

Quantitative Determination of Galantamine in Human Plasma by Sensitive Liquid Chromatography–Tandem Mass Spectrometry Using Loratadine as an Internal Standard

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Abstract

A simple, rapid, sensitive, and selective liquid chromatography–tandem mass spectrometry method is developed and validated for the quantitation of galantamine, an acetylcholinesterase inhibitor in human plasma, using a commercially available compound, loratadine, as the internal standard. Following liquid–liquid extraction, the analytes are separated using an isocratic mobile phase on a reverse-phase C18 column and analyzed by mass spectrometry in the multiple reaction monitoring mode using the respective (M+H)⁺ ions, *m/z* 288 to 213 for galantamine and *m/z* 383 and 337 for the internal standard. The assay exhibit a linear dynamic range of 0.5–100 ng/mL for galantamine in human plasma. The lower limit of quantitation is 0.5 ng/mL, with a relative standard deviation of less than 8%. Acceptable precision and accuracy are obtained for concentrations over the standard curve range. A run time of 2.5 min for each sample makes it possible to analyze more than 400 human plasma samples per day. The validated method is successfully used to analyze human plasma samples for application in pharmacokinetic, bioavailability, or bioequivalence studies.

Introduction

Galantamine, chemically {[4aS-(4a α ,6 β ,8aR*)]-4a,5,9,10,11,12-hexahydro-3-methoxy-11-methyl-6H-benzofuro-[3a,3,2-ef]2benzazepin-6-ol}, a cholinergic drug, was initially isolated from the bulbs of certain *Narcissus* species, but it is now also produced synthetically (1,2). Galantamine is approved for the treatment of mild-to-moderate Alzheimer's Disease (AD) (3). Galantamine acts both as a reversible competitive inhibitor of acetylcholinesterase and as an allosteric modulator of nicotinic acetylcholine receptors (4,5). This dual mechanism may provide additional benefits for patients with AD.

Galantamine exhibits linear pharmacokinetics after oral administration of 4–16 mg twice daily (2). Following oral administration, galantamine is about 90–100% bioavailable, and time to peak plasma concentrations (t_{max}) ranged from 0.5 to 2 h (6). It has a relatively large volume of distribution and low protein binding. Metabolism is primarily through the cytochrome P450 system, specifically CYP2D6 and CYP3A4 isoenzymes (3). Galantamine demonstrates biexponential elimination, with a mean plasma terminal elimination half-life of 5.26 to 5.68 h in healthy subjects (2).

The bioanalytical component of a pharmacokinetic study requires a drug assay with simplicity, selectivity, high sensitivity, small volume requirements, and rapid turnaround time. Only a few methods have been reported for the quantitation of galantamine in biological fluids, which involve high-performance liquid chromatography (HPLC) with UV detection (7,8) or with fluorescence detection (9,10). In these methods, the plasma volume requirement is high, the sensitivity is inadequate for pharmacokinetic studies, and they are time-consuming because of multiple sample preparation and extraction procedures.

Quantitation of drugs in biological fluids by liquid chromatography (LC)–tandem mass spectrometry (MS–MS) is becoming more common, owing to the improved sensitivity and selectivity of this technique (11–24). Recently, Verhaeghe et al. (25) reported an LC–MS–MS method for the quantitation of galantamine in human plasma using a stable isotope labeled internal standard (IS) with a lower limit of quantitation (LLOQ) of 1 ng/mL. The isotope labeled compound is not commercially available, and the cost of custom synthesis prohibits its use. However, without an IS present, there would be no compensation for the variability seen during extraction or injection onto the chromatographic system.

The purpose of this work was to explore the high selectivity and sensitivity of a triple-quadrupole MS system operated in MS–MS mode with an electrospray ionization (ESI) interface for the development and validation of a robust reversed-phase

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LC–MS–MS method in multiple reaction monitoring (MRM) mode for the quantitation of galantamine in human plasma using a commercially available compound, loratadine, as the IS. It was essential to establish an assay capable of quantitating galantamine at concentrations down to 0.5 ng/mL. At the same time, it was expected that this method would be efficient in analyzing large numbers of plasma samples obtained for pharmacokinetic, bioavailability, or bioequivalence studies after therapeutic doses of galantamine.

