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High Performance Liquid Chromatographic Fluorescence Detection Method for the Quantification of Rivastigmine in Rat Plasma and Brain: Application to Preclinical Pharmacokinetic Studies in Rats

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ABSTRACT

A highly sensitive and selective high performance liquid chromatographic fluorescence detection method has been developed and validated for the quantification of rivastigmine in rat plasma and brain. Protein precipitation and one-step liquid–liquid extraction techniques were utilized for the extraction of RSM from brain and plasma, respectively, along with an internal standard. The chromatographic separation was achieved with a column inertsil ODS-3V and a mobile phase consisting of ammonium acetate buffer (20 mM, pH 4.5) and acetonitrile (76:24, v/v) delivered at a flow rate of 1 ml/min. The lower limit of quantitation for the developed method was 10 ng/mL for both matrices. The method was found to be accurate and reproducible and was successfully used to quantify levels of RSM in plasma and brain following *intravenous* administration of RSM in rats.

Key words: HPLC method validation, pharmacokinetics, plasma-brain correlation, rivastigmine

INTRODUCTION

Alzheimer's disease (AD) is the result of degeneration of presynaptic cholinergic system markers and loss of cholinergic neurons. It is the most common cause of cognitive impairment in elderly.[1,2] AD is characterized by the deposition of β-amyloid protein in the form of senile plagues and neurofibrillary tangles in the brain. AD is the commonest form of dementia accounting for 50% cases. It is currently estimated that 40% of nursing home residents have a diagnosis of AD or dementia. The presence of dementia is associated with additional costs, and an increased care requirement in elderly patients. AD causes multiple cognitive defects that progress over a period of time and often accompanied by behavioral disturbances such as aggression, depression, and wandering.[3,4]

Rivastigmine (RSM) is being therapeutically used for the treatment of mild-to-moderate AD and inhibits acetyl

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and butyrylcholinesterase forms in the brain.[5] RSM is a carbamate derivative with central selectivity demonstrated in both animal and human studies, a long duration of acetylcholinesterase inhibition (6–12 h) following single oral dose [Figure 1]. RSM has been shown to improve or maintain AD patient's performance in three major domains such as cognitive function, global function (ADLs), and behavior. The efficacy and tolerability of RSM have been proved by numerous clinical trials.[6]

Several methods have been reported in the literature for quantification of RSM and its metabolites in biological fluids namely, human plasma by LC–MS/MS,[7-9] GC–MS,[10] human serum by GC–MS,[11] rat plasma by LC–MS,[12] and canine plasma by GC–MS.[13] To the best of our knowledge, we were the first group to report a high performance liquid chromatographic (HPLC)-fluorescence detection method for quantification of RSM in rat plasma using simple liquid–liquid extraction (LLE).[14] Subsequently, our research reported a HPLC–UV method for quantification of RSM in rat urine along with identification of a novel metabolite in rat urine using HPLC-DAD and LC–MS/MS.[15]

This work was aimed to report a further higher sensitive and selective HPLC method for quantification of RSM in rat plasma and brain. There is a need for the sensitive HPLC method for the quantification of RSM in rat biological fluids and as well as in brain, as the drug crosses blood–brain barrier (BBB) and reaches brain in significant quantities to elucidate its anticholinesterase activity.[5] Most of the reported methods for quantification of RSM in biological fluids were either LC–MS/MS or GC–MS techniques. Mass chromatographic methods are sensitive, specific, and shorter run time, but they are highly expensive and may not be affordable to many laboratories. Hence, a sensitive and economical HPLC method with fluorescence detection was developed and validated, without a compromising limit of quantitation of RSM in rat biological matrices. In comparison to the published method,[12] the present method has a simple, less cumbersome, and one-step extraction procedure for the quantification of RSM in rat brain. When compared to the reported method,[14] the developed method has higher sensitivity and selectivity for the estimation of RSM in rat plasma and brain tissues using less volume of the biological sample. Further, this method can be extended easily to assess RSM levels in other matrices namely, plasma, serum, brain homogenate of preclinical species, and humans.

