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Quantitative determination of diterpenoid alkaloid Fuziline by hydrophilic interaction liquid chromatography (HILIC)–electrospray ionization mass spectrometry and its application to pharmacokinetic study in rats

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ABSTRACT

A rapid, sensitive and specific hydrophilic interaction liquid chromatography coupled to electrospray ionization mass spectrometric (HILIC–MS) method for the quantification of Fuziline (15α-Hydroxyneoline) in rat plasma was developed and validated. After liquid–liquid extraction with ethyl acetate, Fuziline and Guanfu base A (internal standard) were separated with HILIC Chrom Matrix HP amide column (5 μm, 10 cm × 3.0 mm I.D.) with isocratic elution at a flow-rate of 0.2 mL/min. The analytes were detected by using an electrospray positive ionization mass spectrometry in the selected ion monitoring (SIM) mode. A good linear relationship was obtained in the concentration ranging from 1 to 1000 ng/mL (R^2 = 0.999) with the lower limit of quantification (LLOQ) at 1 ng/mL and limit of detection (LOD) at 0.5 ng/mL. The average recoveries of Fuziline in plasma at the concentrations of 2, 50, 1000 ng/mL ranged from 68.2 to 69.9%. Intra- and inter-batch relative standard deviations ranged from 1.5 to 3.3% and 2.6 to 8.3%, respectively. Fuziline was stable under different sample storage and processing conditions except three-cycle freeze–thaw treatment at 2 ng/mL. This method was successfully applied to the pharmacokinetic studies in Sprague-Dawley rats. The absolute bioavailability of Fuziline after oral administration 4 mg/kg Fuziline in rats was 21.1 ± 7.0%, with clearance rate at 1745.6 ± 818.1 mL/kg/h, and half-life at about 6.3 ± 2.6 h.

1. Introduction

In 2005 Guanfu base A (Fig. 1), isolated from *Aconitum coreanum* (Lè vl.) Rapaics, was approved by China Food and Drug Administration (SFDA) to treat arrhythmia as class I new drug in China [1]. This event aroused great interest to anti-arrhythmia research based on traditional Chinese medicine.

Radix Aconiti Lateralis Preparata (Fuzi) is prepared from the sub root of *Aconitum carmichaeli* Debx or other near-relative species of the same genus [2]. It is widely used as a valuable traditional Chinese medicine for the treatment of arthralgia and heart failure [3]. Modern pharmacological studies revealed that Fuzi played an important role in anti-arrhythmia, anti-inflammatory and improving immunity [4]. Pharmacologically active components are considered to be diterpenoid alkaloids which include hetisine, Fuziline (15α-Hydroxyneoline, Fig. 1), 8-OEt-14-benzoylmesaconine, aconifine, hypaconitine, mesaconitine and beiwutine [5]. Some researches on hypaconitine and mesoconitine have been carried out by HPLC [6], LC–MS [7], UPLC-TOF/MS [8] and LC–MS/MS [9]. And they all aimed to the qualification or quantification of alkaloids in formulation or decoction. Compared with the wide study of Fuzi on the pharmacological activity, no pharmacokinetic study of Fuziline has been carried out according to our literature searching. To support the preclinical pharmacokinetic study, we have developed a sensitive LC–MS method based on hydrophilic interaction liquid chromatography. This type of LC/MS has never been applied to the study of Fuziline. This method was successfully applied to the pharmacokinetic study in rats.

