Scientific Research Publishing

Open Journal of Applied Sciences, 2026, 16(9), 3045-3059
https://www.scirp.org/journal/ojapps
ISSN Online: 2165-3925
ISSN Print: 2165-3917

Determination of Rizatriptan in Human Plasma by LC-MS/MS

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How to cite this paper: Saraner, N., Şenceol, B., Bakıcı, A., Güney, B. and Sağlam, O. (2026) Determination of Rizatriptan in Human Plasma by LC-MS/MS. *Open Journal of Applied Sciences*, **16**, 3045-3059.
https://doi.org/10.4236/ojapps.2026.169168

Received: August 14, 2026
Accepted: September 7, 2026
Published: September 10, 2026

Abstract

A selective LC-MS/MS method was developed and comprehensively validated for the quantification of rizatriptan in human plasma, employing protein precipitation as the sample preparation technique. Chromatographic separation was achieved on a GL Sciences InertSustain AQ C18 column (3 µm, 4.6 × 75 mm) under isocratic conditions and detection was performed by multiple reaction monitoring (MRM) in positive electrospray ionization mode. Rizatriptan-D6 was employed as the internal standard. No significant endogenous interference was observed at the retention times corresponding to rizatriptan or the internal standard, confirming the selectivity of the analytical method. The calibration model demonstrated linearity over the concentration range of 0.25 - 60 ng/mL using weighted ($1/C^2$) linear regression. Accuracy and precision results complied with the predefined acceptance criteria across all evaluated concentration levels and validation batches. The protein precipitation procedure provided consistent extraction performance and recovery of rizatriptan and the internal standard. No significant matrix-related effects or carry-over were observed indicating that neither factor adversely affected the quantitative determination of rizatriptan. Rizatriptan also demonstrated satisfactory stability under all investigated conditions, including bench-top, freeze-thaw, autosampler, whole-blood, stock solution, working solution and long-term storage conditions. The method involved a simple sample preparation procedure and a short chromatographic run time of 2.5 min, allowing plasma samples to be analyzed rapidly. The validated method provides a reproducible approach for the quantification of rizatriptan in human plasma and is suitable for pharmacokinetic, bioequivalence and other quantitative bioanalytical applications.

Keywords

Rizatriptan, Rizatriptan-D6, Human Plasma, LC-MS/MS

1. Introduction

Rizatriptan is an orally administered antimigraine agent belonging to the triptan class and is widely used for the acute treatment of migraine attacks. Its pharmacological effects are primarily mediated through selective agonism of 5-hydroxytryptamine (5-HT)1B/1D receptors, which modulates cranial vascular tone, inhibits neuropeptide release and reduces trigeminovascular nociceptive transmission [1]-[3]. Rizatriptan has a rapid onset of action and established clinical efficacy, making it an important option for the acute treatment of migraine [1] [2].

After oral administration, rizatriptan is rapidly absorbed, with maximum plasma concentrations generally reached within approximately 1 - 1.5 h. Its mean absolute oral bioavailability is approximately 45%, with presystemic metabolism contributing to the incomplete systemic availability. The drug is predominantly metabolized by monoamine oxidase-A (MAO-A) and has a relatively short plasma elimination half-life of approximately 2 - 3 h [1] [2]. These pharmacokinetic characteristics highlight the need for sensitive, selective, accurate and precise analytical methods, particularly for pharmacokinetic and bioequivalence studies.

Several analytical methods have been reported for the determination of rizatriptan in human plasma [4]-[10]. Liquid-liquid extraction (LLE) has been the most frequently reported sample preparation approach in these methods [5]-[9]. Typically, plasma samples are extracted with an organic solvent, followed by separation of the organic phase, evaporation and reconstitution [5]-[9]. Solid-phase extraction (SPE) has also been used for the LC-MS/MS determination of rizatriptan in human plasma [10]. Although these approaches can provide effective sample clean-up and satisfactory recovery, they may involve several processing steps and can therefore increase preparation time and handling requirements when large numbers of samples are analyzed.

Protein precipitation (PP) provides a simpler alternative for plasma sample preparation. However, co-extracted endogenous components may influence electrospray ionization and lead to ion suppression or enhancement. Consequently, recovery and matrix effects need to be assessed when PP is used for LC-MS/MS analysis [11]. Although several LC-MS/MS methods are available for rizatriptan determination in human plasma [4]-[10], a method combining simple protein precipitation with a short chromatographic run remains useful for high-throughput bioanalysis.