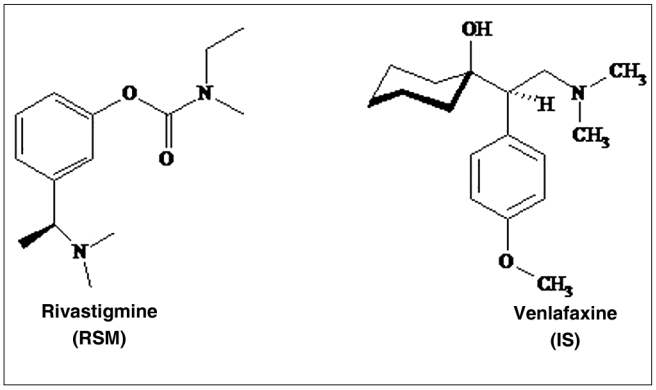

Figure 1: Structural representation of rivastigmine (RSM) and venlafaxine (IS)

MATERIALS ANDS METHODS

Chemicals and reagents

RSM hydrogen tartrate (99%) as a gift sample was obtained from Dr. Reddy's Laboratories Limited, Hyderabad, India. Venlafaxine (internal standard) was kindly donated by Torrent Pharmaceuticals Limited, Ahmadabad, India. HPLC grade acetonitrile, methanol, and analytical grade ammonium acetate were obtained from Merck Chemicals, Mumbai, India. Acetic acid was obtained from S.D. Fine Chemicals, Mumbai, India. Water was purified through a Milli-Q UV plus system procured from Millipore, Bedford, MA, USA. All other chemicals used were of analytical grade.

Chromatographic conditions

HPLC analysis was carried out on Shimadzu LC-20AD Prominence (Shimadzu Corporation, Kyoto, Japan) equipped with LC-20AD pump, RF-10A-XL fluorescence detector, column oven (CTO-10AS VP), auto-sampler SIL-20AC HT, and LC solution version 1.24 SP1. The column oven temperature was maintained at 25°C. The chromatographic separation was achieved by Interstil, ODS-3V, C_{18} (250 × 4.6 mm, 5 µm) column (GL Sciences Inc., Japan). Isocratic elution was performed with ammonium acetate buffer (20 mM, pH 4.5) and acetonitrile 74:26 (v/v) as a mobile phase. The flow rate was maintained at 1 ml/min and the injection volume was 50 µL. Fluorimetric detection was used and excitation and emission wavelengths were of 220 and 293 nm, respectively. The sensitivity of the detector was "medium" and the response kept at 3.

Preparation of stock solutions

Accurately weighed 16 mg of RSM hydrogen tartrate (equivalent to 10 mg of RSM) was weighed and dissolved in 10 mL of methanol to yield a primary standard solution of 1 mg/mL. Venlafaxine (IS) was prepared separately in methanol to yield primary stock solution with a

concentration of 1 mg/mL. Working stock solutions for the calibration curve namely, 0.2, 0.3, 0.5, 1, 2, 5, 10, and 20 μg/mL and quality control standards 0.6, 3.0, and 16 μg/mL were prepared using methanol as a diluent. The IS working stock solution (1 μg/mL) was prepared by diluting the primary stock solution with methanol.

Extraction of RSM from rat biological matrices

Extraction procedure for rat plasma

Whole blood sample collected from the rats and plasma was separated, pooled, and stored at −70°C until analysis. Extraction of RSM from rat plasma was carried out using a LLE technique. Briefly, to an aliquot of 100 μL of rat plasma, 10 μL of IS (1 μg/mL) was added and vortexed for 30 s. Then 1 mL of *Tert*-butyl methyl ether (TBME) was added and vortexed for 10 min and then centrifuged at 10,000 rpm for 5 min. From the supernatant 0.9 mL of organic solvent was aspirated and evaporated in a turbo evaporator (Zymark, Hopkinton, MA, USA) at 50 ± 2°C under a stream of nitrogen. The dried residue was reconstituted with 150 μL of the mobile phase and 50 μL was injected for the HPLC analysis.

