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Short communication

Quantitative determination of amantadine in human plasma by liquid chromatography–mass spectrometry and the application in a bioequivalence study

Wang Ping [a], Liang Yi-Zeng [a,*], Chen Ben-Mei [b], Zhou Neng [a], Yi Lun-Zhao [a], Yu Yan [a], Yi Zhi-Biao [a]

[a] *College of Chemistry and Chemical Engineering, Research Center of Modernization of Chinese Medicines, Central South University, Changsha 410083, PR China*
[b] *Analytical Testing Center of Xiangya Medical School, Central South University, Changsha 410078, PR China*

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Abstract

A sensitive liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) method is developed and validated for rapid determination of amantadine in human plasma. Desloratadine was used as the internal standard (I.S.). Human plasma (0.2 mL) was first alkalified with 100 μL of sodium hydroxide (3 M) and then extracted with 1 mL of *n*-hexane containing 1% isopropanol (v/v) and 10% dichloromethane (v/v) by vortex-mixer for 3 min. The mixture was centrifuged at 14,000 rpm for 5 min. The supernatant was evaporated to dryness and the residue was dissolved in mobile phase. Samples were separated using a Thermo Hypersil-HyPURITYC18 reversed-phase column (150 mm × 2.1 mm i.d., 5 μm). Mobile phase consisted of methanol–acetonitrile–20 mM ammonium acetate (45:10:45, v/v/v) containing 1% acetic acid with pH 4.0. Amantadine and I.S. were measured by electrospray ion source in positive selective ion monitoring mode. The good linearity ranged from 3.9 to 1000 ng/mL and the lowest limit of quantification was 3.9 ng/mL. The extraction efficiencies were approximately 70% and recoveries of method ranged from 98.53 to 103.24%. The intra-day relative standard deviations (R.S.D.) were less than 8.43% and inter-day R.S.D. below 10.59%. The quality control samples were stable when kept at room temperature for 12 h, at −20 °C for 30 days and after four freeze/thaw cycles. The method has been successfully used to evaluation of the pharmacokinetics and bioequivalence of amantadine in 20 healthy volunteers after an oral dose of 100 mg amantadine.

Keywords: Amantadine; LC–ESI-MS; Desloratadine

1. Introduction

Amantadine (1-adamantylamine) is an antiviral agent. It can increase dopaminergic activity in the peripheral and central nervous system. It was widely used in the treatment of influenza A [1,2], hepatitis C [3,4], Parkinsonism [5,6] and multiple sclerosis [7,8]. Amantadine does not possess chromophore for being analyzed by common absorption spectrophotometry. So, many derivatization techniques coupled with chromatography have been established for the analysis of amantadine in biological matrix. Those included GC with radioactive electron-capture detector [9–11], LC-UV [12], LC-fluorimetry [13–18] and capillary electrophoretic-visible diode laser induced fluorescence detection (CE-LIF) [19]. The reported derivatization methods have many disadvantages as follows: (1) the sample pretreatment procedure for derivatization and extraction was complicated, labor-intensive and time-consuming; (2) large volume of plasma samples was used and a lot of reagents for extraction were consumed. Direct analysis of amantadine in serum by LC/MS/MS has been reported [20]. Despite the superiority of MS/MS detector, this method had major drawbacks: (1) relatively high LOQ (50 ng/mL) is proved to be inadequate in the current study; (2) the calibration curve consists of only four standards; (3) QC samples are not in accordance with FDA/EMEA guidelines. In the present study, a sensitive LC–ESI-MS method for the

* Corresponding author. Tel.: +86 731 8830831; fax: +86 731 8825637.
E-mail addresses: error_114@163.com (P. Wang), yizeng_liang@263.net (Y.-Z. Liang).

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determination of amantadine in human plasma was established. The method directly analyzed amantadine without derivatizaton, and the pretreatment was simple and rapid. Application of the proposed method to the studies of pharmacokinetics and bioequivalence of amantadine in the human plasma proved to be specific, sensitive and reproducible.

2. Experimental

2.1. Chemicals and reagents

Amantadine (purity >95.1%) and desloratadine (I.S.) were purchased from National Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China). Acetonitrile (Caledon Company) and methanol (Zhejiang province) were HPLC grade. Other reagents were of analytical grade and all water used was Milli-Q grade (Millipore, Bedford, MA, USA).

