

Determination of memantine in human plasma by liquid chromatography–electrospray tandem mass spectrometry: Application to a bioequivalence study

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Abstract

A simple, sensitive and specific liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the quantification of memantine (**I**) in human plasma is presented. Sample preparation consisted of the addition of amantadine (**II**) as internal standard (IS), liquid–liquid extraction in basic conditions using a mixture of diethyl ether–chloroform (7:3, v/v) as extracting solvent, followed by centrifugation, solvent evaporation and sample reconstitution in methanol. Both **I** and **II** (internal standard) were analyzed using a C18 column and a mobile phase composed of methanol–water–formic acid (80:20:0.1, v/v/v). Eluted compounds were monitored using positive mode electrospray (ES) tandem mass spectrometry. The analyses were carried out by selected reaction monitoring (SRM) using the parent to daughter combinations of m/z 180 > 163 (memantine) and m/z 152 > 135 (amantadine). The peak areas from the analyte and IS were used for quantification of **I**. The achieved limit of quantification (LOQ) was 0.1 ng/mL; the assay exhibited a linear dynamic range of 0.1–50.0 ng/mL with a determination coefficient (r^2) of at least 0.98. Validation results on linearity, specificity, accuracy, precision and stability, as well as on application to the analysis of samples taken up to 320 h after oral administration of 20 mg (two 10 mg capsules) of **I** in healthy volunteers demonstrated the applicability to bioequivalence studies.

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1. Introduction

Memantine (**I**), 1-amino-3,5-dimethyladamantane hydrochloride (CAS – 19982-08-2) is an adamantane derivative administered orally for Alzheimer disease. Memantine is well absorbed, with peak plasma concentrations (C_{\max}) ranging from 22 to 46 ng/mL following a single dose of 20 mg; the time to achieve maximum plasma concentration (T_{\max}) following single doses of 10–40 mg ranges from 3 to 8 h. Daily administration of 20 mg results in steady-state plasma concentrations ranging from 70 to 150 ng/mL, with marked inter-individual variation [1].

Memantine is 45% bound to plasma proteins presenting a distribution volume of approximately 9–11 L/kg, which suggests an extensive distribution into tissues. Memantine is poorly metabolized by the liver and 57–82% of the administered dose is excreted, unchanged, in the urine; with a mean terminal half-life of 70 h [2].

Quantification of drugs in biological matrices by liquid chromatography–tandem mass spectrometry (LC–MS/MS) is becoming more common, due to the improved sensitivity and specificity of this technique [3,4].

Other techniques have been previously used to determine memantine in a variety of matrices. These methods include high performance liquid chromatography (HPLC) [5] and gas chromatography coupled to mass spectrometry (GC–MS) [6] and LC–MS [7]. Some of them were not developed for use in biological matrices, while others have either a very high limit of

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quantification (LOQ) or are too much complex, which limits its application for a larger number of samples.

This paper describes a sensitive and specific liquid chromatography coupled to electrospray (ES) tandem mass spectrometry method for memantine quantification in biological samples [8].

2. Experimental

2.1. Materials and reagents

Memantine hydrochloride was obtained from Sigma (St. Louis, MO, USA) and amantadine (internal standard, IS) was obtained from USP (Rockville, MD, USA). Formic acid and sodium hydroxide were purchased from Merck (Darmstadt, Germany) and HPLC grade methanol from J.T. Baker (Deventer, Netherlands). Diethyl ether and chloroform were of GR grade and purchased from E. Merck. Water was purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other chemicals were of analytical grade and used without further purification.

