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Simultaneous quantitative analysis of fasudil and its active metabolite in human plasma by liquid chromatography electro-spray tandem mass spectrometry

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ABSTRACT

A fast and sensitive method to quantify fasudil hydrochloride (FH) and its active metabolite hydroxyfasudil (M3) in human plasma using HPLC–MS/MS has been developed and validated in present study. The method involved simple sample preparation with methanol as protein precipitation (3:1, v/v) and ranitidine as an internal standard (IS). The analytes and IS were separated using a gradient elution procedure on the analytical column ZORBAX StableBond-C18 (5 μm, 150 mm × 4.6 mm). Detection was performed by an AB 3200 QTRAP tandem mass spectrometer equipped with a Turbo IonSpray ionization source set in positive ion mode. Multiple reaction monitoring (MRM) using the precursor to product ion was *m/z* 292.2/99.2 for fasudil, *m/z* 308.2/99.2 for M3 and *m/z* for 315.3/176.2 for IS. The linear range of the method was from 0.4 to 250 ng/mL for both fasudil and M3. The lower limit of quantification was 0.4 ng/mL for both fasudil and M3. The intra- and inter-day relative standard deviation over the entire concentration range was less than 7.11% for fasudil and 10.6% for M3, respectively. The validated method was successfully applied for the evaluation of pharmacokinetic of fasudil hydrochloride after administration of 30 mg fasudil hydrochloride by continuous intravenous infusion over 30 min in 12 healthy Chinese volunteers.

1. Introduction

Fasudil hydrochloride (1-[5-isoquinolinesulfonyl]-homopiperazine) (FH) has been developed as a new type of anti-vasospasm agent, highly distinct in its mechanism of action from currently used Ca^{2+} antagonists/channel blockers [1,2]. FH is thought to work by inhibiting protein kinases such as protein kinases A, G and C; myosin light chain kinase and Rho-associated protein kinase [3]. The safety and efficacy of FH in patients with subarachnoid hemorrhage (SAH) was reported by Yoshio et al. and Tatsuhiro et al. [4,5]. It has become popular for the prevention of cerebral vasospasm and subsequent ischemic injury after surgery for SAH in recent years. In humans FH is rapidly metabolized by cytosolic enzymes in the liver to its active metabolite hydroxyfasudil (1-[1-hydroxy-5-isoquinolinesulfonyl]-homopiperazine; M3), which preferentially inhibits Rho-kinase and plays a predominant role in vasospasm treatment [6,7]. Thus, there is a need to develop and validate a biological analytical method to separate and quantify fasudil and M3 concentration in human plasma for biological research related to pharmacokinetics and bioequivalence.

However, the biological analytical method for the quantification of fasudil and M3 in human plasma still lacked exhaustive data of method validation. Although Yusei et al. [8] reported a high pressure liquid chromatography tandem to mass spectrometry (HPLC–MS/MS) method for determination of FH and M3 in human serum and cerebrospinal fluid, when they studied chronologic changes of FH and M3 in cerebrospinal fluid of patients with SAH, they did not describe the biological sample preparation, the chromatography parameters or the mass spectrometry parameters. In addition, Peter et al. [9] has briefly described the assay method for FH and M3 in human plasma by HPLC–MS/MS, when they studied the systemic availability of the active metabolite M3 after administration of FH to different sites of the human gastrointestinal tract. In the above method, the plasma preparation required complicated solid-phase extraction procedure and method validation did not provide exhaustive data of method validation.

In present study, we developed a fast and selective method using HPLC–MS/MS with electro-spray ionization for simultaneously quantifying the concentration of FH and M3 in human plasma and provided exhaustive validation data of the biological analysis method. This newly developed method provides high sensitivity, reliable reproduction, small plasma volume and a simple one-step protein precipitation preparation. Furthermore, in this method, the

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developed gradient elution procedure could help to favorably protect the column, and therefore successfully separate more than two thousand human plasma samples through the same column, an important advantage given the need to analyze a great number of samples during a pharmacokinetic study. This method was applied successfully to a clinical pharmacokinetic study of FH and its active metabolite after a single dose of 30 mg FH by continuous intravenous infusion over 30 min in 12 healthy Chinese volunteers.

