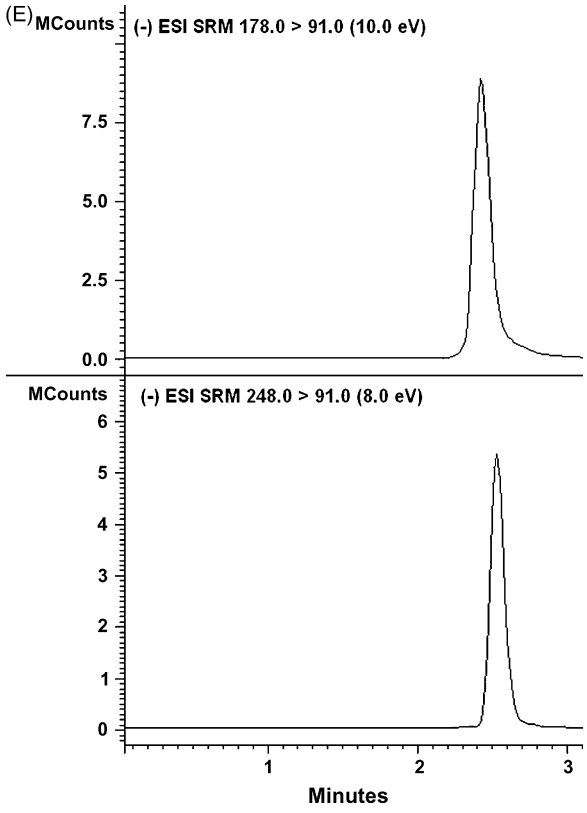

Fig. 2. (*Continued*).

interest. Fig. 2A shows the representative chromatogram of a blank plasma. The LC–MS/MS system was evaluated for the presence of "cross-talk" between the channels used for monitoring fudosteine and IS. Fig. 2C and D clearly shows the absence of any MS/MS response from the analyte into internal standard channel and *vice versa*.

3.3.2. Matrix effects and recovery

When analyzing the supernatant from a plasma sample using protein precipitation, salts and endogenous material are present and can cause ion suppression or enhancement that will lead to higher variation, which is greater than that of solid-phase and liquid–liquid extracts [13]. Table 1 shows the results of the matrix effects of fudosteine and the IS. The mean absolute matrix effect values obtained were 72.0, 74.6, 75.0 and 73.5% for fudosteine at 0.02 (LLOQ), 0.05, 1 and 8 $\mu g\,mL^{-1}$ (low, medium and high QC), respectively ($n = 5$) and 74.4% ($n = 5$) for the IS. The relative matrix effects, expressed as R.S.D. was acceptable with <7.2% for fudosteine and with <5.0% for the IS. This indicated ionization suppression for fudosteine and the IS in the present condition, which were similar and kept consistent at LLOQ and QC concentrations, as well as for five different lots human plasma. It was found that the values of comparing the ratios of fudosteine to the IS for samples spiked postextraction with that for the neat standards at corresponding concentrations was 93.6–100.8% ($n = 5$). The results confirmed that the IS tracked the analyte well in the mass spectrometer ion-source. Despite of the presence of matrix effects, the present LC–MS/MS method was reliable [10]. The extraction recoveries of fudosteine at 0.05, 1 and 8 $\mu g\,mL^{-1}$ were 72.5, 77.0 and 77.2%, respectively ($n = 5$) and that of the IS (1 $\mu g\,mL^{-1}$) was 78.4% ($n = 5$).

3.3.3. Linearity and lower limit of quantification (LLOQ)

A weighted ($1/x$) linear regression was used to perform standard calibration. The mean calibration equation was $y = 0.2981$(R.S.D. = 2.83%, $n = 5$)$x + 0.003033$(R.S.D. = 7.76%, $n = 5$), where y represents the peak area ratios of the analyte to the IS and x represents the plasma concentration of analyte in $\mu g\,mL^{-1}$. Calibration curves showed an excellent linearity in the range 0.02–10 $\mu g\,mL^{-1}$ with the concentration coefficients consistently greater than 0.999. LLOQ samples of five different human plasma, independent from the calibration curves were analyzed and found to be 0.02 $\mu g\,mL^{-1}$ with an accuracy of 107.5%, and within- and

Table 1
Matrix effect for fudosteine and IS ($n = 5$)