Experimental

Chemicals

Galantamine reference standard (99.8% pure) was obtained from Vimta Labs (Hyderabad, India). Loratadine was employed as an IS and was obtained from Cadila Healthcare Limited (Ahmedabad, India). The chemical structures are presented in Figure 1. Drug-free human plasma, containing ethylenediaminetetra acetic acid as an anticoagulant, was obtained from the Usha Mullapudi Cardiac Center (Hyderabad, India). HPLC-grade LiChrosolv methanol and LiChrosolv acetonitrile were from Merck (Darmstadt, Germany). Formic acid and toluene were from Merck (Worli, Mumbai, India). HPLC-grade water from a Milli-Q system (Millipore, Bedford, MA) was used. All other chemicals were of analytical grade.

LC–MS–MS instrument and conditions

The HPLC SIL HTC system (Shimadzu, Kyoto, Japan) was equipped with an LC-AD vp binary pump, a DGU20A5 degasser, a SIL-HTC autosampler equipped with a thermostat, and a CTO-10AS vp thermostated column compartment. The chromatographic

separation was performed on a Waters symmetry, C₁₈ column (5 μ m, 150 \times 4.6 mm i.d.) at a temperature of 30°C. The isocratic mobile phase composition was a mixture of 0.03% formic acid–acetonitrile (20:80, v/v), which was pumped at a flow-rate of 1.5 mL/min with a split ratio of 50:50.

Mass spectrometric detection was performed with an API 4000 triple quadrupole instrument (MDS-SCIEX, Toronto, Canada) using MRM. A turbo ionspray interface in positive ionization mode was used. The main working parameters of the MS are summarized in Table I. Data processing was performed using the Analyst 1.4.1 software package (SCIEX, Toronto, Canada).

Sample processing

A plasma sample (0.2 mL) was transferred to a 15-mL glass test tube, then 20 μ L of IS working solution (0.5 μ g/mL) was added. After vortex mixing for 10 s, 1 mL of toluene was added, and the sample was vortex-mixed for 3 min. The organic layer (0.8 mL) was transferred to a 5-mL glass tube and evaporated to dryness using an evaporator at 40°C under a stream of nitrogen. Then the dried extract was reconstituted in 250 μ L of mobile phase, and a 25- μ L aliquot was injected into the chromatographic system.

Bioanalytical method validation

Standard stock solutions of galantamine (1 mg/mL) and the IS (1 mg/mL) were separately prepared in methanol. Working solutions for calibration and controls were prepared by the appropriate dilution in water–methanol (50:50, v/v, diluent). The IS working solution (0.5 μ g/mL) was prepared by diluting its stock solution with diluent. Working solutions (0.2 mL) were added to drug-free plasma (9.8 mL) as a bulk to obtain galantamine concentration levels of 0.5, 1, 2.5, 5, 10, 25, 50, and 100 ng/mL as a single batch at each concentration. Quality control (QC) samples were also prepared as a bulk based on an independent weighing of the standard drug, at concentrations of 0.5 (LLOQ), 1.5 (low), 40 (medium), and 80 ng/mL (high) as a single batch at each concentration. The calibration and control bulk samples were divided into aliquots in microcentrifuge tubes (Tarson, Calcutta, India) (2 mL) and stored in the freezer at below –50°C until analyses.