2. Materials and methods

2.1. Reagents and chemicals

Fuziline (purity >98%) and Guanfu base A (purity >98%) were supplied by Prof Liu Jinghan, Department of Natural Medicinal Chemistry, China Pharmaceutical University (Nanjing, China). The chemical structures of them are also shown in Fig. 1. Methanol, acetonitrile, formic acid, ethyl acetate, sodium hydroxide were analytical grade and purchased from Sigma–Aldrich (St. Louis, MO,

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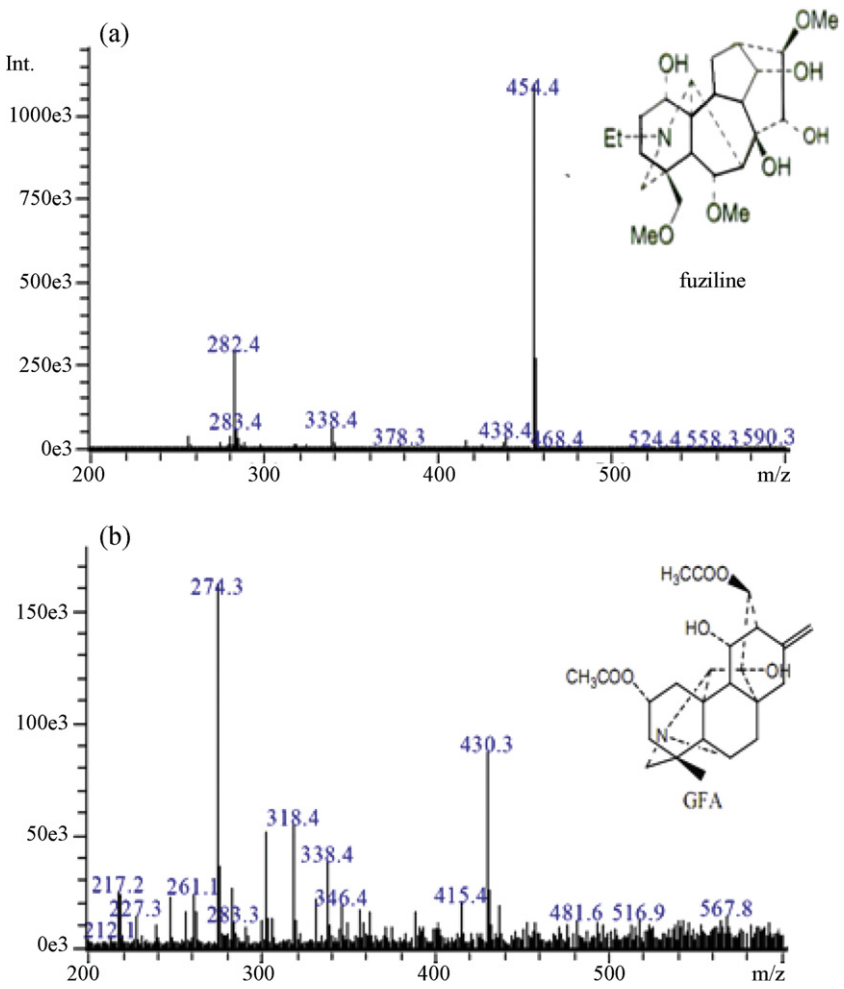

Fig. 1. Chemical structures and mass spectra of protonated molecular ions of internal standard Fuziline(15α-hydroxyneoline) (a) and Guanfu base A (b) in scan mode.

USA). Ultrapure water used throughout the experiments was prepared by Milli-Q Ultrapure water purification system (Millipore, Bedford, USA). Other chemicals and solvents were all of analytical grade.

2.2. Instrument and chromatographic conditions

The HPLC system consisted of a Shimadzu DGU-14 AM online degasser, two Shimadzu LC-10ADvp pumps with a high pressure mixer, a Shimadzu CTO-10Avp column oven and a Shimadzu SIL-HTC autosampler (Shimadzu, Kyoto, Japan). Separation was carried out using a Chrom Matrix HP amide column (5 μm, 10 cm ×3.0 mm I.D.) with mobile phase of water (contained 0.05% formic acid) and acetonitrile at a 60:40 (v/v) ratio. The elution was performed isocratically at a flow rate of 0.2 mL/min and column temperature of 40 °C.