2. Experimental

2.1. Chemical and reagents

Fasudil methylsulphonic acid (purity 99.7%) was kindly supplied by WuHan QiRui Science and Technology Co. (China). Hydroxyfasudil hydrochloride (purity 98.3%) was purchased from Sigma (USA). Ranitidine (internal standard, IS, purity 99.0%) was obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC-grade formic acid was purchased from Tedia reagent corporation (Tedia, USA) and HPLC-grade methanol from Fisher Corporation (USA). Ammonium formate was obtained from Acros Organics (purity 99.0%) (NJ, USA). Human blank plasma samples were provided by the Blood Bank of Beijing AnZhen Hospital.

2.2. Instruments

An Agilent 1100 system consisting of G1322A degasser, G1311A quaternary pump, G1313A autosampler and G1316A thermostatted column compartment (USA) was used to inject and separate sample. An AB 3200 QTRAP triple-quadrupole tandem linear ion trap mass spectrometry equipped with Turbo Electro-Spray Ion (ESI) was used for mass analysis and detection (Applied Biosystems, USA). Data processing was performed on Analyst 1.4.2 software package. Other instruments including electron balance (Sartorius, Germany), WaterPro PS-system (Labconco, USA), refrigerated centrifuge (Sigma, Germany) and nitrogen evaporation device, etc.

2.3. Standard solutions and quality control samples

Stock solutions of FH (1 mg/mL), M3 (100 μg/mL) and ranitidine (IS, 1 mg/mL) were prepared in 50% methanol (methanol:water, 1:1, v/v). The standard working solutions and quality control samples were prepared from different stock solution by making appropriate dilution with methanol–water (1:1, v/v). The nominal plasma concentration of calibration standards were prepared by adding appropriate volumes of working solutions to drug-free plasma to give final concentrations of 0.4, 2.0, 5.0, 50, 100, 200, 250 ng/mL for both FH and M3. Three levels of quality controls (QCs) fixed at 1/1 (low QC), 80/80 (medium QC) and 160/160 (high QC) for FH and M3, respectively, were prepared using the same blank plasma utilized to prepare the calibration-standard curves. The IS of the working solution of a concentration of 10 ng/mL was obtained by diluting stock solutions with methanol. All the prepared solutions were stored at −20 °C until used.

2.4. Plasma sample preparation

A 100 μL aliquot of the IS solution (10 ng/mL) and 500 μL of methanol were added to 200 μL of plasma samples and the tubes were then vortex-mixed for 30 s. Then, the tubes were centrifuged at 11,000 rpm for 5 min. A 500 μL sample of the supernatant was carefully removed, transferred to new tubes, and evaporated to dryness under N_2 at 40 °C. The residue was reconstituted in 100 μL of the mobile phase followed by vortex-mixing for 1 min. The mixture was centrifuged at 11,000 rpm for 5 min and a 20 μL aliquot of the supernatant was injected for LC–MS/MS analysis.

2.5. Chromatographic conditions

The chromatographic separation was performed on 150 mm × 4.6 mm column packed with 5 μm C18 silica particles (ZORBAX SB-C18, Agilent, USA). A guard column, 12.5 mm × 4.6 mm packed with the same C18 material, was used to protect the column. After trying several different HPLC gradient modes using various amounts of methanol and formic acid in the mobile phase, a set of optimum mobile phase conditions was reached. The mobile phase consisted of eluent A (2 mM ammonium formate solution being composed of 0.1% formic acid, v/v) and eluent B (methanol). The detailed gradient program was described: 0–1.8 min 20–80% eluent A (linear gradient, v/v); 1.8–3.0 min constant at 80% eluent B; 3.0–3.1 min changed to 20% eluent B; 3.1–5 min constant at 20% eluent B. The flow rate was set at 1.0 mL/min. The column eluant of approximately 7:10 was split using three-way valves and approximately 300 μL/min entered the mass spectrometer. The injection volume was 20 μL and the column temperature was maintained at 35 °C.