2.2. Instrumentation

The HPLC system included a Shimadzu LC-10Advp pump, an SCL-10Advp system controller, a CTO-10Avp column oven, an FCV-10Alvp low pressure gradient unit, a DGU-14A degasser (Shimadzu, Kyoto, Japan). The mass spectrometer was an LCMS-2010 single quadrupole equipped with electrospray ionization interface (Shimadzu). The Speed Vacplus Model vacuum drier (Savant, USA) was used in the preparation of samples. The data processing was carried out using LCMSSolution software.

2.3. LC conditions

The compounds were separated by using Thermo Hypersil-HyPURITYC18 (150 mm × 2.1 mm, i.d., 5 μm) analytical column. The oven temperature was adjusted at 40 °C. Mobile phase consisted of methanol–acetonitrile–20 mM ammonium acetate (45:10:45, v/v/v) containing 1% acetic acid with pH 4.0 and was isocratically eluted at a flow rate of 0.25 mL/min.

2.4. MS conditions

An LCMS-2010 quadrupole mass spectrometer was interfaced with an electrospray ionization (ESI) probe. The temperatures were maintained at 250, 250, and 200 °C for the probe, CDL, and block, respectively. The voltages were set at 4.5 kV, −50 V, 25 V, 150 V, and 1.5 kV for the probe, CDL, Q-array 1, 2, 3 bias, Q-array radio frequency (RF) and detector, respectively. The flow rate of nebulizer gas was 1.5 L/min. For the quantification of amantadine, the analysis was performed in selection ion monitoring in positive ion mode at *m*/*z* 152 (amantadine, M + H) and 311 (desloratadine, M + H). Tuning of mass spectrometer was accomplished with the help of autotuning function of LCMSSolution software (Version 2.02) using tuning standard solution (polypropylene glycol). Optimization and calibration of mass spectrometer were achieved with autotuning.

2.5. Sample preparation

Human plasma (0.2 mL) was transferred into 2 mL centrifuge tube and 50 μL of I.S. was added. One hundred microliters of sodium hydroxide (3 M) was added to alkalify. The mixture briefly vortexed by vortex-mixer and 1 mL of *n*-hexane containing1% isopropanol (v/v) and 10% dichloromethane (v/v) was added. The mixture was extracted by vortex-mixer for 3 min and centrifuged at 14,000 rpm for 5 min. The upper organic layer was pipetted into clear tube and placed into the Speed Vacplus Model Vacuum drier. The dried residue was dissolved in 0.1 mL of mobile phase and then centrifuged; an aliquot of 5 μL supernatant was injected into analysis column.

2.6. Stock solutions, calibration curve and quality control samples

A stock solution of amantadine at concentration of 126 μg/mL was prepared by dissolving the drug in methanol. Stock solution of amantadine was serially diluted with methanol to give a concentration of 15.6, 31.25, 62.5, 125, 250, 500, 1000, 2000 and 4000 ng/mL to construct working standard solutions. A standard solution of desloratadine at concentration of 100 ng/mL was prepared in mobile phase and used as internal standard for all analysis. Calibration curve samples were freshly obtained by adding 50 μL of working standard solutions to 150 μL blank plasma to yield a concentration of 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, 500 and 1000 ng/mL of amantadine. Three concentration levels (low, medium and high) of quality control (QC) samples need to prepare. Stock solution of amantadine was diluted with methanol to give a concentration of 31.25, 250 and 2000 ng/mL to construct preparative QC solutions. The QC samples were obtained by adding 50 μL preparative QC solutions to 150 μL blank plasma to yield a concentration of 7.8, 62.5 and 500 ng/mL of amantadine. The further procedure of both calibration curve samples and QC samples was as described in Section 2.5. All stock solutions were stored at 4 °C refrigerator until analysis.

2.7. Method validation

To quantify amantadine in human plasma, calibration curve must be established. The calibration curves were constructed using simple linear regression method. The peak area ratios of amantadine to the I.S. were taken as dependent variable, while the concentrations of amantadine as independent variable. It should be noticed that every data point used for construction of calibration curve was an average value of five parallel plasma samples, which were freshly prepared and measured on five separate days. The concentrations of amantadine for unknown human plasma were calculated from the regression equation of the calibration curve. The lowest limit of quantification (LLOQ) was estimated as the amount of amantadine that gave a signal ten times the noise (S/N ≥ 10).