2.2. Instrumentation and chromatographic conditions

An aliquot (10 μL) of the plasma extract was injected into a Polaris C-18 (50 mm \times 3 mm, 3 μm) column (Varian, USA) using a Varian ProStar LC system (Varian, USA). Separation and elution were achieved using methanol–water–formic acid (80:20:0.1, v/v/v) as the mobile phase, at a flow-rate of 0.15 mL/min. The column was at room temperature (25 $^{\circ}\text{C}$) and injection time between injections was 3.0 min. Mass spectrometric detection was performed using a Varian LC 1200 (Varian, USA) triple quadrupole mass spectrometer, equipped with an electrospray source. The temperatures of the desolvation gas and source block were 300 and 45 $^{\circ}\text{C}$, respectively. Nitrogen was used as both, nebulizer (55 psi) and desolvation gas (22 psi). The electrospray source was operated in the positive ionization mode (ES+) at 5000 V, and multiple reaction monitoring mode (SRM), m/z 180 > 163 and m/z 152 > 135 were used for quantification of memantine and amantadine, respectively. Capillary voltage, collision energy and collision gas pressure (argon) were 40, 15.5 V, and 2.8×10^{-5} Torr, respectively, for memantine, and 40, 18.0 V, and 2.8×10^{-3} Torr for amantadine, respectively.

2.3. Preparation standards and quality control (QC) samples

Stock solutions of **I** (100 $\mu\text{g}/\text{mL}$, 1000, 100, and 10 ng/mL) and **II** (100 $\mu\text{g}/\text{mL}$) were prepared in Milli-Q water. Aliquots of the 1000, 100, and 10 ng/mL solutions were used to spike blank human plasma in order to obtain calibration standards of 0.1, 0.2, 0.5, 2.0, 5.0, 10.0 and 50.0 ng/mL . Three levels of quality controls fixed at 0.3, 20.00, and 40.0 ng/mL (low, medium and high) were prepared using the same blank plasma. All spiked plasma was stored at -20°C .

2.4. Sample preparation

The samples were stored as 200 μL aliquots at -20°C ; aliquots were thawed at room temperature before processing. Fifty microliters of 0.05 M NaOH and a 25 μL aliquot of internal standard solution (1 $\mu\text{g}/\text{mL}$ of amantadine) were added to 200 μL of plasma and the tube was briefly shaken. The mixture was then vortex-mixed with 1 mL of diethyl ether–chloroform (7:3, v/v) for 5 min at 1800 rpm. The tube was centrifuged 5 min at 14,000 rpm and the upper organic phase (900 μL) transferred to another tube and evaporated to dryness under a nitrogen stream at room temperature. The residue was dissolved in 100 μL of methanol, the sample was then transferred to the glass autosampler vial and 10 μL was injected into the chromatographic system.

2.5. Recovery

The extraction efficiency of memantine from human plasma was determined by analyzing quality control samples. The recovery at three concentrations was determined by comparing peak areas obtained from the plasma sample and the standard solution spiked with the blank plasma residue. The recovery of IS was also tested using the same procedure.

2.6. Limit of quantification

The limit of quantification was defined as the lowest concentration at which precision and accuracy, expressed by relative standard deviation (R.S.D.), were lower than 20%.

2.7. Analytical curves

The analytical curves were constructed using values ranging from 0.1 to 50 ng/mL of memantine in human plasma. Calibration curves were obtained by weighted linear regression (weighing factor: $1/x$), the ratio of **I** peak area to **II** peak area was plotted versus the ratio of **I** concentration to that of the internal standard, in nanogram per milliliters. The suitability of the calibration model was confirmed by back-calculating the concentrations of the calibration standards.

2.8. Accuracy and precision

Quality controls of memantine (0.3, 20.00 and 40.00 ng/mL) were determined using the corresponding standard curves. The accuracy of the method was shown as relative error (RE) and calculated based on the difference between the mean calculated and nominal concentrations; whereas precision was evaluated by calculating the within- and between-run relative standard deviations.

2.9. Freezing and thawing stability

Freezing and thawing stability for **I** in plasma samples was studied in three cycles with control concentrations in four plasma batches. Samples were frozen at -20°C in three cycles of 24, 36

and 48 h. In addition, the long-term stability of memantine in QC samples was also evaluated by analysis after 5 months of storage at -20°C . Autosampler stability was studied over a 24 h storage period in the autosampler tray with control concentrations.

