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AN LC-MS/MS METHOD FOR THE QUANTIFICATION OF MEMANTINE IN HUMAN PLASMA: DEVELOPMENT, VALIDATION AND APPLICATION TO A PHARMACOKINETIC STUDY

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ABSTRACT

Memantine hydrochloride (MMH), 1-amino-3, 5 dimethyladamantane hydrochloride, is an adamantine derivative administered orally for many neurologic disorders, including Alzheimer's disease. A rapid, simple, sensitive and specific liquid chromatography-tandem mass spectrometry method was developed for the estimation of memantine in human plasma. Sample preparation involved liquid-liquid extraction of memantine and internal standard using tertiary-butyl methyl ether. The samples were analyzed using a monolithic reversed phase column and detected using positive mode ESI tandem mass spectrometry. The LLOQ was 0.2 ng/mL and the assay was linear over the range of 0.2-46ng/mL using a sample volume of 250µL. The method was validated for specificity, linearity, precision, accuracy and stability parameters. The method was applied to the analysis of plasma samples after oral administration of 20 mg (10mg ×2) MMH tablets of marketed product in healthy adult subjects in fasting bioavailability study.

KEY WORDS

LC-MS/MS, Memantine, Bioanalytical, validation.

INTRODUCTION

Memantine hydrochloride (MMH), (1-amino 3-5-dimethyl adamantine HCl), (**Figure 1**) is a low to moderate affinity non-competitive N-methyl-d-aspartate (NMDA) receptor antagonist with strong voltage dependency and rapid blocking/unblocking kinetics [1]. It is believed to block the glutamate receptors which are the current flow-through channels of NMDA receptors. MMH is indicated for the treatment of patients with moderate to severe dementia of the Alzheimer's type [2]. Clinical experience so far confirms the safety of use and good tolerability profile of MMH at the recommended daily dosage of 10-30mg. MMH is well absorbed from the GI tract and its pharmacokinetics is linear over the therapeutic dose range of 5-40 mg [3]. In humans, MMH is 100% bioavailable after an oral dose, undergoes minimal metabolism and exhibits a terminal elimination half-life of 60 to 80 hours (75% or greater of the dose is eliminated intact in the urine) [1]. Peak plasma concentrations occur between 4-6hrs after oral administration. The plasma protein binding is low (45%) and the mean volume of distribution of MMH is 9-11 L/kg [1]. Previously reported methods for determination of MMH include high performance liquid chromatography [4-6], gas chromatography coupled to mass spectrometry (GC-MS) [7] and LC-MS [8-11]. Quantification of drugs in biological matrices by LCMS is becoming more common due to the improved sensitivity and specificity of this technique [12]. The reported LC-MS methods either used a higher sample volume or had longer run-times which limited their application in high-throughput analysis of clinical study samples. The primary objective was to develop and validate a rapid, sensitive and specific LCMS method for MMH estimation in human plasma. We applied this method to analysis of samples of a PK study of MMH in 14

healthy volunteers after oral administration of 20 mg (10mg ×2) MMH tablets of commercial product.

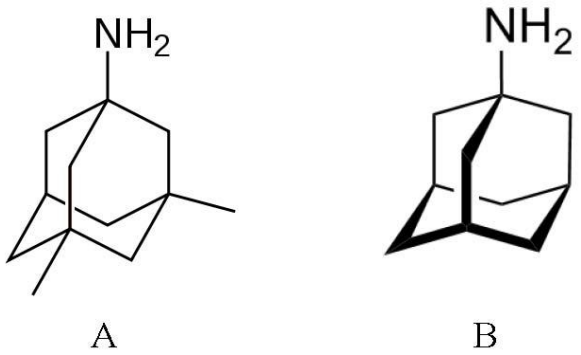


Figure 1: Chemical Structures of (A) memantine and (B) IS (amantadine).

EXPERIMENTAL

MATERIALS & METHODS

MMH was procured from MSN Pharmachem Pvt Ltd, India and amantadine (IS) was obtained from Sigma Aldrich, USA. Acetonitrile (HPLC gradient grade), methanol (LCMS grade), formic acid (puriss grade) and sodium hydroxide were purchased from Sigma Aldrich, USA. Purified water was produced using MilliQ Gradient Millipore system (Millipore, USA). Drug-free human plasma containing K2 EDTA as anticoagulant was used as the blank matrix.