The matrix effects (ME) might have some influence on suppression or enhancement of ionization. In order to evaluate the matrix effects, four concentration levels of working standard solutions, say 3.9 (LLOQ), 7.8, 62.5 and 500 ng/mL and I.S.

were taken as samples (parallel samples being five). They were separately dried and re-dissolved in 100 μL of mobile phase (neat standard, group A). Furthermore, another 25 blank plasma samples (0.2 mL) obtained from five different sources (five per source) were placed into 1.5 mL tubes. The samples were processed as described in Section 2.5 and internal standard was not added. The residue was reconstituted in 100 μL of mobile phase containing the same concentrations amantadine and I.S. as group A (group B).

In order to evaluate the extraction efficiency, other 25 blank plasma samples (0.2 mL), obtained from five different sources (five per source), spiked with working standard solutions and I.S. before extraction (group C) to construct the same concentrations as group A. The samples were processed as described in Section 2.5 and internal standard was not added. The analysis indices for evaluation of matrix effects and extraction efficiency (EE) of three groups of samples (A–C) could be calculated as follows:

$$\mathrm{ME}(\%) = \frac{\mathrm{A2}}{\mathrm{A1}} \times 100; \qquad \mathrm{EE}(\%) = \frac{\mathrm{A3}}{\mathrm{A2}} \times 100$$

where A1 is the mean peak area of group A, A2 is the mean peak area of group B, and A3 is the mean peak area of group C.

The accuracies of the experiment were obtained by comparing the measured concentrations to the added concentrations of amantadine spiked in the blank plasma. The precisions and recoveries of the method were estimated by replicating analysis ($n = 5$) of QC samples at three concentrations levels. Intra-day precision was evaluated by analyzing QC samples five times over 1 day, while inter-day precision was estimated by analyzing QC samples five times in three different days. The precision was defined as the intra- and inter-day relative standard deviation. The accuracy was expressed as mean relative error [MRE% = (mean of the measured concentration − added concentration)/added concentration × 100%]. The recoveries of the method for amantadine at three QC levels were estimated by comparing concentrations of amantadine measured from plasma samples with the amantadine spiked.

All the stability studies were conducted QC samples at three concentration levels with five determinations for each. The stability of amantadine was estimated by placing QC samples at room temperature for 12 h. The freeze/thaw stability of amantadine was also assessed by analyzing QC samples undergoing four freeze (−20 °C)/thaw (room temperature) cycles. The long-term stability of amantadine was evaluated by placing QC samples at −20 °C refrigerator for 1 month.

2.8. Application

In this study, 20 adult healthy males volunteers received the investigation. They have to pass the physical examination criteria and have not taken any medication and alcohol for at least 1 week preceding the experiment and fasted for 12 h with free access to water before the experiments. Volunteers took 100 mg amantadine with a 200 mL of water. Blood samples were extracted from the forearm vein following administration and pipetted into heparinized tubes at 0, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 12, 24, 36, 48 and 72 h. The obtained blood samples were immediately separated by centrifugation at 4000 rpm for 10 min and the