2.6. Mass spectrometric conditions

The mass spectrometer was operated in positive ionization mode. The tuning parameters were optimized for FH, M3 and IS by infusing a solution containing 500 ng/mL of the three analytes at a flow rate of 5 μL/min into the mobile phase (0.3 mL/min) using a post-column "T" connection. The nebulizer (Gas 1), heated auxiliary gas (Gas 2) and curtain gas (nitrogen) were set at 50, 70, 25 psi, respectively. For collision-induced dissociation (CID), nitrogen was used as the collision gas at a back-pressure of approximately 6 psi. The optimized TurboIonSpray voltage and temperature were set at 5500 V and 750 °C, respectively. Quantification was performed using multiple reaction monitoring (MRM) of the transitions m/z 292.2 → 99.2, m/z 308.2 → 99.2 and m/z 315.3 → 176.2 for FH, M3 and IS, respectively, with a dwell time of 200 ms per transition. The optimized collision energy of 40 eV was used for both FH and M3 and 25 eV for the IS. The mass spectrometer was operated at low mass resolution for Q1 scan and at unit mass resolution for Q3 scan, respectively. The value of DP (declustering potential) was set at 60 V for both FH and M3 and 40 V for IS, respectively.

2.7. Method validation

The described procedure was validated according to internationally accepted recommendations for guidance for bioanalytical method validation from center for drug evaluation and research (CDER) in FDA in USA [10] and Shah et al. published the document [11]. The validation parameters of the development method were composed of linearity, selectivity, LLOQ, accuracy, precision, recovery, stability and matrix effects. 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 five consecutive days over the range of 0.4–250 ng/mL for both FH and M3. Least-squares linear regression was used for curve fitting with $1/x$ as the weighting factor. The lower limit of quantification (LLOQ) was defined as the lowest concentration required for a signal-to-noise ratio of at least 10 according to the guidance of biological analytical method and both precision and accuracy were less than or equal to 20%, which was evaluated by analyzing preparation samples of five replicates.

QC samples at three concentration levels (1/1, 80/80, 160/160 ng/mL) for both fasudil and M3 were analyzed to assess the accuracy and precision of the developed method. The QC sam-

ples of method validation were assayed on five consecutive days, and on each day five replicates of QC samples at each concentration level were analyzed. The accuracy and precision were calculated using Microsoft office excel 2003. The accuracy was expressed by relative error (R.E.) and the accuracy to be within ±15%. The intra- and inter-day precision of this method was calculated from the percentage relative standard deviations (R.S.D.%) for the repeated measurements and was required to be below 15%.

The extraction efficiencies of FH and M3 in course of the human plasma sample processing were determined by analyzing QC. The samples were extracted according to the procedure described above. Recovery was determined by comparing the mean of the area or response of five-plasma sample of each QC levels ($n = 5$) spiked before extraction with that spiked after extraction. The recoveries of IS was also determined using the same procedure. The recoveries were required to provide reliable reproduction and have to be higher than 50% according to the guidance of method validation.

To evaluate the matrix effect, i.e., the potential ion suppression or enhancement due to co-eluting plasma components, six different lots of blank plasma were extracted and then spiked with the analyte at three levels (1/1, 80/80, 160/160 ng/mL) and IS is 10 ng/mL. The corresponding peak areas of the analyte in spiked plasma post-extraction (A), were then compared to those of the solution standards in spiked 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 FH, hydroxyfasudil and IS in the plasma extracts and in the mobile phase were same and that no absolute ME was observed. A value of <100% indicates ionization suppression, and a value of >100% indicates ionization enhancement.

The stability validation of FH and hydroxyfasudil were investigated including freeze and thaw stability, short-term temperature stability, long-term stability and post-preparative stability in the developed method. Freezing and thawing stability study for the two analytes in QC samples was determined in three freeze–thaw cycles during 3 days at −20 °C. Short-term stability of the analytes in QC samples after 48 h of storage at room temperature was evaluated. The stability of the two analytes in human plasma extracted with methanol and stored in the autosampler was investigated after 48 h. In addition, long-term stability of FH and hydroxyfasudil in QC samples after 30 days of storage at (−80 °C) was also evaluated. All samples were analyzed using freshly prepared calibration samples.