2.10. Pharmacokinetics and statistical analysis

Pharmacokinetics parameters were calculated from plasma levels applying a non-compartmental statistic using WinNonLin 5.0 software (Pharsight, USA). Following Food and Drug Administration (F.D.A) guideline [8], blood samples were drawn up to a period of three to five times the terminal elimination half-life ($t_{1/2}$) and it was considered as the area under the concentration-time curve (AUC) ratio higher than 80%. The C_{\max} and T_{\max} values were determined by visual inspection of the plasma memantine concentration-time profiles. The area under the concentration-time curve (AUC_{0-t}) was obtained by the trapezoidal method. The total area under the curve ($\text{AUC}_{0-\infty}$) was calculated up to the last measurable concentration and extrapolations were obtained using the last measurable concentration and the terminal elimination rate constant (K_e). The terminal elimination rate constant, K_e , was estimated from the slope of the terminal exponential phase of the plasma of memantine concentration-time curve (by means of the linear regression method). The terminal elimination half-life, $t_{1/2}$, was then calculated as $0.693/K_e$. Results are indicated as mean \pm standard deviation throughout the paper.

Regarding AUC_{0-t} and C_{\max} bioequivalence was assessed by means of an analysis of variance (ANOVA) and calculating the standard 90% confidence intervals (90% CIs) of the ratios test/reference (logarithmically transformed data). The bioequivalence was considered when the ratio of averages of log-transformed data were within 80–125% for AUC_{0-t} and C_{\max} .

3. Results and discussion

3.1. Method development

Among the different possible detection techniques that can be coupled to LC, mass spectrometry has been unparalleled for performing bioanalytical determinations with maximum selectivity and sensitivity [9].

The MS optimization was performed by direct infusion of solutions of both memantine and amantadine (IS) into the ESI source of the mass spectrometer. The critical parameters in the ESI source includes the needle (ESI) voltage, which is directly related to the charged droplet formation and to the amount of

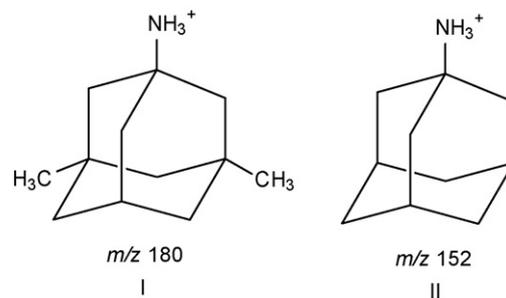


Fig. 1. Chemical structures of memantine I and amantadine II (IS).

gaseous ions formed. Capillary voltage is related to gaseous ion guidance to the inside of the MS and is the last barrier between the atmospheric pressure and the high vacuum of the mass spectrometer. Other parameters, such as the nebulizer and the desolvation gases are meant to be optimized to obtain better spray shape, resulting in better ionization and droplet drying to form, in our case, the protonated ionic I and II (IS) molecules (Fig. 1).

A collisionally-activated dissociation (CAD) product ion spectrum for I and II yielded high-abundance fragment ions of m/z 163 and m/z 135 (Fig. 2).

Scheme 1 shows the proposed dissociation mechanism for the protonated I of m/z 180, a protonated amine by ammonia loss forming the product ion of m/z 163 that is a very stable tertiary carbocation stabilized by p- π interaction.

After the SRM channels were tuned, the mobile phase was changed from an organic phase to a more aqueous phase with acid dopant to obtain a fast and selective LC method. A better signal was obtained using methanol-water-formic acid (80:20:0.1, v/v/v) as mobile phase.

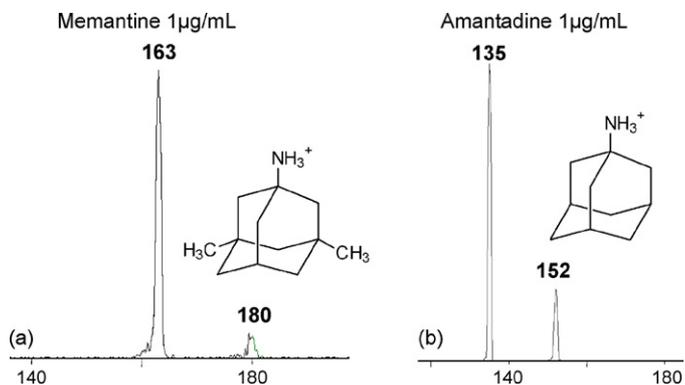
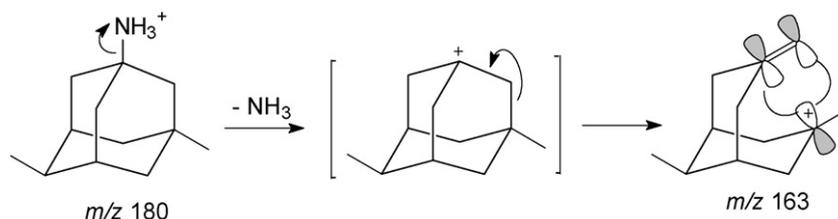