Extraction from rat brain

Protein precipitation method was generally the preferred choice of extraction technique because it is simple, economical, and less cumbersome. An aliquot of 500 μL of a blank rat brain homogenate was taken in a 2 mL Ependorff centrifuge tube and 10 μL of IS (1 μg/mL) solution was added and vortexed for 30 s. Further, 500 μL of cold acetonitrilemethanol mixture (1:1, v/v) was added vortexed for 10 min and then centrifuged at 10,000 rpm (−10°C) for 10 min. Clear supernatant (200 μL) was transferred into a vial and 50 μL was subjected to HPLC analysis.

Recovery

Recovery of RSM in plasma and brain matrices were evaluated by comparing the mean peak areas of low and high QC samples (extracted) to that of mean peak areas of neat reference solutions of same concentration (unextracted). Recovery of IS was also evaluated by the similar manner at 1 μg/mL.

Bioanalytical method validation

A full method validation according to the US-FDA guidelines[16] was performed for the quantification of RSM in rat plasma and brain.

Selectivity

The selectivity of the assay methodology was established using a minimum of six independent sources of rat plasma and brain. These matrices were processed and analyzed using the developed method and checked for interferences at the retention time of RSM and IS.

Sensitivity

The sensitivity of the method was evaluated by spiking the lowest concentration of RSM (10 ng/mL) in both matrices and analyzed by the developed method. The acceptance criteria is the lowest concentration of analyte that should be quantified with accuracy and precision and interferences if any at the retention time (RSM) should be less than 5% of lower limit of quantitation (LLOQ).

Linearity

The linearity of the method was evaluated by the use of a calibration curve in the range of 10−1000 ng/mL for both matrices. The calibration curve was constructed by plotting RSM concentration against RSM-to-IS peak area ratios by least-squares linear regression analysis. The calibration curve requires a coefficient of determination (r^2) of >0.98 as per US FDA guidance for bioanalytical method validation. The acceptance criteria for each back calculated standard concentration should be within ±15% of the nominal concentration except at the LLOQ level where it should not exceed ±20% of relative standard deviation (% RSD) (US DHHS, FDA, CDER, 2001). Each validation run consisted of a double blank, system suitability sample, zero standard, calibration curve consisting of eight non-zero samples covering the total range and QC (quality control) standards at three concentrations (n=6, at each concentration).

Accuracy and precision

Intra- and inter-day accuracy and precision were determined by duplicate analysis of six sets of spiked QC standards of RSM in rat plasma and brain samples. Plasma and brain samples were spiked with four different concentrations of RSM namely, 10, 30, 150, and 800 ng/mL for LLOQ, low, medium, and high QC samples, respectively. These samples were prepared and analyzed on day one and this procedure was repeated for three consecutive days. Acceptance criteria for intra and inter-day accuracy should be within 85–115% of nominal concentration and precision should be less than ±15% of RSD. At the LLOQ level accuracy should be between 80 120% and % RSD not more than ±20% (US DHHS, FDA, CDER, 2001).

Stability

In order to determine the stability of RSM in plasma and brain samples, they were studied at four different stability conditions including bench top, freeze–thaw, auto-injector, and long term. Stability of RSM in both matrices was examined by replicate analysis of RSM in low and high

QC samples. Bench top stability was carried out by keeping replicates (n=6) of QC samples for approximately 12 h at ambient temperature. Freeze–thaw stability was carried out by three freeze–thaw cycles at −70°C by thawing in room temperature for 2−3 h and refreezing for 12 h for each cycle. Auto-sampler stability was tested by analysis of processed and reconstituted QC samples which were stored in the auto-sampler tray for 15 h. Long-term stability of RSM in rat plasma and brain were tested after storage at −70°C for 30 days. The acceptance criteria for the stability are that the deviation compared to the freshly prepared standard should be within ±15% of the nominal concentration.

Dilution effects

The dilution effect was investigated to ensure that samples could be diluted with blank matrix without affecting the final concentration. RSM spiked rat plasma samples prepared at two concentrations (8000 and 16,000 ng/mL) of RSM were diluted with pooled rat plasma at dilution factors of 10 and 20 in six replicates and analyzed. The six replicates should have precision of ≤15% and accuracy of 100 ± 15% similar to other QC samples.