Instrumentation and chromatographic conditions:

Chromatographic separation was achieved by using Merck Chromolith Fast Gradient RP18e column having dimensions of 50*2.0mm using a Waters Acquity UPLC system (Waters, USA). Formic acid (0.1%): Acetonitrile (20:80, v/v) was used as the mobile phase at a flow rate of 0.3 mL/min. The column temperature was set at 40°C and the sample compartment temperature was set at 5°C. 5µL of the sample was injected and the MS detection was performed using a Quattro Premier XE Mass spectrometer (Waters, USA) with an electrospray source. The desolvation temperature and source temperature were set at 400°C and 130°C respectively. The electrospray source was operated in the positive ionization mode and m/z 179.95→162.92 and 151.82 → 134.95 were monitored for quantitation of MMH and amantadine respectively. Data acquisition was performed with MassLynx software (Version 4.1, Waters, USA).

Preparation of standards and Quality Control (QC) samples:

Stock solutions of MMH and amantadine were prepared in methanol (1mg/mL). Further dilutions of MMH to make substocks were done using diluent (water: methanol 50:50) to obtain solutions of 0.005 µg/mL, 0.100 µg/mL, 0.600 µg/mL, 0.240 µg/mL, 0.460 µg/mL, 0.70 µg/mL, 0.850 µg/mL and 1.150 µg/mL. Four levels of QCs (low, medium1, medium2 and high) were also prepared at concentrations of 0.015 µg/mL, 0.200 µg/mL, 0.520 µg/mL and 0.950 µg/mL. The calibration curve standards were prepared by spiking the substocks in blank plasma to obtain concentration of 0.20 ng/mL, 0.40 ng/mL, 2.40 ng/mL, 9.60 ng/mL, 18.40 ng/mL, 28.00 ng/mL, 34.00 ng/mL and 46.00 ng/mL respectively. The QC concentrations of 0.60 ng /mL, 8.00 ng/mL, 20.80 ng/mL and 38.00 ng/mL were prepared by spiking appropriate MMH QC substock solutions in blank human plasma.

Sample preparation:

The plasma samples stored at -80°C were thawed at room temperature before processing. Ten microlitres of internal standard solution (amantadine 0.2 µg/mL) were added to 250 µL of sample aliquoted in a centrifuge tube and mixed for 10 seconds. Twenty microlitre of 1M sodium hydroxide solution was added to the sample and vortexed for 30seconds. The mixture was then vortex-mixed with 2.5 mL of methyl

tertiary butyl ether for 5 minutes at 60rpm. The tube was centrifuged for 5 min at 2800 rpm and the supernatant phase transferred to a test tube and evaporated to dryness under a nitrogen stream at 30°C. The residue was dissolved in 200 µL mobile phase, transferred to a 96 well plate and injected into chromatographic system.

Method Validation procedure:

The method was validated according to the recent US Food and Drug Administration (FDA) guidelines [13]. Specificity, linearity, lower limit of quantification (LLOQ), inter-day and intra-day precision and accuracy as well as recovery and stability of MMH were evaluated. The matrix effect and dilution integrity were also studied.

Specificity

The specificity of the method was determined by screening six different batches of blank human plasma. Blank and LLOQ level standards were prepared from each of the six lots as per the sample preparation method described earlier.

Sensitivity

Six samples at the LLOQ level were processed as per procedure and injected. The % CV and the % accuracy were calculated.

Linearity/Calibration curve

The calibration curves were constructed using values generated from the calibration standards by plotting the analyte to internal standard peak area ratios against concentration. The suitability of the curves was confirmed by back-calculating the concentrations of the calibration standards.

Intra-day and intraday precision and accuracy (P&A)

The intra-day precision and accuracy was performed by processing six samples at each QC level and analyzing against the calibration curve. The % CV and % accuracy at each QC level was calculated. For inter-day precision and accuracy, % CV and % accuracy of the QC samples at each of the five levels (LLOQ, LQC, M1QC, M2QC, and HQC) of two consecutive P & A batches on different days were calculated.

Recovery

Aqueous solutions at the four QC levels were prepared. Recovery of analyte and internal standard from plasma was calculated by comparing the area response of analyte and drug in the extracted sample to the area response of the analyte and drug in the neat solution at the same concentrations.

Matrix effect

The matrix effect was investigated using six different lots of plasma. Three samples each of LQC and HQC levels were processed using each lot of plasma. The %CV of the LQC and HQC samples prepared from different lots of plasma were calculated.

Reinjection reproducibility

The QC samples at low QC and high QC levels of the precision and accuracy batch were re-injected after a gap of 6 hours and analyzed against previously analysed calibration curve. The % change of the initially obtained QC concentrations and those obtained after reinjection were calculated.

Dilution integrity

The effect of a 1:5 dilution on the determination of MMH in human plasma was determined by measuring five samples of spiked DQC (dilution quality control) in human plasma. The DQC concentration (100 ng/mL) was two times the ULOQ concentration. The spiked samples were diluted 1:5 with drug free human plasma and then processed. The QC sample concentrations were calculated using appropriate dilution factor.