2.8. Application to pharmacokinetics study

The developed the LC–MS/MS method was applied to determine the plasma concentrations of FH and M3 from a clinical trial study in which 12 (6 males and 6 females) healthy Chinese subjects after a single intravenous injection (once per day) and 10 healthy Chinese subjects (5 males and 5 females) after multiple intravenous injection (three times per day), respectively. The pharmacokinetic study was approved by the Medical Ethics Committee of Beijing Anzhen Hospital. Informed consent was obtained from all subjects after explaining the aims and risks of the study. The doses of FH chosen for this study were based on findings from preclinical and early clinical studies. Twelve volunteers were randomized into two groups. They were intravenously injected with FH or fasudil methylsulphonic acid equivalent to fasudil base at 30 mg. The 3 mL of venous blood samples were collected into heparinized tubes before and 0.167, 0.333, 0.500, 0.583, 0.667, 0.75, 1.0, 1.25, 1.50, 2.0, 4.0, 6.0, 8.0 and 12.0 h post-dosing, and centrifuged at 3000 rpm (4 °C) for 10 min to separate the upper plasma. The collected plasma samples were stored −80 °C until analysis.

Calculation of the pharmacokinetic parameters for FH and hydroxyfasudil was performed by non-compartmental assessment of data using the computer program DAS 2.1.1 version (Mathematical Pharmacology Professional Committee of China, Shanghai, China). The maximum plasma concentrations (C_{max}) and their time of occurrence (T_{max}) were both obtained directly from the measured data. The area under the plasma concentration–time curve from time zero to the time of the last measurable concentration (AUC_{0-t}) was calculated by the linear trapezoidal method. The terminal elimination rate constant (K_e) was estimated by log–linear regression of concentrations observed during the terminal phase of elimination, and the corresponding elimination half-life ($t_{1/2}$) was then calculated as $0.693/K_e$.

3. Results and discussion

3.1. Establishment of the chromatographic conditions and the MS parameters

For studying the pharmacokinetics of FH and its active metabolite, a simple and rapid preparation procedure with reproductive and satisfactory selectivity and sensitivity is always desirable. In this work, an HPLC–MS/MS method for determination of FH and hydroxyfasudil in human plasma was developed and validated. The chromatographic conditions and MS parameters were optimized such that the gradient elution procedure was used to increase the sensitivity of ion response of the analytes and obtain the baseline separation of FH and hydroxyfasudil. In addition, the solvent A of the mobile phase consisting of 2 mM ammonium formate and 0.1% (v/v) formic acid also enhanced the intensity of ion response of the analytes. An analytical column SB-C18 5 μm (150 mm × 4.6 mm) was selected to successfully complete the separation of more than one thousand of human plasma samples.

In the developed HPLC–MS/MS method, by positive ESI mode, FH, M3 and IS predominantly protonated molecule $[M+H]^+$ at m/z 292.2, 308.2, 315.2 in Q1 full scan mass spectra, respectively. The most suitable collision energy (CE) for the analytes and IS was determined by observing the response of the obtained fragment ion peaks. Fig. 1 displays the product ions spectra of $[M+H]^+$ ions from analytes and IS when the CE was optimized at 40 eV for both FH and M3 and the CE was optimized at 25 eV for IS. The major fragment ions at m/z 99.2, 99.2 and 176.2 are chosen in the MRM for FH, M3 and IS, respectively.

In this method, human sample preparation utilizes a simple one-step protein precipitation with 3 folds volume of methanol before the run, and then was performed using a gradient elution that could provide stable retention time, return quickly to the initial condition (5 min per sample) while keeping the column clean. In addition, minimal eluent entered into the spray needle of ion source by using a three-way valve that better protects the MS analyzer. Thus, the developed method could provide a very high sample throughput (approximately 200 samples per day) in the result of the relatively simple sample preparation procedure and a short chromatographic analysis time.

3.2. Method validation

3.2.1. Assay selectivity and matrix effect

The method selectivity was evaluated by comparing chromatograms of six extracted blank plasma samples of different sources with those of plasma samples spiked with FH, M3 and its IS. Representative total ion chromatograms (TIC) of blank plasma sample and spiked with FH, M3 and IS QC sample by MRM scan were shown in Fig. 2A and C, respectively. In addition, Fig. 2C shows a TIC of a volunteer human sample of 10 min after administration of 30 mg FH by intravenous injection for 30 min. Under optimum method conditions, no interference (matrix effect) was observed