Pharmacokinetics study of RSM in rats

pharmacokinetic study was carried out in male Wistar rats (n=3 for each time point, weight range: 190−210 g). Rats were kept in stainless steel cages and had free access to water and food. RSM was dissolved in isotonic saline and administered through the *intravenous* route at the dose of 1 mg/kg. Rats were anesthetized with diethyl ether, blood samples were collected at predetermined intervals and then sacrificed by a cervical dislocation method for the removal of whole brain. Then brain was washed twice with ice-cold saline and then wiped with the soft tissue paper and stored at −70°C until analysis. The brain tissue was homogenized (10% w/v) with phosphate buffer saline (pH 7.4) under ice and samples were stored at −70°C until analysis.

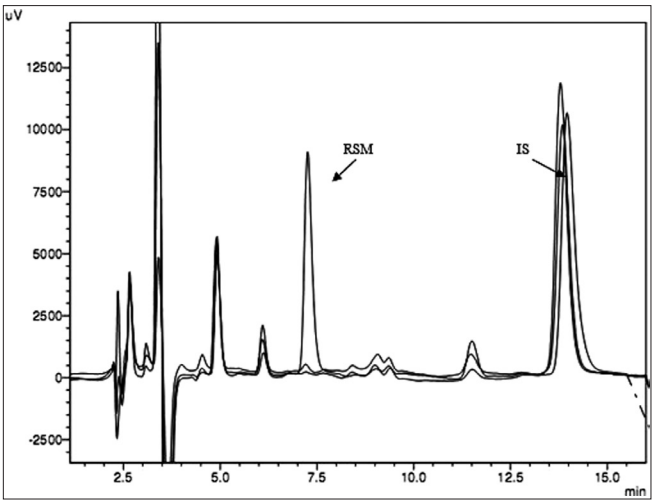

Figure 2: HPLC chromatograms of a 50 µL injection of an extract from (lower tracing) rat blank plasma spiked with IS; (middle tracing) rat plasma spiked with RSM at LLOQ with IS and (upper tracing) a 5 min *in vivo* plasma sample obtained following intravenous dose of RSM at 1 mg/kg to male Wistar rats

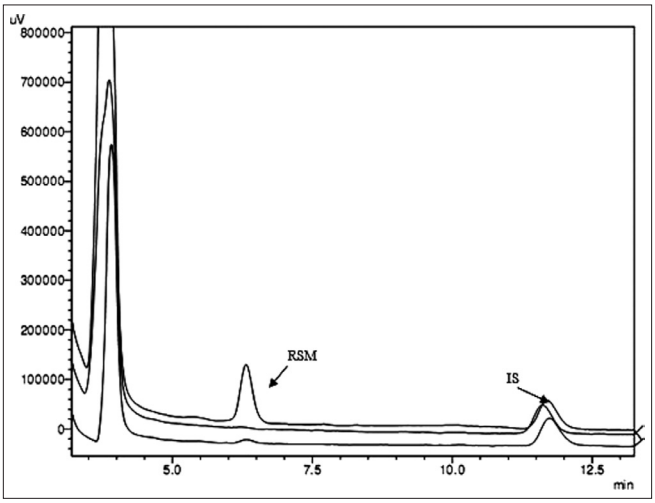

Figure 3: HPLC chromatograms of a 50 µL injection of an extract from (lower tracing) rat brain spiked with RSM at LLOQ with IS; (middle tracing) rat blank brain spiked with IS and (upper tracing) a 5 min *in vivo* brain sample obtained following intravenous dose of RSM at 1 mg/kg to male Wistar rats

RESULTS

Selectivity and sensitivity

The developed method was found to be selective for the quantification of of RSM in rat plasma and brain matrices. Chromatograms were neat, sharp symmetric peaks and there were no endogenous interferences at the retention time of RSM and IS, which indicates that the method is highly selective for the quantification of RSM in rat plasma and brain samples. The overlay of blank chromatograms, LLOQ, and study samples of RSM in rat plasma and brain are shown in Figures 2 and 3. The method was found to be sensitive since it could estimate the RSM in both matrices at 10 ng/mL with acceptable accuracy and precision.

