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A rapid and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the estimation of rivastigmine in human plasma

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Abstract

A rapid and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method has been developed and validated for the estimation of rivastigmine in human plasma. Rivastigmine was extracted from human plasma by using solid-phase extraction technique. Zolpidem was used as the internal standard. A Betabasic-8 column provided chromatographic separation of analytes followed by detection with mass spectrometry. The mass transition ion-pair was followed as *m/z* 251.20 → 206.10, 86.20 for rivastigmine and *m/z* 308.10 → 235.10 for zolpidem. The method involves a rapid solid-phase extraction from plasma, simple isocratic chromatographic conditions and mass spectrometric detection that enables detection at sub-nanogram levels. The proposed method has been validated for a linear range of 0.2–20.0 ng/ml with a correlation coefficient ≥0.9988. The intra-run and inter-run precision and accuracy were within 10.0%. The overall recoveries for rivastigmine and zolpidem were 86.28% and 87.57%, respectively. The total run time was 2.0 min. The developed method was applied for the determination of the pharmacokinetic parameters of rivastigmine following a single oral administration of a 3 mg rivastigmine capsule in 20 healthy male volunteers.

Keywords: Rivastigmine; LC–MS/MS; Human plasma

1. Introduction

Rivastigmine hydrogen tartrate (S)-*N*-ethyl-3-[(1-dimethyl amino)ethyl]-*N*-methyl-phenylcarbamate hydrogen tartrate is an acetylcholinesterase inhibitor of the carbamate type approved for the treatment of Alzheimer's disease [1]. Rivastigmine has received approval for use in 60 countries including all the members of the European Union where it was introduced in 1998 and the US where it was introduced in 2000. The methods aiming at determining this relatively new parasympathomimetic agent are of clinical and pharmacokinetic importance.

Rivastigmine exhibits non-linear pharmacokinetics because of capacity-limited elimination. Thus, maximum plasma drug concentration (C_{max}) and area under the concentration–time curve (AUC) increase more than proportionally with an increasing dose, whereas they are low at low dose [2]. Also rivastigmine has a short plasma half-life, so a sensitive bioanalytical method, which can quantify rivastigmine at sub-nanogram level, is required for the determination of pharmacokinetic parameters of rivastigmine.

Rivastigmine is relatively a new drug, and perhaps for that particular reason, few analytical methods have been reported in the literature for monitoring plasma levels of rivastigmine. The techniques used in these methods include LC–MS/MS [1,3] and GC–MS detection [2,4–6]. Although these assays are sufficiently sensitive, these methods require laborious extraction procedures like liquid–liquid extraction or solid-phase microextraction involving time-consuming and error-prone solvent evaporation and reconstitution steps and long run time. Refer to Table 1 to view the salient features of the reported methods. The reported LC–MS/MS method for the determination of rivastigmine in rat plasma [3] is highly sensitive, but

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Table 1
Comparison of analytical methods developed for estimation of rivastigmine in biological matrix

Sr. no.	Biological matrix (processing volume)	Extraction procedures (extraction time)	Evaporation and reconstitution	Analytical run time	LOQ	Detection technique	Reference
1	Plasma (0.5 ml)	LLE with derivatization, back extraction and re-extraction (more than 40 min/sample)	Done	5.0 min	0.2 ng/ml	LC–MS/MS	[1]
2	Serum (*)	LLE with back extraction and re-extraction (more than 50 min/sample)	Done	*	0.2 μg/ml	GC–MS	[2]
3	Rat plasma and brain homogenates (*)	LLE	Done	*	0.5 pmol/ml plasma and 2.5 pmol/ml brain	LC–MS	[3]
4	Plasma and CSF (*)	*	*	*	0.2 ng/ml	GC–MS	[4]
5	Canine plasma (0.5 ml)	Head space SPE (more than 30 min/sample)	Not required	11.0 min	0.2 ng/ml	GC–MS	[6]
6	Plasma (0.8 ml)	SPE (less than 10 min/sample)	Not required	2.0 min	0.2 ng/ml	LC–MS/MS	Present method

LLE, liquid–liquid extraction; SPE, solid-phase extraction; LOQ, lower limit of quantification.
* Not available.

this method cannot be adopted for human plasma as the range is very low: between 10 and 100 pmol/ml. This method cannot be applied for monitoring of rivastigmine pharmacokinetics in clinical trials and bioequivalence study. Therefore, it was necessary to develop a simple, specific, rapid and sensitive analytical method for the quantification of rivastigmine in human plasma.