The aim of the present study was therefore to develop and validate a rapid and reliable LC-MS/MS method for the determination of rizatriptan in human plasma using protein precipitation. The method was validated with respect to selectivity, carry-over, calibration curve performance, sensitivity, matrix effects, recovery, accuracy, precision, dilution integrity and stability in accordance with the principles described in the ICH M10 Guideline on Bioanalytical Method Validation and Study Sample Analysis [12].

2. Experimental

2.1. Chemicals and Materials

Rizatriptan benzoate (99.77% purity) was used as the reference standard and was procured from BioOrganics & Applied Materials Pvt. Ltd. (India). Rizatriptan-D6 benzoate (94.60% purity), obtained from Clearsynth (India), was selected as the internal standard. Paracetamol and ibuprofen reference standards were also sourced from Clearsynth (India). Acetonitrile, methanol and formic acid were purchased from Merck (Darmstadt, Germany), while dimethyl sulfoxide (DMSO) was obtained from Carlo Erba Reagents (Val-de-Reuil, France). Drug-free K_2EDTA human plasma was supplied by BioIVT (UK) and was used as the biological matrix throughout the method development and validation studies. Ultrapure water was generated in-house using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Stock Solutions, Calibration Standards and QCs

Primary stock solutions of rizatriptan and rizatriptan-D6 (IS) were prepared independently by dissolving accurately weighed reference standards in 0.5 mL of dimethyl sulfoxide (DMSO), followed by dilution to volume with methanol to obtain final concentrations of 1 mg/mL and 0.2 mg/mL, respectively. Thus, the final DMSO:methanol composition of the primary stock solutions was approximately 2.5:97.5 (v/v). The quantities of the reference standards were adjusted according to their certified purities to ensure accurate stock solution concentrations. Stock solutions were stored at −20˚C until use.

Working solutions for calibration standards and quality control (QC) samples were prepared by serial dilution of the corresponding stock solutions with methanol. Calibration standards were freshly prepared by spiking blank human K_2EDTA plasma with appropriate volumes of the working solutions to obtain final concentrations ranging from 0.25 to 60 ng/mL.

Quality control samples were prepared at five concentration levels corresponding to 0.25 (LLOQ), 0.75 (LQC), 3 (MQC-1), 18 (MQC-2), and 48 ng/mL (HQC). In addition, dilution quality control (DQC) samples were prepared at a concentration of 102 ng/mL. All calibration standards and QC samples were vortex-mixed thoroughly and stored at −70˚C until analysis.

2.3. Instrumentation

The analytical measurements were performed on a Shimadzu LCMS-8060 triple quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan) interfaced with a Nexera X2 UHPLC system. The HPLC system consisted of a DGU-20A5R online degasser, LC-30AD binary pumps, SIL-30AC autosampler, CTO-10AS VP column oven and FCV-20AH2 switching valve. Chromatographic separation was conducted using a GL Sciences InertSustain AQ C18 column (3 μm, 4.6 × 75 mm) with the column temperature maintained at 40˚C.

Chromatography was performed under isocratic conditions using a mobile phase consisting of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) mixed at a ratio of 65:35 (v/v). The flow rate was set at 0.8 mL/min, resulting in a total run time of 2.5 min. The autosampler temperature was maintained at 10˚C and the injection volume was 5 µL.

Mass spectrometric detection was performed using an electrospray ionization (ESI) source operating in positive ion mode. High-purity nitrogen generated by a Peak Scientific NL-60 nitrogen generator was used as the nebulizing, drying and heating gas. The nebulizing gas flow was maintained at 3.0 L/min, while the drying and heating gas flows were each adjusted to 10 L/min. The interface voltage was set to 4500 V, whereas the interface, desolvation line (DL), heat block and desolvation temperatures were maintained at 300, 250, 400, and 526˚C, respectively. Quantitative analysis was carried out in multiple reaction monitoring (MRM) mode using the transitions m/z 270.2→201.1 for rizatriptan and m/z 276.1→207.2 for rizatriptan-D6. Instrumental parameters, including dwell time, Q1 pre-bias, collision energy and Q3 pre-bias, were individually optimized to maximize analytical sensitivity and selectivity. Instrument control, data acquisition and data processing were performed using LabSolutions software (Version 5.128 SP2).

