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Determination of rimantadine in rat plasma by liquid chromatography/electrospray mass spectrometry and its application in a pharmacokinetic study

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Abstract

A rapid and sensitive liquid chromatography/mass spectrometry (LC/MS) method was developed and validated for the determination of rimantadine in rat plasma. Rimantadine was extracted by protein precipitation with methanol, and the chromatographic separation was performed on a C_{18} column. The total analytical run time was relatively short (4.6 min), and the limit of assay quantification (LLOQ) was 2 ng/mL using 50 μL of rat plasma. Rimantadine and the internal standard (amantadine) were monitored in selected ion monitoring (SIM) mode at *m/z* 180.2 and 152.1, respectively. The standard curve was linear over a concentration range from 2 to 750 ng/mL, and the correlation coefficients were greater than 0.999. The mean intra- and inter-day assay accuracy ranged from 100.1–105.0% to 100.3–104.0%, respectively, and the mean intra- and inter-day precision was between 1.3–2.3% and 1.8–3.0%, respectively. The developed assay method was successfully applied to a pharmacokinetic study in rats after oral administration of rimantadine hydrochloride at the dose of 20 mg/kg.

Keywords: Rimantadine; Amantadine; LC/MS; Pharmacokinetic study

1. Introduction

Rimantadine hydrochloride (α-methyl-1-adamantane-methylamine hydrochloride), which exhibits equal efficacy and fewer adverse reactions than amantadine hydrochloride, has been clinically used for therapy of infections caused by a broad range of RNA-containing viruses, in particular the influenza A virus [1–3]. Its mechanism of action appears to be the interference of viral replication. In recent years, it has also been reported that rimantadine hydrochloride has some effect on Parkinson's disease [4,5].

The bioanalytical component of a pharmacokinetic study requires an analytical method with simplicity, selectivity, sensitivity and rapid turn-around time. A number of assays have been reported for the determination of rimantadine in biological fluids [6–15], including gas chromatography with electron-capture detection (GC–ECD) [6,7], gas chromatography with mass spectrometry (GC–MS) [8–13], high-performance liquid chromatography with fluorescence detection (HPLC–Flu) [14,15] and high-performance liquid chromatography with ultraviolet detection (HPLC–UV) [16]. The various experiments described above usually require complicated derivatization treatment and liquid–liquid extraction to increase its sensitivity, or special facilities for the use of radioactive compounds. Therefore, a more convenient, sensitive, and simple method is desirable. Lately, liquid chromatography with mass spectrometry (LC/MS) and tandem mass spectrometry (LC/MS/MS) have been increasingly utilized as they can determine many kinds of compounds and provide the benefits of efficient separating power and a lower limit of detection. In this study, a simple and highly sensitive LC/MS method was developed for the determination of riman-

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tadine in rat plasma, utilizing a single sample fore treatment, protein precipitation, and isocratic elution monitored in selected ion monitoring (SIM) mode.

2. Experimental

2.1. Reagents and chemicals

Rimantadine hydrochloride was obtained from Nanjing Jinling Pharmaceutical Co., Ltd. (Jiangsu, China). Amantadine hydrochloride was purchased from NICPBP (National Institute for The Control of Pharmaceutical and Biological Products, China). HPLC-grade acetonitrile and methanol were purchased from Merck KGaA (Darmstadt, Germany). Water was produced by a Milli-Q water system (Millipore, Bedford, MA, USA), and commercial HPLC grade of formic acid was used.

2.2. Animals

The experimental animals used were female Sprague–Dawley rats, weighing 180–220 g, from Shanghai SIPPR/BK experimental animal Co., Ltd. The study was approved by the Animal Ethics Committee of the Nanjing University of Traditional Chinese Medicine. The rats were maintained in air-conditioned animal quarters at a temperature of $22 \pm 2\,^{\circ}C$ and a relative humidity of $50 \pm 10\%$. Water and food (laboratory rodent chow, Nanjing, China) were provided ad libitum. The animals were acclimatized to the facilities for 5 days, and then fasted with free access to water for 12 h prior to each experiment.