Stability

The stability of stock solutions of MMH and amantadine were evaluated after storage for one month at -20°C. Stability of MMH in human plasma was assessed by analyzing MMH samples at two concentrations, low QC and high QC after exposing them to different conditions of time and temperature. The bench top stability was evaluated after exposure of the plasma samples at room temperature for 4 hours. The freeze thaw stability was determined after five freeze-thaw cycles from -80°C to room temperature at regular intervals. The samples were frozen at -80°C for 24 hours and

thawed at room temperature. After complete thawing, the samples were refrozen at -80°C for 12-24hrs at -80°C. This step was repeated four times. After the fifth thawing stage, the samples were analyzed. The long-term stability was assessed after storage of the test samples at -80°C and then analyzed after 3 months of storage. The post-preparative storage stability of MMH was assessed by the auto-sampler stability and dried extract stability. The processed samples in vials were stored in the auto-sampler for 24 hours and then re-injected and analyzed against fresh calibration curve. For dried extract stability, the samples were processed till the evaporation step; the dried extracts were stored at -80°C for 24 hours. After 24 hrs, the frozen dried extracts were retrieved from the -80°C, thawed, reconstituted and analyzed against fresh calibration curve.

CLINICAL STUDY

The study protocol was approved by independent ethics committee and the study was conducted as per pertinent requirements of the current ICMR guidelines and USFDA adopted ICH guidelines for Good Clinical Practice and Declaration of Helsinki (Seoul 2008) and informed consent from subjects were obtained prior to the commencement of study. An open label bioavailability investigation study in healthy, adult, human subjects under fasting condition was conducted. Two tablets of 10 mg MMH were administered to the subjects along with 240ml of water and blood samples were collected at 1.00, 2.00, 3.00, 4.00, 5.00, 6.00, 7.00, 8.00, 10.00, 11.92, 13.00, 14.00, 15.00, 16.00, 17.00, 18.00, 19.00, 20.00, 22.00, 24.00, 28.00, 36.00, 48.00, 72.00, 120.00, 168.00, 216.00 and 264.00 hrs.

RESULTS AND DISCUSSION

Method development: MS and chromatographic development

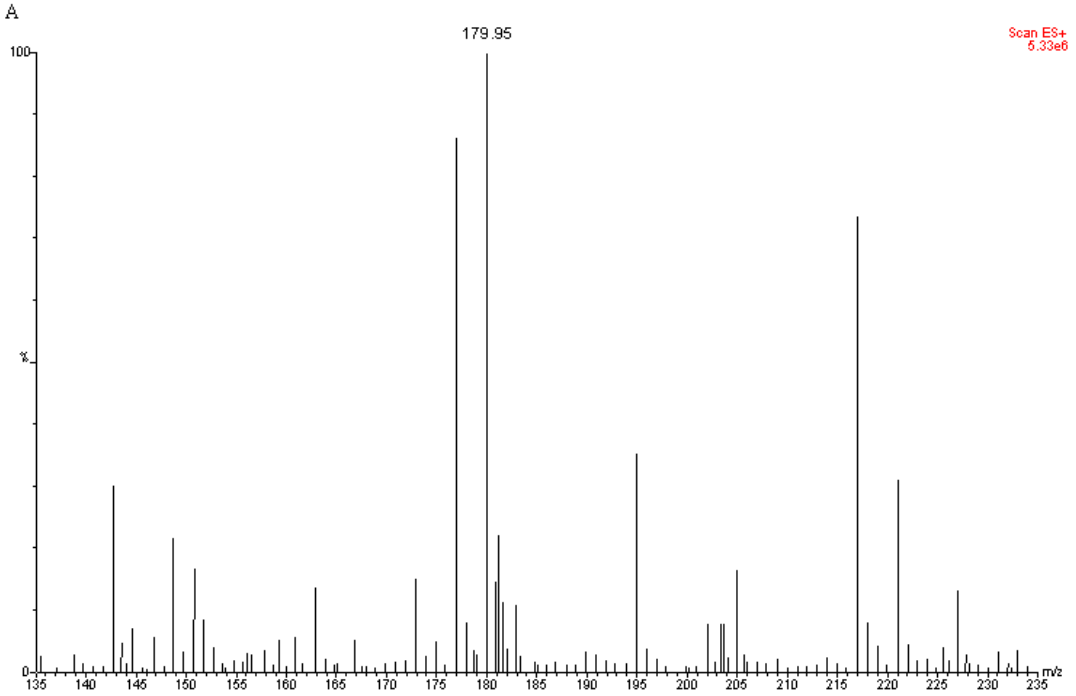
The MS tuning parameters and MS/MS fragmentation were optimized for both MMH and IS by varying the cone voltage and collision energy. Neat solutions of 0.1µg/ml concentration were infused at a pump flow rate of 10µl/min in the ESI positive ion mode. The peak in the mass spectrum at m/z179.95 corresponded to the protonated molecule $(M+H)^+$ of MMH. The most abundant daughter ion fragment at m/z 162.92 in the product ion spectrum was selected to obtain MRM transition to gain maximum sensitivity. Further, an MRM transition for amantadine was also optimized under the same conditions.