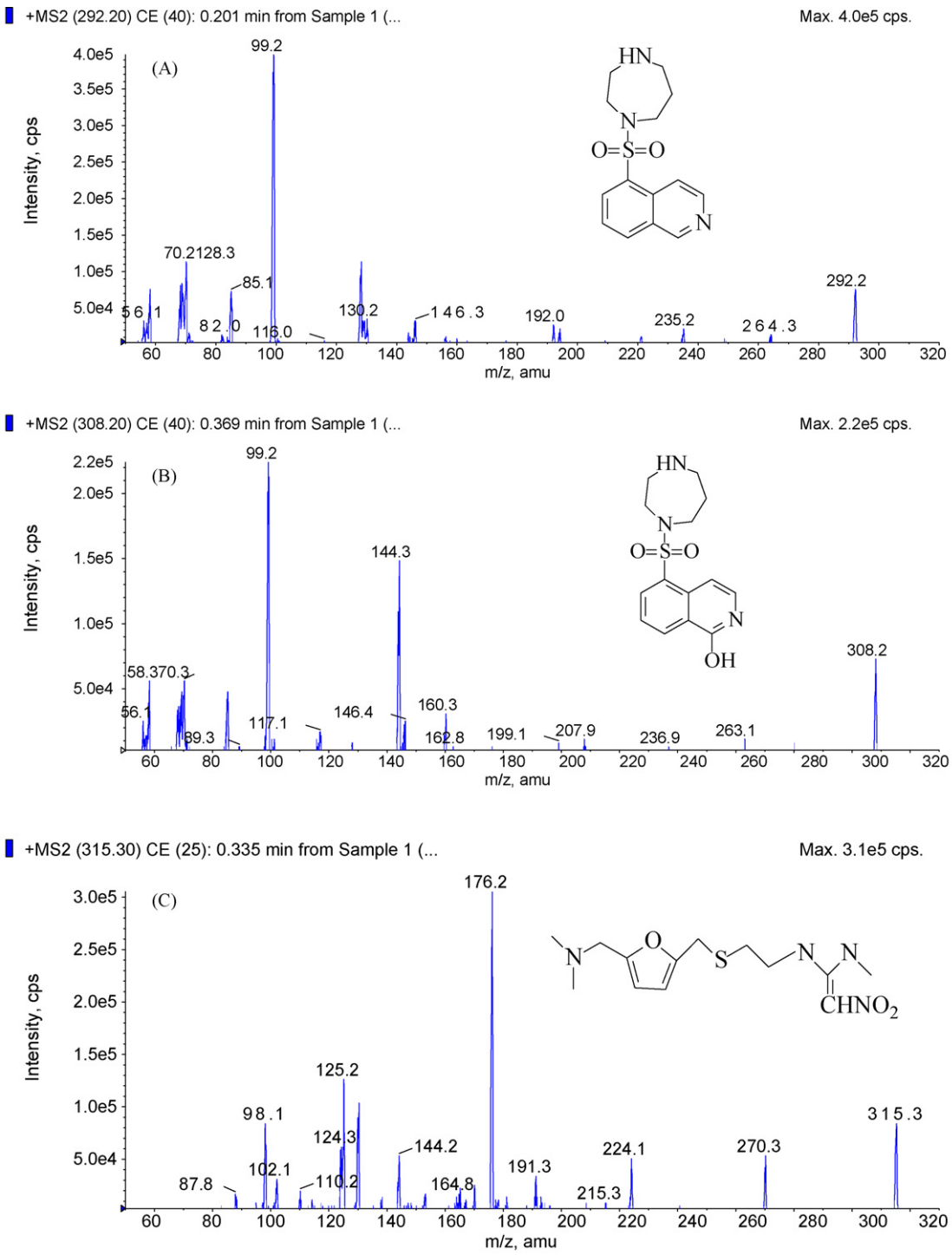

Fig. 1. Product ion spectra of $[M+H]^+$ of fasudil (A), M3 (B) and ranitidine (C).

from endogenous compounds following the extraction of spiked sample plasma over the time window of elution, and gave retention times of 3.41, 3.29, 2.75 min for FH, M3 and IS, respectively. The cross-talk effect at the retention time of compound did not occur because baseline separation can be achieved between the FH and M3.