2.3. Preparation of standard and quality control samples

Stock solutions were prepared by separately dissolving 5.34 mg of rimantadine hydrochloride and 4.96 mg of amantadine hydrochloride in 10 mL of water. Rimantadine standard working solutions were prepared by serial dilution with water. The IS working solution (155 ng/mL) was prepared by dilution with methanol. All the solutions were prepared in glass tubes and stored in the darkness at $4\,^{\circ}C$. Standard calibration samples were prepared by spiking rat plasma with standard working solution, and the range of concentration of the spiked plasma was from 2 to 750 ng/mL. The preparations of quality control (QC) samples were prepared in the same way, and the final concentrations of low, medium and high QC samples were 4, 80 and 600 ng/mL, respectively. Standard calibration samples and QC samples were stored at $-20\,^{\circ}C$ until use.

2.4. Sample extraction

The IS solution (50 μL, 155 ng/mL in methanol) was added to 50 μL of rat plasma sample, and the mixture was then precipitated with 150 μL of methanol. After a vortex mixer for 2 min, the samples were centrifuged at $10{,}000 \times g$ for 10 min. The supernatant fluid was transferred to a glass insert (volume 100 μL) and 5 μL of it was injected into the LC/MS system.

2.5. Instrumentation and LC/MS conditions

An Alliance 2695 HPLC system (Waters, Milford, MA, USA) coupled with a single quadrupole Waters ZQ 2000 mass spectrometer was used. The Mass Lynx 4.0 software was used for instrumental control, and for acquisition and processing of the data. A MS detector with an electrospray ionization (ESI) interface in positive ion mode (ESI^+) was used for quantitative analysis, with acquisition in SIM mode. The *m*/*z* ratios 180.2 for rimantadine and 152.1 for amantadine were recorded simultaneously.

HPLC separation was performed on an Agilent Zorbax SB-C_{18} (150 mm × 2.1 mm, I.D.5 μm) column (Agilent Technologies, Wilmington, DE, USA) with a Security Guard (12.5 mm × 2.1 mm, I.D. 5 μm) column (Agilent Zorbax SB-C_{18}, DE, USA), kept at $30\,^{\circ}C$. The mobile phase, consisting of acetonitrile and deionized water containing 0.1% formic acid (30:70, v/v), was at a flow rate of 0.2 mL/min. The mobile phase entered the probe of the mass spectrometer between 2 and 4.6 min in order to protect the source. At all other times, the mobile phase was automatically discarded, by means of an electrovalve, into the waste container without entering the spectrometer.

The optimized electrospray conditions were: capillary voltage: 2100 V; cone voltage: 30 V; extractor voltage: 4 V; RF lens voltage: 0.3 V; source temperature: $110\,^{\circ}C$; desolvation temperature: $300\,^{\circ}C$; cone gas flow (nitrogen): 20 L/h; desolvation gas flow (nitrogen): 300 L/h.

2.6. Validation

2.6.1. Sensitivity and specificity

The limit of quantification (LLOQ) was defined as the lowest plasma rimantadine concentration that yielded a signal-to-noise (S/N) ratio of greater than 10, within acceptable accuracy and precision (less than 20%). The lower limit of detection (LLOD on column) was defined as the amount of rimantadine that could be detected with a S/N ratio of 3. The specificity of the assay for the analytes versus endogenous substances in the matrix was assessed by comparing the lowest concentration in the calibration curves with the reconstitutions prepared using drug-free plasma from six different rats.

2.6.2. Calibration and sample quantification

The linearity of the calibration curves, ranging from 2 to 750 ng/mL, was validated with five different calibration curves. The calibration curves ($y = bx + a$), were constructed using the weighted regression method ($1/x^2$) of peak area ratios of rimantadine to IS (y) versus actual concentrations (x). Quality control and stability samples were calculated from the resulting area ratios of rimantadine to IS and the regression equation of the calibration curve.

2.6.3. Accuracy and precision

The precision of the method was expressed as the coefficient of variation (CV) of each concentration, and the accuracy was expressed as the relative percentage errors (%). Intra- and inter-

day assay variability of rimantadine was determined by assaying low (4 ng/mL), medium (80 ng/mL) and high (600 ng/mL) QC samples on three consecutive days with five replicate samples on each day.

