



QbD-driven HPLC method for the quantification of rivastigmine in rat plasma and brain for pharmacokinetics study

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ABSTRACT

The goal of the study was to develop a high throughput quantification method with precision and accuracy in plasma and brain matrices for Rivastigmine (RST). For the method development, a risk assessment was performed to first select the critical variables/factors and hence select the critical method parameters which were introduced in Design of Experiment to obtain the optimized analytical method. Protein precipitation and PLC High Performance Liquid Chromatography (HPLC)/ Ultraviolet (UV) were selected as the techniques for the drug extraction and estimation respectively. Further on validation, the method was selective and showed good linearity with recovery ranging between 96.73%–109.81% in plasma and 93.42%–108.39% in the brain. The method validation showed acceptable precision and accuracy and was found to be stable in both the matrices. The method sensitivity in both matrices was demonstrated by the lowest concentration detection at 75 ng.ml⁻¹. Up until now, no HPLC-UV method is available with protein precipitation drug extraction for bioanalysis of RST in plasma and brain matrices. Using this validated method, nasal pharmacokinetics was carried out in Sprague Dawley rats to confirm the method applicability for RST quantification in biological matrices.

INTRODUCTION

In the recent years, there has been an increased surge towards the quality-by-design (QbD) approach in the pharmaceutical industry as a systematic way to achieve quality standards during the product life cycle. QbD uses a quality risk management approach to emphasize the product and process understanding. This approach is in contrast to the classic trial and error methodology, which is purely based upon an expert's opinion wherein only one factor/

variable was modified while keeping the others constant (one factor/variable at a time approach) (Sahu *et al.*, 2018). The QbD principles applied to analytical procedures, also known as Analytical QbD (AQbD), aim to produce data with acceptable quality. Application of Design of experiments (DOE) methodology and risk analysis can help to study the variables affecting a method's uncertainty, to avoid forthcoming failures and hence strategically build a quality method. Box Behnken Design (BBD), Central Composite Design (CCD) and a 3-level complete factorial design are few examples of designs applied to optimize the factors (Carini *et al.*, 2013). The advantage of BBD over CCD and other designs is that It does not operate at extreme levels (that is maximum/lowest level of all parameters at the same time) where there is a potential that practical issues outweigh statistical issues (Mullick *et al.*, 2021; Mutalik *et al.*, 2021).

Rivastigmine (RST) is an US Food and Drug Administration (FDA)-approved acetyl- and butyryl-cholinesterase

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inhibitor used in treating mild to moderate Alzheimer's disease (AD) by increasing synaptic acetylcholine (ACh) levels (Eskander *et al.*, 2005; Lane *et al.*, 2015). AD is a progressive neurological dysfunction caused due to neuronal deterioration which is characterized by dementia has been primarily affecting wide strata of the population worldwide. The early AD pathogenesis begins with reduced production of ACh neurotransmitter, presynaptic loss of limbic and neocortical cholinergic innervations which further progresses leading to the severity of the cognitive decline. This led to proposing the cholinergic hypothesis and invention of cholinesterase inhibitor drugs (Hampel *et al.*, 2018, 2019).

Several analytical techniques were reported for the determination of RST in biological fluids. Spectrophotometric methods were developed for RST estimation in biological matrices using HPLC-UV and HPLC-fluorimetry (Amini *et al.*, 2010; Arumugam *et al.*, 2011b; Karthik *et al.*, 2008); however these HPLC methods were based on liquid-liquid extraction, which were tedious and expensive for routine bio-analysis. Several liquid chromatographic methods using HPLC - Mass spectrometry (MS) were reported for RST analysis in human and rat plasma and urine, which were sensitive and selective with very low quantification levels (Arumugam *et al.*, 2011a; Bhatt *et al.*, 2007; Enz *et al.*, 2004; Frankfort *et al.*, 2006; Pommier and Frigola, 2003). However, such techniques need sophisticated equipment, trained personnel and are not easily affordable in all laboratories. Additionally, most of these MS detections of RST involved liquid-liquid extraction techniques. MS detection of RST in human plasma was reported using the solid phase extraction technique, which is expensive and involves time-consuming processing (Bhatt *et al.*, 2007). Another MS detection of RST in human plasma extracted using protein precipitation used gradient technique for separation of analytes, which requires dwell time to adjust the column conditions and post-gradient column re-equilibration, which results in longer run time (Frankfort *et al.*, 2006). Molecularly imprinted polymers-voltametric sensors have been fabricated for RST measurement in biological samples within a range of 2–1,000 $\mu\text{mol/l}$ (Arvand *et al.*, 2013); Novel miniaturized polyvinyl chloride membrane sensors in all-solid-state graphite and platinum wire supports to aid in electrochemical evaluation of RST in plasma and brain samples (El-Kosasy *et al.*, 2005). However, these methods suffer either from low sensitivity or low drug recovery due to extraction procedures.

In the current work, we assessed the validity of the analytical procedure by applying the AQbD approach in the method development for the quantification of RST in rat plasma and brain matrices. The method optimization was done by applying Box Behnken DOE and validated by US FDA guidelines. The validated method application was studied by conducting nasal pharmacokinetics (PKs) of RST in healthy male Sprague Dawley rats.

MATERIALS AND METHODS

Experimental

Instrumentation

The HPLC system (LC-2010CHT, Serial No. C21255111757—LP, Shimadzu Corp., Kyoto, Japan) used was

equipped with quaternary low-pressure gradient pumps to deliver four solvents, SPD-M-20A photodiode array- UV detector, degasser unit, autoinjector, and column oven. Hyperclone 5 μm Base Deactivated Silica (BDS) C8 130 \AA Liquid Chromatography (LC) column (Dimensions: 250 \times 4.6 mm) was used as the stationary phase. The chromatographic data were acquired and integrated using LC solutions, 5.57 version software. Measurement and pH adjustments of buffer solution were done with help of pH meter (Model Eutech pH 510, Thermofisher Scientific, Bengaluru, India) using a glass electrode (Van London Co., Houston, TX). The aqueous buffer was filtered through 0.22 μ filter membrane using glass vacuum filtration assembly (Millipore, Billerica, MA) and degassed using a bath sonicator (Equitron; Medica Instruments Mfg. Co., Mumbai, India). The complete sample preparations during analysis were done using well calibrated micropipettes (Eppendorf India Ltd, Chennai, India). Vortex mixer, high-speed cooling centrifuge (Model 6000, Kubota Laboratory Centrifuges) were used for sample preparation and -20°C and -80°C freezers were used for storage.

Materials and reagents

RST (base form) and Risperidone (RIS) (used as an internal standard) were provided as gift samples from Zydus Cadila, Ahmedabad, India. Potassium dihydrogen orthophosphate (KH_2PO_4) (purity $\geq 99\%$) was obtained from Sigma Aldrich (Bangalore, India). LC grade of acetonitrile (ACN) was obtained from Merck Ltd (Mumbai, India) and methanol (MeOH) was purchased from Finar Ltd (Ahmedabad, India). Milli-Q water was obtained from a Millipore Direct-Q[®] 3 water purification system (Millipore Corp.) present in our laboratory.

