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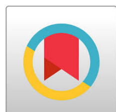
RESEARCH ARTICLE

Validation of a Robust and High-Throughput HPLC-MS/MS Method to Determine Amantadine Levels in Human Plasma



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OBJECTIVES: A simple, robust and high-throughput HPLC-MS/MS method was developed and validated for the determination of amantadine in human plasma.

METHODS: The analyte and its stable isotope-labeled internal standard amantadine-d15 were extracted using protein precipitation method and separation was achieved on an Agilent Eclipse Plus C18 column (50 x 3.0 mm, 3.5 µm). A triple quadrupole mass spectrometer was used for detection. Automation was employed with a Tomtec Quadra 4 Liquid Handling System to improve the throughput.

RESULTS: Lower limit of quantitation was validated to be 15 ng/mL with a reduced sample volume of 20 µL comparing with previously reported methods. Calibration curve was linear over a concentration range of 15-2000 ng/mL. In addition to linearity, other parameters including matrix selectivity, matrix effect, recovery, method accuracy and precision, sensitivity, and stabilities of amantadine under storage conditions were also fully validated following the guidance of United States Food and Drug Administration.

CONCLUSIONS: An HPLC-MS/MS method was developed and validated for the quantitation of amantadine in K_2EDTA human plasma using a simple protein precipitation extraction method from a small sample volume of 20 µL.

KEYWORDS: amantadine, HPLC-MS/MS, human plasma, protein precipitation.

INTRODUCTION

Amantadine was a widely used antiviral drug since the 1960s [1-3]. The antiparkinsonian effect of amantadine has also been recognized and extensively studied [1, 4-6]. In 2017, U.S. Food and Drug Administration approved amantadine hydrochloride (trade name Gocovri) extended-release capsules to treat dyskinesia, a typical movement disorder for patients with Parkinson's disease [7].

Various methods have been reported for the quantitation of amantadine in biological matrices. Rakestraw reported the using of capillary gas chromatography

to determine amantadine concentration [8]. Farajzadeh utilized derivatization and micro-extraction technique to quantitate amantadine in human plasma and urine by gas chromatography coupled with flame ionization detection [9]. Several reports employed high-performance liquid chromatography (HPLC) for the quantitation of amantadine in human or animal matrices[10-15]. These methods suffer from low sensitivity due to the limitation of the detection technique, and low throughput resulting from the complex sample preparation procedures which often involved chemical derivatization. Over the past three decades, high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) technique have evolved tremendously due to its advantages of selectivity, high sensitivity, fast method development duration, etc. Several methods employing HPLC-MS/MS platform for the quantitation of amantadine in human matrix were developed and reported. Torsten and coworkers developed and validated an amantadine LC-MS based method in human serum with an assay range of 50-1000 ng/mL [16]. Wang, Feng and Jie separately reported LC-MS/MS methods with improved sensitivity [17-19]. However, a large sample volume (up to 200 µL) and a complex extraction method (liquid-liquid extraction) were employed for these methods. Although Parkinsonism is known to affect mostly senior patients, Juvenile Parkinsonism has been recognized for many years [20-23]. For bioanalytical methods, a low sample volume is usually recommended because the method can be potentially adapted on the pediatric study. Here, we describe a simple, robust and high-throughput LC-MS/MS method with a validated assay range from 15 ng/mL to 2000 ng/mL using a reduced sample volume of 20 µL that is capable of supporting pediatric clinical studies if needed. When treating Parkinsonism, common amantadine hydrochloride daily dosing levels range from 100 to 400 mg depending on patient response to the drug. Dosing 200 mg amantadine resulted in a mean maximum plasma concentration of 510 ng/mL for young adults and 800 ng/mL for elderly adults in a single-dose pharmacokinetics study of amantadine hydrochloride [24]. In a different study, the amantadine plasma concentration ranged from 91 ng/mL to 4400 ng/mL with a mean daily dosing level of 135.1 mg [25]. Therefore, an assay range of 15 ng/mL to 2000 ng/mL was chosen to cover the expected plasma concentrations from different dosing levels. A 10-fold dilution was validated to extend the upper quantitation limit (ULOQ) up to 20000 ng/mL in case samples with amantadine concentration greater than ULOQ are obtained during clinical sample analysis.