2.6.4. Recovery and matrix effect

The recovery of the protein precipitation procedure was assessed by comparing the peak areas of the samples prepared at three different concentrations (4, 80 and 600 ng/mL), in triplicate, with the mean peak area of spike-after-extraction plasma samples, which represented 100% recovery. To prepare the spike-after-extraction samples, blank human plasma was processed according to the sample preparation procedure as described above. All the supernatant was mixed with the appropriate standard solutions of rimantadine at concentrations corresponding to the final concentration of pretreated plasma samples. Similarly, recovery of IS was evaluated by comparing the mean peak areas of 10 regularly prepared samples to the mean peak areas of three standard solutions spiked in pretreated drug free plasma samples.

The matrix effect, the possible suppression or enhancement of ionization induced by the endogenous substances, was evaluated by comparing the chromatographic peak areas of rimantadine from the spike-after-extraction samples, at three levels in triplicate employing six sources of blank matrix, with the neat standards at the same concentrations.

2.6.5. Stability

The stability of rimantadine was examined under the different conditions described below using three replicates of low, medium and high QC samples. The plasma samples were analyzed after storage at room temperature for 2 h, in an autosampler for 24 h at ambient temperature after protein precipitation, at −20 °C for 2 weeks, and after three freeze-thaw cycles from −20 °C to room temperature. The stability was assessed by comparing the concentrations of QC samples of rimantadine with the theoretical concentrations.

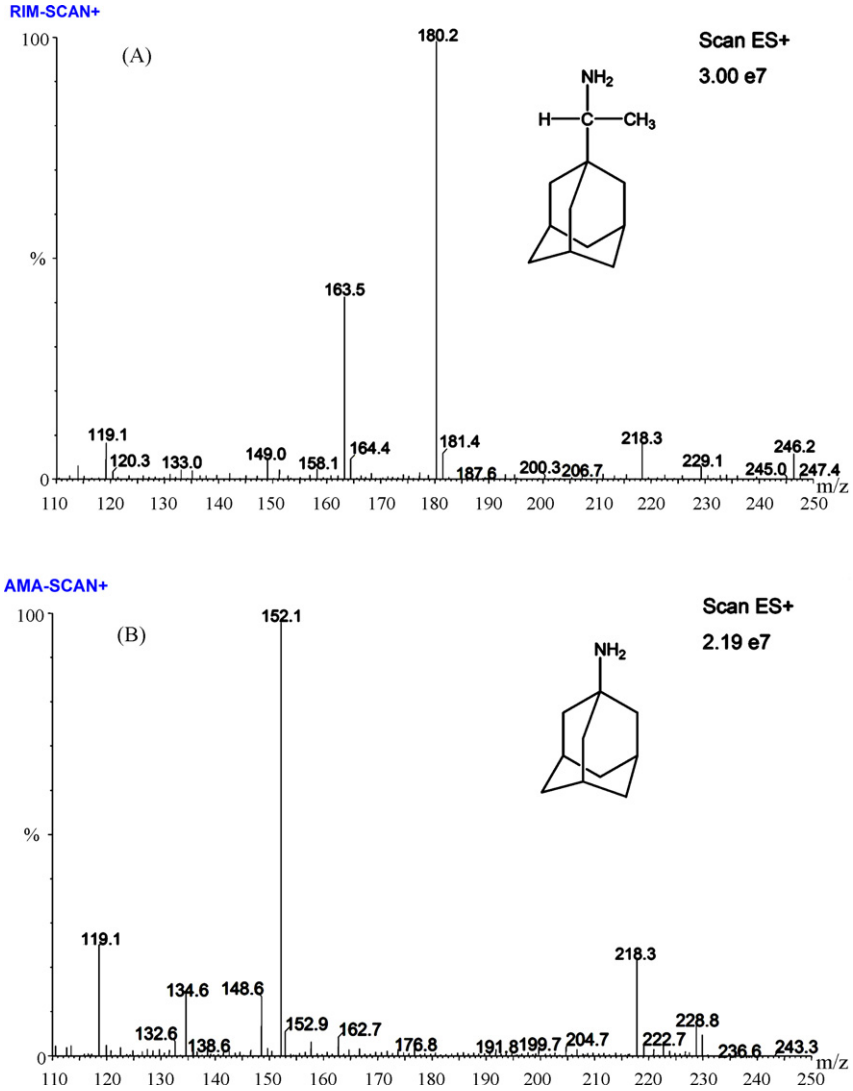

Fig. 1. Positive ion mass spectra of (A) rimantadine ([M + H] $^+$, *m/z* 180.2), and (B) amantadine ([M + H] $^+$, *m/z* 152.1).