Design of experiment

Risk assessment

The quality risk assessment approach was adopted before analytical method optimization to identify, analyze, and predict the potential risks of various chromatographic parameters on the method efficiency. With prior knowledge, a risk estimation matrix was set, as shown in Table 1, thus reducing the number of method parameters to be studied in the experimental phase. Risk assessment further helped in performing DOE using the high-risk method parameters [Critical Method Parameters (CMP)] and studying their impact on the Critical Analytical Attributes (CAA) (Bandopadhyay *et al.*, 2020).

Factor optimization

In this study, BBD, with three CMP (factors) was used to accomplish optimization of the HPLC method. The three factors viz. organic phase (A), flow rate (B), and wavelength (C) are considered, each at three levels including low (-1), medium (0), and high levels ($+1$). BBD was selected over CCD as it required fewer runs to study three factors. BBD was employed to evaluate main effects, interactions between factors, and quadratic terms of independent factors on dependent responses constructed using Design Expert software (Version 9.0.1, Stat-Ease Inc., Minneapolis, MN) through the polynomial equation and further to optimize the factors statistically. The design matrix included 17 experimental runs of which $n = 5$ were replicates of center point to estimate any experimental uncertainty. Table 2 depicts the 17

Table 1. Risk assessment matrix for RST HPLC method.

CAAs	Method parameters					
	Organic phase	Aqueous buffer pH	Flow rate, ml/minute	Injection volume, μ l	Wavelength, nm	Column oven temperature, $^{\circ}$ C
Peak area	1 ^a	3 ^c	1 ^a	2 ^b	1 ^a	2 ^b
Retention time	1 ^a	2 ^b	1 ^a	3 ^c	2 ^b	2 ^b
Peak tailing factor	1 ^a	2 ^b	2 ^b	2 ^b	2 ^b	3 ^c
Theoretical plate	2 ^b	2 ^b	1 ^a	3 ^c	2 ^b	3 ^c

¹ High risk.² Medium risk.³ Low risk.**Table 2.** Experimental data for the BBD.

Run	CMPs			CAAs			
	Factor A	Factor B	Factor C	R_1	R_2	R_3	R_4
	Organic phase, %	Flow rate, ml/minute	Wavelength, nm	Peak area	Retention time, minute	Peak tailing factor	Theoretical plates
1	+1 ^c	-1 ^a	0 ^b	23,728	6.144	1.363	6,022
2	0 ^b	-1 ^a	-1 ^a	45,245	7.211	1.402	6,130
3	0 ^b	0 ^b	0 ^b	19,928	5.539	1.459	5,497
4	0 ^b	0 ^b	0 ^b	19,572	5.535	1.409	5,573
5	0 ^b	+1 ^c	+1 ^c	7,552	4.533	1.468	4,876
6	+1 ^c	0 ^b	+1 ^c	8,269	4.76	1.366	5,402
7	0 ^b	0 ^b	0 ^b	19,692	5.557	1.405	5,560
8	-1 ^a	+1 ^c	0 ^b	15,991	5.479	1.275	5,127
9	+1 ^c	0 ^b	-1 ^a	38,174	4.844	1.411	5,431
10	+1 ^c	+1 ^c	0 ^b	16,478	4.031	1.401	4,767
11	0 ^b	0 ^b	0 ^b	19,509	5.551	1.402	5,518
12	0 ^b	0 ^b	0 ^b	19,865	5.572	1.435	5,684
13	-1 ^a	0 ^b	-1 ^a	37,256	6.732	1.288	5,550
14	-1 ^a	0 ^b	+1 ^c	7,952	6.737	1.253	5,647
15	0 ^b	-1 ^a	+1 ^c	10,400	7.069	1.368	6,146
16	0 ^b	+1 ^c	-1 ^a	32,803	4.584	1.462	5,108
17	-1 ^a	-1 ^a	0 ^b	22,422	8.536	1.211	6,001

-1, 0, +1 are the codes used respectively for low, medium and high levels.

combinations of factors in a randomized order as per BBD and the measure responses. Table 3 depicts the factors selected and their values corresponding to each level.

Statistical analysis of data

The model significance was evaluated statistically using the *F* test and lack of fit test (at $p \leq 0.05$). The significance of regression coefficients was determined by the *F* test. The best fit for the model equation was determined by R^2 as well as adjusted R^2 values. The factor interactions coefficients which were statistically insignificant were disregarded as model terms (Dalvi *et al.*, 2018).

Diagnostic plots

Diagnostic plots including predicted versus actual and residual plots evaluated the model's predictive ability. The desirability function aided in the evaluation of the best condition

for all the responses. The combination of factors that gave the high desirability value was considered as the optimum chromatographic method parameter.

Procedures

Plasma and brain tissue sample processing

All the animal experimental protocols were approved by Institutional Animal Ethics Committee, Kasturba Medical College, Manipal (Approval No. IAEC/KMC/36/2019). Capillary tubes were used for blood withdrawal using the retro-orbital puncture technique from Sprague Dawley rats (250–300 g; male). The blood was taken in centrifuge tubes which contained 10% disodium Ethylenediamine tetraacetic acid (EDTA) solution as an anticoagulant (i.e., 20 μ l anticoagulant required for 200 μ l blood). The collected blood was placed in a cooling centrifuge at 4 $^{\circ}$ C for 10 minutes at 10,000 rpm

Table 3. CMPs and their levels used in BBD.

Code of CMP	CMP studied	Level used (coded)		
		Low (-1)	Medium (0)	High (+1)
A	Organic phase (%)	25	27.5	30
B	Flow rate (ml/minute)	0.8	1	1.2
C	Wavelength (nm)	215	220	225

after which plasma was separated carefully and stored at -20°C . For a collection of the brain, rats were anesthetized and exsanguinated by first incising the abdominal artery and then infusing 0.9% w/v NaCl solution (saline) into the heart, to completely withdraw blood from brain tissue. The whole brain was collected carefully and stored at -20°C till further use.

Preparation of stock solutions

The stock solutions of 1,000 $\mu\text{g/ml}$ RST and internal standard RIS were prepared in MeOH separately. RST aqueous working stock solution was made by diluting accurate quantities of stock solution.

Preparation of calibration control (CC) and quality control (QC) samples

Aqueous dilutions of RST were made by taking the required quantities of working stock and diluting with diluent to get the required concentration of CC standards and QC standards viz. Low-quality control (LQC), Middle-quality control (MQC), and High-quality control (HQC).

Sample extraction method

The protein precipitation technique was used to extract RST from plasma and brain homogenate containing RIS as an internal standard using chilled MeOH. The brain homogenate was prepared with 1:1.5 ratio of brain tissue to phosphate buffer saline (PBS) (pH 7.4) under cold conditions to get a less viscous and fine tissue homogenate. Extraction procedures for blank plasma/brain samples and calibration standards spiked into plasma/brain samples were identical. The plasma/brain calibration standards were prepared as follows: The rat plasma/brain homogenate (190 μl) was placed in pre-labeled microcentrifuge tubes, and 10 μl of respective spiking stock solutions containing RST was added to the respective tube and vortexed for 10 seconds. This was followed by the addition of the precipitating solvent which consisted of internal standard (RIS 10 $\mu\text{g/ml}$) prepared in chilled MeOH. The ratio of the matrix containing RST to precipitating solvent was 1:3. Further, the plasma/brain sample containing only precipitating solvent (chilled MeOH with RIS) was vortexed for 2 minutes and used as a Standard 0. The blank plasma/brain sample was prepared by addition of only chilled MeOH (without RIS). All the above samples were centrifuged for 10 minutes at a speed of 10,000 RPM using cooling centrifuge. After centrifugation, the supernatant was transferred to a clean insert vial appropriately labelled and HPLC analysis was performed (Hegde *et al.*, 2021).