Mass spectrometric analysis was performed on Shimadzu 2010A mass spectrometer (Q-array-Octapole–Quadrupole mass analyzer) equipped with an electrospray ionization (ESI) interface. Vacuum in the mass detector was obtained using a Turbo molecular pump (Edward 28, UK). The MS parameters for ESI were set as follows: CDL voltage 25.0 kV, probe voltage 4.5 kV, Q-array voltage 60 V, RF 150 V, CDL temperature 250 °C, block temperature 200 °C, nebulizing gas flow rate 1.5 L/min, curtain gas at 0.015 mPa. The $[M+H]^+$ ions of Fuziline (*m*/*z* 454.35) and Guanfu base A (*m*/*z* 430.30) were determined for selected ion monitoring (SIM) as best sensitivity in positive ionization mode. The acquisition and processing of data were performed using Shimadzu LC/MS Solution software, version 2.04.

2.3. Preparation of stock solutions, calibration standards and control samples

The stock solutions of Fuziline and Guanfu base A (IS) were prepared in acetonitrile and methanol at concentrations of 1.0 mg/mL, respectively. Working solutions of Fuziline, with concentrations ranging from 10 to 10,000 ng/mL, were prepared by serial dilution with acetonitrile. Internal standard working solution of 1.0 μg/mL was obtained by diluting corresponding stock solution with acetonitrile. All the solutions were stored at 4 °C until use.

For the preparation of calibration standards, 90 μL blank rat plasma was spiked with 10 μL Fuziline working solution to make the plasma concentration of Fuziline at 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000 ng/mL. Quality control (QC) samples were prepared in the same way as calibration standards to yield nominal Fuziline concentrations of 2, 50 and 800 ng/mL, and stored at −20 °C until analysis.

2.4. Sample preparation

The plasma samples were extracted using a liquid–liquid extraction technique. A 100 μL of plasma sample was spiked with 10 μL IS solution (1.0 μg/mL) and mixed. After alkalization with 100 μL of 0.1 mol/L sodium hydroxide solution, 1 mL ethyl acetate was added and the mixture was votexed for 3 min, followed by centrifugation

at 3310 × *g* for 5 min (Thermo Sovall Biofuge Stratos, Germany). An 800 μL supernatant of the upper organic layer was transferred to another Eppendorf tube and evaporated to dryness in a rotary evaporator (SPD2010, Thermo Fisher Scientific, NJ, USA) at 45 °C. The residue was reconstituted in 200 μL acetonitrile and centrifuged at 30,065 × *g* (Thermo Sovall Biofuge Stratos, Germany) and 5.0 μL aliquot was injected for analysis.

2.5. Method validation

The method was validated in terms of specificity, recovery, matrix effect, linearity, accuracy, precision and stability according to the FDA guidelines for validation of bioanalytical method [10].

2.5.1. Specificity

The specificity was assessed by analyzing six different batches of blank rat plasma with and without Fuziline and IS by comparison of corresponding peaks to exclude potential endogenous interference. All the plasma samples were pretreated and analyzed under the same procedure as described above.

2.5.2. Recovery and matrix effect

The recovery for Fuziline and matrix effect from rat plasma extract were determined at 2, 50, 1000 ng/mL ($n = 6$) by comparing three sets of samples: (A) Fuziline spiked into plasma before extraction but IS spiked into dry residue, (B) both Fuziline and IS spiked into the residue after extraction of blank plasma, and (C) Fuziline and IS spiked directly into acetonitrile. Recovery was calculated as percentage of the peak area ratio (Fuziline/IS) of set A compared to that of set B. Similarly, matrix effect was calculated as percentage of the peak area ratio (Fuziline/IS) of set B compared to that of set C.