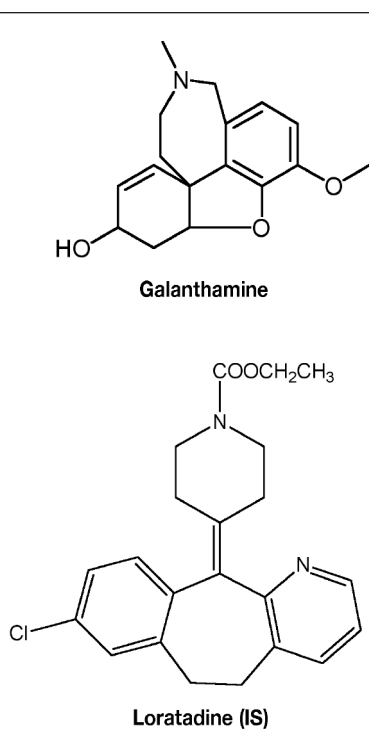


Figure 1. Chemical structures for galantamine and loratadine (IS).

Table I. MS–MS Main Working Parameters

Parameter	Value
Source temperature (°C)	250
Dwell time per transition (msec)	200
Ion source gas 1 (psi)	20
Ion source gas 2 (psi)	20
Curtain gas (psi)	10
Collision gas (psi)	4
Ion spray voltage (V)	5000
Entrance potential (V)	10
Declustering potential (V)	66 (Analyte) and 66 (IS)
Collision energy (V)	31 (Analyte) and 31 (IS)
Collision cell exit potential (V)	14 (Analyte) and 14 (IS)
Mode of analysis	Positive
Resolution	Unit
Ion transition for galantamine (<i>m/z</i>)	288.2 to 213.2
Ion transition for loratadine (<i>m/z</i>)	383.2 to 337.3

A calibration curve was constructed from a blank sample (a plasma sample processed without the IS), a zero sample (a plasma processed with the IS), and eight non-zero samples covering the range 0.5–100 ng/mL, including the LLOQ. The calibration curves were generated using the analyte-to-IS peak area ratios by a weighted ($1/x^2$) least squares regression on five consecutive days. The acceptance criteria for a calibration curve were a correlation coefficient (r) of 0.99 or better and that each back-calculated standard concentration had to be within 15% deviation from the nominal value, except at the LLOQ, for which the maximum acceptable deviation was set at 20%. At least 67% of non-zero standards were required to meet the described criteria, including acceptable LLOQ and the upper limit of quantitation.

The within-batch precision and accuracy were determined by analyzing five sets of QC samples in a batch. The between-batch precision and accuracy were determined by analyzing five sets of QC samples on three different batches. The QC samples were randomized daily, processed, and analyzed in a position either (i) immediately following the standard curve, (ii) in the middle of the batch, or (iii) at the end of the batch. The acceptance criteria for within- and between-batch precision were 20% or better for LLOQ and 15% or better for the other concentrations, and the accuracy was $100 \pm 20\%$ or better for LLOQ and $100 \pm 15\%$ or better for the other concentrations.

Recovery of galantamine from the extraction procedure was determined by a comparison of the peak area of galantamine in spiked plasma samples (five each of low and high QCs) with the peak area of galantamine in samples prepared by spiking extracted drug-free plasma samples with the same amounts of galantamine at the step immediately prior to chromatography. Similarly, recovery of the IS was determined by comparing the mean peak areas of extracted QC samples ($n = 5$) with mean peak areas of IS in samples prepared by spiking extracted drug-free plasma samples with the same amounts of IS at the step immediately prior to chromatography.

Results and Discussion

MS

In order to develop a method with the desired LLOQ (0.5 ng/mL), it was necessary to use MS–MS detection, as the MS–MS methods provide improved limits of detection for trace-mixture analysis (11). The inherent selectivity of MS–MS detection was also expected to be beneficial in developing a selective and sensitive method. The product ion mass spectrum of galantamine and the IS are shown in Figure 2. The predominant ion in the Q1 spectrum was $(M+H)^+$ and it was used as the precursor ion to obtain the product ion spectra. The most sensitive mass transition was from m/z 288 to 213 for galantamine and from m/z 383 to 337 for the IS. The collisionally activated dissociation (CAD) mass spectrum of galantamine showed the formation of characteristic product ions at m/z 197, 209, 213, 225, 231, and 270. The fragmentation behavior was dominated by cleavages in the azepine ring (9). The major product ion at m/z 213 was probably formed by cleavage of the azepine ring, followed by the loss of a

water molecule. The CAD mass spectrum of IS showed the formation of characteristic product ions at m/z 259, 267, 281, 294, and 337. The major product ion at m/z 337 arose from the loss of $\text{CH}_3\text{CH}_2\text{OH}$ of the protonated precursor molecule. The proposed fragmentation pattern is also shown in Figure 2.

