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Development and Validation of an RP-HPLC Bioanalytical Method for Quantification of Rizatriptan Benzoate in Rat Plasma and Brain Homogenate: Application to Pharmacokinetic and Brain-Targeting Studies

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Abstract:

Purpose

This study was conducted to establish and validate a new, rapid, specific, sensitive and reproducible reverse-phase high-performance liquid chromatographic (RP-HPLC) method for the determination of Rizatriptan Benzoate (RZT) in rat plasma and brain homogenate. The validated method was then used to assess the PK profile and brain targeting ability of intranasally administered RZT formulations.

Methods

The chromatographic separation was performed on a C18 analytical column with isocratic mobile phase in the ratio from the acetonitrile and phosphate buffer (pH 3.0) optimal flow rate of approximately 0.4 ml/min. Detection was conducted at the selected wavelength with a UV detector. Biological sample preparation by a protein precipitation method with acetonitrile This analytical method was validated based on selectivity, linearity, precision, accuracy, recovery data and the matrix effect across their corresponding ranges to comply with US Food and Drug Administration (FDA) and International Council for Harmonisation (ICH M10) guidelines. The approved method was used for the analysis of RZT in rat plasma/brain homogenate after nasal administration of RZT solution, solid lipid nanoparticles (SLNs) and SLN-loaded locally gel.

Results

A good chromatographic performance of the developed RP-HPLC method was observed with a retention time of approximately 3.5 min. The calibration curve showed wonderful linearity in the evaluated focus vary (R^2 = 0.9994). Precision and accuracy reported were within the regulatory guideline limits. The extraction recovery was greater than 85% for all three level of quality control samples in plasma or brain homogenate. The matrix factors were between 0.992 and 0.997(preparing method reduces or removes interference related to matrix). RZT was shown to be stable under all investigated storage and processing conditions in stability studies. Implementation of a validated method showed relevant brain accumulation as well as an enhanced pharmacokinetic profile for SLN-loaded in situ gel phase compared to the conventional route used for RZT solution.

Conclusion

The developed RP-HPLC method is simple, reproducible, accurate and appropriate for bioanalytical routine quantification of Rizatriptan Benzoate in biological matrices. The validated methodology can be easily useful on pharmacokinetic studies and bioavailability of nasally delivered drugs in the CNS.

Keywords: Rizatriptan Benzoate; RP-HPLC; Bioanalytical Method Validation; Pharmacokinetics; Brain Targeting; Plasma; Brain Homogenate; Intranasal Drug Delivery.

Introduction

Migraine is one of the most common neurological disorders that impact millions of people around the globe. Migraine is a disease with recurrent attacks of moderate to severe head pain often also associated with nausea, vomiting, photophobia and phonophobia [1, 2]. The blood–brain barrier (BBB) is extremely selective, so the delivery of therapeutic agents across such barrier at a rapid rate and sufficient quantity continues to warrant further investigation, notwithstanding multiple classes of drugs currently being on the market [3].

Rizatriptan Benzoate, a potent and selective 5-HT1B/1D receptor agonist is generally recommended for treating patients with acute migraine attacks. Nevertheless, Rizatriptan is subject to high levels of hepatic first-pass metabolism and low brain penetration when administered orally. Over the years, one such new effective method is intranasal drug delivery which has become an appealing alternative to improve direct nose-to-brain transport for higher therapeutic effect with less exposure to systemic circulation [4, 5].

A prerequisite for pharmacokinetic and brain distribution experiments is the reliable quantification of drugs in biological matrices. In addition to selectivity and specificity, these bioanalytical methods should ensure high sensitivity, linearity, precision & reproducibility with minimum matrix interference. Despite this, due to the methodological simplicity, low cost and strong applicability of RP-HPLC remain one of the most used analytical methodologies [6, 7].

To assure the reliability of pharmacokinetic data generated, regulatory agencies like the US FDA and ICH have developed detailed guidelines on bioanalytical method validation [8, 9]. These criteria for assessment include selectivity, linearity, precision and accuracy of the method (ensure that correct concentration is obtained from sample), extraction recovery (must know what percentage of drug remains after extraction process),matrix effect, stability in matrix and dilution integrity before biological samples can be analyzed using such analytical method [10– 11].

In the literature, many analytical methods have been presented for determination of Rizatriptan in pharmaceutical dosage forms however, only few studies reported on its simultaneous quantification in plasma and brain homogenate using a validated RP–HPLC method which is appropriate for pharmacokinetic studies [12]. Hence, the objective of current research was to develop and validate a simple RP-HPLC method for determination of Rizatriptan Benzoate in rat plasma and brain homogenate, and also to use validated method for pharmacokinetic studies on brain-targeting potential of intranasal formulations.

Materials and Methods

Materials

Rizatriptan benzoate reference standard was obtained from a certified pharmaceutical manufacturera. Acetonitrile and methanol of HPLC grade were purchased from Merck (India). Potassium dihydrogen phosphate was of analytical quality and orthophosphoric acid. Milli-Q purification system was used to make ultrapure water.

Experimental Animals

Healthy adult Wistar albino rats (180–220 g) were obtained from the animal house facility of our institution. This subsection should provide all details of animal housing, acclimatization, ethical approval and experimental conditions appropriate to your study.

Instrumentation

Chromatographic analysis was carried out using a reverse-phase high-performance liquid chromatography (RP-HPLC) system with a binary solvent delivery pump (LC-20AD, Shimadzu Corporation.), autosampler (SIL-20AC, Shimadzu Corporation.), UV detector (SPD-M20A DAD, Shimadzu Corporation.) and data acquisition software. Analytical column (250 × 4.6 mm, 5 μm) : C18].

Chromatographic Conditions

Optimized chromatographic conditions for the determination of rizatriptan benzoate in biological matrices are summarized in Table 1.

Table 1. Optimized chromatographic conditions for the RP-HPLC determination of rizatriptan benzoate in biological matrices.

Parameter	Condition
Column	C18, 250 × 4.6 mm, 5 μm
Mobile phase	Acetonitrile:phosphate buffer
Buffer pH	3.0
Flow rate	1.0 mL/min

Detection	UV
Injection volume	20 µL
Run time	8 min
Retention time	Approximately 3.5 min

Biological Sample Preparation
Rizatriptan benzoate was extracted from biological matrices using protein precipitation. In brief, 200 µL rat plasma was added to acetonitrile and vortexed for 2 min. Afterwards, the supernatant was collected by centrifuging at 10,000r/min for 10 min, and filtered through a membrane filter of 0.22 µm before HPLC analysis. Brain homogenate samples were treated in the same way as described for extraction [13,14]. To aid in better reproducibility, the plasma-to-acetonitrile ratio and method of preparation for the brain homogenate should be described in detail.

Bioanalytical Method Validation
RP-HPLC–UV method was developed and strictly validated according to applicable US FDA bioanalytical method-validation requirements and ICH M10 guidelines [15, 16] for quantification of rizatriptan benzoate level in rat plasma and brain homogenate.
The analytical performance of the developed method was assessed in terms of selectivity, linearity, accuracy, precision, extraction recovery, matrix effect and stability.

Selectivity
To ascertain selectivity, blank rat plasma and brain homogenate prepared from the appropriate matrix, as well as with samples spiked with rizatriptan benzoate later detected in extract to confirm a lack of human endogenous components at the retention time of the analyte.

Linearity
Calibration standards of rizatriptan benzoate prepared in rat plasma and brain homogenate were used for evaluating the linearity of the method. The calibration curve was created by plotting the evaluated response versus the actual analyte concentration.

Accuracy and Precision
Quality-control samples at relevant concentration levels were utilized to determine accuracy and precision. The reliability and reproducibility of the method was evaluated by assessing intra-day as well as inter-day performance.

Extraction Recovery
The extraction recovery of rizatriptan benzoate from rat plasma and brain homogenate was defined by comparing the analytical response obtained from extracted biological samples to those of the corresponding reference solutions prepared at the same concentrations.

Matrix Effect
Matrix-effect studies in plasma and in brain homogenate were conducted to evaluate the possibility of interference from endogenous components of the biologic matrix on the analytical response of rizatriptan benzoate.

Stability
We evaluated the stability of rizatriptan benzoate in plasma and brain homogenate under the sample-handling and storage conditions relevant to this study. Sample stability schemes suffused freeze–thaw, bench-top, long-term storage, and post-preparative assessments.