Concentration ($\mu g\,mL^{-1}$)	Fudosteine		IS[a]		Fudosteine/IS[b]	
	Mean ± S.D. (%)	R.S.D. (%)	Mean ± S.D. (%)	R.S.D. (%)	Mean ± S.D. (%)	R.S.D. (%)
0.02 (LLOQ)	72.0 ± 5.0	7.2	76.8 ± 2.9	3.8	93.6 ± 4.8	5.1
0.05 (Low QC)	74.6 ± 3.3	4.4	75.8 ± 3.8	5.0	98.6 ± 4.4	4.5
1 (Medium QC)	75.0 ± 3.6	4.8	74.4 ± 2.8	3.8	100.8 ± 3.3	3.2
8 (High QC)	73.5 ± 4.7	6.4	74.7 ± 3.0	4.0	98.4 ± 3.1	3.2

[a] The concentration of the IS was 1 $\mu g\,mL^{-1}$ in the methanol to precipitation protein of LLOQ and three QC levels samples.
[b] The ratios of the mean peak area ratios (fudosteine/IS) of the analysts spiked into plasma postextraction to the mean peak area rations (fudosteine/IS) of the neat standards.

Table 2
Accuracy and precision for fudosteine in human plasma from the method validation and study sample analysis

Run	Low QC 0.05 μg mL^{-1}	Medium QC 1 μg mL^{-1}	High QC 8 μg mL^{-1}
Validation run 1			
Mean ± S.D. (μg mL^{-1})	0.05 ± 0.003	1.01 ± 0.02	7.77 ± 0.25
Accuracy (%)	100.7 ± 5.5	100.5 ± 2.3	97.1 ± 3.1
R.S.D. (%)	5.5	2.2	3.2
n	5	5	5
Validation run 2			
Mean ± S.D. (μg mL^{-1})	0.047 ± 0.002	1.02 ± 0.04	8.30 ± 0.28
Accuracy (%)	94.2 ± 3.0	101.9 ± 3.7	103.8 ± 3.5
R.S.D. (%)	3.2	3.6	3.3
n	5	5	5
Validation run 3			
Mean ± S.D. (μg mL^{-1})	0.05 ± 0.002	1.07 ± 0.04	7.98 ± 0.22
Accuracy (%)	100.0 ± 4.7	106.7 ± 4.2	99.8 ± 2.8
R.S.D. (%)	4.7	3.9	2.8
n	5	5	5
Between-run			
Mean ± S.D. (μg mL^{-1})	0.049 ± 0.002	1.03 ± 0.04	8.02 ± 0.31
Accuracy (%)	98.3 ± 5.0	103.0 ± 4.1	100.2 ± 3.9
R.S.D. (%)	5.1	4.0	3.9
n	15	15	15
Sample analysis run 1			
Mean ± S.D. (μg mL^{-1})	0.048 ± 0.002	1.01 ± 0.03	7.83 ± 0.24
Accuracy (%)	96.5 ± 5.0	100.5 ± 3.0	97.9 ± 3.0
R.S.D. (%)	5.2	3.0	3.1
n	3	3	3
Sample analysis run 2			
Mean ± S.D. (μg mL^{-1})	0.048 ± 0.003	1.05 ± 0.03	8.22 ± 0.18
Accuracy (%)	95.7 ± 5.5	104.8 ± 3.3	102.7 ± 2.2
R.S.D. (%)	5.7	3.2	2.1
n	3	3	3
Between-run			
Mean ± S.D. (μg mL^{-1})	0.048 ± 0.002	1.03 ± 0.03	8.02 ± 0.26
Accuracy (%)	96.1 ± 4.3	102.7 ± 3.4	100.3 ± 3.2
R.S.D. (%)	4.5	3.3	3.2
n	6	6	6

between-run precision of 8.0 and 9.2%, respectively. The LLOQ was sufficient for pharmacokinetic studies of fudosteine formulation products in human.

3.3.4. Accuracy and precision

Five replicate samples at each QC concentrations were analyzed in three separate runs. Accuracy was determined by calculating the ratios of the predicted concentrations to the spiked values and with the precision expressed as R.S.D. Table 2 shows the summary of the individual QC data obtain in the three runs used for the validation. 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 the LC–MS/MS method following 96-well protein precipitation in determination of fudosteine in human plasma.