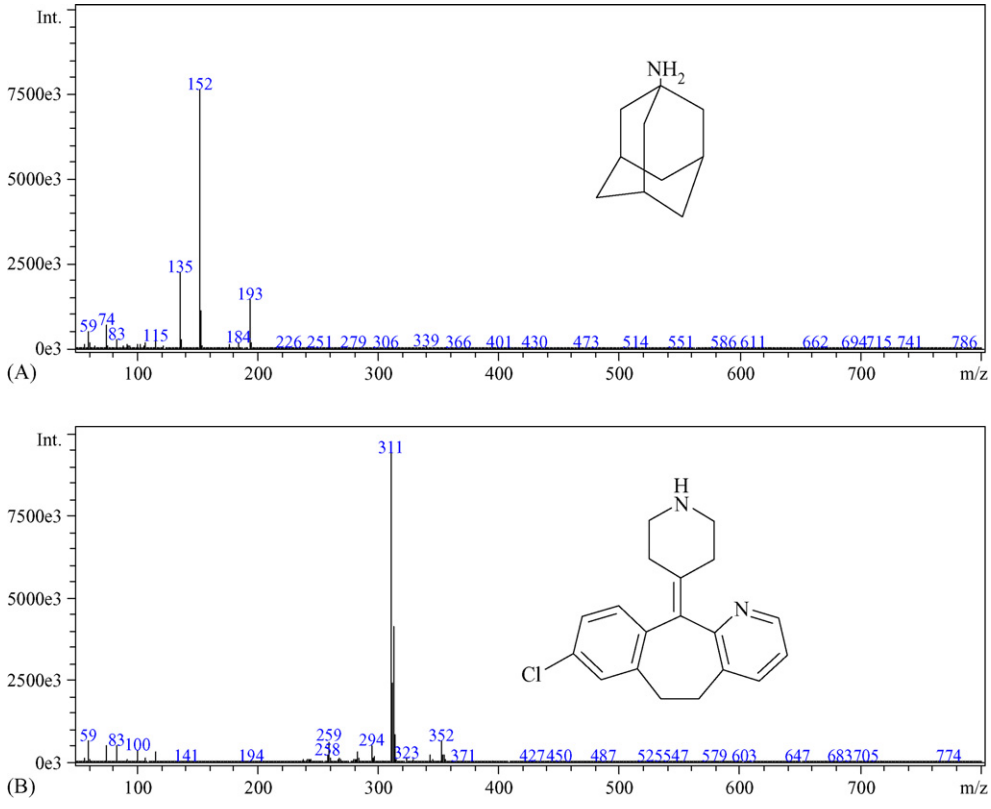

Fig. 1. ESI-MS positive ion scanning spectra and chemical structures of amantadine (A) and desloratadine (B).

supernatants were transferred and stored frozen at −20 °C until analysis.

3. Results and discussion

3.1. Optimization of MS and separation conditions

The choice of ionization mode was guided by base peak with higher intensity in the LC–MS analysis. The mass spectra of amantadine and I.S. obtained from scan mode were characterized by a protonated molecular ion $[M+H]^+$ as base peak. To confirm ionization mode, the mass spectra were measured in ESI and APCI positive and negative mode using injection amantadine and I.S. In both ionization modes, the base peak intensity of positive ion was higher than those of negative ion, and the efficiency of ionization in ESI was higher than APCI. Fig. 1 shows the positive ion mass spectra of amantadine (A) and internal standard (B) by ESI selective ion monitoring. So, selective ion monitoring (SIM) mode ($[M+H]^+$ at *m/z* 152 and 311) was used for quantitative analysis of amantadine and I.S., respectively.

The choice of the chromatographic conditions was selected based on symmetry of peaks shape and short of chromatographic analysis time. The research indicated that the pH of mobile phase and organic modifier percentage have affected on the separation of analytes. The acidity of mobile phase affected the ionization of amantadine. The retention time of analyte and I.S. was also influenced by the acidity of mobile phase. So, mobile phase consisting of methanol–acetonitrile–20 mM ammonium acetate (45:10:45, v/v/v) containing 1% acetic acid with pH 4.0 was used in the experiment.

3.2. Method validation

3.2.1. Matrix effect and selectivity

The data for matrix effects and extraction efficiencies were presented in Table 1.The R.S.D. for matrix effects of the mean peak areas of at four concentrations amantadine and I.S. in five different plasma groups were less than 8%, which strongly indicated little or no difference in ionization efficiency of amantadine and I.S from different groups plasmas. In addition, the extent of the absolute matrix effects was estimated by comparing peak areas of B group (standard and I.S. spiked after extraction) with the corresponding peak areas obtained by injection neat standard and I.S directly, respectively. The ME (%) > 100% indicated ionization enhancement in plasma versus neat standards, while ME (%) < 100% indicated ionization suppression. There was no significant difference in peak areas of the analytes prepared from five different blank plasma samples and from mobile phase. The results indicated that the matrix effects for amantadine and I.S were negligible.

Potential interference from endogenous substances was estimated by analyzing human plasmas of six different sources. Fig. 2(A) shows one of representative chromatogram of six groups of blank plasma, and Fig. 2(B) presents the selective ion chromatogram of the plasma samples at the concentration (500 ng/mL). The retention time of amantadine was about 1.9 min and that of I.S. was approximately 2.6 min. The total run time was 4 min. This realized rapid analysis amantadine in human plasma. This made it possible to analyze more than 400 human plasma samples per day. The endogenous substances from plasma and other impurity did not disturb the separation and measure of samples.