Fig. 2. CAD mass spectra of the (a) memantine and (b) amantadine protonated molecules.



Scheme 1.

3.2. Specificity

The analysis of **I** and **II** using SRM function was highly selective, with no interfering compounds nor significant ion suppression from endogenous substances observed at the retention times for memantine and IS; as shown in Fig. 3. The chromatographic run was executed using a short (50 mm) HPLC column, which is convenient for running a high throughput of samples. There was no chromatographic separation, due to the high degree of similarity shared by the two structures, with the adjusted retention time being as short as 1 min, in order to increase the analytical capability (Fig. 3a and b). Chromatograms obtained from plasma spiked with **I** (0.3 ng/mL) and **II** (250.0 ng/mL) are shown in Fig. 3c and d.

The matrix effect was evaluated directly extracting blank plasma and then spiking with the analyte at the LOQ concen-

tration. There was no difference observed in the signal for the solution and the spiked extract at the LOQ concentration.

3.3. Linearity, precision and accuracy

Calibration curves were plotted as the peak area ratio (drug/I.S.) versus drug concentration. The assay was linear in the concentration range of 0.1–50 ng/mL. The R.S.D.s were less than 10%. The relative error of the mean of the measured concentrations ranged from 7.33 to –9.00%. The determination coefficients (r^2) were greater than 0.98 for all curves (Table 1).

Precision and accuracy for this method were controlled by calculating the intra-batch and inter-batch variation at three concentrations (0.30, 20.00 and 40.00 ng/mL) of QC samples in five replicates. As shown in Table 2, the intra-batch R.S.D.s and REs were less than 10.03%. These results indicate that the method is reliable and reproducible within its analytical range.

3.4. Freezing and thawing stability

The results of the freeze–thaw stability studies are shown in Table 3. Quantification of the analyte in plasma subjected to a number of freeze–thaw (–20 °C to room temperature) cycles showed that the analyte is stable after three cycles. No degradation of the analyte had taken place over a 24 h storage period in the autosampler tray with the final concentrations of memantine ranging from 101.51 to 109.42% of the theoretical values. In addition, the long-term stability of memantine in QC samples after 150 days of storage at –20 °C was also evaluated. The concentrations ranged from 94.0 to 102.7% of the theoretical values. Memantine was therefore stable in human plasma for at least 150 days at –20 °C.

3.5. Recovery

The recovery for the sample preparation, which involved a liquid–liquid extraction with diethyl ether/chloroform (7:3, v/v), was calculated by comparing the peak area ratios of **I** in plasma samples with the peak area ratios of solvent samples and was estimated at control levels of **I**. The recovery of **I**, determined at three different concentrations (0.30, 20.00 and 40.0 ng/mL), were 71.3, 69.4 and 77.4%, respectively; the overall average recovery was 72.7%.

3.6. Application to biological samples

The proposed method was applied to the determination of memantine in plasma samples for the purpose of establishing the bioequivalence of a single 20 mg dose (two 10 mg capsules) in 30 healthy volunteers. Typical plasma concentration versus time profiles are shown in Fig. 4. Plasma concentrations of memantine were in the standard curve range and remained above the 0.1 ng/mL quantitation limit for the entire sampling period. The observed maximum plasma concentration (C_{\max}) that is collect time independent was 32.3 ± 7.48 ng/mL for the standard and 30.3 ± 8.89 ng/mL for the test. The corresponding time of maximum concentration (T_{\max}) was 2.73 ± 1.57 h