Recovery

During this work, a number of attempts were made to standardize an extraction procedure which results in consistent recovery and avoid interferences at the retention time of RSM and IS. protein precipitation method was attempted initially using various protein precipitating agents, but the results were not optimal due to endogenous interferences. TBME was selected for a LLE method

after a testing variety of organic solvents. It consistently extracted more than 90% of RSM from rat plasma without endogenous interferences. The mean relative recovery of RSM was found to be 113.40 ± 10.92 and 111.03 ± 12.42% at LQC and HQC in plasma, respectively. Using a simple protein precipitation technique for quantification of RSM in brain samples the recovery was found to be 102.24 ± 8.92 and 99.03 ± 8.42% at LQC and HQC, respectively. Recovery of IS at 1 μg/mL was found to be 112.50 ± 4.90 and 94.15 ± 5.73% in plasma and brain, respectively.

Linearity

The developed method was linear over the range of 10−1000 ng/mL of RSM in rat plasma and brain matrices. The coefficient of determination was found to be greater than 0.99 for both matrices.

Accuracy and precision

The accuracy and precision of intra- and inter-day studies at LLOQ, low, medium, and high quality control samples of RSM in rat plasma and brain were within acceptable limits. The results indicated that the assay method was accurate and precise for replicate analysis of RSM within the same batch and for different batches and results are shown in Table 1.

Stability

The results showed that RSM was stable at the all tested stability conditions. RSM was stable in rat plasma and brain for about 1 month when stored in the frozen state (−70°C) and these results are presented in Table 2.

Dilution effects

The dilution integrity was confirmed for QC samples that exceeded the upper limit of the standard calibration curve. The results have shown that the precision and accuracy for six replicates of diluted samples were within the acceptance range (data not shown).

Pharmacokinetics study

The method has been successfully employed for the quantitative estimation of RSM in rat plasma and brain samples following *intravenous* administration of RSM in rats. Area under curves (AUC) of RSM in the studied matrices are shown in Figure 4. Results showed that RSM is completely absorbed, rapidly distributed and quickly eliminated from the systemic circulation.

DISCUSSION

The present method was more sensitive with a LLOQ of

Table 1: Intra- and inter-day precision data of the RSM in rat plasma and brain

Quality control	Run	Measured concentration of RSM in plasma (ng/mL)				Measured concentration of RSM in brain homogenate (ng/mL)			
		Mean	SD	RSD (%)	Accu (%)*	Mean	SD	RSD (%)	Accu (%)*
Intra-day variation (six replicates at each concentration)									
LLOQ	1	8.21	1.35	16.44	82.10	9.13	1.36	14.90	91.30
	2	9.35	1.75	18.72	93.50	8.67	1.73	19.95	86.70
	3	8.61	1.70	19.74	86.10	9.05	1.76	19.45	90.50
	4	9.18	1.58	17.21	91.80	8.78	1.62	18.45	87.80
LQC	1	30.47	4.35	14.28	101.57	29.82	2.85	9.56	99.40
	2	32.58	4.60	14.12	108.60	27.72	3.25	11.72	92.40
	3	28.47	3.52	12.36	94.90	30.28	3.51	11.59	100.93
	4	29.96	4.02	13.42	99.87	28.25	3.50	12.39	94.17
MQC	1	149.85	14.58	9.73	99.90	142.27	8.43	5.93	94.85
	2	151.17	13.32	8.81	100.78	149.33	12.19	8.16	99.55
	3	148.51	11.29	7.60	99.01	147.72	17.43	11.80	98.48
	4	151.59	14.21	9.37	101.36	144.21	14.33	9.94	96.14
HQC	1	725.86	80.47	11.09	90.73	691.72	84.52	12.22	86.47
	2	760.44	29.27	9.11	95.06	694.29	75.80	10.92	86.79
	3	764.61	93.17	12.19	95.58	789.21	104.21	13.20	98.65
	4	825.86	100.12	12.12	103.23	747.21	78.74	10.54	93.40
Inter-day variation (24 replicates at each concentration)									
LLOQ		8.84	0.52	5.94	88.38	8.91	0.22	2.45	89.08
LQC		30.37	1.70	5.60	101.23	29.02	1.23	4.23	96.73
MQC		150.28	1.39	0.93	100.19	145.88	3.22	2.21	97.26
HQC		769.19	41.58	5.41	96.15	730.61	46.69	6.39	91.33