LC–MRM is a very powerful technique for pharmacokinetic studies because it provides the sensitivity and selectivity requirements for analytical methods. Thus, the MRM technique was chosen for the assay development. The MRM state file parameters were optimized to maximize the response for the analyte. The parameters presented in Table I are the result of this optimization.

Method development

Liquid–liquid extraction (LLE) was used for the sample preparation in this work. LLE can be helpful in producing a spectroscopically clean sample and avoiding the introduction of non-volatile materials onto the column and MS system. Clean samples are essential for minimizing ion suppression and the matrix effect in LC–MS–MS analyses. Six organic solvents (diethyl ether, toluene, hexane, dichloromethane, chloroform, and butyl *tert*-methyl ether) and their mixtures in different com-

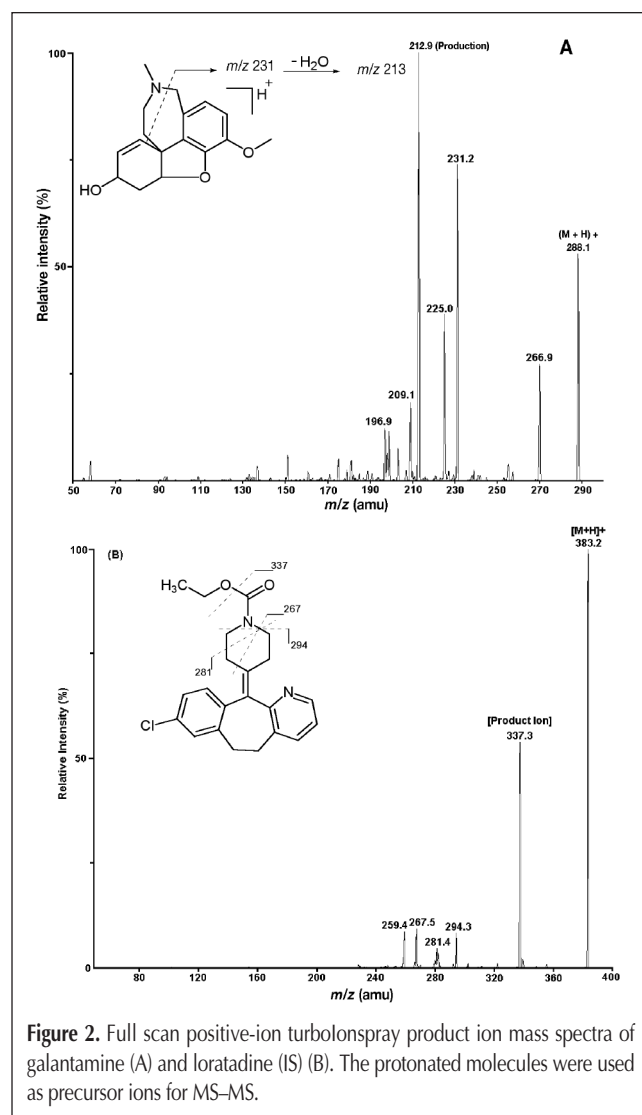


Figure 2. Full scan positive-ion turbolonspray product ion mass spectra of galantamine (A) and loratadine (IS) (B). The protonated molecules were used as precursor ions for MS–MS.

binations and ratios were evaluated. Finally, toluene was found to be optimal as it produced a clean chromatogram for a blank plasma sample. Additionally, toluene yielded the highest recovery for the analyte from the subject or spiked plasma samples.