Calibration Curve
The linearity of the developed RP-HPLC method for Rizatriptan Benzoate as shown in (figure 6) is made to establish a calibration curve over the range. Preparation of standard solutions: A stock solution of Rizatriptan Benzoate was prepared in mobile phase and diluted to working standard solutions at the concentrations (1.0, 2.0, 5.0, and 10.0 µg/mL.) All calibration standards were prepared fresh and analyzed in triplicate at optimized chromatographic conditions [28].
An aliquot of 20 µL of each standard solution was injected in the system RP-HPLC and its chromatographic peak areas were noted. The average peak area corresponding to each concentration was determined and the calibration curve was plotted using mean peak area following a linear regression with calculated nominal concentrations. The least-squares method was used to carry out linear regression analysis for obtaining the regression equation and coefficient of determination (R^2) as an indicator for verifying the linearity of the developed method [29].

Extraction Recovery

Extraction recovery (n = 9, for both plasma and brain homogenate) was assessed to validate the efficiency of the optimized protein precipitation method for the extraction of Rizatriptan Benzoate from rat plasma and brain homogenate [17,18]. The evaluation was conducted using the US FDA Bioanalytical Method Validation Guidance (2018) and ICH Q2(R1) at three concentration levels: Low Quality Control (LQC, 1 µg/mL), Medium Quality Control (MQC, 5 µg/mL), and High Quality Control (HQC, 10 µg/mL). In practice, while the later was obtained with six replicates (n = 6) for each QC concentration.

Samples of two different concentrations were prepared at each QC concentration. The initial set involved pre-extraction spiked samples, where Rizatriptan Benzoate was added to blank rat plasma or brain homogenate followed by the optimized acetonitrile-induced protein precipitation extraction method. The post-extraction spiked samples of this second set, involved extracting blank biological matrices and subsequently injecting Rizatriptan Benzoate at the corresponding QC concentration [19,20].

Each sample was then subsequently analyzed under the optimized RP-HPLC conditions and their relevant chromatographic peak areas were recorded. Extraction recovery was calculated by comparing the peak area from pre-extraction spiked samples with that obtained from the corresponding post-extraction spiked samples according to the following equation:

The recovery (%) was calculated as: Peak area of post-extraction spiked sample/Peak area of pre-extraction spiked sample×100

Mean recovery (%) and percentage relative standard deviation (%RSD) were determined for each QC concentration to evaluate the extraction method's efficacy and reproducibility.

Matrix Effect

To assess the extent to which endogenous components from rat plasma and brain homogenate altered chromatographic response of Rizatriptan Benzoate model. The acetonitrile-based protein precipitation method was then applied to blank plasma and brain homogenate samples. After extraction, for post-extraction spiked samples supernatants were spiked with Rizatriptan Benzoate at selected QC levels. Similarly prepared corresponding neat standard solutions of the same concentrations with Rizatriptan Benzoate were accomplished directly in the mobile phase without biological matrix [21,22].

The measured areas of the chromatographic peaks resulting from HPLC analysis of each sample were recorded and analyzed using the optimized conditions. The matrix effect calculation was done by dividing the peak areas from the post-extraction spiked samples of the matrices compared to those obtained from corresponding neat standard solutions. Equation (7) to build the factor by matrix:

Matrix Factor (MF) = Peak area of neat standard/Peak area of post-extraction spiked sample

An MF that is close to 1.0 suggests minimal endogenous matrix constituent effects, as observed in Rizatriptan Benzoate analysis, which indicates the insignificant matrix-related interference [23].

Stability Studies

Rizatriptan Benzoate was assessed for its stability in both rat plasma and brain homogenate as per the US FDA Bioanalytical Method Validation Guidance 2018. LQC, and HQC samples (n = 6) were assessed for stability and tested in different storage/storage conditions such as bench-top (short-term), freeze–thaw, long-term stability. Stability was expressed as the relative concentration based on the percentage of nominal content remaining after exposure to the stated storage condition (dry versus wet) or post-preparation handling [19,24].

Bench-top (Short-term) Stability

Since the stability of a drug during routine handling in ambient laboratory conditions on bench-top is crucial, we determined the bench-top stability of Rizatriptan Benzoate. Effect of human plasma depending HQC and LQC samples prepared in rat plasma or homogenate The samples were left at room temperature (25 ± 2 °C) for 6 h prior extraction. After the defined storage interval, specimens were treated as per the optimized protein precipitation protocol and then analyzed with the validated RP-HPLC approach. The concentrations measured were compared with fresh QC samples, and stability was calculated as the percentage of initial concentration [25].

Freeze–Thaw Stability

Freeze–thaw stability was performed to evaluate the impact of repeated freezing and thawing cycles on the stability of Rizatriptan Benzoate in rat plasma and brain homogenate. HQC and LQC samples were kept at −20°C and then thawed at the ambient temperature. We performed the freeze and thaw procedure over three full cycles. The samples were extracted following the optimized protein precipitation procedure and analyzed by RP-HPLC after completion of the third cycle. The concentrations found were compared to those of freshly prepared quality control samples from the same batch, and stability expressed as a percentage [26].

Long-term Stability

The stability of Rizatriptan Benzoate during extended storage at frozen temperatures was determined by an evaluation of the long-term stability. LQC and HQC samples in rat plasma and brain homogenate were frozen for 30 days at −20 °C. After the storage period, the samples were melted at room temperature, extracted by the optimized process of protein

precipitation, and analyzed by the validated RP-HPLC method. Concentration stability was calculated as a percentage by comparing the measured concentrations with those of freshly prepared QC samples [27].

Pharmacokinetic Study

The aim of this pharmacokinetic study was to confirm and compare systemic and brain delivery of conventional Rizatriptan Benzoate (RZT) solution, RZT-loaded solid lipid nanoparticles (RZT-SLN), optimized RZT-loaded SLN in situ gel after intranasal administration. We selected an additional intravenous RZT injection group to serve as a reference for systemic pharmacokinetic evaluation [30,31].

Experimental Animals

Healthy male Wistar albino rats (180–220 g) were obtained from the Veterinary College, Khanapara, Guwahati, Assam to conduct all experimental work under standard laboratory conditions: a temperature of 25 ± 2°C, relative humidity of 55 ± 5% and reverse light/dark cycle (12 h each). One week before the beginning of the experiment, animals were acclimatized and allowed free access to a standard pellet diet and water ad libitum [32].

All experimental practices were performed following the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, approved by Institutional Animal Ethics Committee (IAEC), The Assam Royal Global University. The related IAEC approval number is RGU/RSP/IAEC/38/2026(20th February, 2026).

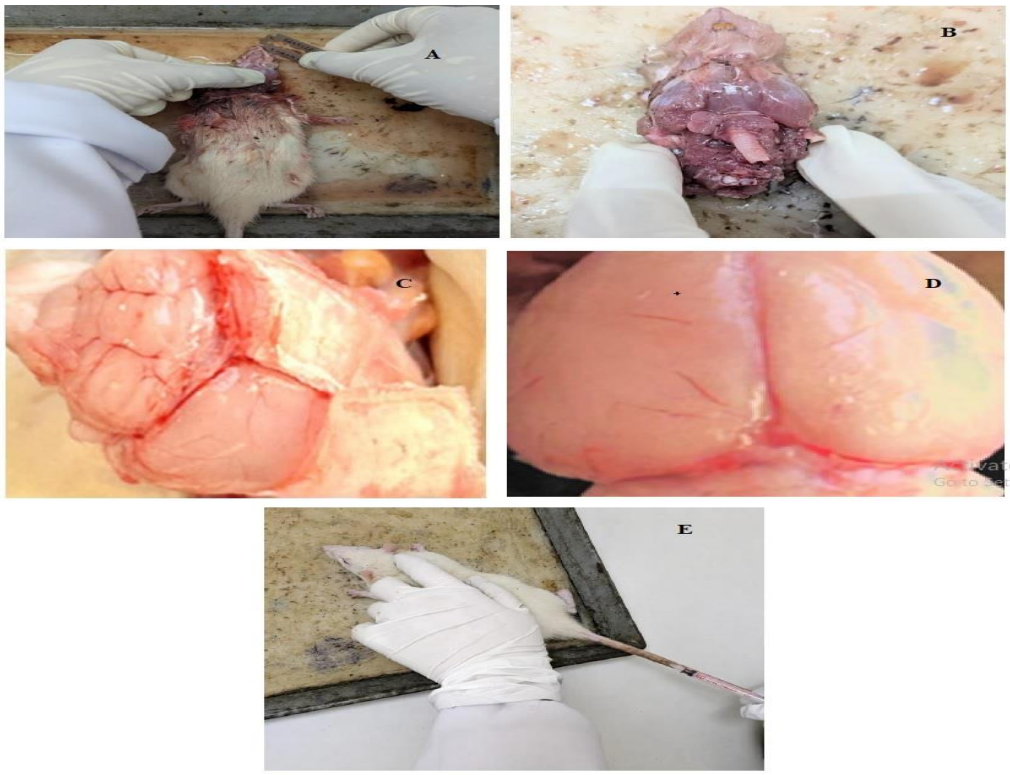

Figure 1. Experimental procedures for brain isolation and blood collection in Wistar albino rats: (A) cranial dissection, (B) exposed brain, (C–D) isolated brain, and (E) tail-vein blood collection.