2.5.3. Linearity

Calibration standards were prepared by spiking 10 μL working solutions into 90 μL drug-free rat plasma to achieve final concentration 1, 2, 5, 10, 20, 50, 100, 200, 500 and 1000 ng/mL. The plasma sample was processed according to the procedure mentioned as before. Ten point calibration curves were constructed by plotting peak area ratio of Fuziline to internal standard versus nominated concentrations. The lower limit of quantification (LLOQ), which was taken as the lowest concentration on the calibration curve, could fulfill the analytical requirement that S/N > 10 and acceptable accuracy and precision.

2.5.4. Accuracy and precision

Intra- and inter-batch variations were used to validate the accuracy and precision by analyzing sample concentrations at 2, 50 and 1000 ng/mL. To evaluate intra-batch variation, six replicates of each concentration were analyzed. Inter-day precision was determined by analysis of 6 replicates of each concentration over 3 consecutive validation days. The precision and accuracy of the method was expressed in terms of relative standard deviation (RSD) and relative error (RE), respectively.

2.5.5. Stability

Six replicates at low, medium and high concentrations were used for stability validation under a variety of storage and handling conditions. Samples were subjected to three freeze–thaw cycles to evaluate freeze–thaw stability. Short-term stability was determined by keeping the samples at room temperature for 6 h. Long-term stability was assessed by analyzing samples stored at −80 °C for 30 days. Post-preparative stability was evaluated by reanalyzing post-extraction samples kept in the autosampler at 10 °C for 24 h.

2.6. Application to pharmacokinetic study in rats

Sprague-Dawley rats were purchased from B&K Universal Group Limited. (Shanghai, China). All the rats were housed in a standard animal laboratory (temperature from 22 to 25 °C, humidity between 30 and 70%) with a 12-h light/dark cycle. Animal care was in accordance with the Guidelines for Animal Experimentation of China Pharmaceutical University (Nanjing, China) and the protocol was approved by the Animal Ethics Committee of the Institution.

Twelve Sprague-Dawley rats were randomly divided into the intravenous and intragastric administration groups (evenly divided between male and female). The rats were fasted overnight but with free access to water before the test. Blank samples were obtained before drug administration. After intravenous administration of Fuziline in saline at 1.0 mg/kg, 250 μL of blood samples were withdrawn from the ophthalmic veins by sterile capillary tube under anesthesia and heparinized at 0.083, 0.33, 0.67, 1, 2, 4, 8, 12, 24 h and immediately centrifuged at 1485 × *g* for 5 min to obtain the plasma. Blood samples were collected at 0.33, 0.67, 1, 2, 3, 4, 8, 12, 24 h after intragastric administration of 4 mg/kg Fuziline in saline and immediately processed same to the i.v. group. The supernatant plasma were collected and frozen at −20 °C until analysis. The pharmacokinetic parameters were calculated using WinNonlin (Version 6.1, Pharsight, Mountain View, CA, USA) according to non-compartmental model. The absolute bioavailability was calculated as follows:

$$F = \frac{AUC_{\text{oral}} \times D_{\text{iv}}}{AUC_{\text{iv}} \times D_{\text{oral}}} \times 100\%$$

3. Results and discussion

3.1. Method development

A simple liquid–liquid extraction method was utilized and ethyl acetate was chosen for its good extraction efficiency. As Fuziline is weak basic, it was necessary to alkalify plasma samples and reduces the interference of acidic endogenous substances. Due to hydroxyl and tertiary ammonium group in Fuziline and Guanfu base A, they have weak retention in conventional reverse phase C18 or C8 column with the retention time less than 2 min in isocratic mobile phase, especially in the present of acid. In the present study, we successfully developed a hydrophilic interaction LC method, using a poly(acrylamide)-immobilized silica column. In the hydrophilic interaction chromatographic mode, acetonitrile was the weak solvent and water was the strong solvent. Long retention can be achieved by increasing the ratio of acetonitrile. The peak shape will become broaden if the ratio of acetonitrile increases high enough to get a good retention time. With the increase of water ratio in the mobile phase mixture, the retention time shifted shorter. In our research, no apparent matrix effect was found in the present chromatography condition, although the retention factor (k') is less than 1. In addition to good retention, the hydrophilic interaction LC–MS method offered three additional advantages: (1) higher ratio of acetonitrile greatly enhances mass spectrometric sensitivity; (2) no carryover; (3) excellent injection-to-injection reproducibility and very long column life. The presence of a low amount of formic acid in the mobile phase improved the sensitivity by promoting ionization of the analysts. Although elution was performed isocratically, both of analytes have good resolution and peak shape and analytical process was completed within 5.5 min. No matrix effect was found and high sensitivity was achieved. This method could also be applied for the separation of other alkaloids from *Aconitum coreanum* (Lè vl.) Rapaics.