The chromatographic conditions, especially the composition of the mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes for the analyte and IS, as well as a short run time. It was found that a mixture of 0.03% formic acid–acetonitrile (20:80, v/v) achieved this purpose and was finally adopted as the mobile phase. The formic acid was found to be necessary in order to lower the pH to protonate the galantamine and thus deliver good peak shape. The percentage of formic acid was optimized to maintain this peak shape while being consistent with good ionization and fragmentation in the MS. Moreover, it was necessary to reconstitute the residues with the mobile phase to produce the expected peak shapes of the analyte. The high proportion of organic solvent eluted the analyte and the IS at retention times of 0.75 and 1.67 min, respectively. A flow rate of 1.5 mL/min produced good peak shapes and permitted a run time of 2.5 min.

For an LC–MS–MS analysis, the utilization of stable isotope-labeled drugs as internal standards proved to be helpful when a significant matrix effect was possible. However, the isotope-labeled analyte was not obtainable commercially, and the cost of custom synthesis was prohibitive to its use. Loratadine was found to be the best for the present study. Clean chromatograms were obtained, and no significant direct interferences in the MRM channels at the relevant retention times were observed. However, in ESI, signal suppression or enhancement may occur because of coeluting endogenous components of the sample matrix. These potential matrix effects were evaluated by spiking blank plasma extracts (after LLE treatment as described previously) at the low and high quality control levels. The resulting chromatograms were compared with those obtained for clean standard solutions at the same concentrations. Five independent plasma lots were used, with five samples from each lot. The results (data not shown) showed that there was no significant difference between peak responses for spiked plasma extracts and clean solutions. This result most likely reflects the efficiency of the sample clean up with LLE. In any event, the use of matrix-matched calibration standards would have minimized any such effects on the quantitation.

Table II. Precision and Accuracy Data of Back-Calculated Concentrations of Calibration Samples for Galantamine in Human Plasma

Concentration added (ng/mL)	Concentration found (mean \pm SD, $n = 5$; ng/mL)	Precision (%)	Accuracy (%)
0.5	0.53 \pm 0.03	5.3	105.1
1	1.02 \pm 0.04	3.9	101.9
2.5	2.61 \pm 0.06	2.4	104.5
5	4.76 \pm 0.33	6.8	95.2
10	9.67 \pm 0.44	4.6	96.7
25	24.19 \pm 1.5	6.2	96.8
50	48.56 \pm 2.04	4.2	97.1
100	102.66 \pm 2.29	2.2	102.7

Assay performance and validation

The eight-point calibration curve was linear over the concentration range 0.5–100 ng/mL. The calibration model was selected based on the analysis of the data by linear regression with or without intercepts and weighting factors ($1/x$, $1/x^2$, and none). The best linear fit and least-squares residuals for the calibration curve were achieved with a $1/x^2$ weighting factor, giving a mean linear regression equation for the calibration curve of: $y = 0.0424 (\pm 0.0015)x - 0.0017 (\pm 0.0023)$, where y is the peak area