Study Design

Animals were randomly divided into various experimental groups (Groups I – V) with 6 animals per group (n = 6; Table 2). Group I was the control group, which received normal saline intranasally; Group II received Rizatriptan solution intranasally as reference formulation; Group III received RZT- loaded SLN intranasally; Group IV RZT-loaded SLN in situ gel administrated intranasal for assessment of nose to brain deliveries and finally, systemic pharmacokinetic behavior comparison between Rizatriptan injection (Group V via intravenous) [33].

Table 2. Experimental design for the pharmacokinetic study

Group	Treatment	Route	Purpose	No. of animals
I	Normal saline	Intranasal	Control	6
II	Rizatriptan solution	Intranasal	Reference formulation	6
III	RZT-loaded SLN	Intranasal	Nanoparticulate formulation	6
IV	RZT-loaded SLN in situ	Intranasal	Nose-to-brain delivery	6

	gel			
V	Rizatriptan injection	Intravenous	Plasma pharmacokinetic comparison	6

Dose Administration
Rizatriptan Benzoate was given as an equivalent maximum-dose of 5mg/kg body weight. Before receiving an intranasal dosage, the rats were lightly anesthetized using inhalational anesthesia to reduce movement and aid in accurate dosing. Each formulation was gently deposited in the opposite nostril using a volume calibrated micropipette in order to encourage even deposition along the nasal mucosa and thereby limit amounts entering the gastrointestinal tract. Vaginal administration and intravenous group (IV): Animals in this group received an equal dose of the drug through tail vein [34].

Sample Collection
Blood and brain samples at the predetermined times of 0.5, 1, 2, 4, 6, 8 or 12 h post administration were collected. For pharmacokinetic analysis, blood was drawn into heparinized tubes at specific time points and centrifuged (5,000 rpm for 10 min) to obtain plasma. Plasma samples were obtained and stored at −20°C until subsequent analyses [35, 36]. To analyse the distribution in the brain, animals were euthanized at specific time points for sampling of the whole brain. Blotted dry with filter paper, washed in ice-cold normal saline to remove any blood present, and weighed accurately the surgical brain tissue.

Brain Tissue Processing
Tissue homogenizer in liquid nitrogen at 80°C to obtain a consistent brain homogenate. The homogenates were then centrifuged at 10,000 rpm for 15 min at 4°C, and the clear supernatants obtained after centrifugation were collected for extraction and quantification of Rizatriptan Benzoate.

Sample Preparation and RP-HPLC Analysis
Plasma and brain homogenate samples were deproteinized with acetonitrile in a procedure recently optimized for the specific extraction of Rizatriptan Benzoate. In short, biological samples were mixed with acetonitrile to precipitate proteins; the mixture was vortexed and centrifuged. This supernatant was then filtered with 0.22 µm membrane filter and a 20 µL aliquot was injected into the RP-HPLC system. Chromatographic separation was carried out on a C18 reversed-phase column (250 × 4.6 mm, 5 µm). The mobile phase consisted of acetonitrile and phosphate buffer (30:70, v/v; pH 3.5) in isocratic conditions at 1.0 mL/min flow rate. Detection was carried out at 225 nm, Rizatriptan Benzoate showed a retention time around 4.5 min. Plasma and brain samples were analyzed using the validated calibration curve [37] for drug quantification.

Pharmacokinetic and Brain Bioavailability Analysis
Brain tissue levels at 12 and 30 min with Rizatriptan Benzoate were therefore expressed as µg/g of brain tissue whereas plasma values were expressed as µg/mL. Subsequently, the obtained concentration–time profiles were analysed with a non-compartmental pharmacokinetic approach [38, 39].
The following pharmacokinetic parameters were determined:
Cmax: maximum observed drug concentration in plasma or brain;
Tmax: time required to reach the maximum observed drug concentration;
AUC_{0}–t: area under the concentration–time curve from time zero to the last measurable concentration.
The relative brain bioavailability (Frel) of the test formulations was calculated using the following equation:
F rel (%)=AUC reference /AUC test × 100
Where AUC test = AUC of the RZT-SLN or RZT-loaded SLN in situ gel, and AUC reference = AUC of conventional Rizatriptan solution [40]. Relative bioavailability based on conventional intranasal RZT solution as reference is denoted above.

Statistical Analysis
All the experimental data has shown as mean ± standard deviation (SD) of six independent observations (n=6). One-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison post hoc test was used for statistical comparisons between treatment groups. Statistical significance was determined at $p < 0.05$. For pharmacokinetic calculations and plotting the concentration–time profiles, appropriate software was used [41,42].

Results And Discussion
Chromatographic Performance
The RP-HPLC method developed offered satisfactory chromatographic separation of Rizatriptan Benzoate in rat plasma and brain homogenate. Chromatography data was obtained and analyzed under optimized chromatographic separation of Rizatriptan Benzoate using a C18 reversed-phase analytical column with an acetonitrile–phosphate buffer mobile phase at a flow rate of 1.0 mL/min. The analyte showed a sharp chromatographic peak with a retention time of ~3.5 min and the total run time was 8 min for the chromatographic separation. This short retention time and run duration signified the

developed method was applicable for rapid bioanalysis of multiple biological samples derived from pharmacokinetic studies. Table 1 shows the optimized chromatographic conditions.
Protein precipitation with acetonitrile facilitated a simple and rapid method for processing plasma and brain homogenate. A centrifugation and membrane filtration procedure was ultimately implemented, resulting in a clear supernatant that was suitable for subsequent chromatographic analysis.

Calibration Curve and Linearity
The calibration data for Rizatriptan Benzoate are presented in Table 3, and the corresponding calibration curve is shown in Figure 2. Increased Rizatriptan Benzoate concentrations of 1.0 to 10.0 µg/mL increased the peak area, and thus demonstrated concentration dependency in detector response. The linear nature of the calibration data was maintained in the concentration range investigated.

Table 3. Calibration data for Rizatriptan Benzoate

Concentration (µg/mL)	Peak Area
1	1,24,840
2	2,39,410
5	6,13,530
10	12,36,960

The resultant linear regression analysis curve is as follows: y = 12374x − 2515 (where y is the chromatographic peak area and x is Rizatriptan Benzoate concentration in µg/mL). The correlation coefficient (R^2 = 0.999) showed an excellent linearity in the concentration range of drug and response from chromatographic, in the range concentration from 1 to 10µg/mL.

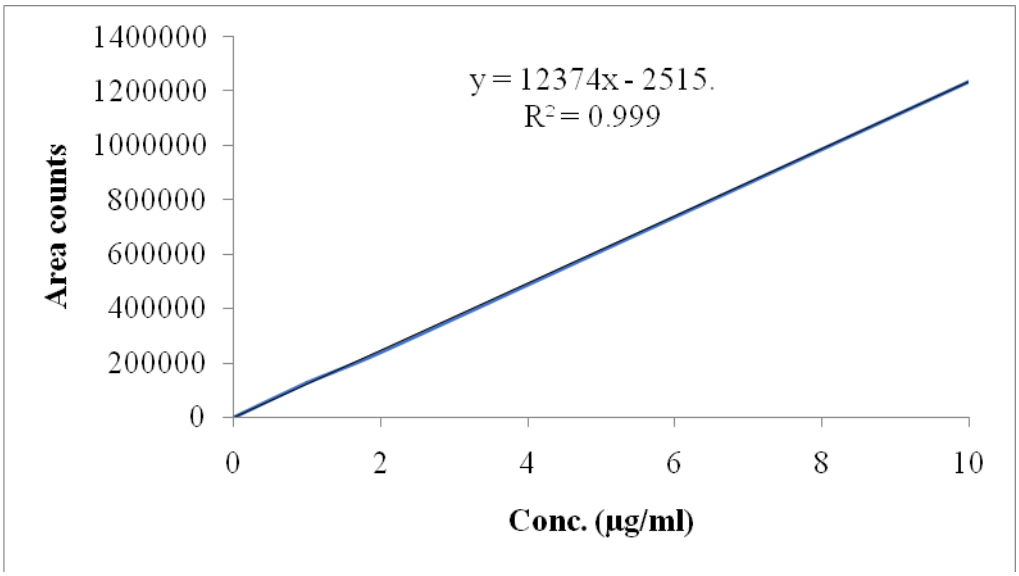

Figure 2. HPLC calibration curve of Rizatriptan benzoate in the concentration range of 1–10 µg/mL (R^2 = 0.999).