MATERIALS AND METHODS

Chemicals and reagents

Both amantadine and the internal standard amantadine-d15 were purchased from Toronto Research Chemicals (North York, ON, Canada) in the form of hydrochloric salt (chemical purity: 98% for both amantadine and amantadine-d15). Please refer to **Figure 1** for structures of both. HPLC grade organic solvents methanol (MeOH) and acetonitrile (ACN) were purchased from BDH Chemicals (Dawsonville, GA, USA). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Fair Lawn, NJ, USA) and was also HPLC grade. GR grade ammonium formate and formic acid were purchased from Acros Organics (Morris Plains, NJ, USA). Biological matrices (blank normal and hemolyzed K_2EDTA human plasma, and K_2EDTA human whole blood) were purchased from BioreclamationIVT (Westbury, NY, USA). Purified water (18.2 MΩ·cm) was obtained from an ELGA Purelab® classic water purification system (Woodbridge, IL, USA).

Equipment

Mettler Toledo (Columbus, OH, USA) MX5 micro balance (capable of weighing 0.000001 gram) and Sartorius (Bradford, MA, USA) CP225D analytical balance (capable of weighing 0.00001 gram) were used for laboratory weighing applications. Beckman Coulter (Indianapolis, IN, USA) Allegra® X-14R benchtop centrifuge was employed to perform sample spins and separation. Tomtec Quadra 4 Liquid Handling system (Hamden, CT,

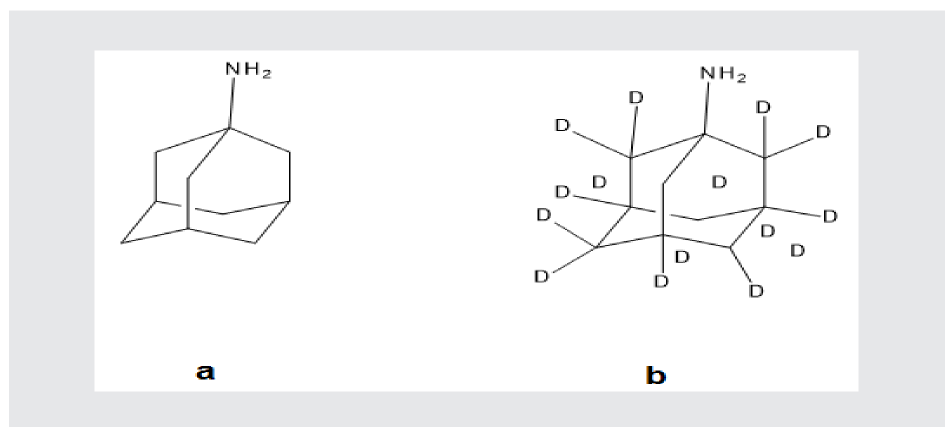

Figure 1. Structures of amantadine (a) and amantadine-d15 (IS) (b)

USA) was used for sample transfer.