2.4. Sample Preparation

Prior to LC-MS/MS analysis, plasma samples were prepared using a protein precipitation procedure. Briefly, 100 µL of human plasma was aliquoted into a polypropylene centrifuge tube, followed by the addition of 50 µL of rizatriptan-D6 working solution (50 ng/mL) as the internal standard. The samples were vortex-mixed for 5 s, after which 500 µL of acetonitrile was added for protein precipitation. The samples were vortexed for 60 s and centrifuged at 5500 rpm for 10 min at 4˚C. Following centrifugation, 200 µL of the resulting clear supernatant was transferred to a collection plate and diluted with 100 µL of water. The plate was subsequently shaken for 5 min and 5 µL of the processed sample was injected into the LC-MS/MS system for analysis.

3. Results and Discussion

3.1. Method Validation

The analytical method was fully validated in human plasma to assess its reliability and reproducibility. The validation parameters included selectivity, calibration curve performance, sensitivity, matrix effects, intra- and inter-batch accuracy and precision, extraction recovery, dilution integrity, carry-over, and stability under various conditions. Stability was evaluated under bench-top, freeze-thaw, autosampler, long-term, whole-blood, stock solution, and working solution conditions. All evaluated parameters met the predefined acceptance criteria. The validated method was subsequently applied to plasma samples obtained from a bioequivalence study for the quantitative determination of rizatriptan.

3.1.1. Specificity/Selectivity and Carry-Over

The selectivity and specificity of the developed LC-MS/MS method were assessed using blank human plasma obtained from eight independent sources, including hemolyzed and hyperlipidemic plasma samples. Potential interference from the internal standard and commonly co-administered medications, namely paracetamol (10 µg/mL) and ibuprofen (10 µg/mL), was also investigated. Rizatriptan at the QC4 concentration and rizatriptan-D6 at the concentration used for the internal standard were evaluated separately, while paracetamol and ibuprofen were tested at 10 µg/mL in plasma. Each test sample and LLOQ sample was prepared and analyzed in triplicate (n = 3). The acceptance criteria for interference were defined as an interfering response of less than 20% of the rizatriptan response at the LLOQ and less than 5% of the internal standard response. No interference exceeding these predefined acceptance criteria was observed at the retention times of rizatriptan or the internal standard in any of the analyzed samples. Representative multiple reaction monitoring (MRM) chromatograms of blank plasma and plasma spiked with rizatriptan at the lower limit of quantification (LLOQ) are presented in Figure 1.

Carry-over was assessed in each validation batch by injecting a processed blank plasma sample immediately following the upper limit of quantification (ULOQ) calibration standard. No significant carry-over was observed for either rizatriptan or the internal standard. The responses obtained from the post-ULOQ blank samples were below 20% of the LLOQ response for rizatriptan and below 5% of the response of the internal standard thereby meeting the predefined acceptance criteria for carry-over.

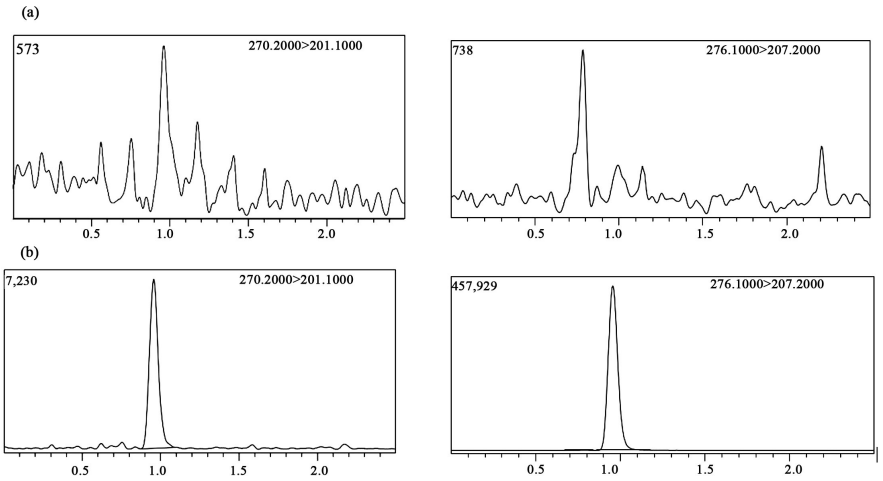

Figure 1. MRM chromatograms of human blank plasma (a), 0.25 ng/mL rizatriptan spiked with internal standard (b).