RSD: Relative standard deviation (SD × 100/mean). *Accu (accuracy) = (mean assayed concentration – nominal concentration)/(nominal concentration) ×100

Table 2: Stability data of RSM in rat plasma and brain

Quality control	Stability	Measured concentration of RSM in plasma (ng/mL)				Measured concentration of RSM in brain homogenate (ng/mL)			
		Mean	SD	Accu (%)*	% CV	Mean	SD	Accu (%)*	% CV
LQC	In injector (12 h)	27.85	3.43	92.83	14.51	30.09	3.43	100.31	11.40
	Bench top (6 h)	32.32	4.32	107.73	13.36	27.88	4.15	92.93	14.89
	Third freeze/thaw	31.84	4.62	95.54	8.42	30.76	2.92	102.55	9.49
	30 day at -70°C	27.26	3.75	90.87	13.76	27.72	3.53	92.40	12.73
HQC	In injector (12 h)	909.46	100.43	113.68	11.04	772.82	97.54	96.60	12.62
	Bench top (6 h)	738.64	78.32	92.33	10.60	783.54	82.26	97.94	10.50
	Third Freeze/thaw	784.39	97.34	98.05	12.41	694.29	76.43	86.79	11.01
	30 day at -70°C	764.32	64.39	95.54	8.42	691.72	74.91	86.47	10.83

% CV (precision); coefficient of variation. *Accu (accuracy); (mean assayed concentration – nominal concentration)/(nominal concentration) ×100

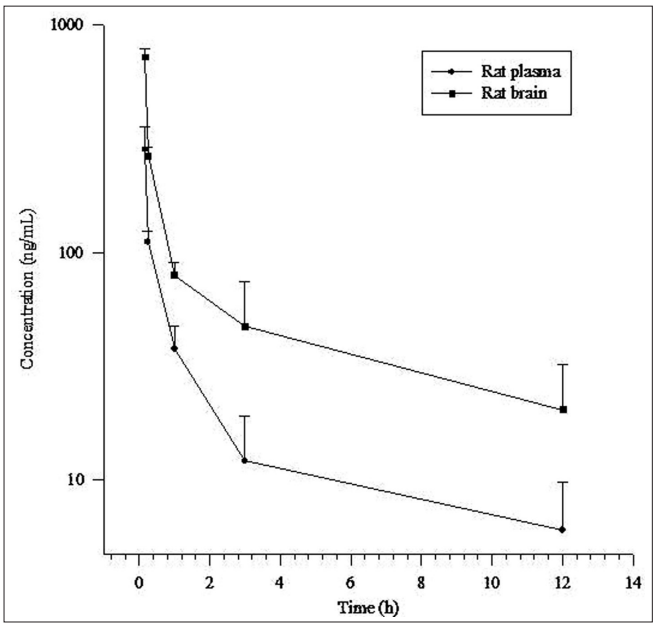

Figure 4: Semi-log plot of time vs. plasma or brain concentrations profile of RSM following intravenous (1 mg/kg) administration of RSM to male Wistar rats (*n*=3 at each time point)

10 ng/mL and employed a simple LLE procedure used for the extraction of RSM in plasma unlike many methods which reported the use of solid phase extraction methods, which would increase the cost of analysis.[7,8] Extraction of RSM in rat brain was carried out by the protein precipitation method, which is the simple, economical, and less time-consuming procedure. The current method used a simple isocratic binary solvent system, commonly used column, and commercially available IS. The developed method requires less sample processing volume (100 µL) thereby allowing us to collect—eight to nine samples from the small rodent-like rat for the preclinical pharmacokinetics study.

CONCLUSION

A highly sensitive, selective, and accurate isocratic HPLC with a fluorescence detection method was developed and validated to quantify RSM in rat plasma and brain. The method has been successfully used in the pharmacokinetic study of RSM in rats. The developed method has been currently employed in estimation of RSM in both matrices, where RSM delivered through novel drug delivery systems to target the brain and the method has been applied for the numerous rat plasma and brain samples.

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