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Development and validation of a rapid high-performance liquid chromatography-tandem mass spectrometry method for the determination of WJ-38, a novel aldose reductase inhibitor, in rat plasma and its application to a pharmacokinetic study

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ABSTRACT

WJ-38 is an aldose reductase inhibitor that is being developed for the treatment of diabetic complications. The present paper describes a sensitive and specific liquid chromatography-tandem mass spectrometry method for the determination of WJ-38 in rat plasma. Partial denaturation of plasma proteins with methanol followed by liquid–liquid extraction using ethyl acetate was used to extract strongly protein-bound WJ-38 from rat plasma. Chromatographic separation was performed on an Inertsil ODS-3 column with an isocratic mobile phase consisting of acetonitrile, water and formic acid (75:25:0.125, v/v/v). Mass spectrometric detection was achieved by a triple-quadrupole mass spectrometer equipped with an ESI interface operating in positive ionization mode. Quantitation was performed using selected reaction monitoring of precursor-product ion transitions at m/z 392 → 246 for WJ-38 and m/z 446 → 321 for glipizide (internal standard). A linear calibration curve was obtained over the concentration range of 10.0–10,000 ng/mL for WJ-38 in rat plasma. The intra- and inter-day precisions were less than 13.6% and the accuracy was within ±5.3%. The extraction recovery of WJ-38 from rat plasma was over 66.0%. The validated method has been successfully applied to a pharmacokinetic study in rats after intragastrical administration of WJ-38.

1. Introduction

Aldose reductase (AR) is primarily known for catalyzing the NADPH-dependent reduction of glucose to sorbitol in the first step of polyol pathway of glucose metabolism [1]. It is well established that hyperglycemia can increase the activity and expression of AR, and thus lead to polyol accumulation and other secondary changes which contribute to the development of diabetic complications such as neuropathy, retinopathy, and cataracts [2–6]. The inhibition of AR, therefore, would be a potential therapeutic strategy to prevent or slow the progression of diabetic complications [3,7–8]. During the past few decades, many AR inhibitors have been developed as drug candidates. However, except for a few successful cases [9–11], most of them have failed due to lack of specificity, in vivo potency and human safety [12]. Currently, there is an urgent need to design and develop well-tolerated and potent AR inhibitors [13].

Synthetic and naturally occurring chalcones have been reported to exhibit AR inhibitory activities [14,15]. Based on the structure of chalcone and the pharmacophore required for AR inhibitor [16], a series of quinolinoneacetic acids have been designed, synthesized and biologically evaluated for their potential as AR inhibitors by Shenyang Pharmaceutical University [17]. Among them, WJ-38, 2-[6-methoxy-3-(2,3-dichlorophenyl)methyl-4-oxo-1(4H)-quinolinyl] acetic acid (see Fig. 1A), was found to be the most active compound. In vitro pharmacological study showed that WJ-38 possessed much stronger AR inhibitory activity (IC_{50} = 0.0459 μmol/L) than clinically used epalrestat (IC_{50} = 0.075 μmol/L) and our previously reported compounds [17–19]. In vivo pharmacological studies in diabetic KKAy mice, streptozotocin-induced diabetic rats and diabetic GK rats revealed that WJ-38-treated diabetic mice and rats had significantly higher motor nerve conduction velocity and markly lower erythrocyte sorbitol levels than the untreated diabetic mice and rats. WJ-38 could also induce the production of endogenous nerve growth factor and increase the creatinine clearance rate. No toxic symptoms or deaths were observed in acute toxicity tests. These results suggest that WJ-38 as a novel AR inhibitor might become an attractive drug candidate for the treatment of diabetic complications.

