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RAPID LC/MS^3 METHOD FOR DETERMINATION OF MEMANTINE IN PIG PLASMA

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ABSTRACT. A simple and sensitive liquid chromatography coupled with tandem mass spectrometry (LC/MS^3) method for the quantification of memantine in pig plasma was developed and validated. The separation was performed on a Zorbax SB-C18 column under isocratic conditions using a mobile phase of 55:45 (v/v) methanol and 0.1% (v/v) formic acid in water at 45 ºC with a flow rate of 1 mL/min. The detection of memantine was performed in multiple reaction monitoring mode using an ion trap mass spectrometer with electrospray positive ionisation, operating in MS^3 mode. The pig plasma samples (0.2 mL) were deproteinized with 6% perchloric acid in water and aliquots of 10 µL from supernatants obtained after centrifugation were directly injected into the chromatographic system. The method shows a good linearity ($r > 0.995$), precision (CV < 12.2%) and accuracy (bias < 12.1%) over the range of 4.86-486 ng/mL plasma. The lower limit of quantification (LLOQ) was 4.86 ng/mL and the recovery was between 92.5-100.8%. The developed and validated method is simple, rapid and specific for the determination of memantine in pig plasma and was successfully applied to a pharmacokinetic study of intravenously administered memantine in pigs.

Keywords: *memantine, LC/MS^3, human plasma, pig plasma*

INTRODUCTION

Memantine, 3,5-dimethyladamantan-1-amine (Figure 1) is a N-methyl-D-aspartate (NMDA) receptor antagonist with neuroprotective effect and is currently used for treating of patients with vascular dementia, Alzheimer disease, hemorrhagic stroke and neuropathic pain [**1-3**].

The process of neuronal degradation in cases of deprived oxygen supply (ischemia), as in cardiac arrest, is closely connected with the activity of the NMDA receptors [**1**]. Despite recent acquisition regarding neuronal vulnerability to hypoxia, at the moment the only clinically available mean of neuroprotection is the therapeutic hypothermia, but this method has limited applicability [**4**]. The similar mechanisms of neuronal injury in Alzheimer disease and in cerebral ischemia suggested the idea of potentially neuroprotective effects of intravenous memantine administered during global cerebral ischemia due experimentally induced cardiac arrest in pigs. In order to evaluate the pharmacological effect of memantine in induced cerebral ischemia and for evaluation of its pharmacokinetics, a fast and reliable analytical method for determination of memantine in pig plasma was needed.

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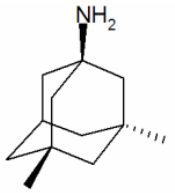

Figure 1. Chemical structure of memantine

Several methods involving gas-chromatography (GC) coupled with mass spectrometry (MS) [**5**] and high-performance liquid-chromatography (LC) with mass spectrometry (MS) [**6-10**] detection have been reported for determination of memantine in biological matrix.

The GC method requires derivatisation of memantine after liquid-liquid extraction (LLE) in order to transform it in a volatile molecule [**5**]. However, both extraction and derivatization are time-consuming steps, increasing the cost of assay and can affect the recovery. The LC/MS or LC/MS/MS methods offer considerable advantages by their powerful performances: speed, selectivity, sensitivity and robustness. However, the sample preparation procedure by solid phase extraction (SPE) [**6**] or LLE [**7-9**] may complicate the analysis in terms of speed and recovery.

The aim of this work was to develop and validate a new simple, specific and efficient LC/MS^3 assay for the quantification of memantine in pig plasma for application in a pharmacokinetic study.