Bioanalytical method validation

The FDA guidance to industry (2001) criteria for bio-analytical technique validation was used to validate the method for the measurement of RST.

System suitability

Six injections of aqueous solution corresponding to the MQC concentration of RST were combined with the RIS working stock solution to determine the system's compatibility. The % CV of the area ratio and the retention time of RST and RIS were computed.

Selectivity

To evaluate any interference from constituents in the rat plasma/brain (matrix effect), screening was done using six plasma/brain matrix lots from different rats. For the specificity, the matrix lots were extracted using precipitating solvent, with and without an internal standard. Any interference from the blank matrix was evaluated by comparing it to the LQC samples (75 ng/ml).

Linearity and sensitivity

The linearity of the method was evaluated using calibration standard range of 75–3,000 ng/ml for plasma and brain matrices. LQC was determined by injecting six samples of the lowest acceptable concentration of the linearity range and % CV and % nominal concentration were calculated.

Accuracy and precision

The RST precision and accuracy were assessed by processing and evaluating quality control standards in both plasma and brain samples, each with six repetitions (LQC, MQC, HQC). Inter-day and intraday batches were used to test precision.

Recovery

The average peak areas of three plasma/brain extracted LQC (75 ng/ml), MQC (750 ng/ml), and HQC (2,500 ng/ml) samples were compared to the aqueous samples of the same concentrations of LQC, MQC, and HQC by replacing plasma/brain matrices with water to assess RST recovery.

Stability studies

RST stock solution stability was determined by storing it at room temperature for 6 hours, to account for the sample preparation time, i.e., the entire time taken for sample work-up and evaluation (Kadian *et al.*, 2016). The stability of stock solutions was assessed by comparing freshly processed samples to stable samples at the MQC level. The stability of RST in rat plasma and brain was determined utilizing two different concentration levels (MQC and HQC). The validation included bench top, post-preparative, and freeze and thawing analytes. All stability samples were compared to freshly produced RST samples at the same concentration level. Stability was calculated as the mean percentage change in the stability samples.

PKs study

Male Sprague Dawley rats were used in the study with body weight ranging between 250 and 300 g ($n = 4$). All study animals had been placed in cages and made accustomed to institutional animal house maintained at temperature of $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$; relative humidity of $55\% \pm 10\%$; 12 hours light/dark cycle. Water and food were given as and when required. The dose of RST intended for nasal PK study was solubilized just before the commencement of the study. The dosing was done using a 20 μl micropipette in rats. RST at a dose of 1.5 mg/kg, at a dose volume of 50 μl ; 25 μl was administered through each nostril. The technique used for blood collection was through retro-orbital vein puncture and the blood was collected in pre-labelled, disodium EDTA anticoagulant containing centrifuge tubes. Blood (approximately 200 μl) was withdrawn at 0, 5, 15, 30, 45, 60, 120, and 240 minutes following nasal administration of RST. For brain PKs, after nasal dosing, brain samples were collected at different time points, which was decided based on plasma concentration versus time profile of RST. At 15, 30, 45, 60, 120, and 240 minutes after dosing and blood sampling, four animals were sacrificed by cervical necrosis method at each time point and the brain was removed, washed with saline, weighed, and stored in -20°C until analysis. Further, the developed and validated method was used to analyse all the PK samples. Plasma concentration and brain concentration versus time profile were plotted to analyze the samples. Different compartment models were used to determine the best fit and to finalize the compartment model which was observed with nasal PKs data of RST. PK parameters like $t_{1/2}$, AUC, t_{max} , C_{max} were computed with help of non-compartmental analysis in PK Solutions software.

RESULTS AND DISCUSSION

Design of experiment

Risk assessment

The risk assessment study included the chromatographic method parameters such as organic phase, aqueous buffer pH, flow rate, wavelength, injection volume, column oven temperature; which would primarily be responsible for any critical variability in the analytical attributes viz. drug peak area, retention time, peak tailing and theoretical plates. The method parameters/factors which showed high-risk impact on CAA included organic phase, wavelength, and flow rate, which were subjected to evaluation by DOE, while CAA included peak area, retention time, peak tailing factor, and theoretical plates were selected as responses.

Factor optimization

Table 3 delineates the independent factors along with the level used. In Table 2, the data for the responses were analyzed and fitted in several mathematical models.

The mathematical models were expressed as follows:

The quadratic model followed the equation:

$$R_i = b_0 + b_1A + b_2B + b_3C + b_4AB + b_5AC + b_6BC + b_7A^2 + b_8B^2 + b_9C^2$$

and, Linear model followed the equation:

$$R_i = b_0 + b_1A + b_2B + b_3C$$

where b_0 is the intercept, R_i is the response, b_i 's ($i = 1, 2, 3 \dots n$) are coefficients of individual linear, quadratic, and cubic effects along with their interactions. Factors A , B , and C are the CMP in the design.

Statistical data analysis

Statistical evaluation for all the responses using BBD was tabulated in Table 4. For every response, the model showing a high F value was selected and validated with help of analysis of variance. Quadratic model was found to be appropriate and significant for the following responses: peak area (R_1) with a F value of 2,589.01 ($p < 0.0001$); retention time (R_2) with a F value of 6,488.79 ($p < 0.0001$) and peak tailing factor (R_3) with F value of 20.42 ($p < 0.0003$). However, it was observed that for theoretical plate number (R_4), linear model was found to be appropriate and significant with a F value of 95.33 ($p < 0.0001$). Adjusted R^2 (R^2_{adj}) value represents the goodness of fit of the model. High values define a better correlation between software predicted values and actual values. The R^2_{adj} value for the responses R_1 , R_2 , R_3 and R_4 were 0.9993, 0.9997, 0.9161, and 0.9465, respectively. The p value determines the significance of each coefficient and the terms with p value less than 0.05 were added to the model equation. The model equation for responses in terms of coded factors is as follows:

Drug peak area

$$(R_1) = +19,713.20 + 378.50^*A - 3,621.38^*B - 14,913.13^*C + 2,398.50^*BC - 572.85^*A^2 + 514.40^*B^2 + 3,772.40^*C^2$$