According to literature [8,11–15], both Fuziline and Guanfu base A have a good mass spectrometric response in positive ESI mode. In our study, a predominant signal with m/z at 454.35 and 430.30 were observed and identified as $[M+H]^+$ for target compound and IS in SIM mode, respectively. The signals were optimized to achieve better sensitivity. With the HILIC technique, matrix effect was minimized and the signals of target analytes were improved significantly, compared with separation by C18 and C8 column (data not shown).

3.2. Method validation

3.2.1. Specificity

The specificity of the method was investigated by analyzing rat plasma of different sources. Typical chromatograms of blank plasma sample, blank plasma sample spiked with Fuziline and IS, and a rat plasma sample after Fuziline administration are shown in Fig. 2. As shown in the figures, the retention time for Fuziline and Guanfu base A were 3.9 and 3.6 min respectively under the

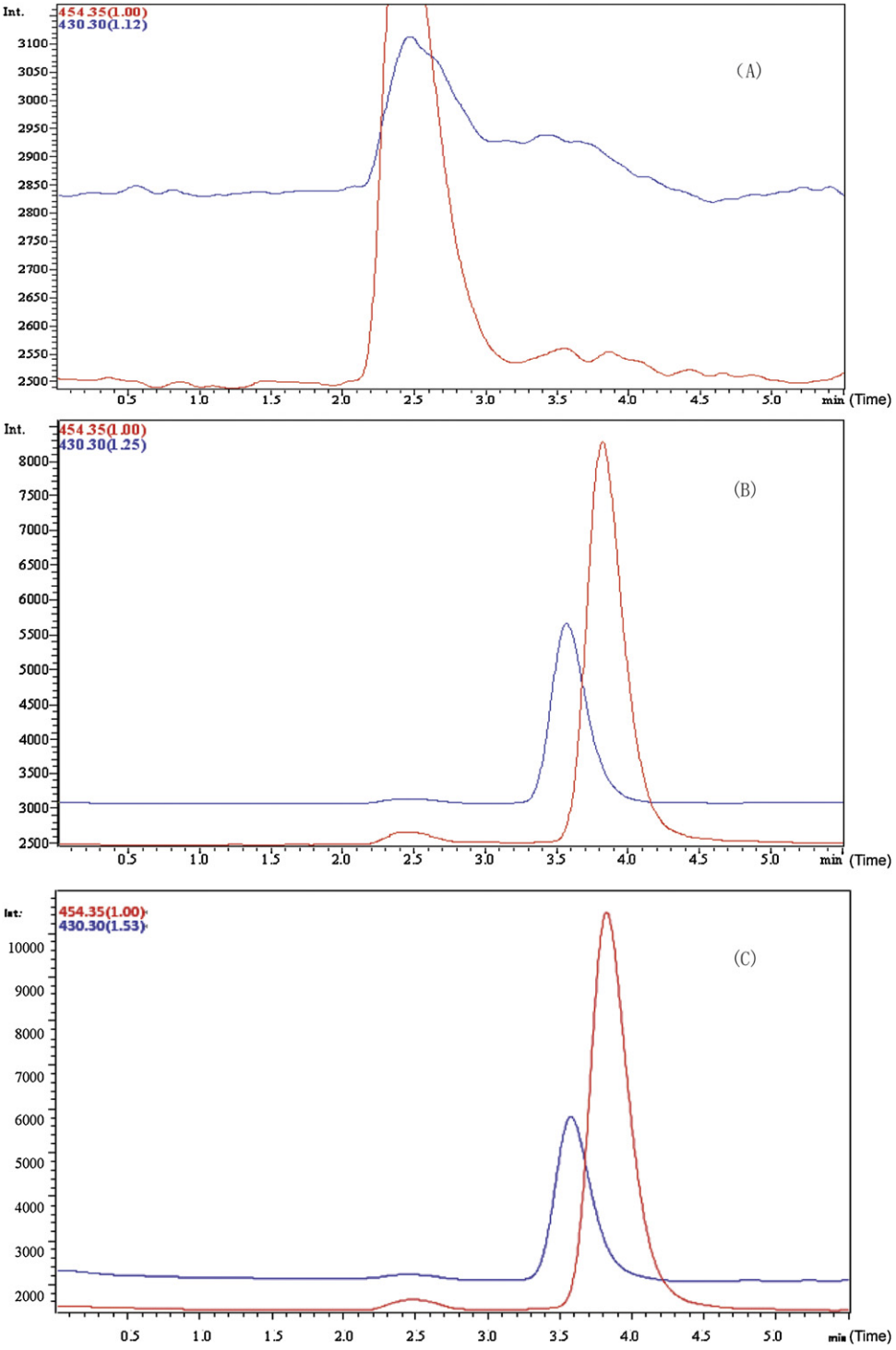

Fig. 2. Representative SIM chromatograms of Fuziline and Guanfu base A in rat plasma: (A) blank plasma; (B) blank plasma spiked with Fuziline (100 ng/mL in plamsa) and Guanfu base A (100 ng/mL in plasma); and (C) plasma at 20 min after intravenous administration of Fuziline (173.4 ng/mL). The retention times of Fuziline and Guanfu base A were 3.9 and 3.6 min, respectively.

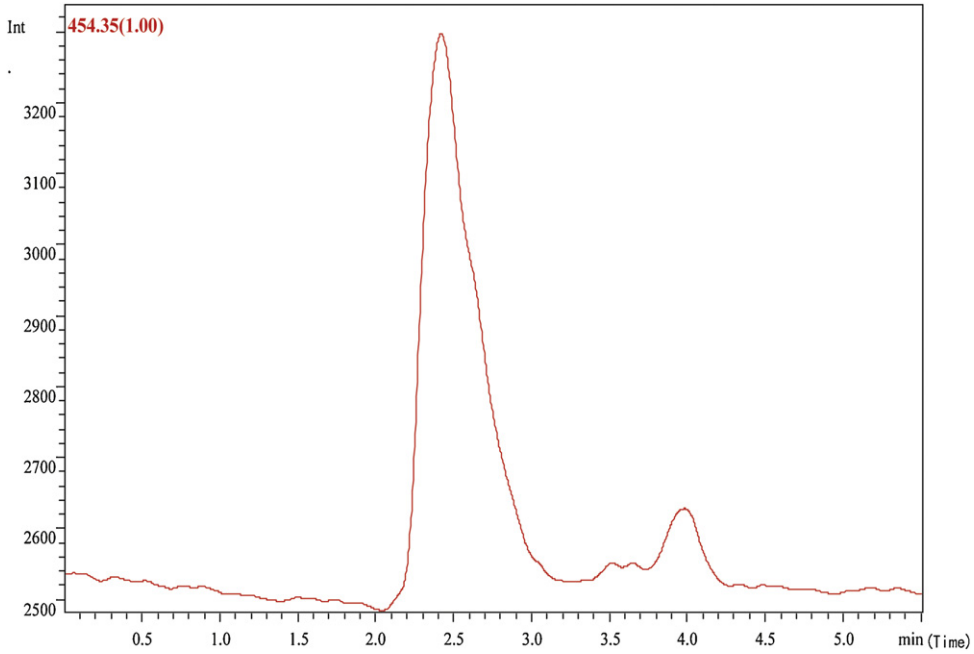

Fig. 3. Chromatography of lower limit of quantification (LLOQ) of Fuziline in plasma (1 ng/mL).