RESULTS AND DISCUSSION

Sample preparation

In LC/MS assays the sensitivity depends on MS detection mode, but the method involved in sample preparation may also influence the chromatographic background level and can generate matrix suppression effects. Usually an extraction step of analyte from matrix prior to analysis (SPE or LLE) has two main advantages: sample purification and sample pre-concentration. As stated before, the extraction step (either SPE or LLE) is laborious, time consuming and usually needs an internal standard to compensate the extraction variability. The protein precipitation (PP), as sample processing method is desirable when one need a high-throughput analysis, and low sample-to sample extraction variability. However, the two main advantages of SPE or LLE extraction mentioned before become drawbacks in case of PP: first- the sample is not really purified so matrix interferences or high background noise may appear; and second - the sample is physically diluted during precipitation, lowering the method sensitivity. Thus, the working parameters in developing an analytical method are related to the performance needed: the sample preparation time and costs, the method speed and sensitivity.

Although after oral administration of memantine in humans, its maximum plasma levels are about 15-25 ng/ml [**6-9**], after intravenous administration (IV) in pigs the maximum plasma levels of memantine are much higher, about

400 ng/mL (pilot study, unpublished data). In that case the calibration curve range was adapted to the concentration range of the samples to be analyzed, in our case 4.86-486 ng/ml. Because the current assay does not require a high sensitivity as in human pharmacokinetic studies, the PP extraction method becomes an attracting alternative to SPE or LLE due the high speed and the high reproducibility of the extraction.

Table 1. Analytical characteristics of reported LC/MS and LC/MS/MS methods for the determination of memantine in biological matrix

Matrix	Pre-treatment/ extraction[a]	Mobile phase constituents[b]	Detection mode[c]	LOQ[d] (ng/mL)	Run time (min)	Reference
Human plasma	SPE	ACN - ammonium acetate buffer	ESI-MS/MS, SRM (m/z 180→163)	0.2	2	**6**
Human plasma	LLE	MeOH- formic acid in water	APCI-MS SIM, m/z 180	0.2 L	6	**7**
Human plasma	LLE	MeOH– formic acid in water	ESI-MS/MS, SRM (m/z 180→163)	0.1	2	**8**
Human plasma	LLE	MeOH– formic acid in water	ESI-MS/MS, SRM (m/z 180→107)	0.1	4	**9**
Rat plasma	Cloud point extraction	MeOH– formic acid in water	APCI-MS SIM, m/z 180	2	6	**10**

[a] SPE, solid-phase extraction; LLE, liquid–liquid extraction;
[b] ACN, acetonitrile; MeOH, methanol;
[c] ESI, electrospray ionisation; APCI, atmospheric pressure chemical ionisation; SIM, selected ion monitoring; SRM, selected reaction monitoring;
[d] LOQ, limit of quantification

In our method we analysed volumes of only 0.2 mL plasma by PP with 7% perchloric acid (0.1 mL) and direct injection into the chromatographic system from supernatant after centrifugation. We obtained a sensitivity corresponding to our needs (LLOQ of 4.86 ng/mL) and absolute recoveries between 92.5-100.8%, this being the first analytical method using the PP as plasma preparation procedure.

LC-MS assay

The analyte detection was optimized in several trials to achieve maximum sensitivity and specificity. The memantine is ionized in ESI source by proton addition, giving a pseudo-molecular ion with m/z 180. After fragmentation, the protonated memantine looses an ammonia molecule (MW 17 amu) and is converted to ion with m/z 163 (Figure 2). However, although in MS^2 mode the method specificity is increased in comparison with MS^1 mode, the M-17 transition is not specific. The reason is that fragmentation of ions occurs in fact in a narrow window of about 2 amu around the selected mass, so in case of

memantine the entire range of m/z 180±2 ions are prone to collision-induced dissociation processes. In that case all the ions in that range having a loss of 16 amu (methane), 17 amu (ammonia) and 18 amu (water) may interfere the analysis.