In this experiment, the estimation of matrix effect was conducted following the procedures described in Section 2.7. By the analysis of six different lots of blank plasma, matrix effect values were calculated. The absolute matrix effect values were 93.8, 91.2 and 95.4% for FH at concentrations of 1, 80, and 160 ng/mL, respectively, and were 92.7, 93.5 and 94.4% for M3 at concentrations of 1, 80, and 160 ng/mL, respectively. The absolute matrix effect for I.S. was 95.7%. The results indicated that no co-eluting substances significantly influenced the ionization of these analytes.

3.2.2. Linearity and lower limit of quantification

The linear regression of the peak area ratios versus concentrations were fitted over the concentration range 0.4–250 ng/mL for both FH and M3. Typical equations of the calibration curves were as follows: FH: $y = 0.0118x + 0.000386$, $r^2 = 0.9997$; M3:

Table 1
Precision and accuracy in the quantification of fasudil and M3 in human plasma sample (5 days, five replicates per day).

Added concentration (ng/mL)	Intra-day			Inter-day		
	Found (mean) (ng/mL)	R.S.D. (%)	R.E. (%)	Found (mean) (ng/mL)	R.S.D. (%)	R.E. (%)
Fasudil						
0.4	0.365	8.51	−8.8	*0.381*	*7.99*	*−4.8*
1.0	0.954	7.11	−4.6	0.921	5.51	−7.9
80	82.6	1.86	3.0	81.9	5.40	2.0
160	153	1.39	−4.4	154	2.96	−3.4
M3						
0.4	0.376	14.0	−6.0	*0.382*	*4.25*	*−4.4*
1.0	0.953	10.6	−4.7	0.957	8.82	−4.4
80	76.3	4.26	−4.7	77.5	8.52	−3.2
160	139	0.322	−13.2	144	3.84	−9.8

R.E., relative error.

$y = 0.00574x + 0.000181$, $r^2 = 0.9991$, where y represents the ratios of FH or M3 peak area to that of IS and x represents the plasma concentrations of FH or M3.

The lower limit of quantification was 0.4 ng/mL for both FH and M3. It was shown in Table 1 that the precision and accuracy at LLOQ were 8.51% and −8.8% (R.E.) or FH, while the values were 14.0% and −6.0% (R.E.) for M3. The precision and accuracy at this concentration level were acceptable, with R.S.D. values below 14.0% and R.E. values within ±8.8%. The results indicated that the present LLOQ of this method was sensitive enough to investigate the pharmacokinetics of FH injection.

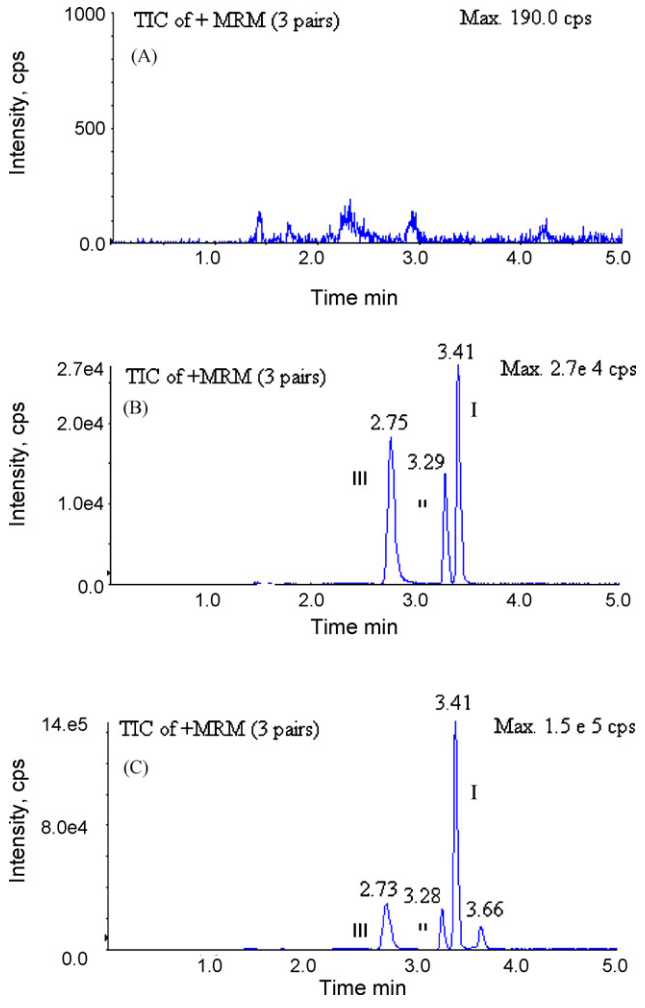

Fig. 2. Total ion chromagrams of representative MRM scan: (A) a blank plasma sample; (B) a blank plasma sample spiked with fasudil (80 ng/mL), M3 (80 ng/mL) and IS (10 ng/mL); (C) a volunteer human sample of 10 min after administration of 30 mg FH by intravenous infusion over 30 min. Peaks I–III refer to the fasudil, M3 and IS.