2.7. Application to a pharmacokinetic study

Rimantadine hydrochloride was suspended in a 0.5% carboxymethyl cellulose sodium salt aqueous solution and mixed well. The preparations were made immediately before drug administration and were intragastrically administrated to six rats at a dosage of 20 mg/kg. Prior to and at multiple intervals (0.16, 0.5, 1, 2, 4, 6, 8, 11, 14, 24 and 36 h) following drug administration, 150 μL of blood samples were collected in the heparinized tubes. Plasma samples were harvested by centrifugation at 3000 × *g* for 10 min and stored at −20 °C. The plasma samples were analyzed within 3 days of storage.

Pharmacokinetic parameters were calculated using the DAS pharmacokinetic software package (version 1.0, Chinese Pharmacological Association, Anhui, China). The area under the plasma rimantadine concentration versus time curve (AUC) and the area under the first moment curve (AUMC) were calculated using the trapezoidal rule extrapolated to infinite time. The extrapolation of AUC and AUMC to infinite time was obtained by adding C_n/λ_z to AUC and $t_n C_n/\lambda_z + C_n/l_z^2$ to AUMC, where C_n was the concentration of the last sampling time (t_n). Mean residence time (MRT) was calculated as AUMC/AUC. The terminal elimination half-life ($t_{1/2}$,λ_z) was calculated as $0.693/\lambda_z$, where λ_z, the elimination rate constant, was calculated using linear regression from the terminal linear portion of the logarithmic plasma concentration–time curve. The C_{max} was the observed maximum concentration, and the T_{max} was the time taken to reach the maximum drug concentration.

3. Results and discussion

3.1. Mass spectrometry and chromatography

The positive mass spectra of rimantadine and amantadine (IS) are shown in Fig. 1. In the full scan mass spectrum, the most abundant ions for rimantadine and amantadine were protonated molecular ions $[M+H]^+$ found at *m/z* 180.2, and 152.1, respectively. The ions $[M+H–NH_3]^+$ found at *m/z* 163.5 and 134.6, which had lower signals than $[M+H]^+$ ions, could also be detected. So the quantification was performed for the protonated molecular ions. The addition of 0.1% formic acid to the mobile phase increased the sensitivity of both of the analytes. The value of capillary voltage should not be higher than 3000 V, otherwise the signal of rimantadine will be much lower and it might be broken into small fragments; the optimal voltage was 2100 V. Ion chromatograms of rimantadine and amantadine, obtained by protein precipitation from rat plasma, are shown in Fig. 2. Both rimantadine and amantadine were eluted within 4.6 min with the retention time of about 2.5 and 3.9 min, respectively. The retention time was short, thus suitable for the high throughput sample determination in a pharmacokinetic study.