In order to obtain the needed specificity of analysis and a maximum signal-to-noise ratio (S/N) of analyte, we used the capability of Ion Trap mass spectrometer to do multiple stages isolation-fragmentation processes, that means MS^n analysis. This feature is specific to Ion Trap MS analyzers, other MS systems (single quadrupole, triple quadrupole, time of flight) don't have that capability. Thus, in a MS^3 stage the ion with m/z 163 obtained in the MS^2 stage was further fragmented and the obtained mass spectra was recorded (Figure 2). The obtained ions with m/z 107, 121 and 135 are specific to memantine and were used for quantification.

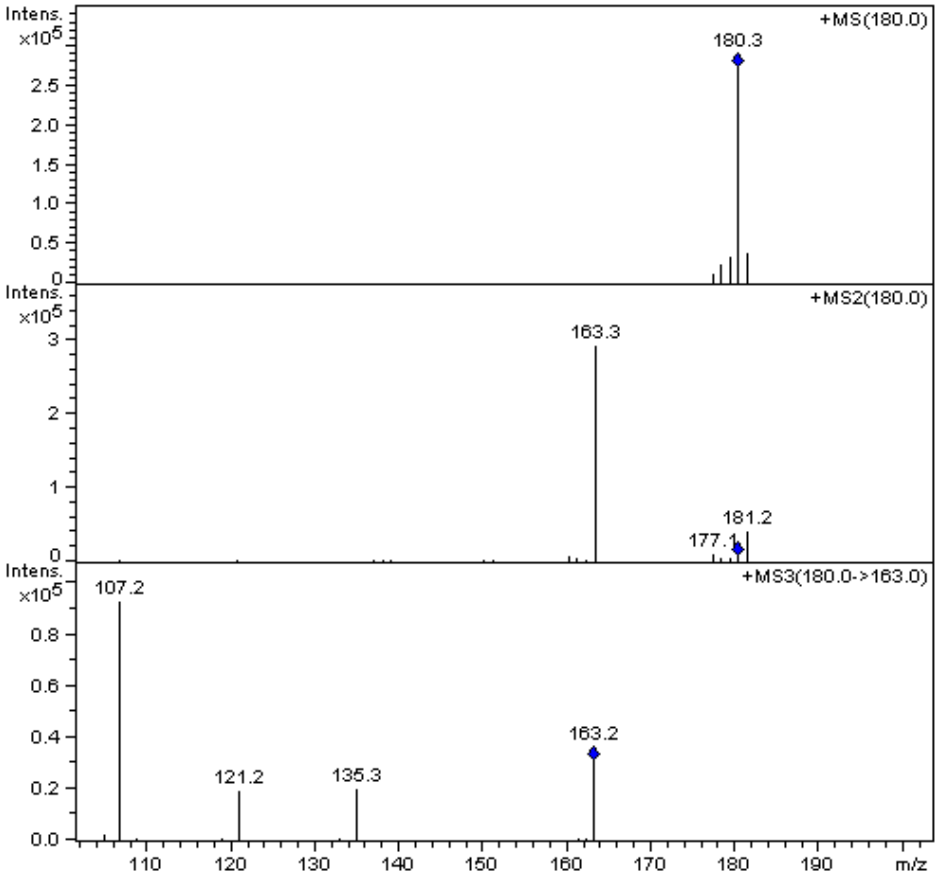

Figure 2. MS spectra of memantine – MS^1 spectra (upper image), MS^2 spectra (middle image) and MS^3 spectra (lower image)

Comparative chromatograms of memantine detected in MS^1, MS^2 and MS^3 mode are presented in Figure 3. By using MS^3 detection mode, due to high specificity, the overall method sensitivity is increased about 6 times in comparison with MS^1 detection.

The detection of memantine was carried out in multiple reaction monitoring (MRM). The extracted ion chromatogram (EIC) of m/z (107, 121, 135) from m/z 180 was analyzed. In the selected chromatographic conditions the retention time of memantine was 1.95 min.