3.2.3. Precision and accuracy

The precision of the method was determined by calculating RSD for QCs at three concentration levels over five validation days using Microsoft EXCEL analysis. The results are shown as Table 1. The intra-day and inter-day precision were ≤7.11% and ≤5.51% for FH, respectively; ≤10.6% and ≤8.82% for M3, respectively. The accuracy ranged from 2.0% to −7.9% (R.E.) for FH and from −3.2% to −13.2% (R.E.) for M3. The results follow the guidance of bioanalytical method validation recommends that the precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ and the accuracy should be within 15% of the actual value except at LLOQ.

3.2.4. Extraction recovery and stability

In this method, the absolute extraction of FH and M3 were determined at three different concentrations (1.0, 80.0, 160.0 ng/mL). Mean extraction recoveries were 72.3 ± 9.41, 77.2 ± 1.83 and 72.6 ± 8.26% for FH and 66.1 ± 4.67, 61.9 ± 3.60, 64.6 ± 2.9% for M3, respectively ($n = 5$). Mean recovery of the IS (10 ng/mL) was 74.6 ± 5.47%. These statistic data indicated the extent of recovery of the analytes of the internal standard were consistent, precise, and reproducible.

As the stability of FH and M3 in human plasma has not been determined, the stabilities of FH and M3 in human plasma were evaluated by analyzing replicates ($n = 3$) at the concentration of 1.0/1.0, 80.0/80.0 and 160/160 ng/mL for FH/M3 when they were exposed to different circumstances such as the process of sample collection and handling, after long-term and short-term storage, after going through freeze and thaw cycles, the analytical process and analyte in stock solution. It was shown as Table 2 that analyte stability was acceptable during all phases of storage and processing. No significant degradation was occurred during chromatography, extraction, short-term or long-term storage for FH and M3 in plasma samples. The results also indicated that the analytes were stable at room temperature for 48 h after the reconstitution of plasma extra residue with mobile phase. In addition, stock solution of FH and M3 in methanol was shown to be stable for 1 month at −20 °C.

3.3. Application in pharmacokinetics study

The LC–MS/MS method described above had been applied successfully to the pharmacokinetic study of FH in healthy Chinese subjects. Using this analytical method, we were able to measure the concentration of FH for up to 4 h and M3 for up to 12 h for all subjects

Table 2
Stability of QC samples of fasudil and M3 in human plasma under different storage conditions ($n = 3$).

Storage conditions	Fasudil (ng/mL)				M3 (ng/mL)			
	Added	Found (mean)	R.S.D. (%)	R.E. (%)	Added	Found (mean)	R.S.D. (%)	R.E. (%)
Three freeze/thaw cycles	1.0	1.02	7.20	2.0	1.0	0.983	7.98	−1.7
	160	166	8.52	3.8	160	163	8.37	1.9
Post-processing stability for 48 h	1.0	0.994	3.69	−0.6	1.0	0.910	3.03	−9.0
	160	151	2.13	−5.6	160	149	4.72	−6.9
Short-term stability for 24 h	1.0	0.975	4.84	−2.5	1.0	0.949	7.6	−5.1
	160	156	1.92	−2.5	160	155	3.8	−3.1
Long-term stability for 30 days	1.0	0.952	11.8	−4.8	1.0	0.943	2.50	−5.7
	160	148	1.0	−7.5	160	154	1.38	−3.8