Full scan and product ion spectra of Memantine and amantadine are shown in **Figure 2**.

Amantadine was chosen as a suitable internal standard as it is structurally related to MMH. Various combinations of acetonitrile, methanol, ammonium acetate and formic acid were investigated with a view to optimize the mobile phase for sensitivity, speed and peak shape. Acetonitrile and formic acid with a composition of 80:20 (v/v) gave better chromatographic results without decreasing response and were selected for the mobile phase. The retention time of both MMH and internal standard was 0.41 min. A relatively short run time (1.5 min) was achieved with the short monolithic column and a high organic content in the mobile phase. The typical chromatograms are shown in **Figure 3**.

Method development: Sample preparation

Various sample preparation trials by solid phase extraction using polymer-sorbent cartridges were carried out with several modifications but it resulted in significant blank interference. Ion exchange cartridges were also evaluated but it did not provide required selectivity. Liquid-liquid extraction using tertiary butyl methyl ether after basifying the plasma with sodium hydroxide gave reasonably good recovery without any blank interference.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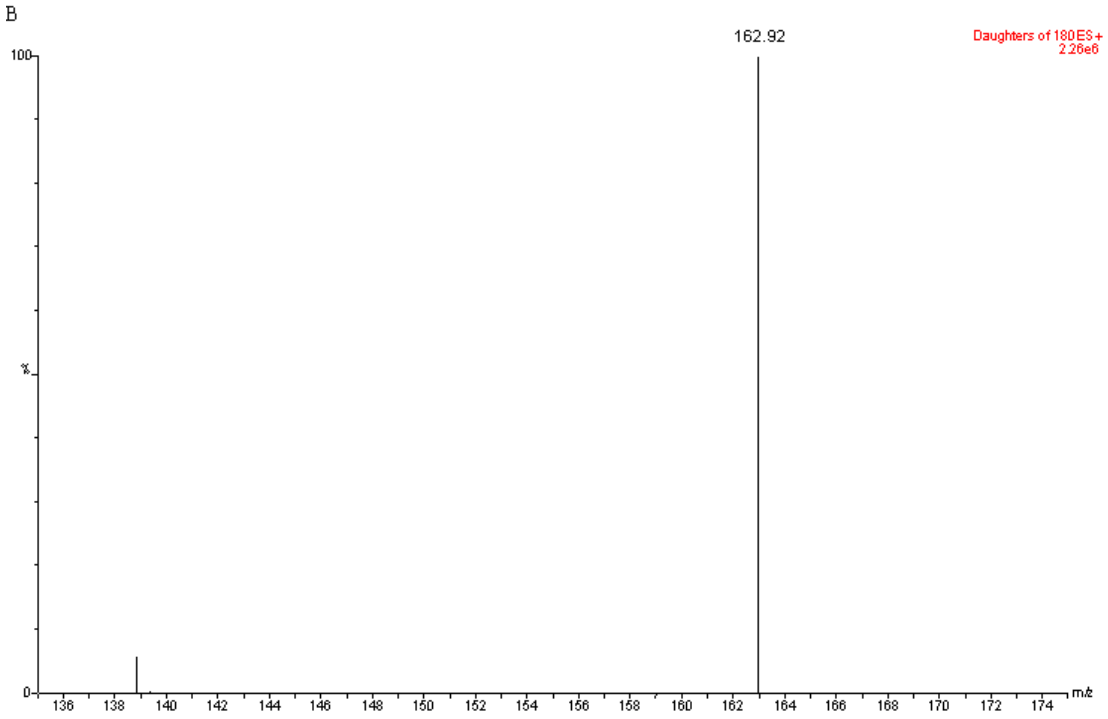
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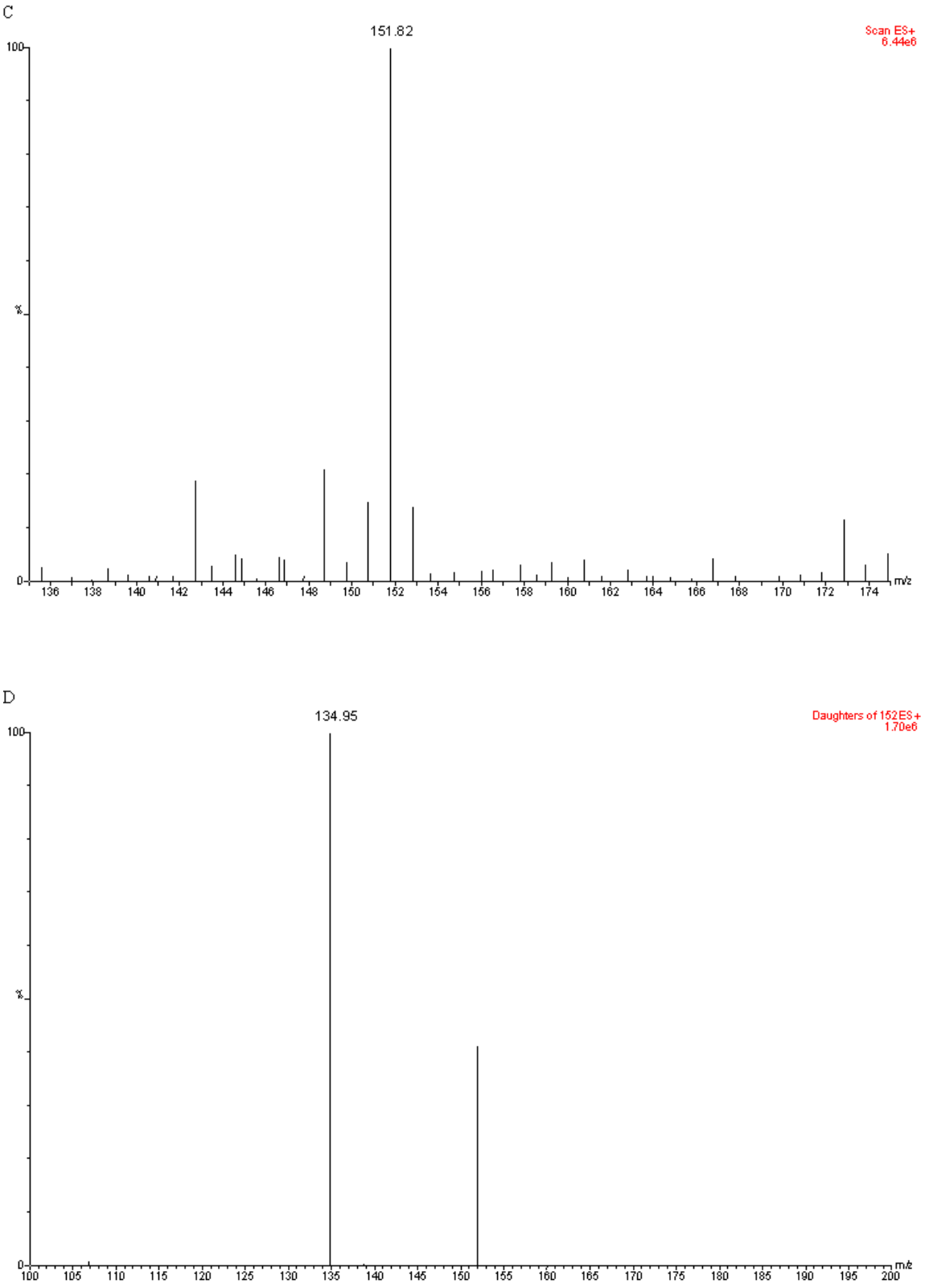

Figure 2: (A) full scan spectra of MMH protonated molecule; (B) product ion spectra of MMH ; (C) full scan spectra of IS; (D) product ion spectra of IS.