Retention time

$$(R_2) = +5.55 - 0.96^*A - 1.29^*B - 0.034^*C + 0.24^*AB + 0.023^*BC + 0.21^*A^2 + 0.29^*B^2$$

Peak tailing factor

$$(R_3) = +1.42 + 0.064^*A + 0.033^*B - 0.10^*A^2$$

Theoretical plate number

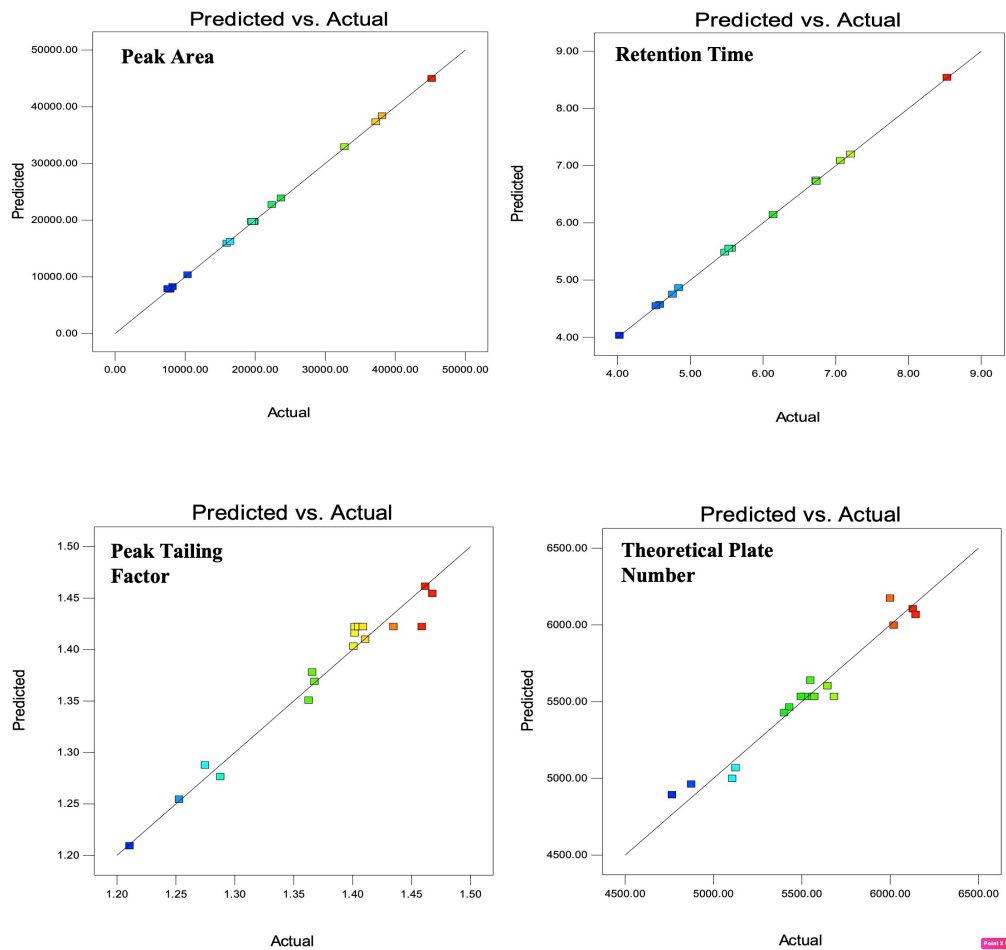
$$(R_4) = +5531.71 - 87.87^*A - 552.62^*B - 18.50^*C$$

Diagnostic plots

Diagnostic plots such as predicted versus actual plots represent the model's appropriateness by depicting the correlation between software predicted values and actual values. As seen in Figure 1, the data points in the plot for all the responses lie close to straight line, thus indicating a good agreement between software predicted values and actual values. Figures 2 and 3 depict the 2D contour plots and perturbation plots respectively which are a graphical representation of the regression equation. The individual effects and interaction effects of the independent factors on the responses can be visualized easily with these plots. In Figure 3, as shown in perturbation plots, the following responses were observed for the independent factors. Peak area significantly decreased with an increase in the wavelength (Factor C) from -1 (215 nm) to $+1$ (225 nm), indicating a negative effect; whereas no significant changes in Retention time, tailing factor and theoretical plate number were observed with increase in wavelength. However, increase in organic phase ratio (Factor A) from -1 (25%) to $+1$ (30%) and increase in flow rate (Factor B) from -1 (0.8 ml/minute) to $+1$ (1.2 ml/minute) showed a substantial negative effect on Retention time. A significant negative effect was also seen in the theoretical plate number where the plate number decreased by increasing the flow rate from -1 (0.8

Table 4. Statistical analysis for BBD.

Response model	Factor	Factor coefficient	<i>p</i> value	<i>R</i> ² adj	Lack of fit	Model <i>F</i> value
<i>R</i> ₁ : Peak area (Quadratic model)	Intercept	19,713.20	<0.0001	0.9993	<i>F</i> value = 4.68 <i>p</i> value = 0.0850	2,589.01 (<i>p</i> < 0.0001)
	<i>A</i> -Organic phase	378.50	0.0079			
	<i>B</i> -Flow rate	-3,621.38	<0.0001			
	<i>C</i> -Wavelength	-14,913.13	<0.0001			
	BC	2,398.50	<0.0001			
	<i>A</i> ²	-572.85	0.0049			
	<i>B</i> ²	514.40	0.0084			
<i>R</i> ₂ : Retention time (Quadratic model)	Intercept	5.55	<0.0001	0.9997	<i>F</i> value = 2.60 <i>p</i> value = 0.1894	6,488.79 (<i>p</i> < 0.0001)
	<i>A</i> -Organic phase	-0.96	<0.0001			
	<i>B</i> -Flow rate	-1.29	<0.0001			
	<i>C</i> -Wavelength	-0.034	0.0016			
	AB	0.24	<0.0001			
	BC	0.023	0.0498			
	<i>A</i> ²	0.21	<0.0001			
<i>R</i> ₃ : Peak tailing factor (Quadratic model)	Intercept	1.42	0.0003	0.9161	<i>F</i> value = 0.54 <i>p</i> value = 0.6774	20.42 (<i>p</i> < 0.0003)
	<i>A</i> -Organic phase	0.064	<0.0001			
	<i>B</i> -Flow rate	0.033	0.0039			
	<i>A</i> ²	-0.10	<0.0001			
<i>R</i> ₄ : Theoretical plates (linear model)	Intercept	5,531.71	<0.0001	0.9465	<i>F</i> value = 1.96 <i>p</i> value = 0.2698	95.33 (<i>p</i> < 0.0001)
	<i>A</i> -Organic phase	-87.87	0.0199			
	<i>B</i> -Flow rate	-552.62	<0.0001			

**Figure 1.** Predicted versus actual plots indicating the model's appropriateness for the responses *R*₁, *R*₂, *R*₃, and *R*₄.