The calibration procedure was implemented on the corresponding plasma and brain homogenate samples for biological sample analysis with the validated analytical method. The calibration response obtained thus allowed the quantitative measurement of Rizatriptan Benzoate in the biological matrices to be employed for pharmacokinetic assessment.

Selectivity
The RP-HPLC method developed here exhibited good selectivity, since there was no significant background from the endogenous components of rat plasma and brain homogenate at the retention time of Rizatriptan Benzoate. There were no interfering peaks observed in blank matrices and only a single distinct peak of the analyte (spiked samples). Therefore, optimized sample preparation and chromatographic conditions were sufficient for the separation and quantitative determination of Rizatriptan Benzoate in biological matrices.

Accuracy and Precision
The RP-HPLC method developed is simple, specific, sensitive and reproducible for the determination of Rizatriptan Benzoate in rat plasma and brain homogenate. The low %RSD values at both LQC, MQC and HQC levels indicated

good intra-day as well as inter-day precision and reproducibility. The findings confirmed the methodology as suitable for robust pharmacokinetic analysis.

Extraction Recovery

The extraction recovery of Rizatriptan Benzoate at LQC, MQC and HQC (n = 6) was investigated using the optimized protein-precipitation method as shown in Figure 3-5. The values are summarized in Tables 4 and 5. Values for mean extraction recovery were from 88.6% to 93.5% in rat plasma and from 85.4% to 92.1% in brain homogenate. Plasma and brain homogenate data Recovery increased as concentration of QC was increased in both matrices with the highest recovery at HQC (93.5% plasma; 92.1% in brain homogenate). At LQC in brain homogenate, the lowest recovery was 85.4%. However, despite this variation, the recovery was consistent enough for all QC levels in our investigation.

In addition, the extraction reproducibility (%RSD: Plasma 0.96%–1.45%) (Brain homogenate 1.10%–1.62%) was also satisfactory. The somewhat inferior recovery in brain homogenate versus plasma may be due to increased complexity of the analysis matrix with lipids, phospholipids and other cellular components that can affect protein precipitation and analyte extraction.

In general, the noted recovery and low variability demonstrated that the optimized extraction procedure was efficient and reproducible for Rizatriptan Benzoate recovery from both biological matrices. Method accurate and precise based on the recovery obtained at different QC levels, indicating a feasibility for quantitative analysis of plasma and brain samples from pharmacokinetics and brain-distribution studies.

Table 4. Extraction recovery of Rizatriptan Benzoate from rat plasma (n = 6)

QC Level	Concentration (µg/mL)	Pre-extraction Peak Area	Post-extraction Peak Area	Recovery (%)	%RSD
LQC	1	46,072	52,000	88.6	1.45
MQC	5	132,240	145,000	91.2	1.18
HQC	10	266,475	285,000	93.5	0.96

Table 5. Extraction recovery of Rizatriptan Benzoate from rat brain homogenate (n = 6)

QC Level	Concentration (µg/mL)	Post-extraction Peak Area	Pre-extraction Peak Area	Recovery (%)	%RSD
LQC	1	75,000	64,050	85.4	1.62
MQC	5	200,000	179,600	89.8	1.25
HQC	10	400,000	368,400	92.1	1.10

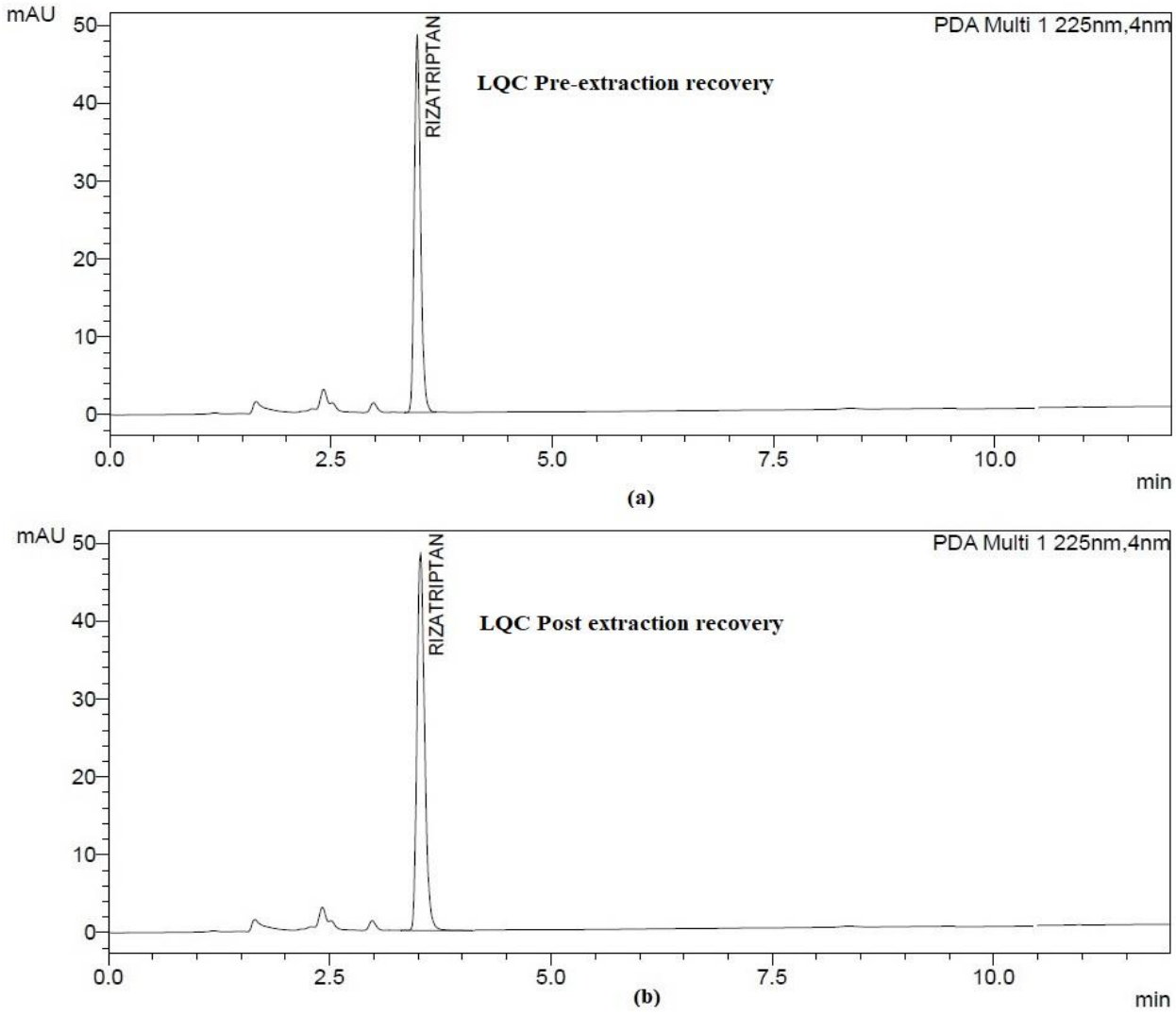

Figure 3. RP-HPLC chromatograms for extraction recovery evaluation of Low Quality Control (LQC, 1 µg/mL) samples of Rizatriptan Benzoate: (a) pre-extraction spiked sample and (b) post-extraction spiked sample. Comparable peak responses at a retention time of approximately 3.5 min indicate efficient analyte recovery.