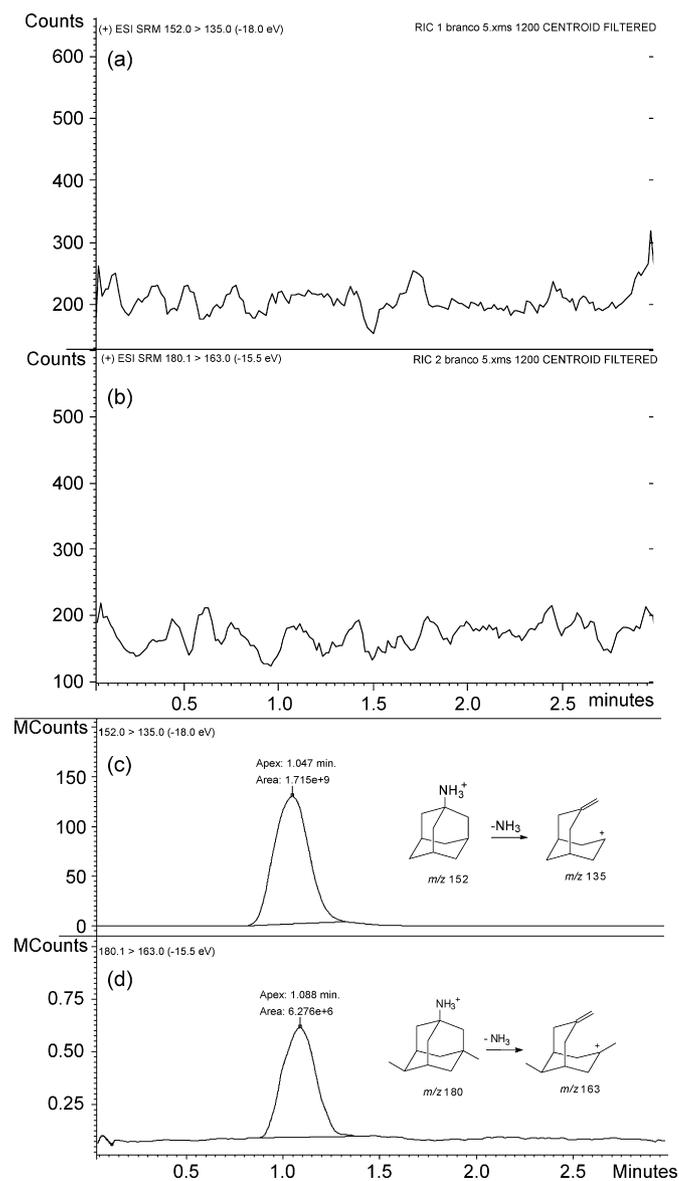


Fig. 3. Representative SRM chromatograms of memantine in human plasma: (a) **I** and (b) **II** blank human plasma; (c) **I** and (d) **II** spiked human plasma containing 0.30 ng/mL memantine and internal standard **II**.

Table 1
Calibration curves from one batch of the validation section

Spiking plasma concentration (ng/mL)	Concentration measured (mean) (ng/mL)	R.S.D. ^a (%) (<i>n</i> = 3)	Relative error ^b
0.10	0.104	6.29	4.00
0.20	0.201	0.53	0.50
0.50	0.505	0.11	1.00
2.00	2.062	0.32	3.10
5.00	4.918	2.29	−1.65
10.00	9.771	8.59	−2.29
50.00	47.220	5.19	−5.56

^a Standard deviation/mean concentration measured.

^b [(Mean concentration measured – spiked plasma concentration)/spiked plasma concentration] × 100.

Table 2
Precision and accuracy (analysis with spiking plasma samples at three different concentrations)

Spiking plasma concentration (ng/mL)	Within-run			Between-run		
	Concentration measured (mean ± S.D.) (ng/mL)	R.S.D. ^a (%) (<i>n</i> = 5)	Relative error ^b (%)	Concentration measured (mean ± S.D.) (ng/mL)	R.S.D. ^a (%) (<i>n</i> = 15)	Relative error ^b (%)
0.30	0.29 ± 0.029	10.03	−3.18	0.32 ± 0.028	9.21	0.65
20.00	19.51 ± 0.664	3.41	−2.47	20.87 ± 1.564	7.50	4.32
40.00	39.48 ± 3.483	8.82	−1.29	41.265 ± 7.91	7.91	3.16

^a Standard deviation/mean concentration measured.