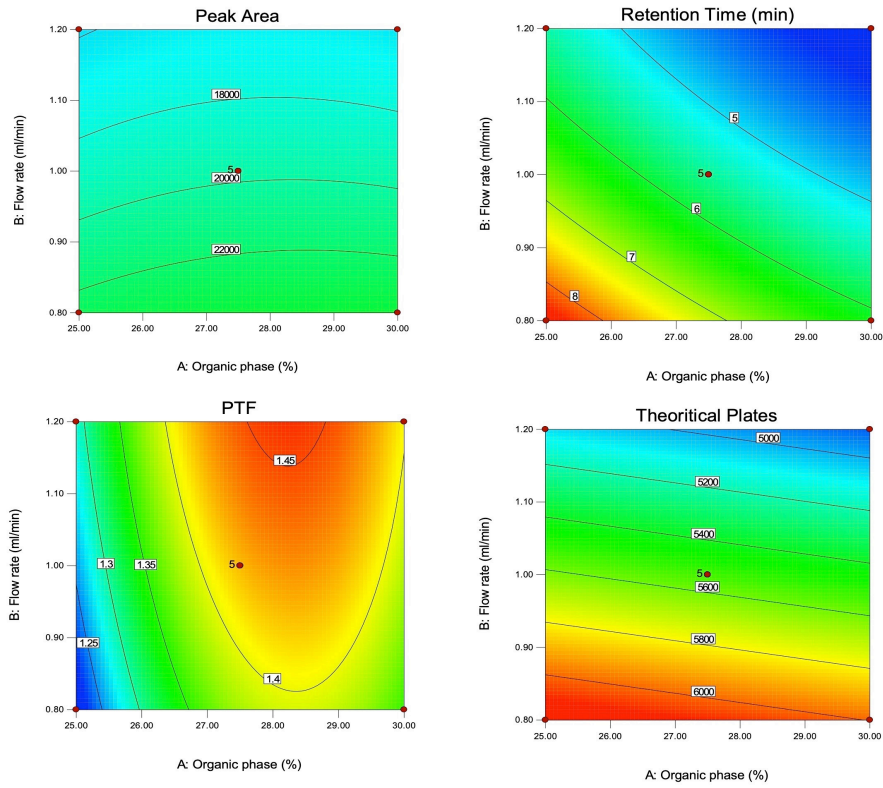


Figure 2. 2D contour plots depicting effect of organic phase (Factors A), flow rate (Factor B), wavelength (Factor C) for the responses R_1 , R_2 , R_3 and R_4 .

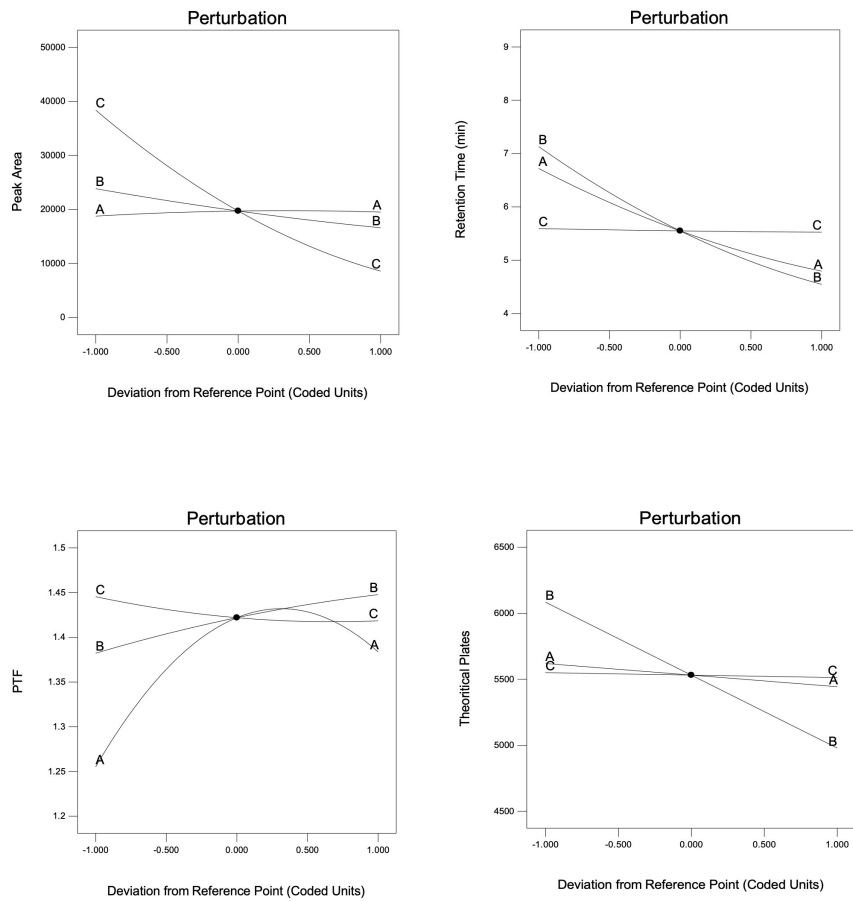


Figure 3. Perturbation plots depicting effect of organic phase (Factors A), flow rate (Factor B), wavelength (Factor C) for the responses R_1 , R_2 , R_3 , and R_4 .

ml/minute) to +1 (1.2 ml/minute). A slight increase in peak tailing factor was observed as the flow rate increased whereas a mixed response was observed with the effect of the organic phase on peak tailing factor. There was an initial significant increase in peak tailing factor as organic phase was increased, after reaching the mid value, there was a slow decrease in the peak tailing factor. As seen in perturbation plots, the effect of flow rate and organic phase was observed in 2 D contour plots in Figure 2. The desirability values usually range between 0 and 1 for optimized solutions, indicating the strength of the method. If values are closer to 0, then the predicted method is deemed to be weak and if values are closer to 1, then the method is deemed to be strong. An optimized solution, indicated by the desirability value of 0.996 was observed with the following chromatographic conditions: Mobile phase consisting of pH 4.5 phosphate buffer and ACN as organic phase in the ratio of 72.5 and 27.5, flow rate of 0.8 ml/minute, column temperature of 40°C and detection at 215 nm. For the check point analysis, the predicted values of the factors and responses obtained from the software are compared with the actual experimental values ($n = 3$) and the % error was tabulated in Table 5, which was found to be within $\pm 15\%$.

Sample extraction technique

Previous reports suggest the use of protein precipitation technique for RST extraction from brain matrices using ACM:MeOH mixture (Mullangi *et al.*, 2011), whereas there are very few reports suggesting the RST extraction from plasma using protein precipitation technique (Bhatt *et al.*, 2007). Further, few studies have used HPLC with UV detection for RST estimation in biological matrices, which is commonly available and economical (Amini *et al.*, 2010). In the above-mentioned published article (Mullangi *et al.*, 2011), the protein precipitation technique was used for the extraction of the drug in the brain matrix; whereas liquid-liquid extraction technique was used to extract the drug from the plasma. In the present study, the protein precipitation technique was for the drug extraction technique from the plasma and brain matrices. Additionally, with the protein precipitation technique, the recovery in both matrices was good and both drugs were quantified using the HPLC-UV method. Although HPLC with fluorescence detection is more sensitive than HPLC-UV technique, the objective of this work was to optimize the chromatographic conditions using HPLC-UV, which is more commonly available and economical, to detect the drug in the plasma and brain matrices using a simple extraction technique with good recover

In the present work, for the estimation of RST in brain, brain tissue was homogenized using chilled PBS (pH 7.4). The RST

samples from the plasma and brain were extracted using the protein precipitation method, which is a faster and less expensive approach in comparison to liquid-liquid extraction and solid-phase extraction. For RST extraction, solvents such as ice cold ACN and MeOH were used individually and in combinations to precipitate the protein. Since ACN was present in mobile phase, initial trials were taken using ACN as precipitating agent alone and in combination with MeOH, but the presence of ACN resulted in broad drug and internal standard peaks along with matrix interference. MeOH gave sharp, well-defined peaks for RST and RIS (internal standard). RIS was desired as an internal standard as it showed good recovery after being extracted from the biology matrices and also gave a good peak shape at a desirable retention time and did not interfere with the retention time of RST. RIS could also be sufficiently quantified at the wavelength of 215 nm. To efficiently recover RST from the biological matrices, ice cold MeOH was used at 1:3 ratio, 1 part of plasma/brain homogenate was added with 3 parts of ice cold MeOH containing RIS (internal standard).