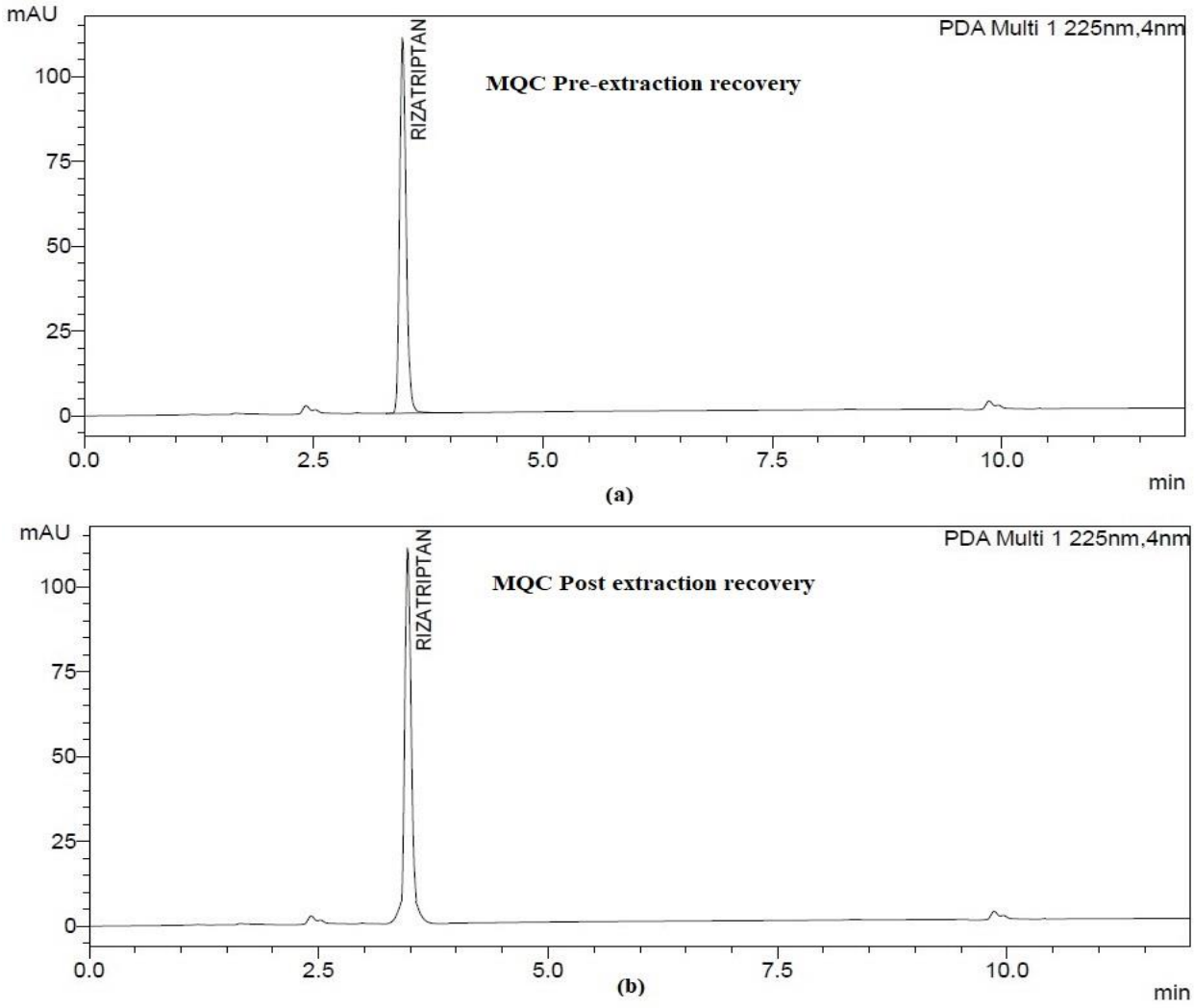

Figure 4. RP-HPLC chromatograms for extraction recovery evaluation of Medium Quality Control (MQC, 5 µg/mL) samples of Rizatriptan Benzoate: (a) pre-extraction spiked sample and (b) post-extraction spiked sample. Comparable peak responses at a retention time of approximately 3.5 min demonstrate consistent extraction efficiency.

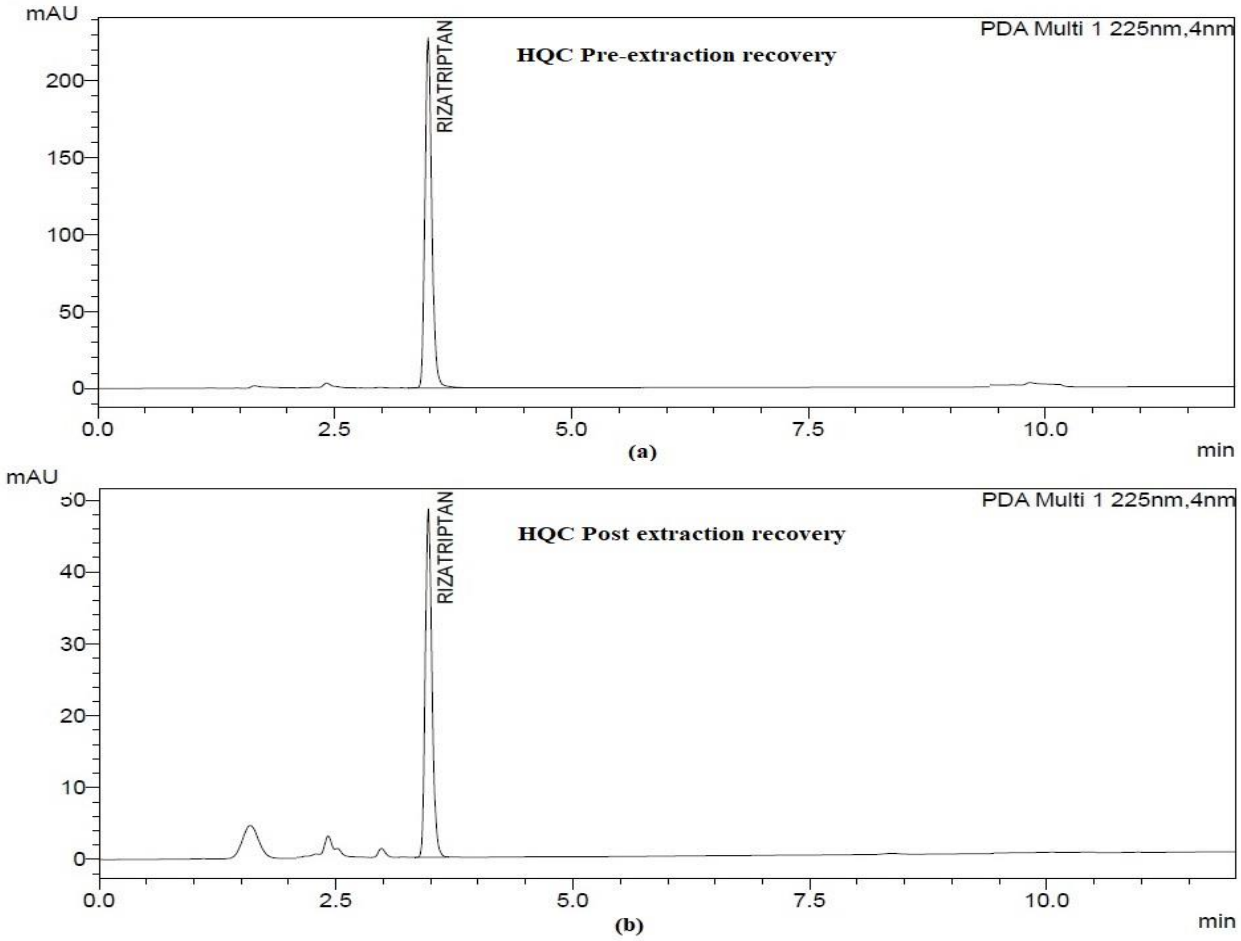

Figure 5. RP-HPLC chromatograms for extraction recovery evaluation of High Quality Control (HQC, 10 µg/mL) samples of Rizatriptan Benzoate: (a) pre-extraction spiked sample and (b) post-extraction spiked sample. Similar peak responses at a retention time of approximately 3.5 min confirm efficient and reproducible analyte recovery.