LC-MS/MS instruments and methods

Shimadzu (Kyoto, Japan) HPLC system (LC-20AD pumps/SIL-20AC HT autosampler) coupled with Sciex API 4000 triple quadrupole mass spectrometer was used for chromatographical separation and detection of amantadine and amantadine-d15. Analyst software (version 1.6.1 or higher, Applied Biosystems, Foster city, CA, USA) was used to support the LC-MS system and perform instrument control and raw data acquisition. Acquired raw data were exported to Watson LIMS system (version 7.3, Thermo Fisher Scientific, Inc., Philadelphia, PA) for regression and processing. Gradient separation was achieved on an Agilent Eclipse Plus C18 HPLC column (50 x 3.0 mm, 3.5 μm) using 5 mM ammonium formate in H_2O as mobile phase A and ACN as mobile phase B. The gradient started at 15% of B, and B percentage was increased to 35% at 1.6 minute, during which amantadine and amantadine-d15 were eluted from column (retention time was about 1.2 minute for both analyte and IS). B fraction was elevated to 100% at 1.7 minute and the column was flushed with 100% B until 3.7 minute. At 3.8 minute, B was restored back to 15% and maintained at 15% to 5.3 minute, when the injection cycle finished. Electrospray ionization (ESI) was used to produce protonated amantadine and protonated amantadine-d15 in positive ion mode and both ions were selected using multiple reaction monitoring (MRM) detection mode with MS transitions of m/z 152.0 → 135.1 for amantadine and m/z 167.2 → 150.1 for amantadine-d15, respectively. Signal response (peak area ratio of analyte and IS) was acquired for data processing and quantitation. General MS setting and compound dependent MS settings were optimized by directly infusing amantadine into the MS system in order to achieve the highest sensitivity.

Calibration standards and quality control samples

The Standard stock and QC stock solutions of amantadine were prepared separately by dissolving two separately-weighted amantadine hydrochloride reference standard into diluent (DMSO:MeOH, 3:7, v/v) to obtain a final concentration of 0.500 mg/mL for amantadine free base. The standard spiking solutions were subsequently diluted from standard stock solution at 15.0, 30.0, 90.0, 250, 500, 1000, 1600, 2000 ng/mL from STD-1 to STD-8, respectively. Five concentration levels of amantadine QC samples in human plasma (LLOQ: 15.0 ng/mL, QC Low: 45.0 ng/mL, QC Mid: 150 ng/mL, QC High: 1500 ng/mL, QC AQL 10×: 15000 ng/mL) were prepared during method validation. The QC samples were stored at both -20 °C and -70 °C for validation and stability testings. The internal standard

stock solution was prepared in diluent at a concentration of 1.00 mg/mL (amantadine-d15 free base). IS spiking solution was diluted from IS stock solution to a final concentration of 500 ng/mL.

Sample preparation

Calibration standards were prepared freshly in each run batch by spiking 20 µL of the standard spiking solution into 20 µL of human plasma. For double blank (without IS), blank (with IS), QC samples and study samples, 20 µL of plasma was mixed with 20 µL of diluent (DMSO:MeOH, 3:7, v/v)). 20 µL of IS spike (500 ng/mL of amantadine-d15 in diluent) was added into each of the samples above except for double blank (20 µL of diluent will be spiked in as substitute) and mixed well. Both amantadine and IS were extracted using 500 µL of MeOH with a vigorous vortexing for approximately 60 seconds. After the samples were centrifuged for about 5 minutes at 2500 g, 20 µL of the supernatant was diluted by adding 400 µL of reconstitution solution (0.1% formic acid in MeOH/H_2O, 1:4, v/v) in a 96-well HPLC plate using Tomtec. The amantadine and IS were diluted 588-fold using the protein precipitation extraction method, thus leading to highly clean extracted samples.

Validation Procedures

During validation, eight calibration standards were freshly prepared in each validation run batch as described in section calibration standards and quality control samples. Six replicates of QC samples at four levels of concentration (LLOQ: 15.0 ng/mL, QC Low: 45.0 ng/mL, QC Mid: 150 ng/mL, QC High: 1500 ng/mL) were prepared and analyzed in three different batches to evaluate intra- and inter- run accuracy and precision. The recovery of the sample preparation was evaluated by comparing the mean area ratio of the extracted QC samples with the mean area ratio of unextracted samples at three different concentration levels in three replicates at each concentration level. The internal standard was added post-extraction for all recovery samples. The matrix effect was to evaluate the suppression or enhancement of ionization of amantadine by the presence of matrix components in the biological samples. Six lots of K_2EDTA human plasma were randomly selected and extracted in single replicate following the method. Each of the posted extracted blank samples from six lots were spiked with analyte and IS spike solution at two concentration levels (QC Low and QC High) before the reconstitution step. Three replicates of neat samples were prepared in the same manner as matrix samples except that purified water was used to substitute plasma. During the analysis, it may be necessary to dilute the samples if the analyte concentrations are above the upper limit of quantitation (ULOQ, 2000 ng/mL). In order to verify that the dilution of a high concentration sample would yield an analytical response that is within the dynamic range and acceptable tolerance limits of the assay, pooled human plasma was fortified with amantadine at a concentration 10 times above the QC High sample concentration (15000 ng/mL) of the method. Six replicate samples were prepared by diluting the samples 10-fold with blank pooled plasma and assayed against the calibration curve.