The chromatographic conditions for the Bioanalysis of RST were as follows: Stationary phase: Hyperclone 5 μm BDS C8 130 Å LC column (Dimensions: 250 \times 4.6 mm); Mobile phase: pH 4.5 phosphate buffer and ACN as organic phase in the ratio of 72.5 and 27.5; Column temperature: 40°C; Flow rate: 0.8 ml/minute; Injection volume: 40 μl ; UV wavelength: 215 nm; Internal standard: RIS; Precipitating solvent: Chilled MeOH.

Bioanalytical method validation

System suitability

The developed techniques satisfied the system appropriateness requirements by having a % CV of 2.4% for the area ratio and 0.21% for the analyte retention time.

Selectivity

The protein precipitation method was demonstrated to be effective and reliable for quantifying RST in plasma and brain matrices. In both matrices, there were no interfering peaks during the retention periods of RST or RIS. Figures 4 and 5 depict chromatogram overlays of diluent, blank matrix, matrix spiked with only internal standard, LQC, and HQC samples, and PK study samples for both plasma and brain. The peak shape was good under the optimized chromatographic conditions at retention times of 7.2 ± 0.09 and 13.2 ± 0.1 minutes for RST and RIS (internal standard), respectively, in plasma and 7.15 ± 0.1 and 13.17 ± 0.1 minutes for RST and RIS, respectively in the brain.

Table 5. Check point analysis of optimized batch.

Values/error	Organic phase, %	Flow rate, ml/minute	Wavelength, nm	Peak area	Retention time, minute	Peak Tailing factor (PTF)	Theoretical plate
Predicted value	27.5	0.8	215	44,933	7.19	1.42	6,102
Experimental value ^a	27.5	0.8	215	45,111.66 \pm 116.28	7.19 \pm 0.01	1.39 \pm 0.02	6,012.66 \pm 54.26
% Error				0.397	0.08	-1.41	-1.46

^aAverage of triplicate values.

% Error = ((Experimental value - Predicted value)/Predicted Value*100).

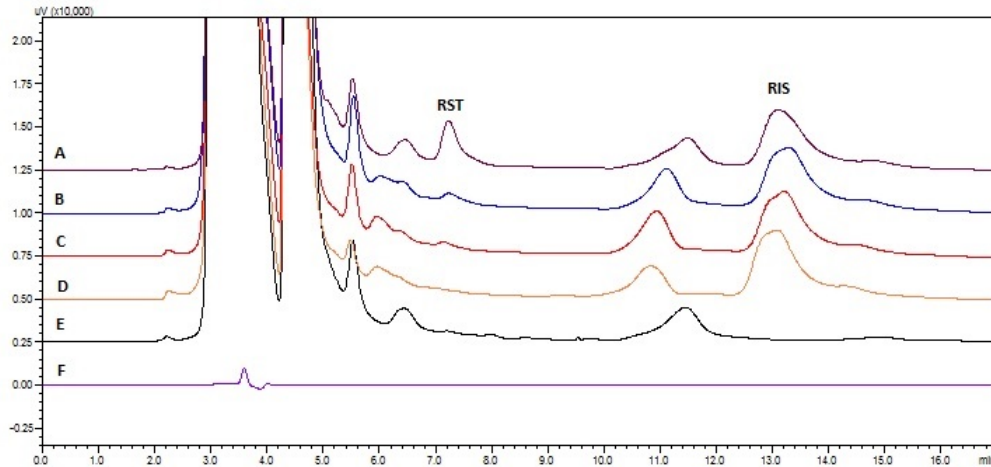


Figure 4. Chromatograms of diluent and plasma samples eluted (A) plasma spiked with HQC; (B) PK sample; (C) plasma spiked with LQC; (D) plasma spiked with only internal standard; (E) blank plasma; (F) diluent.

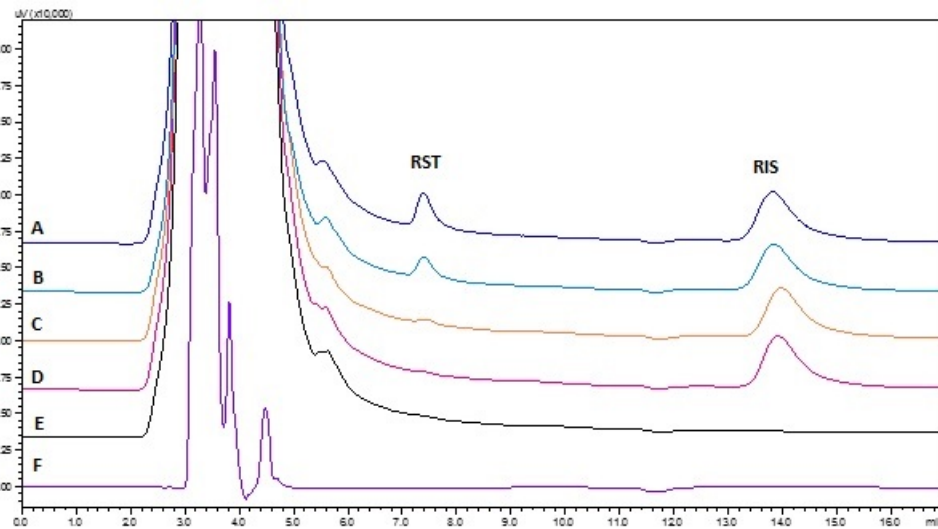


Figure 5. Chromatograms of diluent and brain samples eluted: (A) brain spiked with HQC; (B) PK sample; (C) brain spiked with LQC; (D) brain spiked with only internal standard; (E) blank brain; (F) diluent.

Linearity

The standard calibration curves were created using the peak area ratios of RST/RIS as the vertical axis and the RST concentration (ng/ml) as the horizontal axis. The slope, intercept, and R^2 values were calculated using linear regression analysis for plasma and brain. In plasma, the slope was 0.00007, intercept was 0.0011 and R^2 was 0.9971. In the brain, the slope was 0.00005, the intercept was 0.0025 and R^2 was 0.9973. Calibration curves for RST concentrations of 75–3,000 ng/ml in plasma and brain were linear, accurate, and exact. The concentration of RST at each calibration level was determined by back calculation using the calibration curves. The findings fulfilled the $R^2 > 0.98$ acceptance criterion.

Accuracy and precision

The inter-day and intraday precision and accuracy for RST at each concentration level in both matrices are represented

in Tables 6 and 7. RST QC samples, such as LQC, MQC, and HQC samples, were determined to be within the acceptable standards of 85%–115% for plasma samples and 80%–120% for brain samples.

Recovery

Absolute recovery of RST was observed in all QC samples. The mean recovery for RST from the rat plasma and brain at LQC, MQC, and HQC concentrations were found to be in the range of 90%–101% in comparison to the aqueous samples of the same concentrations. % CV for LQC levels of RST in plasma and brain were found to be 3.36% and 2.03%, respectively.