Matrix effect

In order to evaluate the possible effect of endogenous matrix components on the chromatographic response of Rizatriptan Benzoate, each peak area corresponding to post extraction plasma or brain homogenate samples was compared with that from the same pure standard prepared in mobile phase. Table 6 shows the results. The peak area of pure Rizatriptan Benzoate standard was 300,000 while the peaks areas of post-extraction plasma and brain homogenate samples were 299100 and 297600 respectively (Figure 6). These matrix factors were 0.997 for plasma and 0.992 for brain homogenate. Additionally, the matrix factors were near unity for each of the biological matrices, suggesting little suppression or enhancement of the Rizatriptan Benzoate response by endogenous matrix constituents. The interim definition for matrix factor was a little less in brain homogenate, perhaps because it has higher matrix complexity than plasma. Together, the results indicate that matrix effects were minimal and are therefore not expected to impair the accuracy and reliability of Rizatriptan Benzoate quantification in the biological samples investigated.

Table 6. Comparison of peak areas of post-extraction plasma and brain homogenate samples with the pure standard for evaluation of matrix factor.

Sample	Peak Area	Matrix Factor
Pure standard (mobile phase)	300,000	—
Post-extraction plasma sample	299,100	0.997
Post-extraction brain homogenate sample	297,600	0.992

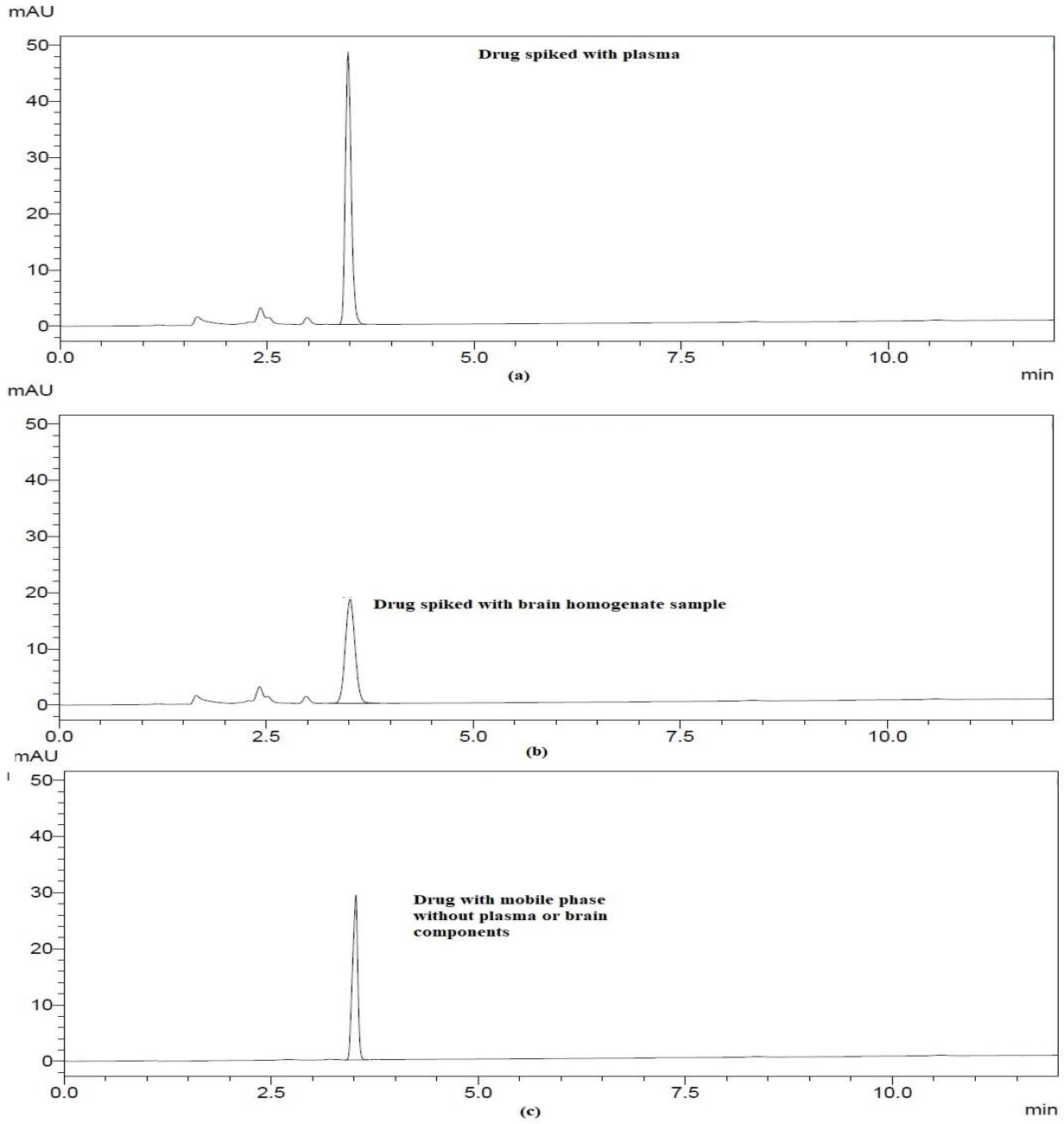

Figure 6 RP-HPLC chromatograms of Rizatriptan Benzoate for matrix effect assessment showing (a) post-extraction spiked plasma sample, (b) post-extraction spiked brain homogenate sample, and (c) neat standard solution in mobile phase.

Results of Stability Studies

The stability of Rizatriptan Benzoate was found to be good in rat plasma and brain homogenate under all the investigated conditions. Stability ranged from 96.5–98.6% when stored on the bench-top for 6 h, ranged from 95.8–98.2% after three freeze–thaw cycles and was between 94.8 and 97.4% at −20°C after 30 days of storage, respectively All results stayed within the premised acceptance range of ±15% indicating that negligible degradation occurred during routine sample handling, repeated freeze-thaw cycles and prolonged frozen store (Table 7). The observed slightly lower stability in brain homogenate and after long-term storage likely do not represent a significant loss of analyte integrity. All the results demonstrate a good stability of Rizatriptan Benzoate in biological matrices and support our ability to use the validated RP-HPLC method for reliable analysis of pharmacokinetic and brain-distribution samples.

Table 7. Results of Stability studies of Rizatriptan Benzoate in rat plasma and brain homogenate (n = 6).

Stability condition	Plasma LQC (%)	Plasma HQC (%)	Brain LQC (%)	Brain HQC (%)
Bench-top (6 h, room	97.8	98.6	96.5	97.9

temperature)				
Freeze–thaw (3 cycles)	96.9	98.2	95.8	97.3
Long-term (30 days, −20°C)	95.6	97.4	94.8	96.9

Pharmacokinetic Studies

Thus the validated RP-HPLC method was successfully utilized for quantification of Rizatriptan Benzoate from rat plasma and brain homogenate after intranasal administration of Rizatriptan solution, RZT-loaded solid lipid nanoparticles (RZT-SLN) and RZT-SLN in situ gel. It was also proved that the pharmacokinetic profiles showed distinct differences in both exposure and duration of drug at the target site (brain) between the formulations.

Rizatriptan Benzoate RP-HPLC Analysis in Brain Homogenate

Figure 7 shows a representative RP-HPLC chromatogram of an intranasally administered dose of the optimized RZT-SLN in situ gel from rat brain homogenate. A clear and resolved peak of Rizatriptan Benzoate was observed at a retention time of around 3.5 min without any significant background interference from endogenously present brain components at the analyte retention region. The selectivity of the validated RP-HPLC method for quantitative determination of Rizatriptan Benzoate in brain homogenate has been confirmed.

Rizatriptan Benzoate Detection in Brain after Intranasal Administration: Analytical Evidence of Brain Exposure The increased and sustained brain concentrations seen with the RZT-SLN in situ gel are consistent with our hypothesis that combining a lipid-based NP with an in situ gelling system that provides slow release and/or prevents drug efflux as the carrier slowly dissolves can enhance distribution in brain. This is because SLN could serve as a drug carrier for partitioning and transport of the drug across the nasal mucosa, while gel can enhance nasal residence time by inhibiting rapid mucociliary clearance. The effects together could improve drug transport along the olfactory and trigeminal pathways, thus increasing the exposure in the brain to a greater extent.