RESULTS AND DISCUSSION

HPLC-MS/MS method development

Prior to the screening of HPLC columns, the multiple reaction monitoring (MRM) transition parameters for amantadine and amantadine-d15 (IS) were manually optimized by infusing a 200 ng/mL amantadine and IS solution into the electrospray ionization source. The exact Q1 and product mass, as well as the source-dependent and compound-dependent parameters were manually optimized to achieve the optimum response. Ionspray voltage, source temperature, nebulizing gas and drying gas were set to be 5000 volts, 600 °C, 65 psi and 55 psi, respectively. Declustering potential, entrance potential, collision energy and collision cell exit potential were kept at 80.0 volts, 10.0 volts, 26.0

volts and 10.0 volts, respectively. With the optimized parameters in hand, different mobile phase combinations were compared and columns were screened.

Literature searching results indicate that columns with various chemistry can be used for amantadine retention and separation in human matrix, such as Phenomenex Luna C8(2) (100 x 2.0 mm i.d., 3-microm)[16] and Thermo Hypersil-HyPURITYC18 reversed-phase column (150 mm x 2.1 mm i.d., 5 microm) [17]. A silica based column was also used for amantadine separation in poultry tissues and egg [26]. During method development, a total of five different columns were evaluated: Agilent Eclipse Plus, C18, 50 x 3.0 mm, 3.5 µm; Phenomenex Max-RP, 50 × 2.0 mm, 4 µm; Waters XBridge, C18, 50 x 2.1 mm, 3.5 µm; Waters Atlantis T3, 50 x 2.1 mm, 3 µm; Agilent Zorbax Bonus-RP 50 x 2.1 mm, 3.5 µm. Screening result indicated that under ambient temperature, Agilent Eclipse Plus C18 column gave the best sensitivity while maintaining good peak shape and retention in combination with the 5mM ammonium formate in H_2O as mobile phase A and ACN as mobile phase B (Please refer to **Figure 2** for selected chromatograms). Autosampler was also kept at ambient temperature. In order to improve the throughput of the method, automation was employed for sample preparation by using Tomtec Quadra 4 Liquid Handling System for supernatant transfer and reconstitution. By introducing the Tomtec system, the sample preparation time is shortened significantly when large quantity of study samples is processed, and helps reduce human error and improve efficiency.

Method validation results

Method validation was performed according to the FDA 2001 guidance [27], Viswanathan et. al. recommendations on best practice for bioanalytical validation [28], and applying relevant aspects of good laboratory practices (GLP) as a quality standard. The following validation parameters were evaluated: method selectivity, matrix effect, sensitivity, linearity (range), accuracy of the calibration standards, accuracy and precision of the quality control (QC) samples (intra-run and inter-run), recovery of the sample preparation, dilution integrity, re-injection reproducibility, processed sample stability, QC freeze/thaw stability, QC benchtop stability, whole blood stability, hemolysis effect, carryover evaluation and maximum batch size evaluation. The lowest limit of quantitation was validated to be 15 ng/mL for the current method.

Linearity of calibration curves

The calibration curve was linear over the targeted range of 15-2000 ng/mL for amantadine using a weighing factor of the reciprocal of the concentration squared ($1/x^2$). During validation, the correlation coefficients (R^2) were between 0.9972 to 0.9993, indicating very good linearity. Back-calculated concentrations for all standards were all within ±15% (±20% for LLOQ), and the results are presented in **Table 1**.