Stability studies

The stability studies of RST included stock solution stability and stability in plasma and brain matrix. After 6 hours at room temperature, the RST stock solution was determined to be

Table 6. Intraday accuracy and precision of RST in plasma and brain.

Matrix	QC levels	SD	%CV	% Nominal conc.
Plasma	LQC	2.59	3.36	102.74
	MQC	25.28	3.72	90.71
	HQC	86.13	3.41	101.07
Brain	LQC	1.51	2.03	99.47
	MQC	15.76	2.04	102.78
	HQC	43.39	1.46	118.74

Table 7. Inter-day accuracy and precision of RST in plasma and brain.

Matrix	QC levels	SD	%CV	% Nominal conc.
Plasma	LQC	2.62	3.47	100.45
	MQC	15.99	2.12	100.73
	HQC	103.89	4.18	99.48
Brain	LQC	2.96	4.21	93.72
	MQC	20.84	3.10	89.68
	HQC	36.49	1.24	117.42

Table 8. Stability analysis of RST in plasma and brain.

Stability	MQC		HQC	
	Plasma	Brain	Plasma	Brain
Mean % change				
Bench top stability	-2.72	7.18	-6.41	-1.42
Post-preparative stability at 4°C	-4.48	-2.82	1	-1.84
Freeze thaw stability	-4.08	5.61	-5.57	-2.3

Table 9. PK parameters for intranasal route for RST.

Biological matrix	Parameters	Values ^a
Plasma	C_{max} , ng/ml	343.027 ± 14.896
	T_{max} , minute	5.00 ± 0.00
	AUC ₀₋₂₄₀ , µg.minute/ml	30.549 ± 1.559
	AUC _{0-∞} , µg.minute/ml	75.121 ± 8.521
	$t_{1/2}$, minute	347.102 ± 27.611
	MRT, minute	479.170 ± 41.642
	C_{max} , ng/ml	555.949 ± 9.390
Brain	T_{max} , minute	15.00 ± 0.00
	AUC ₀₋₂₄₀ , µg.minute/ml	45.361 ± 0.493
	AUC _{0-∞} , µg.minute/ml	103.753 ± 3.240
	$t_{1/2}$, minute	336.443 ± 16.162
	MRT, minute	449.434 ± 20.671

^a The values are presented as mean ± SD, $n = 4$.

stable with a mean percentage change of 0.29 compared to freshly prepared MQC. RST was shown to be stable in both plasma and brain samples after 3 freeze-thaw cycles. When processed samples were kept in auto-sampler at 4°C and on bench-top for 24 hours, RST was found to be stable in both matrices under all the stability conditions and all the results were within the 15% tolerance limit.

All the above data were represented as Mean percentage change in comparison to freshly prepared samples and tabulated in Table 8.

PKs study

The validated method aided in PK estimation of RST in plasma and brain samples at defined time intervals and

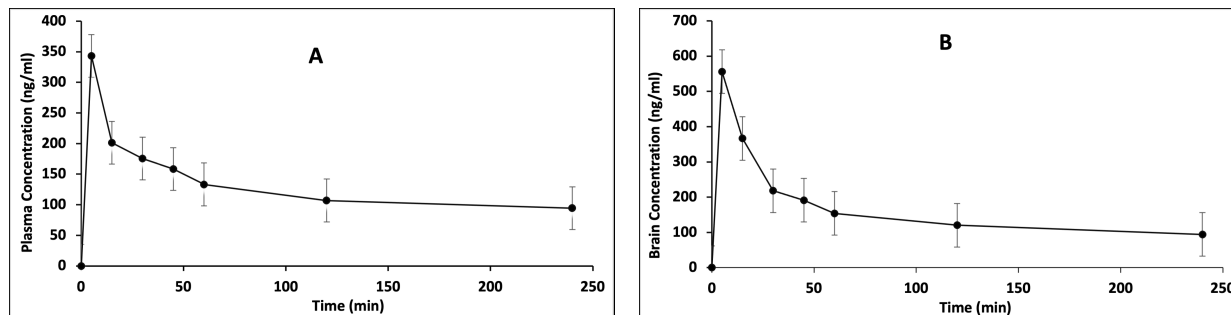


Figure 6. PKs profiles of RST via intra-nasal route. (A) mean plasma concentration of RST versus time (1.5 mg/kg, $n = 4$). (B) mean brain concentration of RST versus time (1.5 mg/kg, $n = 4$).

in determining the PK parameters for RST following nasal administration. The PK parameters are tabulated in Table 9. The mean plasma concentration of RST ($n = 4$) versus time profile is shown in Figure 6A and mean brain concentration of RST brain is shown in Figure 6B. Nasal administration of RST in rats resulted in a peak concentration of (C_{max}) of 343.027 ± 14.896 ng/ml in plasma and 555.949 ± 9.390 ng/ml in brain and exhibited T_{max} at 5 minutes in plasma and 15 minutes in brain. The plasma $AUC_{(0-240)}$ was observed to be 30.549 ± 1.559 $\mu\text{g}\cdot\text{minute}/\text{ml}$ while $AUC_{(0-\infty)}$ was observed to be 75.121 ± 8.521 $\mu\text{g}\cdot\text{minute}/\text{ml}$. The brain plasma $AUC_{(0-240)}$ was observed to be 45.361 ± 0.493 $\mu\text{g}\cdot\text{minute}/\text{ml}$ while $AUC_{(0-\infty)}$ was observed to be 103.753 ± 3.240 $\mu\text{g}\cdot\text{minute}/\text{ml}$. RST showed a mean residence time (MRT) of 479.170 ± 41.642 minutes and $t_{1/2}$ equivalent to 347.102 ± 27.611 minutes in plasma and MRT of 449.434 ± 20.671 minutes and $t_{1/2}$ equivalent to 336.443 ± 16.162 minutes in the brain. These results were compared with previously reported results for nasal administration of RST (Arumugam *et al.*, 2008; Wavikar *et al.*, 2017). Thus, the chromatographic method demonstrates its appropriateness to estimate RST in rat plasma and brain.

CONCLUSION

This study reports the impact of QbD in the development and routine use of the bioanalytical method over the traditional approach to method development for estimation of RST in rat plasma and brain. This research is of high importance as no validated bioanalytical method has been previously reported for the RST estimation in these biological matrices using a highly affordable HPLC/UV system wherein the drug was extracted by a simple and fast protein precipitation approach. This validated method was found to be selective, linear, accurate, precise, and able to detect RST at the lowest concentration of 75 ng/ml. Nasal PK studies using RST solution were studied as an application of the method which further helped to elucidate the drug's estimation in the brain. This validated method has been observed seen to be simple, sensitive, cost-effective, and reproducible for routine estimation of RST. This study also emphasizes that the QbD approach could represent novelty for the controlled development of a robust and reliable bioanalytical method for its pre-clinical or clinical applications.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important

intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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The authors report no financial or any other conflicts of interest in this work.

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All the animal experimental protocols were approved by Institutional Animal Ethics Committee, Kasturba Medical College, Manipal (Approval No. IAEC/KMC/36/2019).