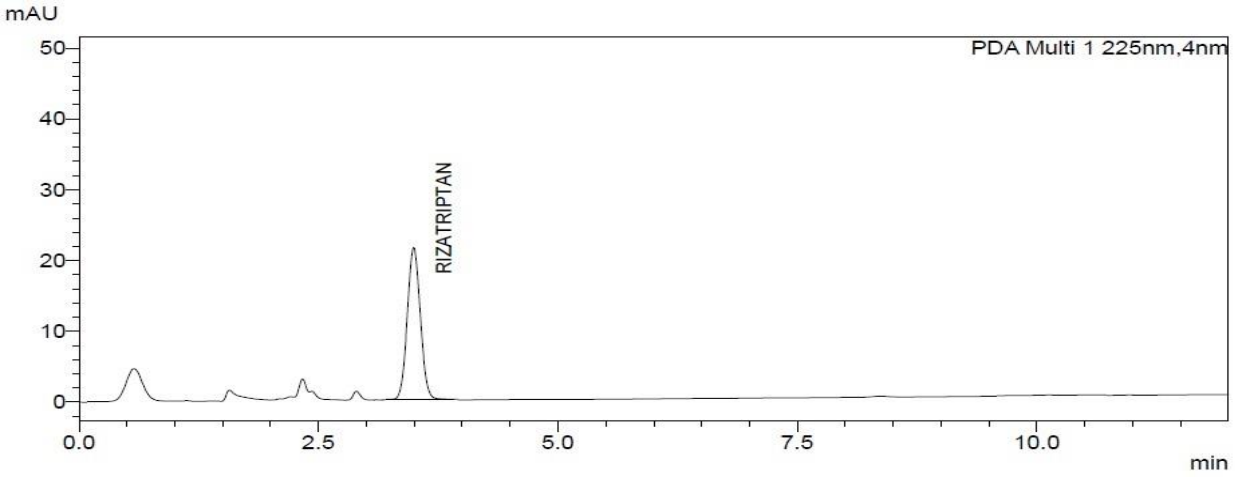

Figure 7. RP-HPLC chromatogram showing the detection of Rizatriptan Benzoate in rat brain homogenate after intranasal administration of the optimized RZT-loaded SLN in situ gel formulation, confirming efficient nose-to-brain drug delivery.

Brain Concentration–Time Profile

Table 8 and Figure 8 present the brain concentration–time profiles following intranasal administration of RZT solution, RZT-SLN, and RZT-SLN in situ gel. Within 0.5 h, Rizatriptan Benzoate was measurable in the brain tissue of all treatment groups indicating a rapid brain exposure following intranasal administration.

The conventional RZT solution reached the highest brain concentration of 1.25 µg/g at 1 h, then decreased to 0.05 µg/g at 12 h ($P < 0.05$), showing a rapid distribution followed by relatively minor retention in the brain. The peak concentration (PC) of RZT-SLN was 2.84 µg/g at 1 h, which was about a 2.3-fold enhancement in comparison the to conventional solution. This enhancement could be related to nanoscale carrier that increase drug interaction with nasal mucosa and reduce barrier transport across the nasal epithelium.

The highest concentrations in the brain was 3.92 µg/g (at 2 h) after administration of RZT-SLN in situ gel, significantly delayed Tmax compared with solution and RZT-SLN formulations suggested a sustained drug input into the brain. Notably, the formulation provided measurable brain concentrations for 12 h (0.35 µg/g), demonstrating prolonged exposure of the CNS. This sustained profile could be the result of both enhanced transport of drug into CNS due to nanoparticle formulation and prolonged nasal residence provided by an in situ gel.

Table 8. Brain concentration–time profile of Rizatriptan Benzoate following intranasal administration.

Time (h)	RZT Solution (µg/g)	RZT-SLN (µg/g)	RZT-SLN Gel (µg/g)
0.0	0.00	0.00	0.00
0.5	0.82	1.95	1.48
1.0	1.25	2.84	2.95
2.0	0.95	2.15	3.92
4.0	0.56	1.52	2.84
6.0	0.30	0.96	1.78
8.0	0.15	0.52	0.98
12.0	0.05	0.18	0.35

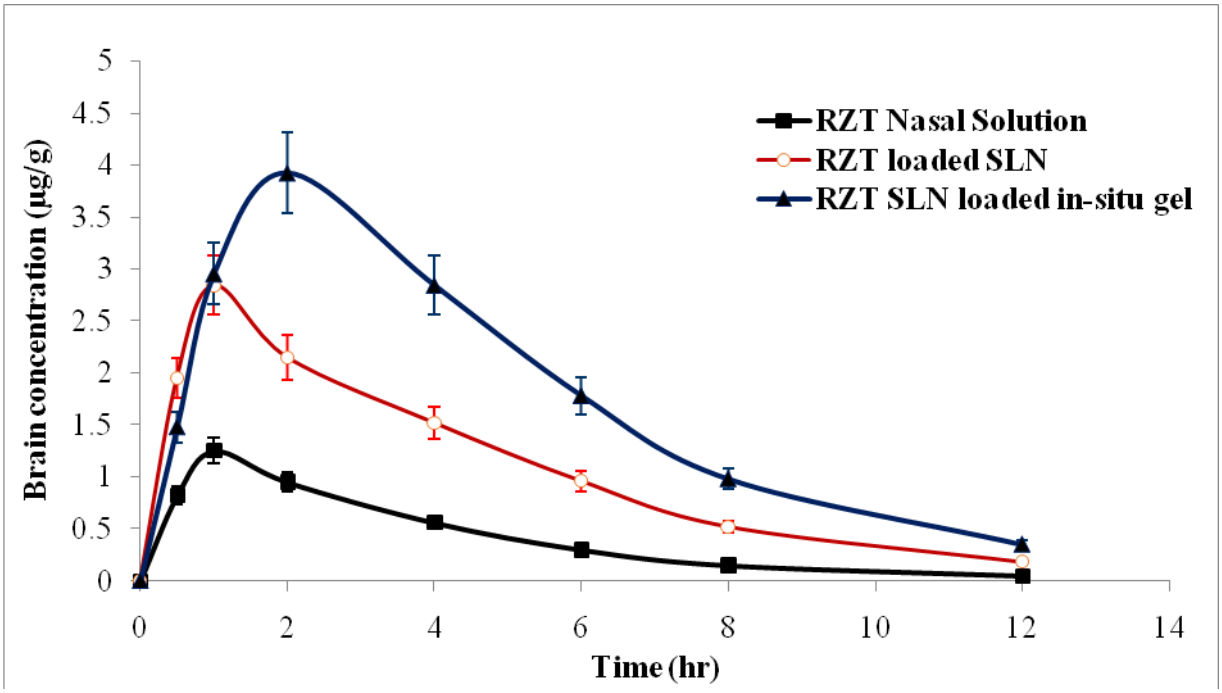

Figure 8. Comparative brain concentration–time profiles of Rizatriptan benzoate (RZT) following intranasal administration of RZT nasal solution, RZT-loaded solid lipid nanoparticles (SLN), and RZT SLN-loaded in-situ gel in Wistar rats. Data are expressed as mean ± SD (n = 6).

Brain Pharmacokinetic Parameters

The data of pharmacokinetics also provided evidence for the higher brain exposure achieved by RZT-SLN in situ gel. The Cmax, Tmax and AUC₀–t of conventional RZT solution were 1.25 ± 0.12 µg/g, 1 h and a value of 3.85 ± 0.35 µgh/g respectively The incorporation of Rizatriptan Benzoate to SLNs increased the Cmax (2.84 ± 0.21µg/g), AUC₀–t (8.92 ± 0.75 µgh/g) without change in Tmax (1 h).

The Cmax, Tmax and AUC₀–t of optimized RZT-SLN in situ gel were 3.92 ± 0.30 µg/g, 2 h and 12.64 ± 1.02 µgh/g respectively; and the bioavailability comparing with the traditional solution was increased by about 3.1 time ($P < 0.05$) for Cmax, about 3.3 times for AUC°–□ ($P<0.01$). These data show that this optimized formulation leads to significantly higher and longer-lasting brain exposure. This increased brain targeting might be due to the synergistic functions of SLN and in situ gel elements. While lipid nanoparticle system can improve drug solubilization, mucosal mucoadhesion and permeability through nasal epithelium, in situ gel helps sustain the nasal residence time and prolong drug release. Thus, the dual system may facilitate direct nose to brain delivery and is also expected to prolong the fast tail-off in drug concentration seen post dosing of a conventional solution.

In general, the pharmacokinetic results show the RZT-SLN in situ gel is a better formulation than the others since it had higher Cmax, AUC₀–t, longer Tmax and detectable drug concentrations over time. These results underpin the feasibility of utilizing an optimized, intranasal drug formulation that may substantially improve and prolong brain delivery of Rizatriptan Benzoate when compared to a conventional nasal solution.