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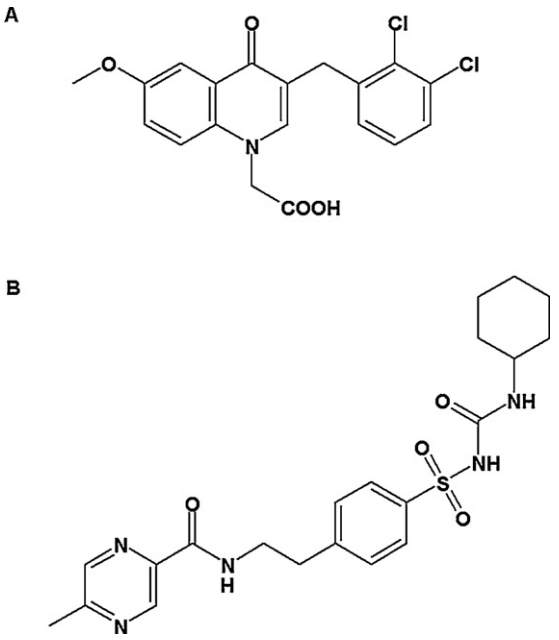

Fig. 1. Chemical structures of WJ-38 (A) and IS (B).

To support the pre-clinical pharmacokinetic study of WJ-38, a reliable analytical method is necessary for the determination of WJ-38 in rat plasma. High-performance liquid chromatography with UV detection (HPLC–UV) was found not suitable for the present study because it required a long chromatographic run time (>15 min) to ensure adequate separation of WJ-38 from endogenous substances as well as a large volume of plasma sample (>0.5 mL) to achieve a lower limit of quantitation (LLOQ) of 10.0 ng/mL. Consequently, a rapid and sensitive liquid chromatography-tandem mass spectrometry (LC–MS/MS) method was developed for the determination of WJ-38 in rat plasma. Although the superior sensitivity and selectivity of MS/MS reduces the need for sample preparation, effective extraction of strongly protein-bound WJ-38 (over 97% of WJ-38 was bound to plasma proteins) from rat plasma before LC–MS/MS analysis still remains a challenge. In the present study, special emphasis was focused on the optimization of sample preparation during method development. The developed method was fully validated in terms of selectivity, linearity, precision, accuracy, extraction recovery and stability, and then applied to investigate the pharmacokinetics of WJ-38 in rats after intragastrical administration of WJ-38.

2. Experimental

2.1. Chemicals and reagents

WJ-38 (99% purity) was synthesized and purified in Key Laboratory of New Drugs Design and Discovery of Liaoning Province, Shenyang Pharmaceutical University. Glipizide (internal standard, IS, see Fig. 1B) was obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC-grade acetonitrile and formic acid were purchased from Concord Technology Co. Ltd (Tianjin, China) and Kermel Chemical Reagents Co. Ltd (Tianjin, China), respectively. All other reagents were of analytical grade. Doubly distilled water was used throughout the study.

2.2. Instrumentation and analytical conditions

The LC–MS/MS system consisted of a Shimadzu SIL-HT_A autosampler, a Shimadzu LC-10ADvp pump (Kyoto, Japan) and a Thermo Finnigan TSQ Quantum Ultra triple-quadrupole mass spectrometer (San Jose, CA, USA) equipped with an ESI interface. The separation was carried out on an Inertsil ODS-3 column (100 mm × 2.1 mm I.D., 3 μm, GL Sciences, Japan) with a Diamonsil EasyGuard C18 guard cartridge (8 mm × 4 mm I.D., 5 μm, Dikma, China) at 20 °C. The mobile phase consisted of acetonitrile, water and formic acid (75:25:0.125, v/v/v). The flow rate was set at 0.25 mL/min. The eluate from the analytical column was directed to waste for the first 1 min via a divert valve and then introduced directly into the ESI source which was operated in positive ionization mode. The electrospray voltage was set at 4.2 kV and the capillary temperature was maintained at 340 °C. Nitrogen was used as the sheath gas (30 Arb) and auxiliary gas (5 Arb) for nebulization and desolvation. Argon was used as the collision gas (1.0 mTorr) for collision-induced dissociation. Selected reaction monitoring (SRM) was conducted by monitoring the precursor-product ion transitions of m/z 392 → 246 for WJ-38 and m/z 446 → 321 for IS. The collision energies for WJ-38 and IS were 20 eV and 27 eV, respectively. Data acquisition and processing were performed using Thermo Scientific Xcalibur 2.0.7 SP1 data system and LCquan 2.5.6 quantitation software (Waltham, MA, USA).