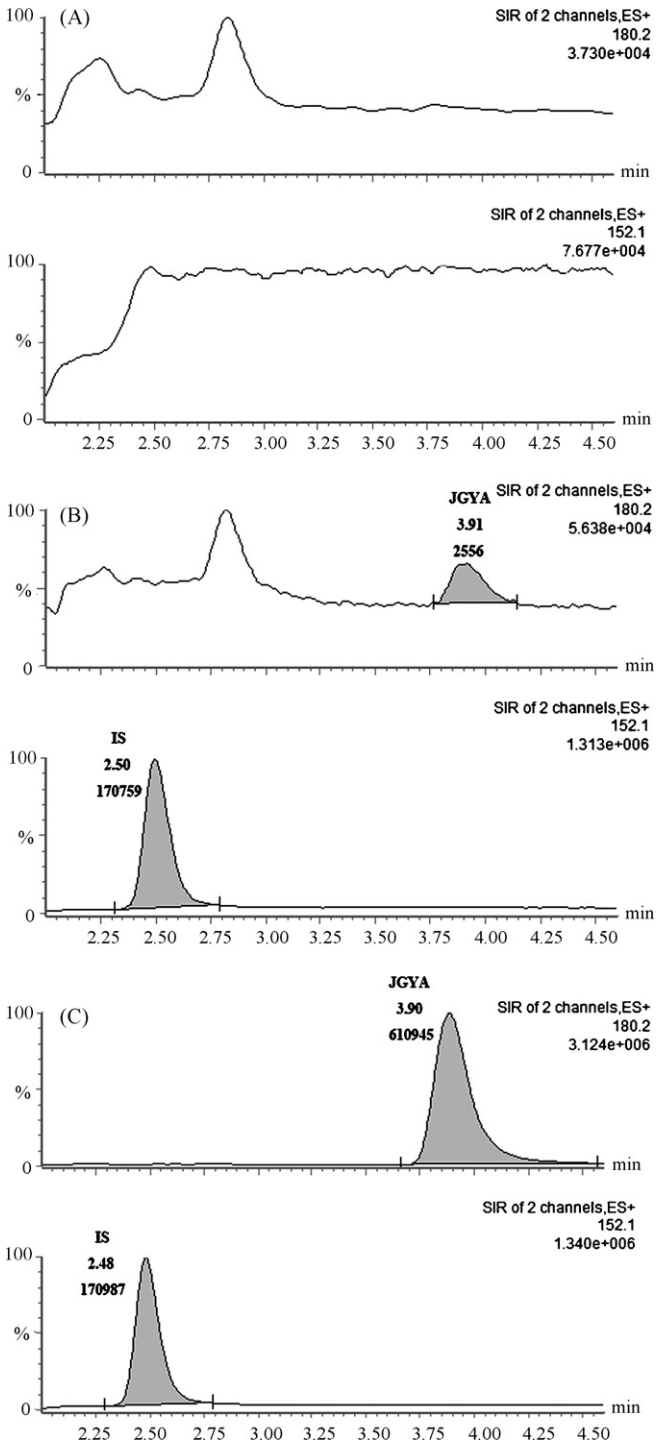

Fig. 2. Typical chromatograms of rimantadine and amantadine obtained by extraction of (A) blank rat plasma, (B) plasma spiked with rimantadine (2 ng/mL, LLOQ) and amantadine (155 ng/mL), (C) plasma obtained 4h after intragastric administration of rimantadine hydrochloride to a rat (dose 20 mg/kg).

3.2. Sample extraction

It has been reported that liquid–liquid extraction with dichloromethane [16], toluene [6,7], or freshly distilled *n*-hexane [14], and solid-phase extraction [10] are usually used for rimantadine pre-treatment. In addition, derivatization and plasma alkalization with NaOH or Na_2HPO_4 buffer are usually applied to increase the extraction recovery of the compound from the biological samples. Therefore, the fore treatments of rimantadine in the plasma samples were laborious and time consuming. The protein precipitation method with methanol used

Table 1
Summary of calibration curves used in method validation

Curve no.	Slope	Intercept	r
1	0.0071	−0.0001	0.9998
2	0.0078	0.0007	0.9996
3	0.0079	0.0009	0.9994
4	0.0080	0.0004	0.9994
5	0.0078	0.0006	0.9994
Mean ± SD	0.0077 ± 0.0003	0.0005 ± 0.0004	0.9995 ± 0.0002

in this study was simpler, more rapid and convenient, and yet was able to produce a higher level of sensitivity (LLOQ was 2 ng/mL) using only 50 μL of rat plasma.

3.3. Method validation

3.3.1. Specificity

Specificity was assessed by comparing the chromatograms of six different batches of blank rat plasma with the corresponding spiked plasma. Fig. 2 shows the typical chromatograms of a blank plasma sample, a blank plasma sample spiked with rimantadine at the LLOQ and IS, and a plasma sample collected at 8 h after drug administration. No significant interference from endogenous substances with analyte or IS was detected.