^b [(Mean concentration measured – spiked plasma concentration)/spiked plasma concentration] × 100.

Table 3
Freeze and thaw stability of the samples

Sample concentration (ng/mL)	Initial (0 h)		Cycle 1 (24 h)		Cycle 2 (36 h)		Cycle 3 (48 h)	
	Concentration measured (mean ± S.D.) (ng/mL)	R.S.D. ^a (%) (<i>n</i> = 5)	Concentration measured (mean ± S.D.) (ng/mL)	R.S.D. ^a (%) (<i>n</i> = 5)	Concentration measured (mean ± S.D.) (ng/mL)	R.S.D. ^a (%) (<i>n</i> = 5)	Concentration measured (mean ± S.D.) (ng/mL)	R.S.D. ^a (%) (<i>n</i> = 5)
0.30	0.27 ± 0.016	6.00	0.305 ± 0.015	4.84	0.29 ± 0.028	9.82	0.29 ± 0.031	10.61
20.00	18.06 ± 0.503	2.78	20.54 ± 1.083	5.27	18.03 ± 1.069	5.93	18.02 ± 1.077	5.98
40.00	35.67 ± 0.894	2.51	40.37 ± 1.082	2.68	38.79 ± 3.58	9.28	35.78 ± 0.806	2.25

^a Standard deviation/mean concentration measured.

for reference drug and 4.18 ± 4.88 h for generic drug. The value of area under the curve from time 0 to the last sampling time (AUC_{0-t}) was 1879.6 ± 498.5 ng h/mL for the standard and 1868.9 ± 532.3 ng h/mL for the test, and area under the curve from 0 to ∞ ($AUC_{0-\infty}$) was 1928.1 ± 515.23 ng h/mL for the standard and 1918.85 ± 551.18 ng h/mL for the test. The elimi-

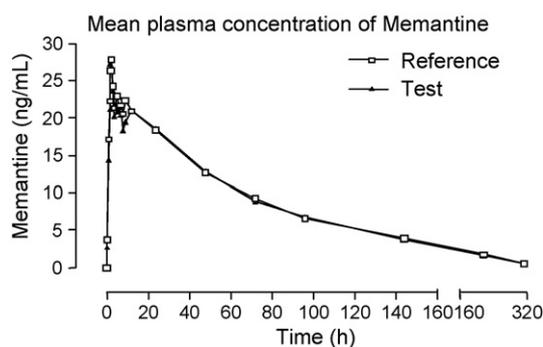


Fig. 4. Mean plasma concentrations of test vs. reference after a 20 mg single oral dose (30 healthy volunteers).

nation half-life ($t_{1/2}$) was 57.39 ± 8.68 h for the reference drug and 58.32 ± 12.2 h for generic drug. The pharmacokinetic data obtained were similar to those reported by Periclou et al. [7]. In addition, the mean ratio of $AUC_{0-t}/AUC_{0-\infty}$ was higher than 90% with following the Food and Drug Administration Bioequivalence Guideline [8].

The ratio test/reference (T/R) and 90% confidence intervals (90 CIs) for overall analysis were comprised within the previously stipulated range (80–125%). The ratio T/R and 90 CIs (in parenthesis) were 92.2% (84.52–100.49%) for C_{max} , 97.8% (91.49–104.57%) for AUC_{0-t} and 98.5% (92.08–105.33%) for $AUC_{0-\infty}$. Therefore, it can be concluded that the two memantine formulations (reference and test) analyzed are bioequivalent in terms of rate and extent of absorption.

4. Conclusion

In conclusion, the use of LC–MS/MS allows an accurate, precise and reliable measurement of memantine concentrations in human plasma for up to 320 h after oral administration of 20 mg

to healthy volunteers. The described method has proven to be fast and robust, with each sample requiring less than 3 min of analysis time. The assay method is also highly specific due to the inherent selectivity of tandem mass spectrometry and has significant advantages over other techniques previously described for measuring memantine in biological fluids. The sensitivity of the assay is sufficient to follow accurately the pharmacokinetics of this drug.

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