This paper describes the development and validation of an LC–MS/MS method for the quantification of rivastigmine in human plasma, which reduces sample preparation and analysis time relative to other commonly employed techniques and has a limit of quantification (LOQ) 0.2 ng/ml. Zolpidem was used as an internal standard.

2. Experimental

2.1. Chemicals and reagents

The working standards of rivastigmine hydrogen tartrate and zolpidem tartrate were obtained from Torrent Research Centre (Ahmedabad, India). High purity water was prepared in-house using a Milli-Q water purification system obtained from Millipore (Bangalore, India). Gradient grade methanol and acetonitrile were purchased from Ranbaxy (New Delhi, India). Suprapure formic acid was purchased from Merck (Darmstadt, Germany). Drug-free (blank) buffered human plasma was obtained from Green Cross Laboratory (Ahmedabad, India) and was stored at −20 °C prior to use.

2.2. Calibration curves

Stock solutions of rivastigmine and internal standard, zolpidem, were prepared in methanol at free base concentration of 1 and 2 mg/ml, respectively. Secondary and working standard solutions were prepared from stock solutions by dilution with water. These diluted working standard solutions were used to prepare the calibration curve and quality control (QC) samples in human plasma.

A nine-point standard calibration curve for rivastigmine was prepared by spiking the blank plasma with appropriate amount of rivastigmine. The calibration curve ranged from 0.2 to 20.0 ng/ml. Quality control samples for rivastigmine were prepared at three concentration levels: 0.6 ng/ml low quality control (LQC), 6.0 ng/ml medium quality control (MQC) and 15.0 ng/ml high quality control (HQC), in a manner similar to preparation of the standard solutions from the stock solution.

2.3. Sample preparation

A 0.8 ml aliquot of human plasma sample was mixed with 25 μl of internal standard working solution (150 ng/ml of zolpidem). The sample mixture was loaded into an Oasis HLB (1 cm^3/30 mg), an extraction cartridge that was pre-conditioned with 1.0 ml methanol followed by 2.0 ml water. The extraction cartridge was washed with 2.0 ml water followed by 1.0 ml 20% methanol. Rivastigmine and zolpidem were eluted with 0.5 ml of acetonitrile; 10.0 μl of the eluate was injected into the LC–MS/MS system.

2.4. Instrumentation

Chromatographic separation was carried out on Surveyer HPLC with Betabasic-8 (5.0 μm, 100 mm × 4.6 mm) purchased from Thermo electron corporation, UK. A mobile phase consisting of 0.1% formic acid in acetonitrile and 0.1% formic acid in water (70:30%, v/v) was delivered with a flow rate of 1.0 ml/min (flow was splitted 30% to the source and 70% to the waste). The column oven temperature was kept at 45 °C. The total run time for each sample analysis was 2.0 min. The sample injection volume was 10.0 μl. Mass spectra were obtained using a TSQ Quantum mass spectrometer, a triple stage quadrapole mass

Table 2
Ion source and analyte-dependent parameters

Ion source		
Spray voltage		3500 V
Capillary temperature		350 °C
Sheath gas		40 (arbitrary)
Auxillary gas		20 (arbitrary)
Polarity mode		Positive
	Analyte dependent	
	Rivastigmine	Zolpidem
Precursor ion (*m/z*)	251.20	308.10
Product ion (*m/z*)	206.10, 86.20	235.10
Tube lens off set (*V*)	63	96
Q1 Pw[a] (amu)	0.3	0.7
Q3 Pw[b] (amu)	0.7	0.7
Collision energy	20	40

[a] Quadrapole 1 peak width.
[b] Quadrapole 3 peak width.

analyzer with electron multiplier detector, equipped with electrospray ionization (ESI) source (Thermo Finnigan Ltd., UK). The mass spectrometer was operated in the selected reaction-monitoring (SRM) mode. Sample introduction and ionization was ESI in the positive ion mode. The MS parameters for analytes are listed in Table 2. The data acquisition was ascertained by Xcalibur 1.4 software.