2.3. Preparation of calibration standards and QC samples

A standard stock solution of WJ-38 was prepared at 1 mg/mL in methanol and a series of standard working solutions ranging from 10.0 to 10,000 ng/mL were subsequently prepared by diluting the stock solution to appropriate concentrations with methanol. QC working solutions at concentrations of 20.0, 500 and 8000 ng/mL were made from a separately prepared 1 mg/mL stock solution of WJ-38. A stock solution of IS was prepared at 1 mg/mL in methanol and diluted with methanol to yield a 500 ng/mL IS working solution.

Calibration standards were freshly prepared by spiking standard working solutions into blank rat plasma to yield plasma concentrations of 10.0, 20.0, 50.0, 200, 500, 2000, 5000 and 10,000 ng/mL. QC samples were prepared by the same procedure as above at plasma concentrations of 20.0, 500 and 8000 ng/mL.

2.4. Sample preparation

A 100-μL aliquot of plasma sample was pipetted into a 10-mL glass tube, and then 100 μL of methanol and 100 μL of IS working solution were added. The mixture was extracted with 2 mL of ethyl acetate by vortex-mixing for 1 min and shaking on an orbital shaker for 10 min. After centrifugation at 3500 rpm for 5 min, the upper organic phase was transferred into another tube and evaporated to dryness under a gentle stream of nitrogen at 37 °C. The residue was reconstituted in 200 μL of mobile phase and vortex-mixed for 30 s. A 20-μL aliquot of the final solution was injected into the LC–MS/MS system for analysis.

2.5. Method validation

The LC–MS/MS method was validated according to the US FDA guideline on bioanalytical method validation [20].

2.5.1. Specificity, carryover and matrix effect

The specificity of the LC–MS/MS method was investigated by analyzing blank rat plasma from six different sources to ensure that there were no interfering peaks at the retention time of either WJ-38 or IS. Carryover was assessed by six consecutive injections of an extracted blank sample after the injection of an extracted upper limit of quantitation (ULOQ) sample. The matrix effect was evaluated at three QC levels of WJ-38 and one concentration level of IS by comparing the peak areas of the analyte spiked after extraction

from six different batches of rat plasma with those of the neat standard solutions at the same concentration.

2.5.2. Linearity and LLOQ

The linearity of the method was evaluated by analyzing calibration standards in duplicate at each concentration level over 3 consecutive days. Calibration curves (peak area ratio of WJ-38 to IS versus the nominal concentration) were fitted by least-squares linear regression using $1/X^2$ as weighing factor. The correlation coefficient should be more than 0.99. The LLOQ was defined as the lowest concentration of WJ-38 on the calibration curve with a relative standard deviation (RSD) lower than 20% and deviations from the nominal concentration within ±20% by six replicate analyses.

2.5.3. Accuracy and precision

The precision and accuracy of the method were assessed by analyzing QC samples in six replicates at three QC levels on 3 validation days. The intra- and inter-day precisions were calculated using one-way analysis of variance (ANOVA) in terms of RSD and they should not exceed 15%. The accuracy was calculated as percentage deviation of the measured concentration from the nominal concentration (often expressed as relative error, RE) and it should be within ±15%.