Precision and accuracy

The overall accuracy (presented as %bias) was between -7.3% to 3.3% for intra-run

Table 1. Precision and accuracy of calibration standards (N=4)

	Amantadine, ng/mL							
N=4	STD1 15	STD2 30	STD3 90	STD4 250	STD5 500	STD6 1000	STD7 1600	STD8 2000
Mean	15.2	29.4	86.9	257	4.97	994	1610	2040
SD	0.435	1.82	1.78	8.22	21.1	30.2	35.6	47.6
%CV	2.9	6.2	2.0	3.2	4.2	3.0	2.2	2.3
%Bias	1.3	-2.0	-3.4	2.8	-0.6	-0.6	0.6	2.0

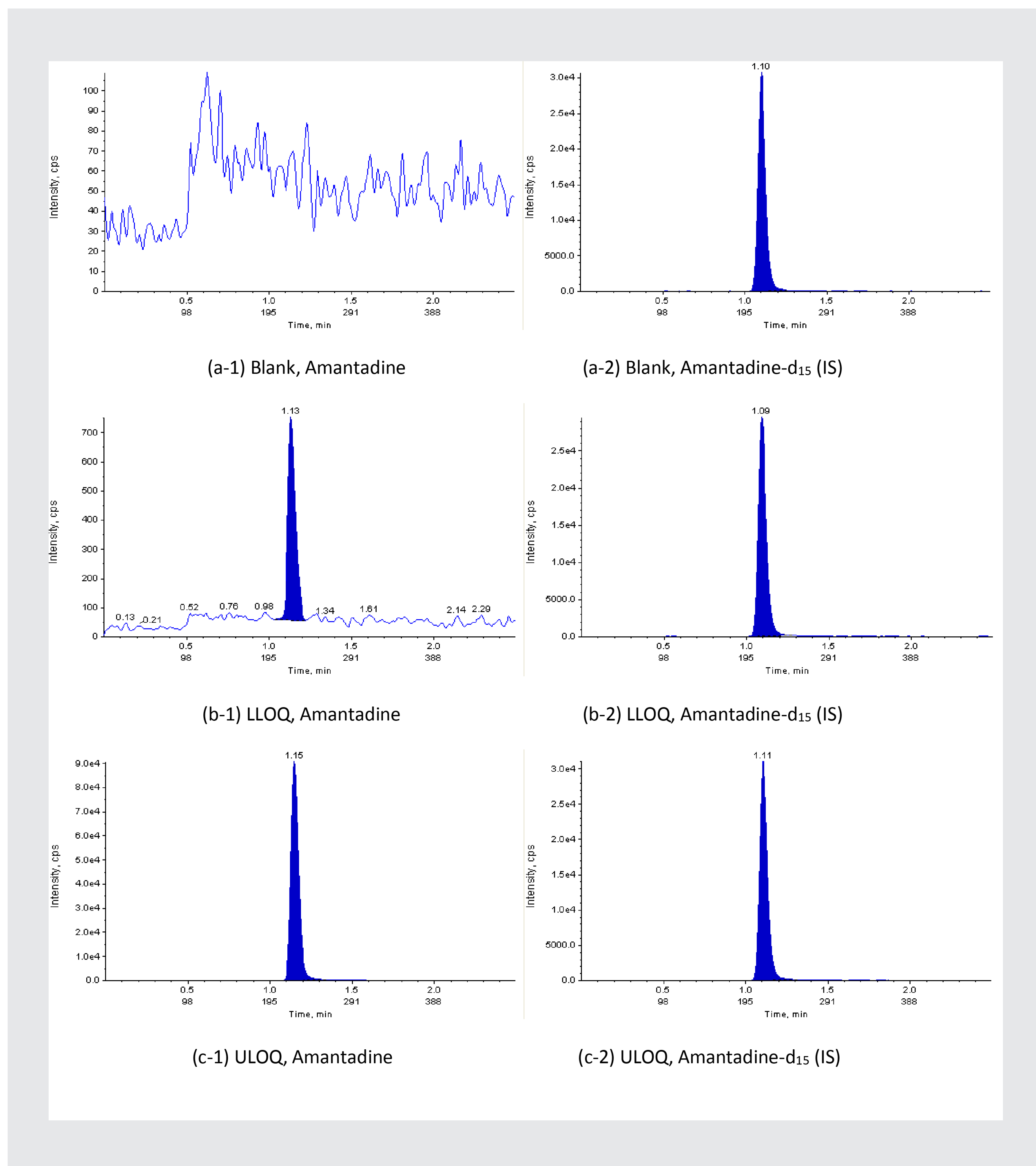

Figure 2. Chromatographs of blank (a), LLOQ (b), and ULOQ samples (c)

Table 2. Precision and accuracy of quality control samples (N=6)

Day ID	N=6	Amantadine, ng/mL LLOQ 15	LOW 45	MID 150	HIGH 1500
Intra-Day 1	Mean	15.2	44.7	155	1520
	SD	0.571	1.95	2.53	31.9
	%CV	3.8	4.4	1.6	2.1
	Bias	1.3	-0.7	3.3	1.3
Intra-Day 2	Mean	13.9	42.1	145	1450
	SD	0.879	0.874	2.45	53.5
	%CV	6.3	2.1	1.7	3.7
	%Bias	-7.3	-6.4	-3.3	-3.3
Intra-Day 3	Mean	14.5	43.7	146	1470
	SD	0.763	1.94	5.24	61.1
	%CV	5.3	4.4	3.6	4.2
	%Bias	-3.3	-2.9	-2.7	-2.0
Inter-Day results	Mean	14.6	43.5	149	1480
	SD	0.895	1.91	5.7	55.3
	%CV	6.1	4.4	3.8	3.7
	%Bias	-2.7	-3.3	-0.7	-1.3

accuracy and -3.3% to 0.7% for inter-run accuracy; and precision (presented as %CV) was within 6.6% for intra-run precision and 6.3% for inter-run precision. The results are presented in **Table 2**, indicating high robustness of the method.

Dilution integrity

The measured concentration is within 8.7% of the nominal concentration value and has a %CV of 2.3%, thus, a 10-fold dilution was validated and the upper limit of quantitation was increased to 20000 ng/mL.

Recovery

The recovery was measured to be 101.0%, 102.6% and 97.0% at QC Low, QC Mid and QC High concentration, respectively, thus yielding an average recovery of 100.2%. The results indicate that the recovery was consistent through the assay range and amantadine was fully extracted during sample extraction.

Matrix effect and matrix selectivity

The internal standard-normalized matrix effect across six different lots of blank plasma were very consistent (%CV was within 2.9%). Moreover, comparing with neat samples, both IS-normalized matrix effect (presented as area ratio) and absolute matrix effect (presented as area) from matrix samples were very consistent without any signal enhancement or suppression, indicating high-robustness of the method. For matrix selectivity, six lots of blank plasma were screened and no baseline interference (larger than 20% of the

LLOQ for analyte and 5% for IS, respectively) was detected at the retention time of the analyte and IS for all lots.

Stability of the analyte

Various stability tests were conducted to mimic the possible sample handing conditions between sample collection and bioanalysis. Amantadine is found to be stable for at least 6 hours in human K_2EDTA plasma at ambient temperature. After three freeze/thaw cycles (from a nominal of -20 or -70 °C freezer to room temperature), no stability issue was observed for amantadine. Hemolysis effect (amantadine stability in 2% hemolyzed plasma)