3.3.2. Linearity of calibration curves and lower limit of quantification

The calibration curve data used in the method validation are summarized in Table 1. The assay was linear over a concentration range from 2 to 750 ng/mL, with a typical correlation coefficient greater than 0.999. If the concentration of rimantadine exceeded 750 ng/mL, the rat samples were diluted by blank plasma, and the determined concentration multiplied the diluted multiples. The mean slope of the calibration curves used in method validation was 0.0077 ± 0.0003, with % CV being less than or equal to 4.1% and the mean intercept was close to zero.

The lower limit of quantification was 2.0 ng/mL. At the LLOQ level, the intra-day precision was 1.9% and the accuracy was 105.5%. With the present LLOQ, the concentrations of rimantadine in rats could be determined 36 h after intragastric administration of 20 mg/kg rimantadine hydrochloride, which was sensitive enough to be used to investigate the pharmacokinetic behaviors of the drug.

3.3.3. Accuracy and precision

Values for the intra- and inter-day accuracy and precision are shown in Table 2. The intra- and inter-day accuracy ranged from 100.1–105.0% (CV less than or equal to 2.3%) and 100.3–104.0% (CV less than or equal to 3.0%), respectively. The results, calculated using a one-way ANOVA, indicated that the values were within the acceptable range and the assay was accurate and precise.

Table 3
Recovery of rimantadine (mean ± standard deviation (SD)) and amantadine (internal standard)

Sample	Spiked conc. (ng/mL)	Recovery (%)	Matrix effect (%)
Rimantadine	4	102.5 ± 2.5	103.7 ± 4.8
	80	100.8 ± 2.3	102.5 ± 5.0
	600	98.9 ± 1.1	97.9 ± 8.1
Amantadine	155	99.4 ± 3.0	101.6 ± 2.9

3.3.4. Recovery and matrix effect

The recoveries of rimantadine determined at 4, 80, and 600 ng/mL were 102.5 ± 2.5%, 100.8 ± 2.3%, and 98.9 ± 1.1%, respectively, while the recovery of the IS determined at 155 ng/mL was 99.4 ± 3.0% (Table 3).

The matrix effect of blank plasma in the six batches spiked after the sample preparation with 4, 80 and 600 ng/mL of rimantadine were found to be within the acceptable range (Table 3). The same evaluation was performed on the IS and no significant peak area differences were observed (Table 3). Thus, we concluded that ion suppression or enhancement from plasma matrix was negligible for this assay.

3.3.5. Stability

Results of the short-term stability, freeze/thaw stability, autosampler stability, and long-term stability are shown in Table 4. The mean percentages of deviation of calculated versus theoretical concentrations were less than or equal to 3.9% for short-term stability, less than or equal to 3.0% for freeze/thaw stability, less than or equal to 6.1% for autosampler stability, and less than or equal to 3.2% for long-term stability. The good stability of rimantadine simplified the precautions needed for laboratory manipulations during the analytical procedures.

3.4. Application of the method

The developed assay method was applied to a pharmacokinetic study after intragastric administration of 20 mg/kg of rimantadine hydrochloride to rats. The LLOQ of the assay was sufficient to characterize the pharmacokinetics of rimantadine in rats. The mean concentration of rimantadine versus the time profile is shown in Fig. 3. To our knowledge, in previous research,

Table 2
Intra- and inter-day accuracy and precision of rimantadine assay in rat plasma

Concentration (ng/mL)	Intra-day (overall mean, $n = 5$)			Inter-day (overall mean, $n = 15$)		
	Conc. found (ng/mL)	Accuracy (%)	CV (%)	Conc. found (ng/mL)	Accuracy (%)	CV (%)
4	4.0	100.1	2.3	4.0	100.3	2.2
80	84.0	105.0	1.3	83.1	103.9	1.8
600	620.4	103.4	2.3	624.0	104.0	3.0

Table 4
Stability of rimantadine ($n = 3$, mean ± SD)