2.5.4. Extraction recovery, stability and dilution integrity

The extraction recovery was evaluated at three QC levels of WJ-38 and one concentration level of IS using six replicates at each concentration by comparing the peak areas of the analyte obtained from plasma samples with the analyte spiked before extraction to those spiked after extraction. The stability of WJ-38 and IS stock solutions was evaluated after storage at 4 °C for 15 days. The peak areas obtained by triplicate injections of the diluted stock solutions were compared with those of freshly prepared solutions. The stability of WJ-38 in rat plasma was evaluated by analyzing QC samples in three replicates at low and high concentration levels under a variety of storage and processing conditions. The long-term stability was assessed after storage of QC samples at −20 °C for 30 days. The freeze-thaw stability was determined after exposing QC samples to three freeze-thaw cycles. The bench-top stability was assessed by keeping QC samples at room temperature (25 °C) for 4 h. The post-preparative stability was evaluated by placing processed QC samples in an autosampler as long as 24 h. Dilution integrity was assessed by six replicate analysis of QC samples at 5 times the ULOQ, i.e. 50,000 ng/mL. These samples were diluted 10-fold with blank rat plasma prior to analysis.

2.6. Pharmacokinetic study

Eighteen Sprague-Dawley rats (9 male and 9 female) weighing 180–200 g were obtained from Laboratory Animal Centre of China Medical University. Before intragastrical administration of WJ-38 at the dose of 16.7, 50 or 150 mg/kg, the rats were fasted for 12 h, but allowed free access to water. Blood samples were collected from the eye retro-orbital sinus of rats into heparin-coated Eppendorf tubes at 0 (before administration), 0.167, 0.333, 0.5, 0.75, 1, 2, 3, 5, 7, 9, 12 and 24 h after administration. The whole blood was immediately centrifuged at 3500 rpm for 10 min at 20 °C. The obtained plasma was stored at −20 °C until analysis.

3. Results and discussion

3.1. Method development

3.1.1. Sample preparation

Preliminary in vitro protein binding studies have shown that WJ-38 is more than 97% bound to plasma proteins. Therefore, breaking the strong binding interaction to completely release WJ-38 from protein binding sites is critical for sample preparation. Protein precipitation (PPT) was attempted in the early method development stage. It was found that plasma extract from PPT with either methanol or acetonitrile as protein precipitant was not clean enough to be able to achieve the LLOQ required. Subsequently, partial denaturation of plasma proteins with methanol followed by liquid–liquid extraction was tested. Several organic solvents (diethyl ether, ethyl acetate and dichloromethane) under neutral or acidic conditions were investigated. Degradation of WJ-38 was observed when plasma was acidified by the addition of hydrochloric acid before extraction with organic solvents, indicating that WJ-38 might be unstable in strong acidic condition. Finally, LLE with ethyl acetate under neutral conditions was adopted because it gave higher and more consistent recoveries without any degradation.

It should be noted that the amount of methanol added to plasma prior to LLE had a significant influence on the extraction efficiency. Different volumes of methanol (10, 50, 100, 150, 200 and 250 μL) were tested. It was found that the recovery increased as the volume of methanol was increased (Supplementary Fig. S1). About 23% of WJ-38 in plasma was extracted when adding 10 μL of methanol to plasma and then extracting with ethyl acetate. However, when 200 μL (or 250 μL) of methanol was added, a three-fold increase in extraction recovery was observed. The results indicated that 200 μL of methanol was sufficient to liberate WJ-38 from protein binding sites. Based on the above findings, 200 μL of methanol (100 μL of methanol together with 100 μL of IS working solution) was added to plasma to release WJ-38 bound to proteins prior to LLE.

3.1.2. LC–MS/MS optimization

Considering that WJ-38 contains multiple chromophores that absorb ultraviolet light, we attempted to develop an HPLC–UV method for the determination of WJ-38 in rat plasma during the early phase of method development. Due to the low sensitivity of UV detection, a large volume of plasma sample (>0.5 mL) was required to achieve an LLOQ of 10.0 ng/mL. Moreover, the lack of specificity of UV detection resulted in a relatively long chromatographic run time (>15 min) to ensure complete separation of WJ-38 from endogenous substances. Therefore, a sensitive and high-throughput LC–MS/MS method was chosen for the present study.