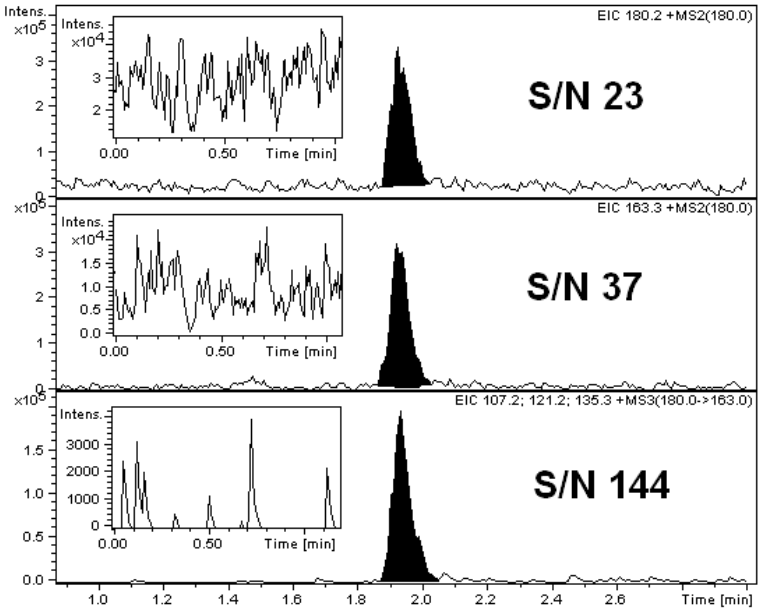

Figure 3. Chromatograms of memantine, same concentration, different MS detection modes: MS^1 (upper image), MS^2 (middle image) and MS^3 (lower image). In insert, the typical background noise pattern for each case, in absolute scale (ion abundance)

Assay validation

The method was validated in accordance with international regulations [**11,12**]. A representative chromatogram of pig plasma spiked with memantine at LLOQ is shown in Figure 4. No interfering peaks from the endogenous plasma components were observed at the retention time of memantine.

The calibration curves were linear over the concentration range of 4.86 – 486 ng/mL in pig plasma, with a correlation coefficient greater than 0.995. The LLOQ was 4.86 ng/mL. The values obtained for intra-day and inter-day precision and accuracy during the validation are shown in Tables 2 and 3, respectively. All values for accuracy and precision were within guidelines recommended limits (<15%) [**11,12**]. The absolute recovery values were between 92.5-100.8%.

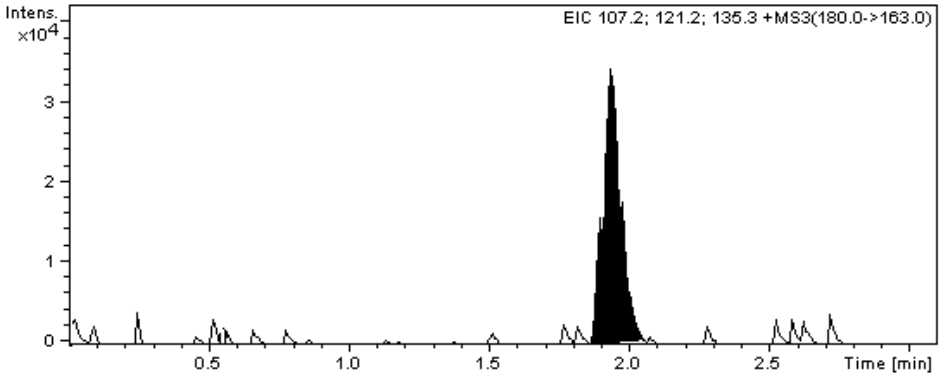

Figure 4. Representative chromatogram of pig plasma sample spiked with memantine at lower limit of quantification (4.86 ng/ml) (retention time – 1.95 min)