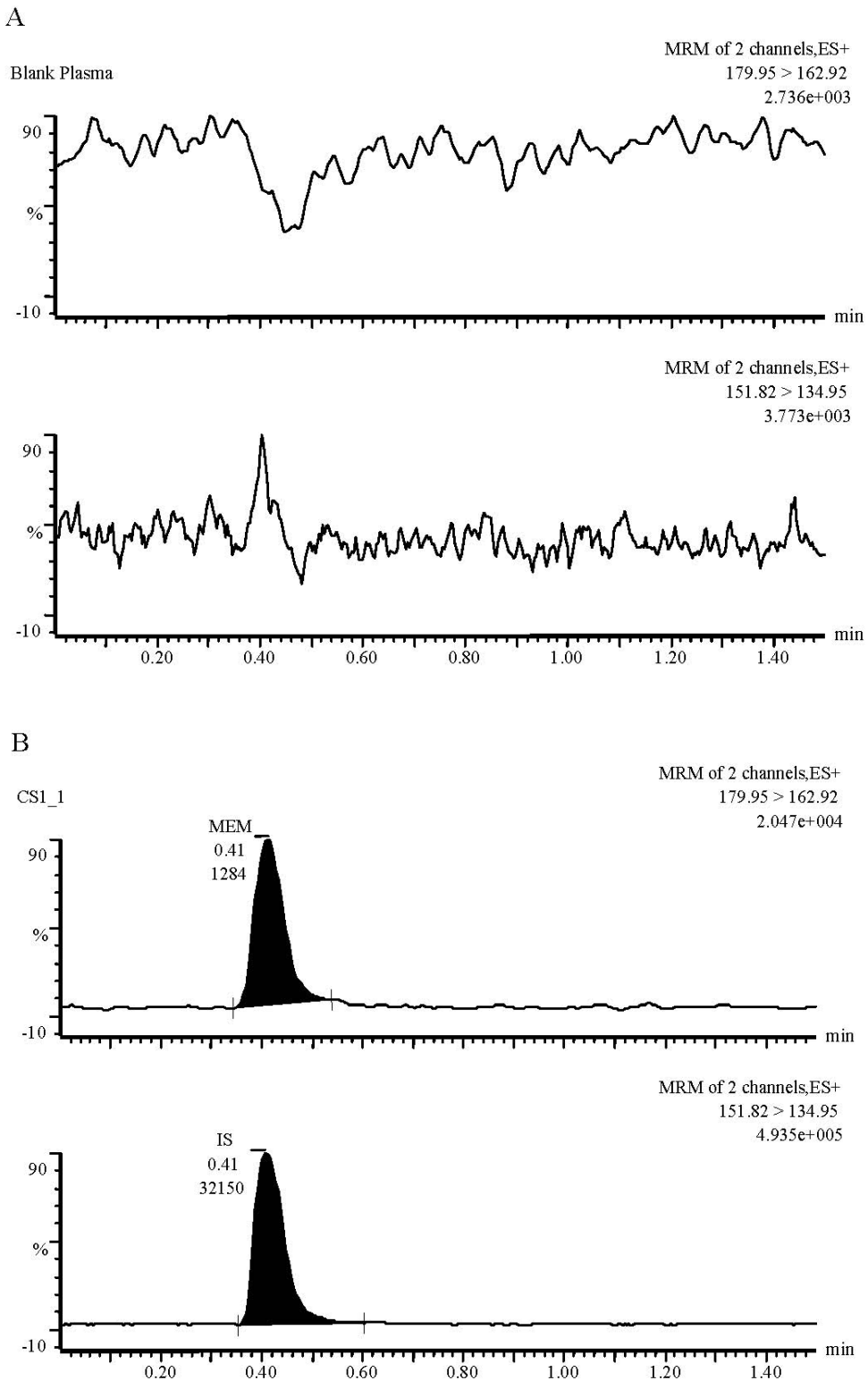

Figure 3: Representative chromatograms of (A) blank plasma sample; and (B) plasma sample spiked with memantine at lower limit of quantification (LLOQ).

Method Validation

Selectivity

All the six lots of tested plasma had no significant interfering peaks at the retention time of analyte or internal standards. All six lots met the acceptance criteria.

Sensitivity

The mean accuracy of the six samples prepared at the LLOQ concentration level was 108.79% with a CV of 10.69%.

Linearity

The linear regression was fitted over the concentration range of 0.2 to 46 ng/mL of MMH in human plasma by a 1/x weighted least squares linear regression. For the four consecutive batches, the calibration curve standards showed an overall accuracy of 90.21 -114.70 % with RSD of less than 10%. The results are shown in **Table 1**. Good linearity was obtained in the validated concentration range. The correlation coefficients of the 1/x weighted calibration curves from consecutive batches were in the range of 0.990-0.996. A typical calibration curve is shown in **Figure 4**.