Ionization of WJ-38 was attempted with ESI as well as APCI sources. ESI source was finally chosen because it provided more stable and stronger mass spectral signal than APCI source. In positive ESI mode, WJ-38 and IS formed protonated molecules $[M+H]^+$ at m/z 392 and 446, respectively. The $[M+H]^+$ ions were selected as the precursor ions and subsequently fragmented in MS/MS mode. Fig. 2 displays the product ion mass spectra of WJ-38 and IS. The most intense fragment ions at m/z 246 for WJ-38 and m/z 321 for IS were selected for the SRM analysis.

The optimization of chromatographic conditions was mainly guided by the requirement for minimizing matrix effects and reducing the analytical run time. It was found that WJ-38 was strongly retained on the column when using a various percentage of methanol or acetonitrile and water as mobile phase. When a little formic acid was added into the mobile phase, WJ-38 was eluted within a reasonable time. The optimal mobile phase consisted of acetonitrile, water and formic acid (75:25:0.125, v/v/v). The chromatographic run time was only 3.5 min and the co-eluting matrix components had no effect on the ionization of WJ-38 and IS.

3.2. Method validation

3.2.1. Specificity, carryover and matrix effect

Fig. 3 shows the typical SRM chromatograms of a blank plasma, a blank plasma spiked with WJ-38 and IS, and a plasma sample from

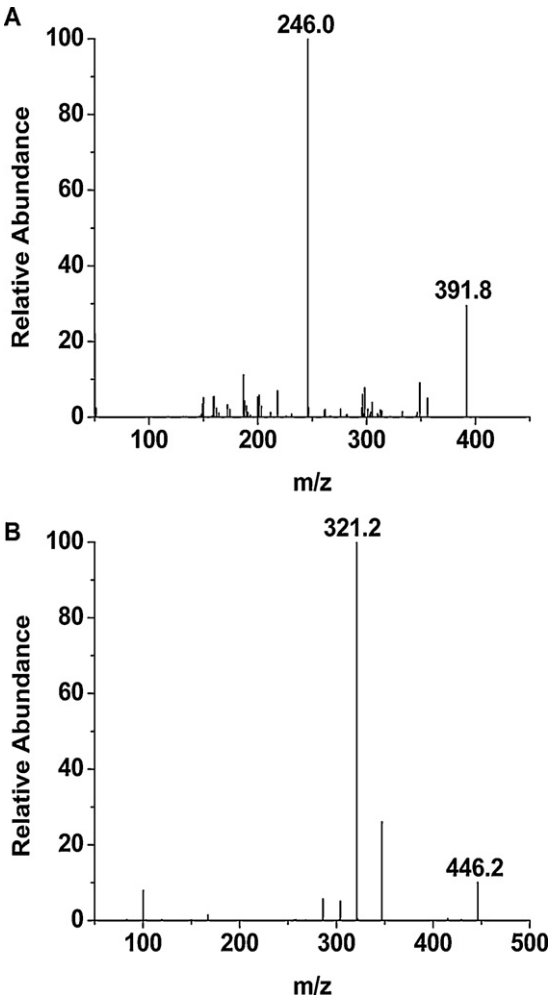

Fig. 2. Product ion mass spectra of WJ-38 (A) and IS (B).

a rat 12 h after an intragastrical administration of 50 mg/kg of WJ-38. No endogenous peaks were observed at the retention time of either WJ-38 or IS. The carryover study showed that the WJ-38 peak area obtained from the blank sample injected after an ULOQ sample was less than 2% of the peak area of the LLOQ, indicating that the carryover from residues in rotary sampling/switching valves is negligible. Furthermore, no carryover from late-eluting residues on the column was observed over six consecutive blank injections after an ULOQ injection. The matrix effects of WJ-38 at the concentrations of 20.0, 500 and 8000 ng/mL were 102.5 ± 4.4%, 97.2 ± 3.8% and 97.5 ± 5.1%, respectively. The matrix effect of IS was found to be 102.7 ± 5.8%.