Table 1
Extraction recovery and matrix effect data for Fuziline and Guanfu base A.

Nominal concentration (ng/mL)	Recovery (%), $n = 6$	Matrix effect (%), $n = 6$
Fuziline		
2	69.9	94.3
50	68.2	99.9
100	68.2	108.6
Guanfu base A		
100	72.3	104.7

described conditions. No additional peaks due to endogenous substances interfering with analyte detection proved the specificity of the method.

3.2.2. Recovery and matrix effect

The results of recovery and matrix effect of Fuziline and IS were summarized in Table 1. At low, medium and high concentrations, no apparent ionization interference was found and recovery rates were acceptable.

3.2.3. Linearity and sensitivity

The calibration curve exhibited a good linearity over a concentration range of 1–1000 ng/mL ($y = 0.012x + 0.002$). The coefficient of determination (R^2) was 0.999. The LLOQ for Fuziline in rat plasma was 1 ng/mL (Fig. 3) and LOD 0.5 ng/mL. At this LLOQ, signal-noise ratio was greater than 10, and the accuracy and precision were 105.2% and 9.5%, respectively. The LLOQ is sufficient for rat pharmacokinetic studies following intravenous and intragastric administration of Fuziline.

3.2.4. Precision and accuracy

The intra-batch and inter-batch accuracy, which are expressed as percentage error, was calculated by comparing the averaged measurements and the nominal values. The intra- and inter-batch precisions were assessed by calculating the relative standard deviation. Accuracy and precision data for Fuziline at three different concentration levels (2, 50, 1000 ng/mL) are presented in Table 2. The intra- and inter-batch variations, as well as the accuracy, were within the acceptable range, except for accuracy at 2 ng/mL of intra-batch precision study. Since the intra-batch and inter-batch accuracy samples and standard curves were prepared by different people, the negative bias at most concentrations might be the working solution of accuracy sample prepared low from the start. The accuracy and precision is good enough from another batch of QC samples (see supplemental data), which revealed that the method was reliable and reproducible for the quantitative analysis of Fuziline in rat plasma.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.11.017.

3.2.5. Stability

Stability of Fuziline under various storage conditions was evaluated at 2, 50, 1000 ng/mL (Table 3). The results indicated that Fuziline was stable under typical sample storage and processing

Table 2
Intra-batch and inter-batch precision and accuracy for the quantification of Fuziline ($n = 6$).

Nominal concentration (ng/mL)	Intra-batch		Inter-batch	
	Precision (RSD%)	Accuracy (RE%)	Precision (RSD%)	Accuracy (RE%)
2	3.3	−15.4	8.3	−11.6
50	1.5	−6.7	3.9	−10.1
1000	1.5	0.9	2.6	−1.9

Table 3
Stability of Fuziline in rat plasma under different storage conditions ($n = 6$).

Storage conditions	Nominal concentration (ng/mL)	Concentration determined (ng/mL)	Precision (RSD%)	Accuracy (RE%)
Three freeze–thaw cycles	2	1.68 ± 0.05	2.9	−16.1
	50	46.3 ± 1.0	2.1	−7.5
	1000	1010.5 ± 14.6	1.4	1.1
Short-term (6 h at 20 °C)	2	1.73 ± 0.09	5.2	−13.7
	50	44.7 ± 1.5	3.4	−10.6
	1000	973.0 ± 24.2	2.5	−2.7
Long-term (30 d at −80 °C)	2	1.71 ± 0.11	6.4	−14.7
	50	46.5 ± 1.0	2.1	−7.1
	1000	1024.1 ± 12.5	1.2	2.4
Autosampler (24 h at 10 °C)	2	1.77 ± 0.11	6.0	−11.7
	50	44.7 ± 1.5	3.5	−10.7
	1000	972.2 ± 23.4	2.4	−2.8