2.5. Validation

The method has been validated for selectivity, sensitivity, linearity, precision, accuracy, recovery, stability and matrix effect. Selectivity was performed by analyzing the blank plasma samples from different sources (or donors) to test for interference at the retention time of rivastigmine and internal standard, zolpidem. Sensitivity was determined by analyzing five replicates of blank human plasma and plasma spiked with the analyte at the lowest level of the calibration curve. The intra-run and inter-run accuracy were determined by replicate ($n = 5$) analysis of quality control samples that were extracted from the sample batch at LOQ, LQC, MQC and HQC level. Inter-run precision and accuracy of the calibration standards were assessed using five calibration curves used for assay validation.

Accuracy is defined as the percent of relative error (%RE) and was calculated using the formula $\%RE = (E - T) \times 100/T$, where E is the experimentally determined concentration and T is the theoretical concentration. Assay precision was calculated by using the formula $\%R.S.D. = (S.D./M) \times 100$, where %R.S.D. is the percent of relative standard deviation, M is the mean of experimentally determined concentrations and S.D. is the standard deviation of M.

The extraction efficiencies of rivastigmine and zolpidem were determined by comparing the peak area of extracted analytes to the peak area of non-extracted standards (analyte spiked post-extraction in blank plasma).

The processed sample stability was evaluated by comparing the extracted plasma samples, which were injected immediately (time 0), with the samples that were re-injected after keeping in the autosampler at 5 °C for 24.0 h. The stability of spiked human plasma stored at room temperature (Bench-top stability) was evaluated for 5 h and compared with freshly prepared extracted samples. The freeze–thaw stability was conducted by comparing the stability samples that had been frozen and thawed three times, with freshly spiked quality control samples. The stability of spiked human plasma stored at −70 °C (long-term stability) was evaluated by analyzing low, medium and high quality control samples that were stored at −70 °C for 42 days together with freshly spiked calibration standard and quality control samples. All stability evaluations were based on back-calculated concentrations. Analytes were considered stable if the deviation of the mean test responses were within 15% of freshly prepared or comparison sample.

Matrix effect was checked with six different lots of plasma. Three samples each of LQC and HQC were prepared from different lots of plasma (in total, 36 QC samples) and checked for accuracy in all the QC samples. This was performed with the aim to see the matrix effect of these different lots of plasma on the back-calculated value of QC's nominal concentration. It is considered there is no matrix effect if the deviation of the mean test responses were within 15% of freshly prepared or comparison samples.

3. Results and discussion

3.1. Method development

To develop a rapid, sensitive and simple assay method for the extraction and quantification of rivastigmine during method development, different options were evaluated to optimize detection, extraction and chromatography parameters. Rivastigmine accepted the proton in an acidic mobile phase and produced a protonated precursor ion ($[M+H]^+$) at *m/z* 251.20. Electro spray ionization and atmospheric pressure chemical ionization (APCI) were evaluated to get better response of analytes. It was found that the best signal was achieved with ESI positive ion mode. Two product ions *m/z* 206.10 and 86.20 of rivastigmine were monitored, which gave better sensitivity and selectivity. The selected fragments of each compound, as product ion to be monitored, are indicated in Fig. 1.

Further optimization in chromatography conditions resulted in improvement in signal and reduction in run time. It is observed that reduction in buffer pH from 7.0 to 2.5 resulted in improved response and peak symmetry. Use of Betabasic C-8 (100 mm × 4.6 mm i.d., 5 μ) column enabled use of high flow rate, which resulted in run time as low as 2.0 min with better peak symmetry and signal of analytes.