3.2.2. Extraction efficiency

Duh et al. [17] used sodium hydroxide to alkalinize the same volume urine samples and the same volume toluene was used to extract. However, the extraction efficiency has not been reported. In the present experiment, different concentration and different volume of sodium hydroxide were researched with respect to extraction efficiency. Different organic solvents were tested for extraction of amantadine from plasma, such as *n*-hexane, methanol, isopropanol, acetonitrile and dichloromethane. The best extraction solvent was *n*-hexane containing 1% isopropanol (v/v) and 10% dichloromethane (v/v) in the present experiment. The method was better than previously reported methods [10–19] based on procedure for pretreatment.

Table 1 shows extraction efficiencies of amantadine. The extraction efficiencies observed ($n = 5$) were 69.04, 68.84, 68.63 and 70.98% (3.9, 7.8, 62.5 and 500 ng/mL, respectively) and 67.11% for I.S. (100 ng/mL).

3.2.3. Linearity and sensitivity

The nine-point linear regression equation from calibration curve samples was obtained as follows: $y = 0.0048x + 0.0035$ with the correlation coefficient of 0.9979. The results indicated that a very good linearity between y and x was attainable over

Table 1
Extraction efficiencies and matrix effect of amantadine and I.S. ($n = 5$)

Nominal concentration (ng/mL)	Peak area[a]	Peak area[b]	Peak area[c]	Matrix effect	Mean recovery (%)
3.9 (LLOQ)	16,475 (7.94)[d]	15,930 (7.63)	10,998 (10.20)	96.69	69.04
7.8	32,838 (7.56)	31,932 (7.11)	21,982 (9.11)	97.24	68.84
62.5	248,731 (6.36)	255,273 (5.92)	175,194 (8.33)	102.63	68.63
500	1,925,641 (4.28)	1,989,380 (3.54)	1,412,062 (7.89)	103.31	70.98
I.S.	1,433,423 (6.91)	1,419,805 (4.57)	952,831 (9.75)	99.05	67.11

[a] Neat standard and I.S.
[b] Standard and I.S. spiked after extraction.
[c] Standard and I.S. spiked before extraction.
[d] Number in parentheses was R.S.D.