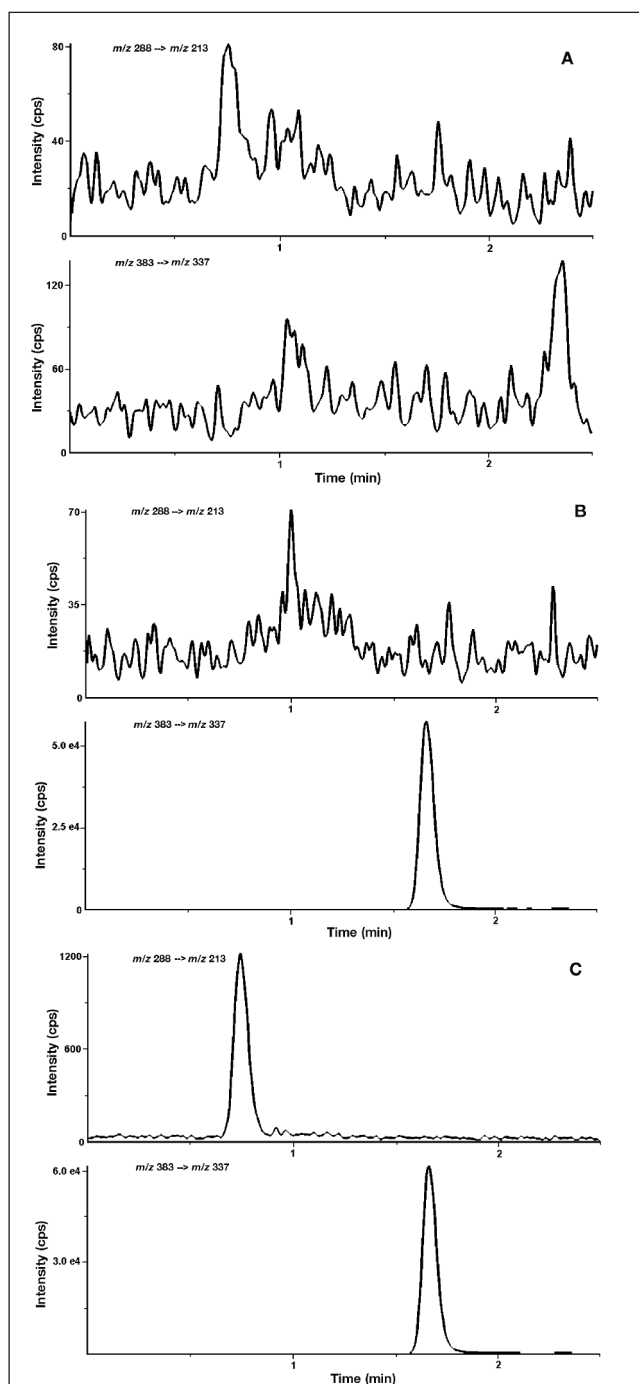


Figure 3. MRM chromatograms for galantamine and the IS resulting from analysis of: blank (drug and IS free) human plasma (A); blank (drug-free spiked with IS) human plasma (B); 500 pg/mL (LLOQ) of galantamine spiked with the IS (C).

ratio of the analyte to the IS, and x is the concentration ratio of the analyte to the IS. The mean correlation coefficient of the weighted calibration curve generated during the validation was 0.998 ± 0.001 . Table II summarizes the calibration curve results.

The selectivity of the method was examined by analyzing ($n = 5$) blank human plasma extract (Figure 3A) and an extract spiked only with the IS (Figure 3B). As shown in Figure 3A, no significant direct interference in the blank plasma traces was observed from endogenous substances in drug-free human plasma at the retention time of the analyte. Similarly, Figure 3B shows the absence of direct interference from the IS to the MRM channel of the analyte. Figure 3C depicts a representative ion-chromatogram for the LLOQ (0.5 ng/mL). Excellent sensitivity was observed for a 25- μ L injection volume with a split ratio of 50:50. The LLOQ corresponded to approximately 6.2 pg on-column. The MRM chromatograms obtained for an extracted plasma sample of a healthy subject, who participated in a bioequivalence study conducted on 18 subjects, is depicted in Figure 4. Galantamine was identified and quantitated as 19.8 ng/mL.

Validation parameters at the LLOQ

The LLOQ was defined as the lowest concentration in the standard curve that can be measured with acceptable accuracy and precision and was found to be 0.5 ng/mL in human plasma. The mean response for the analyte peak at the assay sensitivity limit