In order to achieve cleanliness in extract, solid-phase extraction was optimized for extraction of analytes from plasma. Both the analytes showed good retention when eluted with basic condition on cartridges. It was observed that washing the solvent with 20% methanol strength resulted in reduced interference without losing recovery of analytes. In order to eliminate the time-consuming and error-prone solvent evaporation and reconstitution steps for concentration of samples after elution with

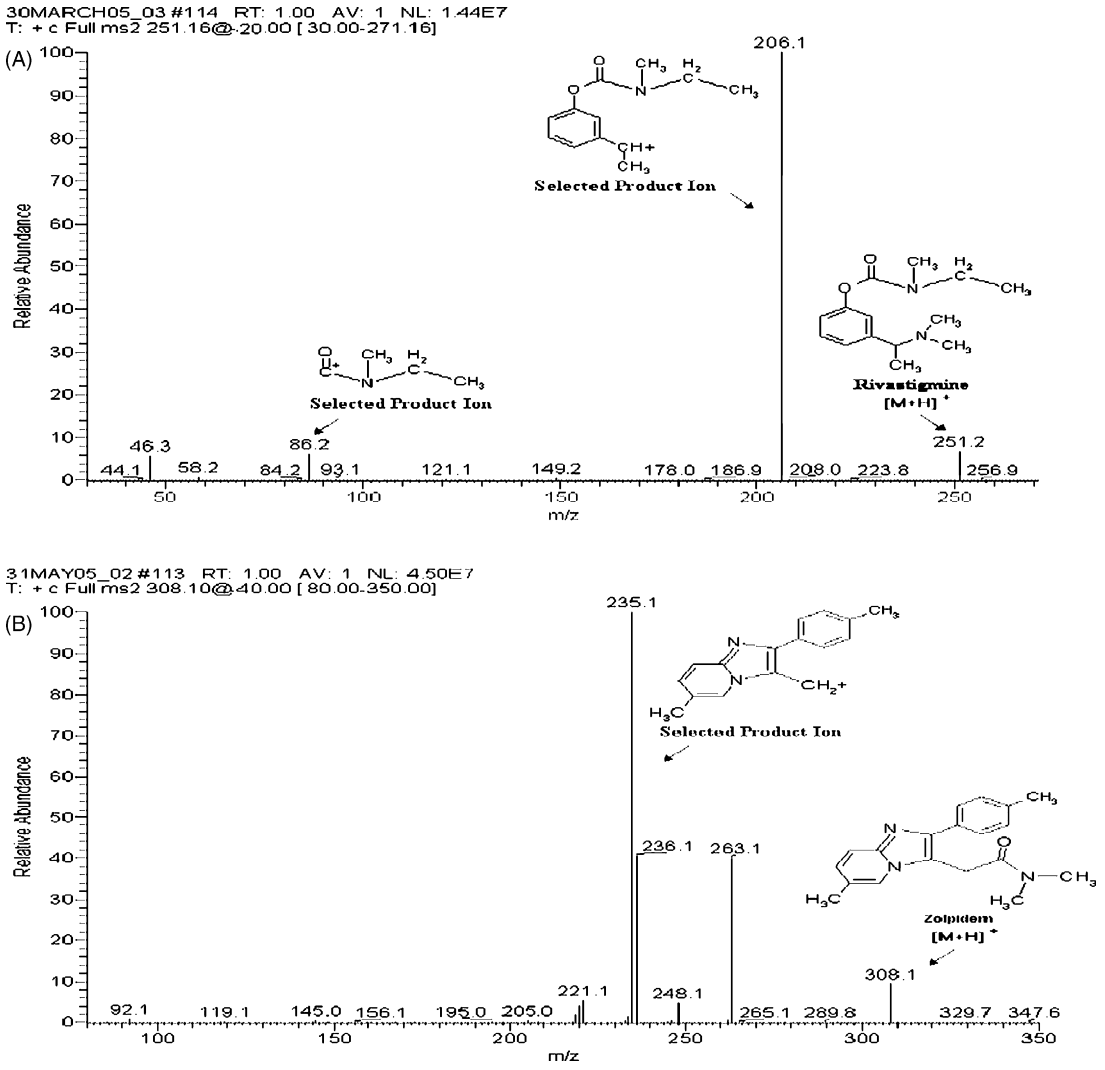

Fig. 1. ESI production mass spectra for the precursor ions of (A) rivastigrmine and (B) zolpidem.

acetonitrile, the elution volume of acetonitrile was reduced to 0.5 ml to concentrate the samples in eluate. The optimized detection and sample extraction chromatography are enabled to reduce processing and analysis time without compromising the sensitivity.