3.2.2. Linearity and LLOQ

Calibration curves showed good linearity over the concentration range of 10.0–10,000 ng/mL for WJ-38 in rat plasma. A typical linear regression equation of the calibration curve was $y = 0.0846 + 0.0144x$, $r^2 = 0.9916$, where y represents the ratio of peak area of the analyte to that of the IS and x represents the plasma concentration of WJ-38. The LLOQ was 10.0 ng/mL, at which the RSD was 14.2% and the RE was 6.0%.

3.2.3. Accuracy and precision

The intra- and inter-day precisions and accuracy of the method are summarized in Table 1. The intra- and inter-day precisions ranged from 5.2 to 13.6%, and the accuracy was between −5.3 and 4.3%. All the values were within the acceptable range.

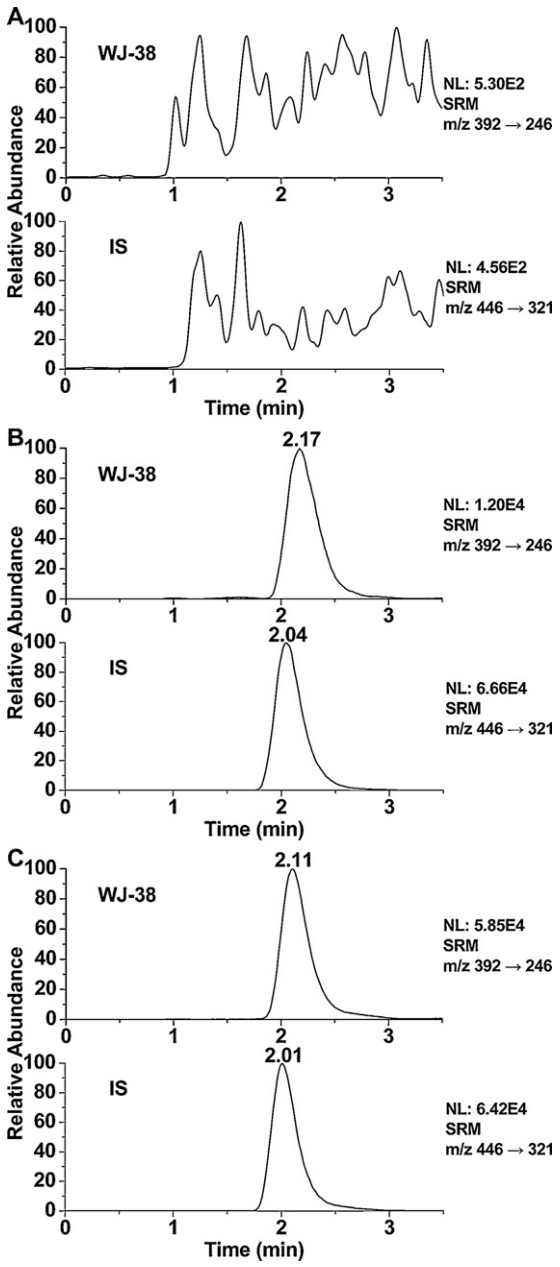

Fig. 3. Representative SRM chromatograms of a blank plasma (A); a blank plasma spiked with 10.0 ng/mL WJ-38 and 500 ng/mL IS (B); a plasma sample from a rat 12 h after an intragastrical administration of 50 mg/kg of WJ-38 (C).

Table 1
Precision and accuracy for the determination of WJ-38 in rat plasma.

Concentration (ng/mL)	Intra-day RSD (%)	Inter-day RSD (%)	RE (%)
20.0	6.0	13.6	−5.3
500	8.9	5.2	4.3
8000	7.3	10.3	−3.1

3.2.4. Extraction recovery, stability and dilution integrity

The extraction recoveries of WJ-38 at three QC levels were 68.8 ± 5.0%, 67.1 ± 7.6% and 66.0 ± 6.1%, respectively. The extraction recovery of IS was 72.7 ± 7.1%. The deviations of the peak areas derived from the stored stock solutions in comparison with those of freshly prepared solutions were within ±3.5%, indicating that the stock solutions of WJ-38 and IS are stable for at least 15 days at 4 °C. Table 2 lists the stability data of WJ-38 in rat plasma. It can be

Table 2
Stability of WJ-38 in rat plasma.