Table 9. Brain Pharmacokinetic Parameters

Parameter	RZT Solution	RZT-SLN	RZT-SLN Gel
Cmax (µg/g)	1.25 ± 0.12	2.84 ± 0.21	3.92 ± 0.30
Tmax (h)	1.0	1.0	2.0
$AUC_{0–t}$ (µg·h/g)	3.85 ± 0.35	8.92 ± 0.75	12.64 ± 1.02

Relative Brain Bioavailability

Relative brain bioavailability was estimated from the brain $AUC_{0–t}$ values using the conventional RZT solution as the reference formulation. The RZT-SLN formulation produced an approximately 2.31-fold increase in relative brain bioavailability, whereas the optimized RZT-SLN in situ gel produced a 3.28-fold increase compared with the RZT solution. The greater brain exposure observed with the RZT-SLN formulation may be attributed to the nanoscale lipid carrier, which can enhance drug interaction with the nasal mucosa and facilitate transport across the nasal epithelium. The further increase obtained with the in situ gel may result from prolonged nasal residence and sustained drug release, thereby maintaining drug availability for nose-to-brain transport. Overall, the higher relative brain exposure of the RZT-SLN in situ gel supports the potential of this formulation to enhance and prolong delivery of Rizatriptan Benzoate to the brain compared with the conventional solution.

Plasma Concentration–Time Profile

The concentration–time profiles of Rizatriptan Benzoate in the plasma are given in Table 10 and Figure 9. Intravenous administration resulted in an immediate plasma concentration of 6.20 µg/mL at 0 h, declining to a terminal concentration of 0.08 µg/mL at 12 h; the intranasal RZT-SLN produced slower nasal absorption with a delayed Tmax (2 h) and maximum plasma concentration (Cmax = 2.85 g/mL), followed by endogenous elimination (six times lower than the IV route: C12h = 0.32 g/mL), supporting prolonged absorption from the intranasal formulation probably due to longer nasal residence time and sustained drug release from SLN in situ gel system. In general, the formulation provides a more prolonged plasma exposure compared to intravenous dosing and complements the increased brain exposure than that shown in the brain pharmacokinetic study.

Table 10. Plasma concentration–time profile of Rizatriptan Benzoate following intravenous RZT solution and intranasal RZT-loaded SLN in situ gel administration

Time (h)	Intravenous RZT Solution (µg/mL)	Intranasal RZT-SLN In Situ Gel (µg/mL)
0.0	6.20	0.00
0.25	5.42	0.82
0.50	4.65	1.46
1.0	3.42	2.18
2.0	2.18	2.85
4.0	1.12	2.32
6.0	0.58	1.58
8.0	0.26	0.92
12.0	0.08	0.32

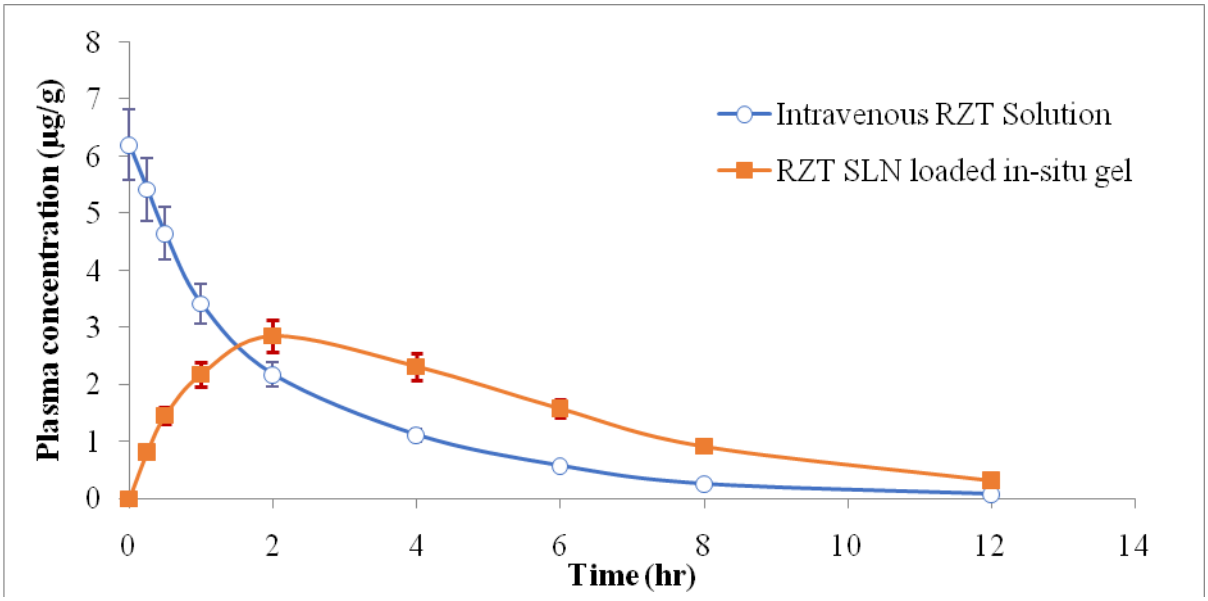

Figure 9. Plasma concentration–time profile of Rizatriptan benzoate (RZT) following intravenous administration of RZT solution and intranasal administration of RZT solid lipid nanoparticle (SLN)-loaded in-situ gel in Wistar rats. Data are presented as mean ± SD (n = 6).

Despite the lower plasma Cmax, the faster-absorbed intranasal formulation yielded a higher AUC0–t (18.21 ± 1.46 µgh/mL) versus intravenous RZT (14.42 ± 1.18 µgh/mL), indicating prolonged systemic exposure. The prolonged Tmax and reduced Cmax can be explained by prolongation of the nasal residence time due to in situ gel formation and controlled drug release from SLN matrix. These results together with the better brain exposure demonstrated by the formulation support the possibility for sustained intranasal delivery as well as increased brain targeting of Rizatriptan.

Table 11. Plasma pharmacokinetic parameters of Rizatriptan Benzoate following intravenous administration of RZT solution and intranasal administration of RZT-loaded SLN in situ gel.

Parameter	Intravenous RZT Solution	Intranasal RZT-SLN In Situ Gel
Cmax (µg/mL)	6.20 ± 0.42	2.85 ± 0.24
Tmax (h)	0.0	2.0
AUC0–t (µg·h/mL)	14.42 ± 1.18	18.21 ± 1.46

Statistical Analysis

Statistical comparison of the pharmacokinetic parameters demonstrated that both the RZT-SLN and RZT-loaded SLN in situ gel formulations produced significantly higher Cmax and AUC0–t values than the conventional RZT solution ($p < 0.05$). Moreover, the optimized SLN in situ gel exhibited significantly greater brain drug exposure than the SLN formulation alone, confirming the additional benefit provided by the thermoresponsive gel system in prolonging nasal residence and enhancing brain targeting.

Conclusion

A rapid validated RP-HPLC–UV method, enabling the determination of Rizatriptan Benzoate in rat plasma and brain homogenate in one run was developed and successfully validated. This optimized analytical method exhibited appropriate chromatographic performance, linearity, selectivity, extraction recovery, matrix effect, precision, accuracy and stability to allow for pharmacokinetic study. In the present study, a Rizatriptan-loaded solid lipid nanoparticle (RZT-SLN) in situ nasal gel was developed in order to enhance Rizatriptan brain exposure. The formulation led to a significantly increased brain Cmax and AUC₀–t, demonstrating an improved and sustained availability of drug in the tissue. In the plasma pharmacokinetic study, Tmax was delayed, Cmax decreased and systemic exposure prolonged following intranasal administration. Overall, these results indicate that SLN-based in situ gel can prolong nasal residence and sustained drug delivery as well the high brain delivery of Rizatriptan with much avoided rapid systemic exposure. In conclusion, the formulated intranasal solution of Rizatriptan may provide a potential novel route for brain targeted delivery and merits translation in clinical studies.

Declarations

Funding

The authors declare that no external funding was received for this study.

Conflict of Interest
The authors declare no conflict of interest.

Ethical Approval
The animal study protocol was approved by the Institutional Animal Ethics Committee (IAEC), The Assam Royal Global University. The related IAEC approval number is RGU/RSP/IAEC/38/2026 (Dated. 20th February, 2026).

Author Contributions
The original research work was carried out by Priyanka Das and Bipul Nath, who contributed to the conceptualization, methodology, investigation, validation, data analysis, and preparation of the original manuscript. Payal Dasgupta, Purusuttam Hazarika, Kunal Dutta, and SK Imran Hassan contributed to the review and editing of the manuscript. All authors approved the final version of the manuscript.

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