Table 2. Intra-day precision, accuracy and recovery (n = 5) for memantine

$C_{nominal}$ ng/mL	Mean C_{found} ng/mL (± S.D.)	CV %	Bias %	Recovery % (± S.D.)
4.86	4.73±0.58	12.2	-2.5	97.1±8.3
14.57	16.32±0.49	3.0	12.1	100.0±2.6
97.10	92.06±3.49	3.8	-5.2	96.2±3.3
194.20	178.85±6.73	3.8	-7.9	92.5±2.9

Table 3. Inter-day precision, accuracy and recovery (n = 5) for memantine

$C_{nominal}$ ng/mL	Mean C_{found} ng/mL (± S.D.)	CV %	Bias %	Recovery % (± S.D.)
4.86	4.94±0.37	7.5	1.7	100.0±5.7
14.57	16.09±0.66	4.1	10.5	100.8±6.5
97.10	96.05±3.52	3.7	-1.1	99.8±7.3
194.20	180.09±11.42	6.3	-7.3	98.1±7.7

Method application

The validated method for determination of memantine in pig plasma was successfully applied in a pharmacokinetic study of intravenously administered memantine in pigs.

CONCLUSION

Our developed LC/MS^3 assay is simple, rapid, specific, accurate and not expensive. This is the first published analytical method for analysis of memantine in biological matrix using protein precipitation as plasma processing method. This new fast and specific method was successfully applied in a pharmacokinetic study of intravenously administered memantine in pigs.

EXPERIMENTAL SECTION

Reagents

Memantine was reference standard from USP (Rockville, MD, USA). Methanol of gradient grade for liquid chromatography, formic acid and 70% perchloric acid of analytical-reagent grade were purchased from Merck KGaA (Darmstadt, Germany). Bidistilled, deionised water *pro injections* was purchased from Infusion Solution Laboratory of University of Medicine and Pharmacy Cluj-Napoca (Romania). The pig blank plasma was from drug-untreated pigs.

Apparatus

The following apparatus were used: 204 Sigma Centrifuge (Osterode am Harz, Germany); Analytical Plus and Precision Standard Balance (Mettler-Toledo, Switzerland); Vortex Genie 2 mixer (Scientific Industries, New York, USA); Ultrasonic bath Elma Transsonic 700/H (Singen, Germany). The HPLC system used was an 1100 series Agilent Technologies model (Darmstadt, Germany) consisting of one G1312A binary pump, an in-line G1379A degasser, an G1329A autosampler, a G1316A column oven and an Agilent Ion Trap Detector 1100 SL.

Chromatographic and spectrometric conditions

Chromatographic separation was performed on a Zorbax SB-C18 (100 mm x 3.0 mm i.d., 3.5 μm) column (Agilent Technologies) under isocratic conditions using a mobile phase of a 55:45 (v/v) mixture of methanol and 0.1% (v/v) formic acid in water at 45 ºC with a flow rate of 1 mL/min. In order to maintain the ESI source clean, the column effluent was diverted to waste for the first 1.5 minutes after injection. The detection of memantine was performed in multiple reaction monitoring (MRM) mode using an ion trap mass spectrometer with an electrospray ion (ESI) source, positive ionisation (capillary 3500 V, nebulizer 60 psi (nitrogen), dry gas nitrogen at 12 L/min, dry gas temperature 350ºC). The extracted ion chromatogram (EIC) of m/z (107, 121, 135) from m/z 163 from m/z 180 was analyzed (MS^3 mode).

Standard solutions

A stock solution of memantine (0.971 mg/mL) was prepared by dissolving an appropriate quantity of memantine in methanol. Two working solutions (19.42 μg/mL and 486 ng/mL, respectively) were prepared by appropriate dilution in drug-free pig plasma. These solutions were used to prepare eight plasma calibration standards with the concentrations between 4.86 and 486 ng/mL, respectively. Quality control (QC) samples of 14.6 ng/mL (lower), 97.1 ng/mL (medium) and 194.2 ng/mL (higher) were prepared by adding the appropriate volumes of working solutions to drug-free pig plasma.