Concentration (ng/mL)	Long-term stability		Freeze-thaw stability		Bench-top stability		Post-preparative stability	
	20.0	8000	20.0	8000	20.0	8000	20.0	8000
RE (%)	6.2	2.1	−4.3	−0.4	−2.2	0.9	−2.7	1.4
RSD (%)	6.5	3.7	9.4	4.8	3.1	3.2	8.6	5.5

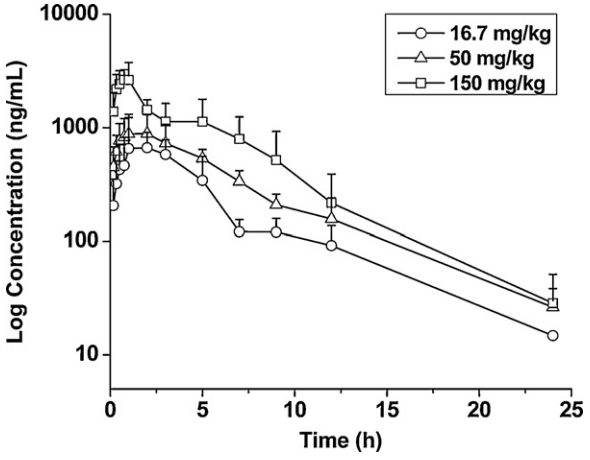

Fig. 4. Semi-logarithmic plot of mean plasma concentration versus time after intragastrical administration of WJ-38 to rats.

Table 3
Pharmacokinetic parameters of WJ-38 in rats.

Parameters	Dose (mg/kg)		
	16.7	50	150
C_{max} (ng/mL)	854 ± 548	1006 ± 403	2974 ± 1001
T_{max} (h)	1.8 ± 0.8	1.4 ± 0.7	0.7 ± 0.2
AUC_{0-t} (ng h/mL)	3681 ± 1486	5919 ± 1360	13,045 ± 6207
$AUC_{0-\infty}$ (ng h/mL)	3953 ± 1614	6241 ± 1253	13,278 ± 6280
$t_{1/2}$ (h)	3.0 ± 0.7	3.3 ± 1.1	3.2 ± 0.5

seen that WJ-38 is stable under all the tested conditions. The precision and accuracy for analyzing diluted QC samples (1/10 dilution) were 8.1% and −4.6%, respectively. The results suggest that plasma samples whose concentrations are greater than the ULOQ can be re-analyzed by appropriate dilution.

3.3. Pharmacokinetic and statistical analysis

The validated method was applied to the analysis of plasma samples obtained from rats after intragastrical administration of 16.7, 50 or 150 mg/kg of WJ-38. Fig. 4 shows a semi-logarithmic plot of mean plasma concentration of WJ-38 versus time. A non-compartmental model was used to calculate the pharmacokinetic parameters with DAS 2.1.1 software (Mathematical Pharmacology Professional Committee of China, Shanghai, China). The mean pharmacokinetic parameters of WJ-38 are summarized in Table 3. There was no statistical significant difference in $t_{1/2}$ among the various doses applied. Linear correlations between the dose and C_{max} and AUC values were observed, indicating that pharmacokinetics of WJ-38 are linear over the dose range of 16.7–150 mg/kg.

4. Conclusion

A specific and sensitive LC–MS/MS method was developed and validated for the quantification of strongly protein-bound WJ-38 in rat plasma. Special emphasis was placed on the optimization of sample preparation so as to completely release WJ-38 from protein binding sites and effectively extract it from plasma. The chromatographic run time was only 3.5 min and the LLOQ was 10.0 ng/mL using 100 μL of rat plasma. The method has been successfully applied to the pharmacokinetic study of WJ-38 in rats.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2012.02.024.

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