3.1.2. Linearity

Linearity of the proposed LC-MS/MS method was evaluated over the validated concentration range of 0.25 - 60 ng/mL. Calibration standards at eight concentration levels (0.25, 0.5, 2, 6, 12, 36, 54 and 60 ng/mL) were freshly prepared and analyzed in each validation batch. Weighted linear regression models using dif-

ferent weighting factors were evaluated based on the distribution of residuals and the accuracy of the back-calculated calibration-standard concentrations. The $1/C^2$ weighting factor was selected for quantitative analysis because it provided a more homogeneous distribution of residuals across the calibration range and satisfactory back-calculated accuracy compared with the other evaluated models. Calibration curves constructed during intra- and inter-assay validation runs showed strong correlation, with coefficient of determination (r^2) values consistently greater than 0.9979 for all analytes. The back-calculated concentrations of the calibration standards are presented in Table 1, whereas a representative calibration curve is shown in Figure 2. Freshly prepared calibration standards were included throughout method validation to verify calibration performance. The calibration model demonstrated excellent linearity throughout the investigated concentration range and fulfilled all predefined acceptance criteria, confirming its suitability for quantitative determination of rizatriptan in human plasma.

Table 1. Results and regression parameters of the calibration curve for linearity.

Concentration (ng/mL)	Batch 1		Batch 2		Batch 3		Concentration (mean ± SD; ng/mL)	CV (%)	RE (%)
	Conc. (ng/mL)	RE (%)	Conc. (ng/mL)	RE (%)	Conc. (ng/mL)	RE (%)			
0.25	0.257	2.840	0.258	3.088	0.260	4.094	0.258 ± 0.002	0.643	3.340
0.5	0.472	−5.581	0.471	−5.844	0.461	−7.761	0.468 ± 0.006	1.271	−6.395
2	2.003	0.145	1.973	−1.367	1.978	−1.119	1.984 ± 0.016	0.817	−0.780
6	5.847	−2.543	5.941	−0.985	5.845	−2.590	5.878 ± 0.055	0.933	−2.039
12	12.142	1.184	12.193	1.611	12.028	0.235	12.121 ± 0.085	0.697	1.010
36	35.563	−1.215	35.534	−1.296	35.805	−0.542	35.634 ± 0.149	0.418	−1.017
54	54.985	1.824	55.372	2.540	55.929	3.572	55.429 ± 0.474	0.856	2.646
60	62.007	3.345	61.351	2.251	62.467	4.112	61.942 ± 0.561	0.906	3.236

SD, standard deviation; CV, coefficient of variation; RE, relative error.

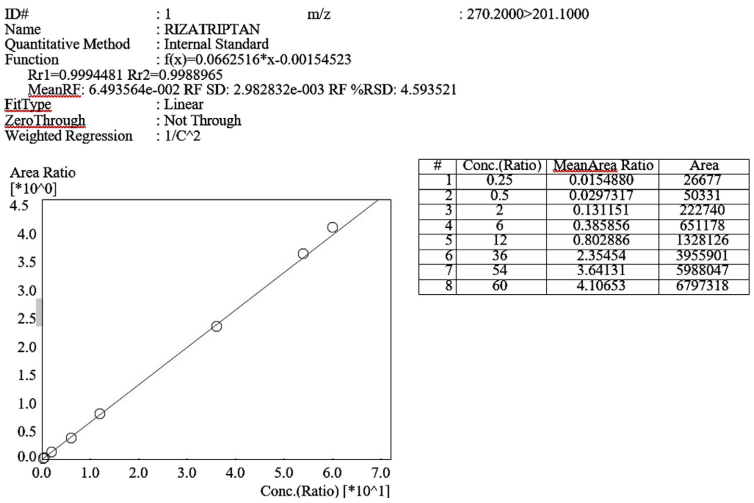

Figure 2. Calibration curve for rizatriptan.