3.2. Selectivity

Utilization of predominant product ions for each compound enhanced mass spectrometric selectivity. The mass transition ion-pair was selected as *m/z* 251.20 → 206.10, 86.20 for rivastigmine and *m/z* 308.10 → 235.10 for zolpidem. The product ions selected were specific for rivastigmine and zolpidem.

Chromatographic selectivity of the method was demonstrated by the absence of endogenous interfering peaks at the retention times of rivastigmine and zolpidem in six different lots of extracted blank plasma. Representative chromatograms of extracted blank plasma, extracted plasma samples containing 0.2 ng/ml rivastigmine (low standard) and plasma samples from subjects are presented in Figs. 2–4, respectively.

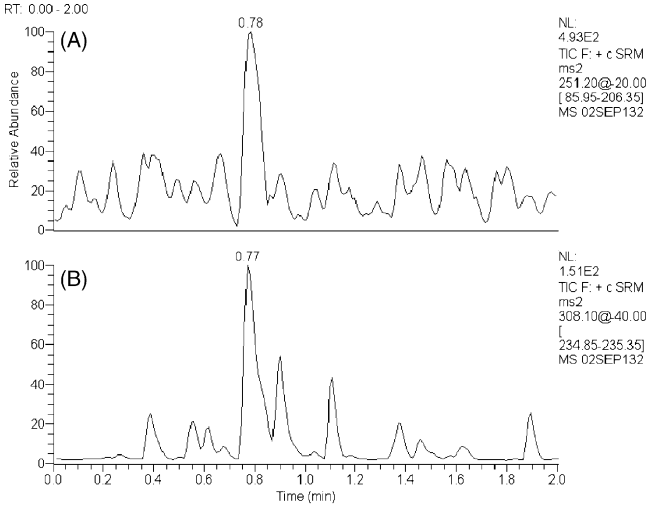

Fig. 2. Representative chromatograms of extracted blank plasma sample (A) rivastigmine and (B) zolpidem.

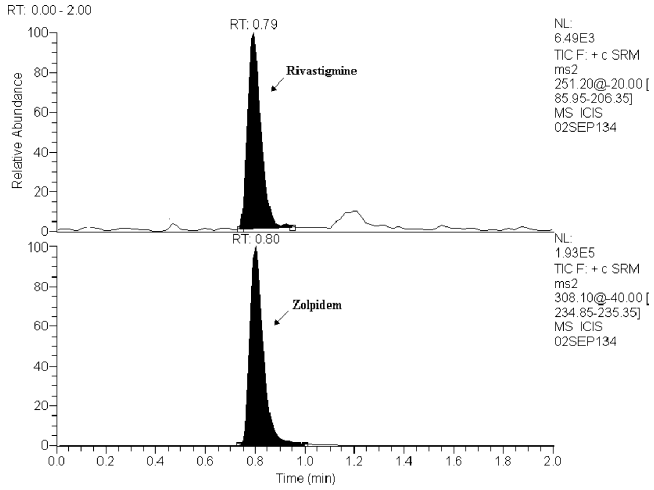

Fig. 3. Representative chromatograms of an extracted plasma sample at the LOQ.

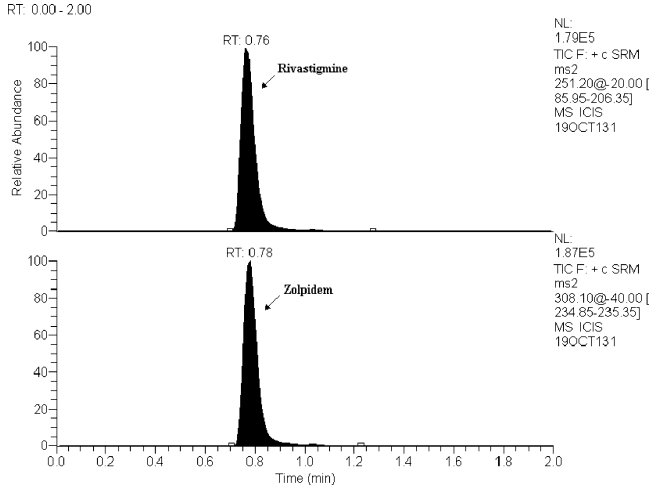

Fig. 4. Representative chromatogram of extracted subject sample.