3.3.5. Stability

The stability of fudosteine was studied under various conditions. The mean values and standard deviations of the ratios between the concentrations found and initial concentration was used for stability evaluation. Fudosteine 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 protein precipitation and after three freeze–thaw cycles with the values 97.3–101.1, 99.1–101.6, 98.3–103.5 and 97.7–98.5%, respectively, at the three concentrations studied.

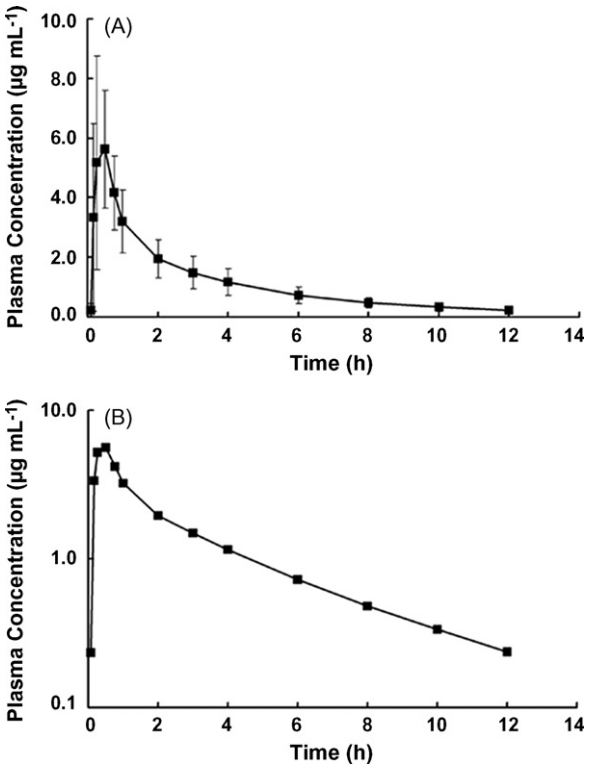

Fig. 3. Mean plasma concentration–time curve in 10 male, adult, healthy Chinese volunteers when administered oral doses of 400 mg fudosteine. (A) linear and (B) log-transform scale.

3.4. Application to clinical pharmacokinetic study

The assay has successfully been utilized to analyze samples obtained from subjects administered oral doses of fudosteine. Mean plasma concentration–time curves are shown in Fig. 3. The concentration–time data were analyzed by non-compartmental method and the mean values of pharmacokinetic parameters are C_{max} 6.39 ± 2.67 μg mL^{-1}, T_{max} 0.42 ± 0.18 h, $t_{1/2}$ 3.89 ± 0.65 h, MRT 4.55 ± 0.74 h, AUC_{0-12} 14.10 ± 4.88 μg h mL^{-1}, $AUC_{0-\infty}$ 15.40 ± 5.13 μg h mL^{-1}. Jiao et al. [5] and Ding et al. [3] reported the pharmacokinetic studies of fudosteine in 10 and 12 healthy Chinese volunteers after single-dose administration of 400 mg, with an even proportion of both sexes, respectively. The mean AUC observed in Jiao's study [5] is 23.95 μg h mL^{-1} and in Ding's study [3] 29.00 μg h mL^{-1}. In our study on ten healthy male volunteers, the mean AUC is 15.40 μg h mL^{-1}, about 45% lower than Jiao's and Ding's. C_{max} is 6.39 μg mL^{-1} in our report, which is lower than Jiao's study (10.13 μg mL^{-1}) and Ding's report (11.00 μg mL^{-1}). The discrepancies of AUC and C_{max} from other studies seemed to be linked to the sexual difference and the between-study difference (for example, in the study population, the study conduct, the study formulation and inter-individual variability). Therefore, further study should be focused on the disposition and pharmacokinetics of fudosteine between different genders. The present method proved to be suitable for pharmacokinetic study to determine the concentrations of fudosteine in human plasma.

4. Conclusion

A rapid and simple LC–MS/MS assay has been developed for determination of fudosteine in human plasma using protein precipitation in 96-well format for sample preparation. The structure analog, erdosteine was used as the IS and could tracked the fudosteine well in the mass spectrometer ion-source for their sim-

ilar ionization suppression. The assay was validated and all results meet the purpose of the high-throughput bioanalysis of fudosteine in human plasma.

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