3.1.3. Accuracy and Precision

The accuracy and precision of the developed LC-MS/MS method were assessed using quality control samples at five concentration levels (0.25, 0.75, 3, 18 and 48 ng/mL). Six replicate samples at each concentration level were analyzed in three independent validation batches. Quantification was performed using a weighted ($1/C^2$) linear regression model based on the peak area ratio of rizatriptan to the internal standard.

The intra-batch and inter-batch validation results are summarized in Table 2. The back-calculated concentrations of all quality control samples were in close agreement with their respective nominal concentrations. Intra-batch accuracy (RE%) ranged from −5.22% to 7.24%, while precision (CV%) ranged from 0.63% to 2.65% across the three validation batches, satisfying the predefined acceptance criteria in all cases. Likewise, inter-batch accuracy ranged from −4.81% to 3.62%, and precision ranged from 1.45% to 3.09%. These results demonstrate that the developed analytical method provides accurate, precise, and reproducible quantification of rizatriptan across the validated concentration range, with all results meeting the predefined acceptance criteria.

Table 2. Within-batch precision and accuracy of the method for determining rizatriptan in plasma samples.

		Intra-batch (n = 6)				Inter-batch (n = 18)		
Nominal concentration (ng/mL)		Concentration (mean ± SD; ng/mL)	RE (%)	CV (%)	Sample	Concentration (mean ± SD; ng/mL)	RE (%)	CV (%)
0.25	Batch01	0.255229 ± 0.004648	2.092	1.821	LLOQ	0.259060 ± 0.006294	3.624	2.430
	Batch02	0.262507 ± 0.005806	5.003	2.212				
	Batch03	0.259444 ± 0.006881	3.777	2.652				
0.75	Batch01	0.739295 ± 0.011037	−1.427	1.493	LQC	0.750692 ± 0.012794	0.092	1.704
	Batch02	0.754490 ± 0.007498	0.599	0.994				
	Batch03	0.758292 ± 0.011661	1.106	1.538				
3	Batch01	2.843479 ± 0.066666	−5.217	2.345	QC Medium1	2.855566 ± 0.044004	−4.814	1.541
	Batch02	2.856863 ± 0.033386	−4.771	1.169				
	Batch03	2.866356 ± 0.026598	−4.455	0.928				
18	Batch01	18.49196 ± 0.288971	2.733	1.563	QC Medium2	18.252051 ± 0.265512	1.400	1.455
	Batch02	18.03789 ± 0.134934	0.211	0.748				
	Batch03	18.22629 ± 0.114344	1.257	0.627				
48	Batch01	51.47560 ± 0.497987	7.241	0.967	HQC	49.574102 ± 1.530959	3.279	3.088
	Batch02	48.20023 ± 0.469270	0.417	0.974				
	Batch03	49.04646 ± 0.750248	2.180	1.530				

SD: standard deviation; RE: relative error; CV: coefficient of variation.

3.1.4. Matrix Effect

The potential matrix effect associated with different sources/lots of human plasma

on the quantitative determination of rizatriptan was evaluated in accordance with the principles and acceptance criteria [12]. The evaluation included eight independent plasma sources/lots, including haemolysed and lipaemic plasma, to assess potential matrix-related variability in the analytical response.

Low- and high-quality control samples (LQC and HQC; 0.75 and 48 ng/mL, respectively) were prepared using each individual plasma source, with four replicates at each concentration level. The resulting samples were processed using the validated protein precipitation procedure and quantified against a freshly prepared calibration curve. For each plasma source, accuracy was assessed based on the relative error (RE%), while precision was expressed as the coefficient of variation (CV%).

As summarized in **Table 3**, the analytical performance of rizatriptan was consistent across all investigated plasma sources. At the LQC level, the mean calculated concentrations ranged from 0.772 to 0.795 ng/mL, corresponding to RE values between 2.95% and 6.04%. The associated CV values ranged from 0.71% to 1.95%. At the HQC level, the mean calculated concentrations ranged from 52.068 to 53.512 ng/mL, with RE values ranging from 8.47% to 11.48% and CV values ranging from 0.35% to 1.22%.

For all individual plasma sources, the accuracy values were within ±15% of the nominal concentrations and the precision values were ≤15%, thereby fulfilling the predefined acceptance criteria. These results indicate that matrix-related variability, as assessed by QC accuracy and precision across the individual plasma sources, did not adversely affect the quantitative determination of rizatriptan. No individual plasma source showed a meaningful deviation in analytical performance at either QC concentration level.