3.3. Linearity

The peak area ratios (area of rivastigmine/area of zolpidem) of calibration standards were proportional to the concentration of analytes in each assay over the nominal concentration range of 0.2–20.0 ng/ml for rivastigmine. The calibration curves appeared linear and were well described by least squares lines. A weighting factor of 1/concentration was chosen to achieve homogeneity of variance. The correlation coefficients were $\geq$0.9988 ($n = 5$) for rivastigmine. The mean ($\pm$S.D.) slopes of the calibration curves ($n = 5$) for rivastigmine were 0.158804 ($\pm$0.011510).

3.4. Sensitivity (lower limit of quantification)

The LOQ is defined as the lowest concentration of the calibration standard yielding accuracy of $\pm$20% and precision of $\leq$20%. The LOQ for rivastigmine was 0.2 ng/ml. These data are tabulated in Table 3 for rivastigmine. The intra-run precision at the LOQ plasma samples containing rivastigmine was 8.06%. The intra-run accuracy at the LOQ plasma samples containing rivastigmine was 4.06%.

3.5. Precision and accuracy

The intra-run precision was $\leq$8.06% and intra-run accuracy was $\leq$4.74 for rivastigmine (Table 3). The inter-run precision and accuracy were determined by pooling all individual assay results of replicate ($n = 5$) QC samples over the three separate batch runs. The inter-run precision was $\leq$8.30. The inter-run accuracy was $\leq$4.76 for rivastigmine (Table 4).

3.6. Recovery

Five replicates at low, medium and high quality control concentrations for rivastigmine were prepared for recovery determination. The mean recovery for rivastigmine was 86.28% with a precision of 7.48%. The mean recovery for zolpidem was 87.57%.

Table 3
Intra-run ($n = 5$) precision and accuracy of rivastigmine in human plasma

Analyte	Spiked concentration (ng/ml)	Mean calculated concentration (ng/ml)	%R.S.D.	%RE
Rivastigmine	0.200	0.208	8.06	4.06
	0.600	0.577	6.01	−3.83
	6.000	5.943	4.81	−0.95
	15.000	14.29	4.69	−4.74

Table 4
Inter-run ($n = 5$) precision and accuracy of rivastigmine in human plasma

Analyte	Spiked concentration (ng/ml)	Mean calculated concentration (ng/ml)	%R.S.D.	%RE
Rivastigmine	0.200	0.210	8.30	4.76
	0.600	0.609	6.64	1.50
	6.000	5.981	4.37	−0.31
	15.000	14.327	5.40	−4.49

Table 5
Stability results for rivastigmine ($n = 5$)

Stability	Spiked concentration (ng/ml)	Mean (±S.D.) obtained concentration (ng/ml)	%RE
Process[a]	0.600	0.570(±0.016)	−5.06
	15.000	14.745(±0.388)	−1.70
Bench top[b]	0.600	0.598(±0.053)	−0.27
	15.000	14.357(±1.137)	−4.23
Freeze/thaw[c]	0.600	0.608(±0.043)	1.27
	15.000	14.524(±0.908)	−3.18
Long-term[d]	0.600	0.656(±0.020)	9.31
	6.000	5.929(±0.129)	−1.18
	15.000	15.386(±0.508)	2.57

[a] After 24 h in autosampler at 5 °C.
[b] After 5 h at room temperature.
[c] After three freeze/thaw cycles at −70 °C.
[d] −70 °C for 42 days.