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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REFERENCES

- Amini H, Ahmadiani A. High-performance liquid chromatographic determination of rivastigmine in human plasma for application in pharmacokinetic studies. *Iran J Pharm Res*, 2010; 0(2):115–21.
- Arumugam K, Subramanian GS, Mallayasamy SR, Averineni RK, Reddy MS, Udupa N. A study of rivastigmine liposomes for delivery into the brain through intranasal route. *Acta Pharm*, 2008; 58(3):287–97.
- Arumugam K, Chamallamudi MR, Gilibili RR, Mullangi R, Ganesan S, Kar SS, Averineni R, Shavi G, Udupa N. Development and validation of a HPLC method for quantification of rivastigmine in rat urine and identification of a novel metabolite in urine by LC-MS/MS. *Biomed Chromatogr*, 2011a; 25(3):353–61.
- Arumugam K, Chamallamudi M, Mallayasamy S, Mullangi R, Ganesan S, Jamadar L, Ranjithkumar A, Udupa N. High performance liquid chromatographic fluorescence detection method for the quantification of rivastigmine in rat plasma and brain: application to preclinical pharmacokinetic studies in rats. *J Young Pharm*, 2011b; 3(4):315–21.
- Arvand M, Fallahi P. Voltammetric determination of rivastigmine in pharmaceutical and biological samples using molecularly imprinted polymer modified carbon paste electrode. *Sens Actuators B Chem*, 2013; 188:797–805.

Bandopadhyay S, Beg S, Katare OP, Sharma T, Singh B. Integrated analytical quality by design (AQbD) approach for the development and validation of bioanalytical liquid chromatography method for estimation of valsartan. *J Chromatogr Sci*, 2020; 58(7):606–21.

Bhatt J, Subbaiah G, Kambli S, Shah B, Patel M, Saxena A, Baliga A, Nigam S, Parekh H, Yadav G. A high throughput and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the estimation of bisoprolol in human plasma using multiplexing technique. *J Chromatogr B Anal Technol Biomed Life Sci*, 2007; 852(1–2):374–81.

Carini JP, Kaiser S, Ortega GG, Bassani VL. Development, optimisation and validation of a stability-indicating HPLC method of achyrobichalcone quantification using experimental designs. *Phytochem Anal*, 2013; 24(3):193–200.

Dalvi AV, Uppuluri CT, Bommireddy EP, Ravi PR. Design of experiments-based RP–HPLC bioanalytical method development for estimation of Rufinamide in rat plasma and brain and its application in pharmacokinetic study. *J Chromatogr B Anal Technol Biomed Life Sci*, 2018; 1102–1103:74–82.

El-Kosasy AM, Shehata MA, Hassan NY, Fayed AS, El-Zeany BA. Membrane electrodes for the determination of glutathione. *Talanta*, 2005; 66(3):746–54.

Enz A, Chappuis A, Dattler A. A simple, rapid and sensitive method for simultaneous determination of rivastigmine and its major metabolite NAP 226-90 in rat brain and plasma by reversed-phase liquid chromatography coupled to electrospray ionization mass spectrometry. *Biomed Chromatogr*, 2004; 18(3):160–6.

Eskander MF, Nagykerly NG, Leung EY, Khelghati B, Geula C. Rivastigmine is a potent inhibitor of acetyl- and butyrylcholinesterase in Alzheimer's plaques and tangles. *Brain Res*, 2005; 1060(1–2):144–52.

Frankfort SV, Ouwehand M, van Maanen MJ, Rosing H, Tulner LR, Beijnen JH. A simple and sensitive assay for the quantitative analysis of rivastigmine and its metabolite NAP 226-90 in human EDTA plasma using coupled liquid chromatography and tandem mass spectrometry. *Rapid Commun Mass Spectrom*, 2006; 20(22):3330–6.

Hampel H, Mesulam MM, Cuello AC, Farlow MR, Giacobini E, Grossberg GT, Khachaturian AS, Vergallo A, Cavado E, Snyder PJ, Khachaturian ZS. The cholinergic system in the pathophysiology and treatment of Alzheimer's disease. *Brain*, 2018; 141(7):1917–33.

Hampel H, Mesulam MM, Cuello AC, Khachaturian AS, Vergallo A, Farlow MR, Snyder PJ, Giacobini E, Khachaturian ZS. Revisiting the cholinergic hypothesis in Alzheimer's disease: emerging evidence from translational and clinical research. *J Prev Alzheimers Dis*, 2019; 6(1):2–15.

Hegde AR, Padya BS, Soman S, Mutalik S. A simple, precise, and sensitive HPLC method for quantification of letrozole in rat plasma: development, validation, and preclinical pharmacokinetics. *J Anal Sci Technol*, 2021; 12(1):25.

Kadian N, Raju KSR, Rashid M, Malik MY, Taneja I, Wahajuddin M. Comparative assessment of bioanalytical method validation guidelines for pharmaceutical industry. *J Pharm Biomed Anal*, 2016; 126:83–97.

Karthik A, Subramanian GS, Surulivelrajan M, Ranjithkumar A, Kamat SB. Fluorimetric determination of rivastigmine in rat plasma by a reverse phase—high performance liquid chromatographic method. Application to a pharmacokinetic study. *Arzneimittelforschung*, 2008; 58(5):205–10.

Lane RM, Darreh-Shori T. Understanding the beneficial and detrimental effects of donepezil and rivastigmine to improve their therapeutic value. *J Alzheimers Dis*, 2015; 44(4):1039–62.

Mullangi R, Ranjithkumar A, Arumugam K, Mallayasamy SR, Ganesan S, Jamadar L, Udupa N, Chamallamudi MR. High performance liquid chromatographic fluorescence detection method for the quantification of rivastigmine in rat plasma and brain: application to preclinical pharmacokinetic studies in rats. *J Young Pharm*, 2011; 3(4):315–21.

Mullick P, Mutalik SP, Hegde AR, Pandey A, Jagadish PC, Kini SG, Jain S, Mutalik S. Simultaneous estimation of apremilast and betamethasone dipropionate in microsponge-based topical formulation using a stability indicating RP-HPLC method: a quality-by-design approach. *J Chromatogr Sci*, 2021; 59(10):928–40.

Mutalik SP, Mullick P, Pandey A, Kulkarni SS, Mutalik S. Box–Behnken design aided optimization and validation of developed reverse phase HPLC analytical method for simultaneous quantification of dolutegravir sodium and lamivudine co-loaded in nano-liposomes. *J Sep Sci*, 2021; 44(15):2917–31.

Pommier F, Frigola R. Quantitative determination of rivastigmine and its major metabolite in human plasma by liquid chromatography with atmospheric pressure chemical ionization tandem mass spectrometry. *J Chromatogr B Anal Technol Biomed Life Sci*, 2003; 784(2):301–13.

Sahu PK, Ramisetty NR, Cecchi T, Swain S, Patro CS, Panda J. An overview of experimental designs in HPLC method development and validation. *J Pharm Biomed Anal*, 2018; 147:590–611.

Wavikar P, Pai R, Vavia P. Nose to brain delivery of rivastigmine by in situ gelling cationic nanostructured lipid carriers: enhanced brain distribution and pharmacodynamics. *J Pharm Sci*, 2017; 106(12):3613–22.

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