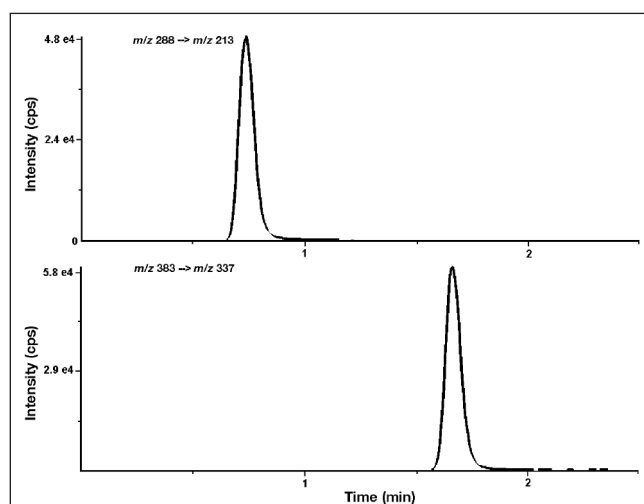


Figure 4. MRM ion-chromatograms resulting from the analysis of a subject plasma sample after the administration of a 4 mg oral dose of galantamine. The sample concentration was determined to be 19.8 ng/mL.

(0.5 ng/mL) was approximately 14-fold greater than the mean response for the peak in five blank human plasma samples at the retention time of the analyte. The between-batch precision at the LLOQ was 7.1%, and the between-batch accuracy was 105.1% (Table III). The within-batch precision was 7.5%, and the accuracy was 93.5% for galantamine.

Validation parameters at the middle and upper concentrations

The middle and upper quantitation levels of galantamine ranged from 1.5–80 ng/mL in human plasma. For the between-batch experiments, the precision ranged from 3.2% to 5.6% and the accuracy from 95.4% to 96.5% (Table III). For the within-batch experiments, the precision and accuracy for the analyte met the acceptance criteria ($< \pm 15\%$). The extraction recovery of galantamine was $80.1 \pm 3.5\%$, and the recovery of the IS was $66.4 \pm 1.1\%$ of the concentration used in the assay (0.5 μ g/mL). Recoveries of the analyte and IS were high and were consistent, precise, and reproducible. Therefore, the assay has proved to be robust in high throughput bioanalysis.

Stability studies

The stability of the analyte and IS in human plasma under different temperature and timing conditions, as well as their stability in the stock solutions, was evaluated as follows. QC samples were subjected to short-term room temperature conditions, to long-term storage conditions ($< -50^{\circ}\text{C}$), and to freeze-and-thaw stability studies. All the stability studies were conducted at two concentration levels (1.5 and 80 ng/mL as low and high values) with five determinations for each.

For short-term stability determination, stored plasma aliquots were thawed and stored at room temperature for a period of time exceeding that expected to be encountered during routine sample preparation (around 24 h). Samples were extracted and analyzed as previously described, and the results are given in Table IV. These results indicated reliable stability behavior under the experimental conditions of the regular analytical procedure. The stability of QC samples stored in the autosampler for 24 h was also assessed. The results showed that solutions of galantamine and the IS could remain in the autosampler for at least 24 h without showing significant loss in the quantitated values, indicating that samples should be processed within this period of time (Table IV). The data representing the stability of galantamine in plasma samples at two QC levels over three freeze-and-thaw cycles are given in Table IV. These tests indicate that the analyte is stable in human

Table III. Precision and Accuracy of the LC–MS–MS Method for Determining Galantamine Concentrations in Plasma Samples

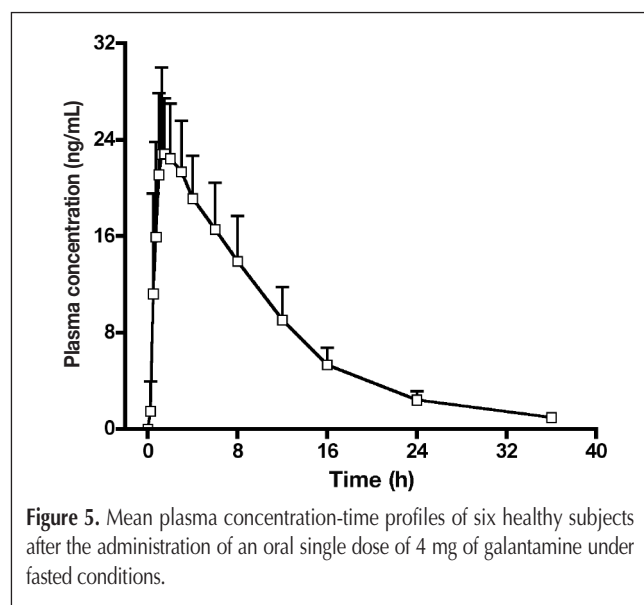
Concentration added (ng/mL)	Within-batch precision ($n = 5$)			Between-batch precision ($n = 3$)		
	Concentration found (mean \pm SD; ng/mL)	Precision (%)	Accuracy (%)	Concentration found (mean \pm SD; ng/mL)	Precision (%)	Accuracy (%)
0.5	0.47 \pm 0.04	7.5	93.5	0.53 \pm 0.04	7.1	105.1
1.5	1.40 \pm 0.05	3.7	93.3	1.45 \pm 0.05	3.2	96.5
40	38.77 \pm 2.82	7.3	96.9	38.15 \pm 1.16	3.0	95.4
80	81.92 \pm 4.07	5.0	102.4	77.13 \pm 4.34	5.6	96.4