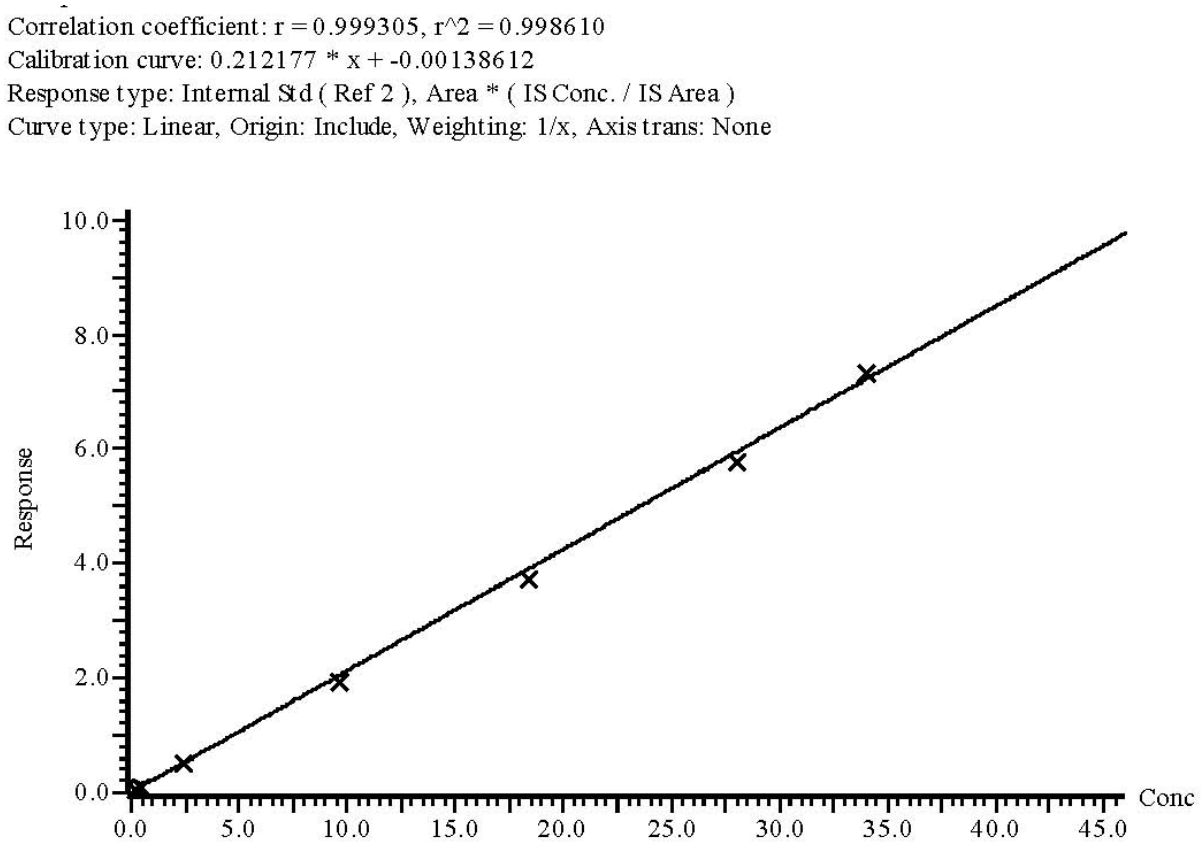

Figure 4: Representative Calibration Curve for the validated range of 0.2 to 46ng/ml.

Table 1: Precision and accuracy of calibration standards of memantine in human plasma from four consecutive validation batches.

Nominal calibration standard concentration (ng/ml)	Mean Back-calculated calibration standard concentration (ng/ml)	%CV (n=4)	%Accuracy (n=4)
0.199	0.228	4.19	114.70
0.399	0.404	6.04	101.19
2.392	2.454	8.32	102.60
9.567	8.881	9.35	92.83
18.337	16.541	3.12	90.21
27.904	27.494	4.49	98.53
33.383	33.146	6.50	99.29
45.842	45.427	3.14	99.09

Intra-day and inter-day precision and accuracy

The resulting intra and inter-day precision and accuracy data for each spiked QC concentration are presented in **Table 2**. These results indicate that the method is reliable and reproducible within its analytical range.

Table 2: Intraday and Interday Precision and accuracy of QC samples for Memantine in human plasma

QC levels	Nominal QC concentration (ng/ml)	Intraday Precision and accuracy (n=6)			Interday Precision and accuracy (n=12)		
		Mean (ng/ml)	%CV	%accuracy	Mean (ng/ml)	%CV	%accuracy
LLOQ	0.199	0.217	10.69	108.79	0.195	15.47	97.948
LQC	0.597	0.624	11.27	104.46	0.582	10.52	97.411
M1QC	7.966	7.910	3.76	99.30	7.424	8.10	93.200
M2QC	20.712	19.650	5.28	94.87	19.458	3.88	93.945
HQC	37.838	35.875	9.43	94.81	35.579	6.73	94.029

Recovery

The recovery of MMH was 88.78%, 71.89%, 80.55% and 90.17% at the low QC, medium 1 QC, medium 2 QC and high QC levels respectively. The mean global recovery of MMH across the four levels of QCs was 82.85±10.20%. The results showed that MMH had consistent recovery across all four levels. The mean recovery of the internal standard was 72.3%.