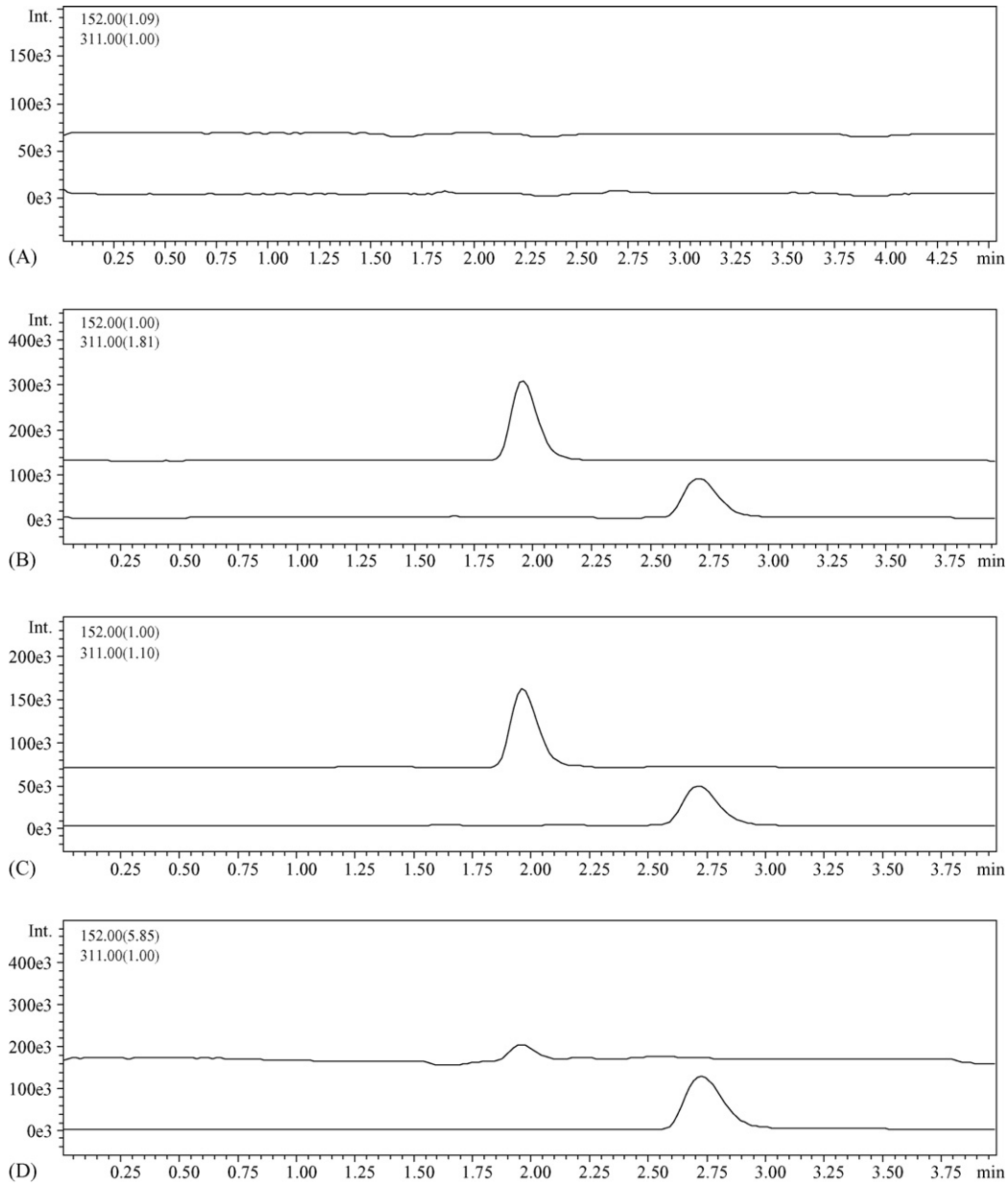

Fig. 2. Selective ion chromatograms of amantadine and desloratadine (I.S.). (A) Blank plasma; (B) blank plasma spiked with amantadine (500 ng/mL) and IS; (C) human plasma sample after administration of amantadine and spiked with I.S.; (D) blank plasma spiked with amantadine (3.9 ng/mL) and I.S.

3.9–1000 ng/mL. The lowest limit of quantification (LLOQ) for determination of amantadine in human plasma was 3.9 ng/mL with accuracy ranged from −4.21 to 5.38 and precision ≤10.02%. It is sufficient for pharmacokinetic study. The results were presented in Table 2. The method was better than previously reported methods [10–19] by virtue of sensitivity.

3.2.4. Precision and accuracy

The method showed very good precision and accuracy. The intra- and inter-day precision (R.S.D.) and accuracy (MRE) were studied in the QC samples at three concentrations levels. The results indicated that the R.S.D. and MRE for intra- and inter-day analysis were below 11%. The results were summarized in Table 3. The method was sufficient to the study of pharmacokinetic and bioequivalence.

3.2.5. Samples stability

Amantadine was shown to be stable under conditions of storage and analysis processing. Amantadine was also stable in human plasma when stored at room temperature at least 12 h, at −20 °C for at least 30 days. Amantadine was stable under the influence of four freeze/thaw cycles. Table 3 shows the stability data of amantadine kept various storage conditions and freeze–thaw cycles.

3.3. Application

The method was applied to determine plasma concentration of amantadine after an oral administration of amantadine (100 mg) to 20 healthy volunteers. Mean plasma concentration-time profiles of amantadine were presented in Fig. 3. The main

Table 2
Average precision, accuracy and linear regression parameters of amantadine determination in human plasma

Added concentration (ng/mL)	Mean measured concentration ($n = 5$) (ng/mL)	Precision R.S.D. (%)	Accuracy mean relative error[a]
3.9	4.11	10.02	5.38
7.8	8.13	7.82	4.06
15.6	14.97	5.32	−4.21
31.25	32.93	6.24	5.10
62.5	64.58	4.76	3.22
125	128.30	3.96	2.57
250	257.65	4.37	2.97
500	490.13	5.51	−2.01
1000	1015.32	4.58	1.51
Calibration curve:			
Slope	0.0048		
Intercept	0.0035		
Correlation coefficient	0.9979		

[a] Mean relative error = (mean measured concentration − added concentration) × 100/added concentration.