plasma for three freeze-and-thaw cycles, when stored at below -50°C and thawed to room temperature.

Table IV also summarizes the long-term stability data for galantamine in plasma samples stored for a period of 30 days at below -50°C . The stability study of galantamine in human plasma showed reliable stability behavior, as the means of the results of the tested samples were within the acceptance criteria of $\pm 15\%$ of the initial values of the controls. These findings indicated that storage of galantamine in plasma samples at below -50°C was adequate, and no stability-related problems would be expected during routine analyses for pharmacokinetic, bioavailability, or bioequivalence studies.

The stability of the stock solutions was tested and established at room temperature for 2 h, 24 h, and under refrigeration ($\sim 4^{\circ}\text{C}$) for 6 months. The recoveries for galantamine and IS were 102.2, coefficient of variance (CV) 1.9%; 99.5, CV 1.8%; 101.5, CV 1.1% and 100.2, (CV 0.9%); 102.3, CV 2.6%; and 99.5, CV 2.1%, respectively. The results revealed optimum stability for the prepared stock solutions throughout the period intended for their daily use.

Table IV. Stability of Galantamine in Human Plasma			
Sample concentration (ng/mL; n = 5)	Concentration found (ng/mL)	Precision (%)	Accuracy (%)
<i>Short-term stability for 24 h in plasma</i>			
1.5	1.52	7.7	101.1
80	78.57	6.5	98.2
<i>Three freeze-and-thaw cycles</i>			
1.5	1.47	4.2	97.9
80	78.74	3.8	98.4
<i>Autosampler stability for 24 h</i>			
1.5	1.41	4.4	93.0
80	76.52	5.9	95.6
<i>Stability for 30 days at $< -50^{\circ}\text{C}$</i>			
1.5	1.36	7.6	90.5
80	76.10	6.5	95.1



Application

The validated method has been successfully used to quantitate galantamine concentrations in human plasma samples after the administration of a 4-mg oral dose of galantamine under fasted conditions. Mean plasma concentration versus time profiles for six subjects, each receiving a single dose, is presented in Figure 5.

Conclusion

In summary, a method for the quantitation of galantamine from human plasma by LC-MS-MS in positive ionization mode using MRM is described. The current method has shown acceptable precision and adequate sensitivity for the quantitation of galantamine in human plasma samples obtained for pharmacokinetic, bioavailability, or bioequivalence studies. Furthermore, it was utilized for the analysis of hundreds of human plasma samples. The method described is simple, rapid, sensitive, selective, and fully validated according to commonly accepted criteria (26). The simplicity of the assay, and using rapid LLE with a commercially available IS, and a sample turnover rate of 2.5 min per sample, make it an attractive procedure in high-throughput bioanalysis of galantamine. The validated method allows quantitation of galantamine in the 0.5–100 ng/mL range.

Acknowledgments

The authors wish to acknowledge the support received from Mr. Venkateswarlu Jasti, CEO, Suven Life Sciences, Hyderabad.

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Manuscript received September 12, 2006;
revision received October 13, 2006.