Table 3. Accuracy and precision of rizatriptan in different human plasma matrix sources for matrix-effect evaluation.

Matrix source	LQC Mean (ng/mL)	LQC RE (%)	LQC CV (%)	HQC Mean (ng/mL)	HQC RE (%)	HQC CV (%)
Haemolysed plasma	0.772	2.95	0.71	52.288	8.93	0.46
Lipaemic plasma	0.779	3.86	1.22	52.068	8.47	0.35
Plasma source 1	0.774	3.19	1.28	52.801	10.00	0.48
Plasma source 2	0.786	4.77	0.74	52.909	10.23	1.16
Plasma source 3	0.784	4.54	1.95	52.438	9.25	0.57
Plasma source 4	0.792	5.61	1.69	52.906	10.22	0.69
Plasma source 5	0.780	3.98	1.30	53.512	11.48	1.10
Plasma source 6	0.795	6.04	1.19	53.018	10.45	1.22

LQC, low-quality control; HQC, high-quality control; SD, standard deviation; CV, coefficient of variation; RE, relative error.

3.1.5. Recovery

The extraction recovery of rizatriptan was evaluated to assess the efficiency, consistency and reproducibility of the protein precipitation procedure used for

plasma sample preparation. Recovery was investigated at three quality control (QC) concentration levels (0.75, 18 and 48 ng/mL). For each concentration level, the analytical response obtained from plasma samples processed according to the established protein precipitation procedure was compared with the response obtained from corresponding post-extraction spiked samples prepared at the same nominal concentrations.

The protein precipitation procedure provided consistent extraction performance across the investigated concentration range. As shown in Table 4, rizatriptan showed an overall mean extraction recovery of 99.74% ± 0.37%, with a coefficient of variation (CV) of 0.37%. The individual mean recovery values were 99.56%, 100.17% and 99.50% at 0.75, 18 and 48 ng/mL, respectively, demonstrating consistent recovery across the evaluated concentration range with low variability between the QC levels.

The isotopically labeled internal standard, rizatriptan-D6, also exhibited satisfactory extraction performance, with a mean recovery of 101.63% (Table 4). The consistent recovery observed for both rizatriptan and the internal standard supports the reproducibility of the protein precipitation procedure. Overall, the recovery results demonstrated consistent extraction performance across the evaluated QC levels and met the predefined acceptance criterion of CV ≤ 15%.

Table 4. Recovery of rizatriptan at three QC concentration levels (n = 6).

QC concentration (ng/mL)	Mean recovery (%)
0.75	99.56
18	100.17
48	99.50
Overall mean ± SD (%)	99.74 ± 0.37
CV (%)	0.37
Internal standard (rizatriptan-D6)	101.63

SD, standard deviation; CV, coefficient of variation.

3.1.6. Dilution Integrity

Dilution integrity was evaluated to demonstrate the ability of the developed LC-MS/MS method to accurately and precisely quantify plasma samples containing rizatriptan at concentrations above the established calibration range. Quality control samples were prepared at 102 ng/mL, corresponding to 1.7-fold the upper limit of quantification (ULOQ) and stored under the validated storage conditions. Prior to analysis, the samples were diluted with blank human plasma at dilution factors of 1:2 and 1:20. The diluted samples were quantified against freshly prepared calibration standards and the resulting concentrations were corrected for the corresponding dilution factors.

Accurate and precise quantification was achieved following both dilution procedures. Samples diluted 20-fold yielded a mean back-calculated concentration of 97.02 ng/mL, corresponding to an RE of −4.89% and a CV of 0.75%. Following 2-

fold dilution, the mean back-calculated concentration was 112.75 ng/mL, with an RE of 10.54% and a CV of 1.08%.

These results demonstrate that plasma samples containing rizatriptan at concentrations exceeding the validated calibration range can be reliably quantified following appropriate dilution with blank human plasma, without compromising the accuracy and precision of the analytical method.