Table 3. Stability of amantadine

Stability	N=6	Amantadine, ng/mL LOW 45	MID 150	HIGH 1500
Bench-top, 6 hrs	Mean	43.6	NA	1460
	SD	0.977		17.5
	%CV	2.2		1.2
	Bias	-3.1		-2.7
Freeze-thaw (three cycles, from -20 °C to room temperature)	Mean	44.5	NA	1510
	SD	0.852		32.0
	%CV	1.9		2.1
	%Bias	-1.1		0.7
Freeze-thaw (three cycles, from -70 °C to room temperature)	Mean	44.1	NA	1470
	SD	0.459		45.4
	%CV	1.0		3.1
	%Bias	-2.0		-2.0
2% Hemolyzed plasma	Mean	45.6	NA	1560
	SD	1.53		24.3
	%CV	3.4		1.6
	%Bias	1.3		4.0
Processed sample stability (32 hrs)	Mean	45.4	156	1550
	SD	1.85	3.19	47.5
	%CV	4.1	2.0	3.1
	%Bias	0.9	4.0	3.3
Reinjection stability (185 hrs)	Mean	45.4	157	1550
	SD	1.01	2.93	32.9
	%CV	2.2	1.9	2.1
	%Bias	0.9	4.7	3.3

NA; not applicable.

Table 4. Stability of amantadine

Conditions	Concentration		Amantadine peak area ratio 0 min	60 min	120 min
Ice water bath	LOW 15 ng/mL	Mean	0.056213	0.054936	0.057150
		SD	0.002210	0.001336	0.001970
		%CV	3.9	2.4	3.4
		% Difference from t_0	NA	-2.3	1.7
	HIGH 1500 ng/mL	Mean	1.864261	1.802159	1.809209
		SD	0.083451	0.032141	0.049790
		%CV	4.5	1.8	2.8
		%Difference from t_0	NA	-3.3	-3.0
Room temperature	LOW 15 ng/mL	Mean	0.056213	0.054697	0.054485
		SD	0.002210	0.002248	0.002776
		%CV	3.9	4.1	5.1
		%Difference from t_0	NA	-2.7	-3.1
	HIGH 1500 ng/mL	Mean	1.864261	1.810729	1.818478
		SD	0.083451	0.55574	0.056715
		%CV	4.5	3.1	3.1
		%Difference from t_0	NA	-2.9	-2.5

NA; not applicable.

and whole blood stability (at room temperature and ice-water bath) were also evaluated. 2% Hemolyzed plasma did not affect the quantitation of amantadine, nor any stability issue was observed for amantadine in human blood for up to 2 hours, at either room temperature or ice-water bath (4 °C). After the samples were being processed, it was good for reinjection for at least 185 hours and the processed samples were stable for at least 32 hours against a freshly prepared standard calibration curve. The amantadine plasma and blood stabilities are summarized in **Table 3** and **Table 4**, respectively.

Interference of analyte to IS

The interference of the analyte amantadine on the IS amantadine-d15 was evaluated by preparing three replicates of the upper limit of quantitation (ULOQ, 1500 ng/mL for amantadine) and processing and analyzing the samples without the addition of the IS. No interference peak was observed in any of the three replicates samples for IS.

Carryover evaluation

To evaluate carryover, a double blank was placed after STD-8 sample in each validation run batch. All of the carryover samples had analyte peak areas that were less than 20% the peak area of the LLOQ when using 5 mM ammonium formate in ACN/MeOH, 1:1, v/v) as needle washing solvent.

Batch-Size evaluation

Largest batch in validation includes 133 samples and passed acceptance criteria very

well. It indicated that the method was good for production with a batch size of at least 133 samples.

CONCLUSION

A simple, high-throughput and robust HPLC-MS/MS method was developed and validated for quantitation of amantadine in K_2EDTA human plasma. Protein precipitation was used to extract amantadine and its internal standard amantadine-d15. Comparing with previously reported amantadine LC-MS methods, we significantly improved the throughput while maintaining high sensitivity and method robustness using a reduced sample volume of 20 µL. Considering amantadine and IS were diluted 588-fold using the current extraction method, if higher sensitivity is needed, it can be easily achieved by reducing the sample dilution fold. Validation results indicate the robustness of the method and it can be readily applied to clinical studies in the future.

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