Matrix effect

The % CV of the QC sample concentrations from the six different lots of plasma was 9.23 at the low QC level and 2.39 at the high QC concentration. The results indicated that there was no significant lot-to-lot variation for plasma matrices.

Re-injection reproducibility

The % change in obtained QC concentrations after initial analysis and after reinjection was 0.72% at the LQC level, -1.30% at M1QC level, -2.70% at the M2QC level and -2.83% at the HQC level. The method met the acceptance criteria for reinjection reproducibility after 6 hrs.

Dilution Integrity

The six DQC samples processed after 1/5 dilution had an accuracy of 97.36% with a CV of 1.95%. The dilution integrity at 1/5 dilution met the acceptance criteria.

Stability

Acceptable stability was indicated in stock solution after storage at -20° C for 110 days and 84 days for

MMH and internal standard respectively. Stability of MMH in plasma was demonstrated at bench top conditions for 4 hours and over five freeze/thaw cycles. MMH was found to be stable in plasma after long term storage at -80°C for 90days. The processed samples in the auto-sampler were stable for 24 hrs at 5°C whereas the processed samples at the dry extract stage were stable at -80°C for 24 hrs. No significant degradation of MMH was observed in any of the stability test conditions and the results were consistent. The results are summarized in **Table 3**.

Table 3: Summary of stability data of Memantine in human plasma

Stability condition	Stability duration	QC Level	Mean Accuracy	Mean Precision (%CV)
Bench top	4hrs	LQC	98.74	7.67
		HQC	90.03	4.13
Freeze thaw	3cycles	LQC	102.82	9.40
		HQC	85.11	3.53
Long term	90 days	LQC	101.47	7.34
		HQC	85.53	0.68
Auto-sampler	24hrs	LQC	102.31	9.21
		HQC	89.43	10.11
Dried Extract	24hrs	LQC	110.05	7.89
		HQC	96.91	2.10

Clinical application

The above validated LC-MS/MS method was successfully used to estimate pharmacokinetic parameters for MMH in human plasma. A mean plot of MMH concentration in plasma versus time was plotted from the data generated as shown in **Figure 5**. The pharmacokinetic parameters were calculated using Winnonlin software (Version 5.3 Pharsight, USA). The mean values were Cmax 28.80 ng/ml, Tmax 17.50hrs and AUC (0→t) 2120.35 ng.hr/mL.

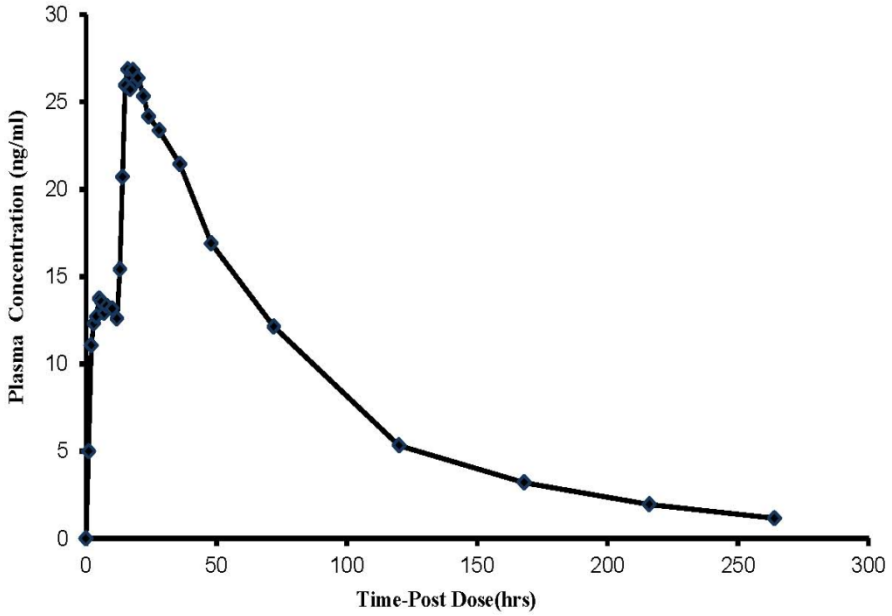

Figure 5: Mean Plasma concentration-time profile of Memantine following administration of 2x10mg bid to healthy subjects in fasting condition (n=14).

CONCLUSION

A simple, rapid and sensitive LC-MS/MS method for the determination of MMH in human plasma was developed. The method allows high sample through-put due to its short run time and relatively simple sample preparation procedure. This method is reliable, convenient and meets the criteria for application in clinical pharmacokinetic studies.

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