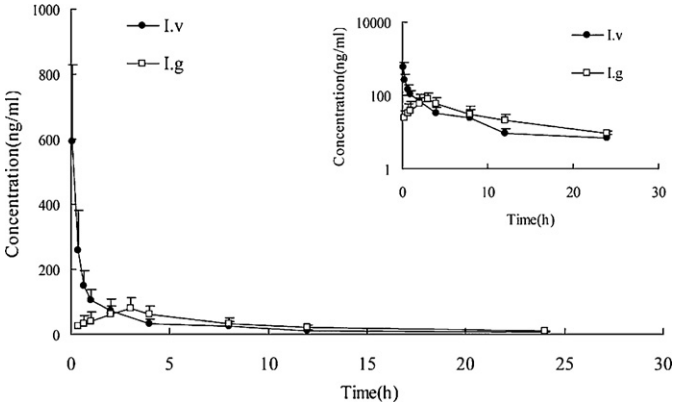

Fig. 4. Mean plasma concentration–time profile of Fuziline after i.g. administration of 4 mg/kg and i.v. administration of 1 mg/kg to rats, respectively. The inserted represented for the semi-log graph.

Table 4
Pharmacokinetic parameters after i.v. dose of 1 mg/kg Fuziline and i.g. administration of 4 mg/kg to rats.

Parameters	i.v. administration	i.g. administration
$T_{1/2}$ (h)	5.0 ± 1.9	6.3 ± 2.6
MRT (h)	5.1 ± 1.6	11.0 ± 4.1
AUC_{0-t}(ng h/mL)	679.5 ± 240.0	447.3 ± 190.0
$AUC_{0-\infty}$ (ng h/mL)	733.1 ± 239.9	595.0 ± 229.5
V or V/F (mL/kg)	2522.1 ± 1886.7	14,663 ± 3727.4
CL or CL/F (mL/kg/h)	305.1 ± 146.3	1745.6 ± 818.1
F (%)		21.1 ± 7.0

conditions, except at 2 ng/mL after three-cycle freeze–thaw treatment, with accuracy less than 85%.

3.3. Pharmacokinetic study

This method was successfully applied to determine the plasma concentration of Fuziline in rats following intravenous and intragastric administrations. The mean plasma concentration–time profiles of Fuziline after intravenous and intragastric administration are shown in Fig. 4. The pharmacokinetic parameters based on non-compartmental method are summarized in Table 4. Our research firstly clarifies the pharmacokinetic behavior of Fuziline in rats.

For intravenous administration, the mean $C_{5\,min}$ value of Fuziline was 582.2 ± 229.0 ng/mL. The mean plasma elimination half-life was 6.3 ± 2.6 h. As for oral administration, C_{max} value was 72.1 ± 28.9 ng/mL, with corresponding mean T_{max} value at 2.8 ± 0.7 h. The mean plasma elimination half-life was 5.0 ± 1.9 h. The absolute bioavailability of Fuziline after oral administration in rats was 21.1 ± 7.0%.

4. Conclusion

A sensitive, accurate and fast LC–MS method was developed and validated for the quantification of Fuziline in rat plasma for the first time. This method guarantees a relatively short analysis time and acceptable sensitivity, precision, accuracy, selectivity, recovery and stability. A simple liquid–liquid extraction method was applied for the pretreatment of plasma samples and showed good reproducibility. Besides, the pharmacokinetic parameters and absolute bioavailability were calculated for the first time, providing valuable information for future development of Fuziline researches.

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