	Rimantadine nominal conc. (ng/mL)		
	4	80	600
Room temperature (4 h)			
Measured conc. (ng/mL)	3.9 ± 0.2	81.8 ± 2.3	603 ± 9.0
Accuracy (%)	98.4 ± 3.9	101.4 ± 2.9	100.6 ± 1.5
Three freeze/thaw cycles			
Measured conc. (ng/mL)	3.9 ± 0.1	83.0 ± 2.4	612.6 ± 4.1
Accuracy (%)	98.5 ± 1.6	103.7 ± 3.0	102.1 ± 0.7
Autosampler rack for 24 h			
Measured conc. (ng/mL)	3.9 ± 0.2	83.2 ± 2.4	637 ± 20.3
Accuracy (%)	98.5 ± 6.1	104.0 ± 3.0	106 ± 3.4
Stored at −20 °C for 2 weeks			
Measured conc. (ng/mL)	3.8 ± 0.1	83.4 ± 1.4	596.9 ± 1.4
Accuracy (%)	95.5 ± 3.2	104.3 ± 1.7	99.5 ± 0.2

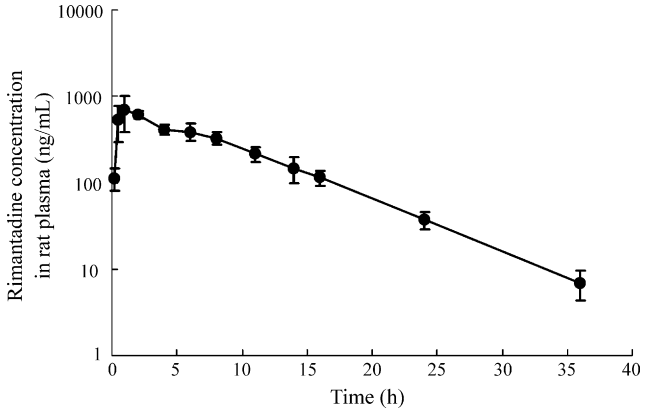

Fig. 3. Average plasma concentration of rimantadine vs. time profile (mean ± SD) in rats ($n = 6$) obtained after intragastric administration of rimantadine hydrochloride (dose 20 mg/kg).

blood samples were only drawn up to 12 h after drug administration in rats [16], which therefore did not give information about $t_{1/2}$. According to the China State of Food and Drug Administration (SFDA) guidance for Nonclinical Pharmacokinetic Studies of Chemical drugs [17], it needs 3–5 elimination half-lives for study. The LLOQ for this method satisfied this requirement, and moreover, it was the first study to provide the parameter $t_{1/2}$ of rimantadine in rats. The partial main pharmacokinetic parameters of this study and previous report are shown in Table 5. The differences of C_{max}, $AUC_{0–t_n}$ and $MRT_{0–t_n}$ might come from the differences in dosage and blood drawing times. Different batches of rats or individual variation might lead to the difference in T_{max}. From Table 5, we could infer that there might be non-linear pharmacokinetics when rimantadine hydrochloride is given to rats in a higher dosage, and this presumption needs further experimental verification.

Table 5
Comparison of partial pharmacokinetic parameters of rimantadine after intragastric administration of rimantadine hydrochloride in rats between this study and previous study ($n = 6$)

Parameters	Application of this study	Reference [16]
Dosage (mg/kg)	20	100
LLOQ (μg/L)	2	50
C_{max} (μg/L)	733 ± 217	1860 ± 320
T_{max} (h)	1.3 ± 0.5	2.5 ± 0.6
Last time point (h)	36	12
$t_{1/2,\lambda z}$ (h)	4.86 ± 0.94	–
$AUC_{0–t_n}$ (μg/Lh)	6113 ± 1452	7970 ± 1130
$MRT_{0–t_n}$ (h)	7.71 ± 1.39	5.78 ± 1.46

4. Conclusion

A rapid and sensitive LC/MS assay method was developed for the determination of rimantadine in rat plasma. Rimantadine was extracted by protein precipitation with high recovery. The assay showed a linear dynamic range of 2–750 ng/mL, with excellent intra- and inter-day accuracy and precision. This assay method was successfully applied to a pharmacokinetic study following intragastric administration of rimantadine hydrochloride (20 mg/kg) in rats.

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