Sample preparation

Standards and plasma samples (0.2 mL) were deproteinized with a 7% perchloric acid aqueous solution (0.1 mL). After vortex-mixture (10 s) and centrifugation (3 min at 12000 rpm), the supernatants (0.15 mL) were transferred in autosampler vials and 10 μL were injected into the HPLC system.

Method validation

The specificity of the method was evaluated by comparing the chromatograms obtained from the plasma samples containing memantine with those obtained from plasma blank samples.

The concentration of memantine was determined automatically by the instrument data system using peak areas and the external standard method. The calibration curve model was determined by the least squares analysis: y = b + ax, weighted (1/y) linear regression, where y - peak area of the analyte and x - concentration of the analyte (ng/mL).

The intra-day precision (expressed as coefficient of variation, CV %) and accuracy (expressed as relative difference between obtained and theoretical concentration, bias %) were determined by analysis of five different samples (n = 5) from each QC standards (at lower, medium and higher levels) on the same day. The inter-day precision and accuracy were determined by analysis on five different days (n = 5) of one sample from each QC standards (at lower, medium and higher levels). The lower limit of quantification (LLOQ) was established as the lowest calibration standard with an accuracy and precision less than 20%. The relative recoveries (at LLOQ, lower, medium and higher levels) were measured by comparing the response of the spiked plasma with the response of standards in solvent with the same concentration of memantine as the plasma (n = 5) [**11-15**].

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REFERENCES

1. R.G. Geocadin, M.A. Koenig, R.D. Stevens, M.A. Peberdy, *Crit Care Clin*, **2006**, *22*, 619.
2. S.A. Lipton, *Nature Rev*, **2006**, *5*, 160.
3. S. Varanese, J. Howard, A. Di Rocco, *Mov Disord*, **2010**, *25(4)*, 508.
4. R.S. Green, D.W. Howes, *Can Med Assoc J*, **2007**, *176*, 759.
5. H.J. Leis, G. Fauler, W. Windischhofer, *J Mass Spectrom*, **2002**, *37*, 477.
6. S.K. Dubey, A. Patni, A. Khuroo, N.R. Thudi, S. Reyar, A. Kumar, et al., *E-J Chem*, **2009**, *6(4)*, 1063.
7. M.Y. Liu, S.N. Meng, H.Z. Wu, S. Wang, M.J. Wei, *Clin Ther*, **2008**, *30(4)*, 641.
8. A.A. Almeida, D.R. Camposa, G. Bernasconi, S. Calafatti, F.A.P. Barros, M.N. Eberlin, et al., *J Chrom B*, **2007**, *848*, 311.
9. R.N. Pan, T.Y. Chian, B.P. Kuo, L.H. Pao, *Chromatogr.*, **2009**, *70(5-6)*, 783.
10. W. Liu, K. Bi, X. Liu, J. Zhao, X. Chen, *Chromatogr.*, **2009**, *69(9-10)*, 837.
11. Guidance for Industry, Bioanalytical Method Validation. U.S. Department of Health and Human Services, Food and Drug Administration. Federal Register, **2001**, *66*.
12. Guidance on the Investigation of Bioavailability and Bioequivalence. The European Agency for the Evaluation of Medicinal Products, Committee for Proprietary Medicinal Products, **2001**, CPMP/EWP/QWP/1401/98.
13. M. Achim, D. Muntean, L. Vlase, I. Bâldea, D. Mihu, S. E. Leucuța, *Studia Univ. Babeş-Bolyai, Chemia*, **2009**, *54(3)*, 7.
14. D. S. Popa, L. Vlase, S. E. Leucuța, F. Loghin, *Farmacia*, **2009**, *57(3)*, 301.
15. D. Mihu, L. Vlase, S. Imre, C.M. Mihu, M. Achim, D.L. Muntean, *Studia Univ. Babeş-Bolyai, Chemia*, **2009**, *54(3)*, 151.