3.7. Stability

The results of the stability studies are enumerated in Table 5. The bench-top stability, process stability and freeze and thaw stability of rivastigmine in plasma were investigated by analyzing quality control samples in replicates ($n = 5$) at LQC and HQC levels. Process stability results indicated that the difference in back-calculated concentration from time 0 to 24 h is ≤5.06%, which allowed to conclude that processed samples are stable at least for 24 h at 5 °C in autosampler. Bench-top stability results allowed us to conclude that rivastigmine is stable for at least 5 h at room temperature in plasma samples. Freeze and thaw stability results indicated that the repeated freezing and thawing (three cycles) did not affect the stability of rivastigmine. Long-term stability of rivastigmine in plasma at −70 °C was performed at LQC, MQC and HQC levels; it was found to be stable for at least 42 days at −70 °C.

3.8. Matrix effect

Three quality control samples at each level along with the set of calibration standards were analyzed, and the %bias of the samples analyzed was found within ±15 for each QC level for rivastigmine (Table 6).

Hence, this clearly proves that the elution of endogenous matrix peaks during the run has no effect on the quantification of rivastigmine. Therefore, the method of extraction of rivastigmine from plasma was rugged enough and gave accurate and consistent results when applied to real patient samples.

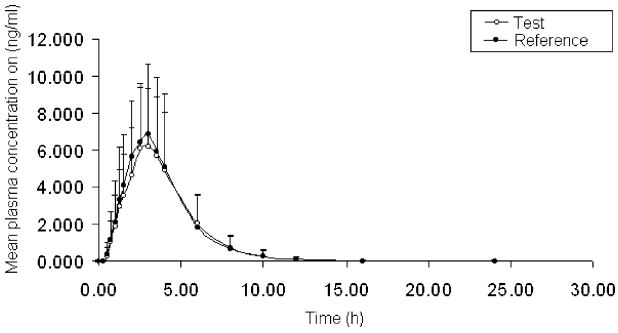

Fig. 5. Mean rivastigmine plasma concentration–time profile following a 3 mg oral dose of rivastigmine to human subjects.

3.9. Application of method

The proposed method was applied to the determination of rivastigmine in plasma samples from ongoing projects for the development of an immediate release formulation. Plasma samples were periodically collected up to 24 h after a single oral dose administration of a 3 mg capsule to 20 healthy male volunteers in each phase. The time periods at which the plasma samples were drawn were 0.00, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 6.00, 8.00, 10.00, 12.00, 16.00 and 24.00 h after a single oral dose administration of a 3 mg capsule. A total of 720 human plasma samples from 20 male volunteers were analyzed along with calibration standards and QC samples. Four calibration curves were made for sample quantification.

Table 6
Matrix effect ($n = 3$) for rivastigmine

Sr. no.	Plasma lot no.	LQC (0.6 ng/ml)			HQC (15.0 ng/ml)		
		Mean calculated concentration	%R.S.D.	%RE	Mean calculated concentration	%R.S.D.	%RE
1	LOT-A1	0.595	5.24	−0.78	14.434	2.99	−3.78
2	LOT-A2	0.598	4.94	−0.33	14.299	0.79	−5.14
3	LOT-A3	0.585	3.36	−2.44	14.344	0.89	−4.38
4	LOT-A4	0.604	5.69	0.67	14.645	1.8	−2.37
5	LOT-A5	0.599	6.65	−0.17	14.482	0.59	−3.45
6	LOT-A6	0.572	3.53	−4.61	14.182	0.17	−5.46

No interference peak was found in pre-dose samples for all volunteers. The mean (±S.D.) plasma maximum concentrations obtained for the rivastigmine test and reference formulations were 6.196 (±3.140) ng/ml and 6.879 (±3.778) ng/ml, respectively. The mean rivastigmine plasma concentration–time profile following a 3 mg oral dose of rivastigmine to human subjects is shown in Fig. 5. The area under the curve (AUC) measured from 0 h to the last sampling point was higher than 90% of the value of AUC extrapolated from 0 to infinity, which indicates the suitability of the LOQ of the analytical method for pharmacokinetic study.

4. Conclusions

A simple, specific, rapid and sensitive LC–MS/MS method has been developed for the determination of rivastigmine in human plasma. The proposed method provided excellent specificity and reproducibility with a limit of quantification of 0.2 ng/ml for rivastigmine.

It is concluded that this sensitive and specific method is applicable for the quantitative determination of rivastigmine in human plasma in pharmacokinetic and bioavailability studies of rivastigmine.

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