Table 3
Precision, accuracy and stability data for amantadine of QC samples in human plasma

Nominal concentration (ng/mL)	Mean found concentration ($n = 5$) (ng/mL)	Precision R.S.D. (%)	Accuracy MRE[a] (%)
Intra-run			
7.8	7.76 (0.65)[b]	8.43	−0.51
62.5	62.38 (4.97)	7.96	−0.19
500	492.66 (21.02)	4.27	−1.47
Inter-run ($n = 3$ days, five replicates per day)			
7.8	7.69 (0.81)	10.59	−1.45
62.5	64.53 (5.57)	8.63	3.24
500	501.31 (29.70)	5.93	0.26
Short-term stability for 12 h in plasma at room temperature			
7.8	7.66 (0.78)	10.22	−1.79
62.5	63.23 (4.16)	6.58	1.17
500	492.12 (27.71)	5.63	−1.58
Long-term storage at −20 °C for 30 days			
7.8	7.76 (0.73)	9.41	−0.51
62.5	60.98 (4.04)	6.63	−2.43
500	509.04 (28.96)	5.69	1.81
Four freeze/thaw cycles			
7.8	7.56 (0.56)	7.40	−3.08
62.5	62.86 (5.10)	8.12	0.58
500	498.90 (38.07)	7.63	−0.22

[a] Mean relative error = (mean measured concentration − added concentration) × 100/added concentration.
[b] Number in parentheses was S.D.

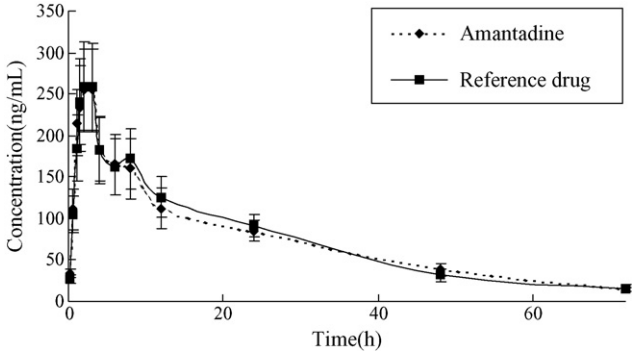

Fig. 3. Mean plasma concentration–time profile of 20 healthy volunteers after an oral administration of 100 mg dose of amantadine. Solid line: reference drug; dashed line: test drug.

pharmacokinetic parameters of amantadine in 20 volunteers were calculated. Table 4 shows the pharmacokinetic parameters of amantadine and reference drug. The bioequivalence of drugs was determined with respect to AUC_{0-t}, C_{max}, T_{max}, $T_{1/2}$ and

Table 4
Pharmacokinetic properties of two oral formulations (amantadine and reference drug) of single-dose 100 mg amantadine in healthy subjects ($N = 20$)

Property	Amantadine (T)	Reference drug (R)	T/R
C_{max} (ng/mL)	391.34 (127.03)	401.83 (128.55)	0.97
T_{max} (h)	2.65 (0.63)	2.48 (0.52)	1.07
AUC_{0-t} ([ng h]/mL)	5322.93 (1132.41)	5419.61 (1145.20)	0.98
$T_{1/2}$ (h)	12.05 (3.47)	11.69 (3.26)	1.03
C_{max}/AUC_{0-t} (h^{-1})	0.07	0.07	1.00

Values are mean (S.D.).

C_{max}/AUC_{0-t}. As can be seen from Table 4, the pharmacokinetic parameters of test (amantadine) drug were very close to those of reference drug. In this study in 20 healthy volunteers, a single, 100 mg dose of test drug (amantadine) was found to be bioequivalent to reference drug based on the rate and extent of absorption.

4. Conclusion

A rapid, simple and specific LC–ESI-MS method for determination of amantadine in human plasma has been described. Method validation has been proved by a variety of tests for matrix effects, extraction efficiency, selectivity, linearity, sensitivity, precision, recovery and stability. The method has several advantages compared to the previously reported methods [9–19], as it provides better sensitivity, simpler sample pretreatment and smaller volume plasma samples and extract. Compared with the reported LC/MS/MS method [20], the proposed method was more suitable for the pharmacokinetics and bioequivalence study of amantadine. The method has been successfully applied to evaluation of the pharmacokinetics and bioequivalence of amantadine in 20 healthy volunteers after an oral dose 100 mg amantadine.

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