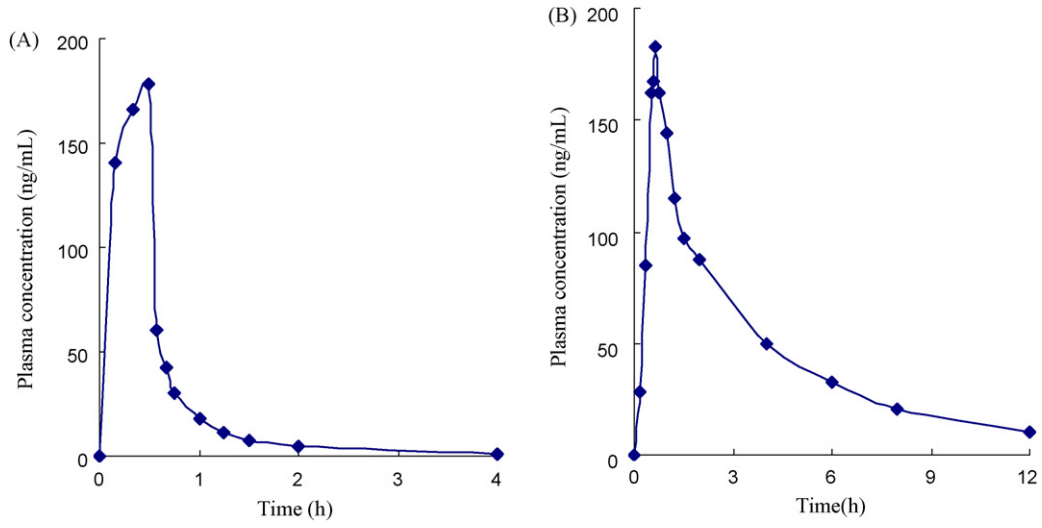

Fig. 3. Mean plasma concentration–time curve of fasudil (A) and M3 (B) after a single dose of 30 mg FH by continuous intravenous infusion over 30 min to 12 Chinese healthy volunteers.

Table 3
Major pharmacokinetic parameters for fasudil and M3 after administration of 30 mg FH by continuous intravenous infusion over 30 min to 12 healthy volunteers.

Parameter	Fasudil	M3
C_{max} (mg/L)	188 ± 52	190 ± 35
T_{max} (h)	0.5 ± 0.00	0.646 ± 0.138
AUC_{0-t} (mg h/L)	69.0 ± 30.2	555 ± 112
K_e (h^{-1})	2.17 ± 0.45	0.248 ± 0.036
$t_{1/2}$ (h)	0.335 ± 0.087	2.85 ± 0.43

after a single of FH injection equivalent to 30 mg fasudil base. Fig. 3 shows the profile of the mean FH and M3 plasma concentration versus time. Meanwhile, the main pharmacokinetics parameters for FH and M3 are presented in Table 3. When the same doses of FH were administered, it was observed that the value of $t_{1/2}$ in this study is a little longer compared with the pharmacokinetic results reported previously [12], but that T_{max} did not differ between them, which might be resulted from the different sensitivities of the analytical methods. The value of C_{max} that we obtained is similar to the previous published pharmacokinetic studies [12].

4. Conclusion

A rapid and reproducible LC–MS/MS method with high selectivity was developed and validated for the simultaneous determination of FH and its active metabolite M3 in human plasma. This method was sensitive enough to accurately measure FH and its metabolite in human plasma after administration of 30 mg FH by continuous intravenous infusion over 30 min. In this method, the developed gradient elution procedure could afford high loading capacity to successfully separate more two thousands of human plasma samples on same column. Furthermore, this method offered advantages of wide linear concentration range, short run time and simple sample preparation. It was successfully applied to characterize the pharmacokinetics of FH to 12 healthy Chinese volunteers.

To contrast the developed method with two previous papers [8,9], it is obvious that this method have significant advantages over existing systems. First, we developed a simple one-step sample preparation only using methanol as precipitation. Secondly, we used gradient elution procedure to increase sensitivity and shorten the retention time. Thirdly, minimal eluent entered into the spray needle of ion source by using a three-way valve so that it contributes to protect mass analyzer. Finally, the developed method could provide a very high sample throughput (approximately 200 samples per day) as the result of the relatively simple sample preparation procedure and a short chromatographic analysis time. Furthermore, in the current study, it is the most important that the exhaustive validation data of the biological analysis method on the FH and its active metabolite M3 was firstly provided. There are possibilities that the basic pharmacokinetic data from healthy Chinese volunteers will provide helpful reference in developing future research on the patient that will accept FH treatment.

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