3.1.7. Stability

The stability of rizatriptan was evaluated in human plasma under conditions representative of routine bioanalytical sample handling, processing and storage. Stability assessments were performed in accordance with the principles and acceptance criteria outlined in the ICH M10 Guideline on Bioanalytical Method Validation and Study Sample Analysis [12]. The stability investigations included bench-top (short-term), freeze-thaw, autosampler, long-term frozen storage, whole-blood, stock solution, working solution and internal standard solution stability. Throughout all stability assessments, rizatriptan remained stable under the investigated storage and handling conditions. Measured concentrations consistently satisfied the predefined acceptance criteria for accuracy and precision, indicating that routine sample handling and storage did not compromise quantitative determination of the analyte. These findings further demonstrate the robustness of the developed LC-MS/MS method for routine bioanalytical applications.

Bench-top (short-term) stability

Short-term stability of rizatriptan in human plasma was evaluated at the LQC (0.75 ng/mL) and HQC (48 ng/mL) levels. Six replicates at each concentration level were thawed unassisted at room temperature and maintained under these conditions for 4 hours prior to sample preparation. Following the stability period, the samples were processed and analyzed together with freshly prepared calibration standards and QC samples. The results are summarized in Table 5.

Table 5. Results of the short-term (bench-top) stability assessment of rizatriptan in human plasma.

QC level	Nominal concentration (ng/mL)	Condition	Mean concentration ± SD (ng/mL)	RE (%)	CV (%)
LQC	0.75	After 4 h	0.730 ± 0.012	−2.73	1.67
LQC	0.75	Freshly prepared	0.738 ± 0.013	−1.58	1.77
HQC	48	After 4 h	49.415 ± 0.817	2.95	1.65
HQC	48	Freshly prepared	49.356 ± 0.422	2.83	0.85

LQC, low-quality control; HQC, high-quality control; SD, standard deviation; CV, coefficient of variation; RE, relative error.

After 4 h at room temperature, the LQC samples showed a mean concentration of 0.730 ± 0.012 ng/mL, with an RE of −2.73% and a CV of 1.67%. The corresponding freshly prepared LQC samples showed a mean concentration of 0.738 ± 0.013 ng/mL, with an RE of −1.58% and a CV of 1.77%. At the HQC level, the mean concentration after 4 h was 49.415 ± 0.817 ng/mL, corresponding to an RE

of 2.95% and a CV of 1.65%, whereas the freshly prepared HQC samples yielded a mean concentration of 49.356 ± 0.422 ng/mL, with an RE of 2.83% and a CV of 0.85%.

All results were within the predefined acceptance criteria of ±15% for accuracy and ≤15% for precision. The results obtained after 4 hours were comparable to those of the freshly prepared QC samples, demonstrating that rizatriptan was stable in human plasma for at least 4 hours at room temperature.

Autosampler stability

The stability of rizatriptan in plasma extracts was evaluated after storage in the autosampler at 10˚C for 24.5 hours. LQC and HQC samples were prepared in six replicates (n = 6) for the stability assessment. The QC samples were stored in the autosampler at 10˚C for 24.5 hours. At the end of the storage period, the stored QC samples were analyzed in the same analytical run as freshly prepared calibration standards and freshly prepared QC samples. The freshly prepared calibration standards were used to construct a new calibration curve, against which both the autosampler-stored and freshly prepared QC samples were quantified. The results are summarized in Table 6.

The freshly prepared and autosampler-stored QC samples showed similar results at both concentration levels. For the LQC, the RE and CV values were 0.65% and 1.96%, respectively, after 24.5 h in the autosampler. For the HQC, the corresponding values were 4.46% and 0.72%, respectively. All results were within the acceptance criteria of ±15% for RE and ≤15% for CV. Therefore, rizatriptan was considered stable in plasma extracts for at least 24.5 h at 10˚C.

Table 6. Autosampler stability of rizatriptan in plasma extracts at 10˚C for 24.5 h.

QC level	Nominal concentration (ng/mL)	Freshly prepared QC Mean ± SD (ng/mL)	CV (%)	RE (%)	Autosampler-stored QC Mean ± SD (ng/mL)	CV (%)	RE (%)
LQC	0.75	0.756 ± 0.006	0.78	0.87	0.755 ± 0.015	1.96	0.65
HQC	48	49.468 ± 0.507	1.03	3.06	50.140 ± 0.362	0.72	4.46

LQC, low-quality control; HQC, high-quality control; SD, standard deviation; CV, coefficient of variation; RE, relative error.

Freeze-thaw stability

The freeze-thaw stability of rizatriptan in human plasma was evaluated over three freeze-thaw cycles. QC samples at low and high concentrations were frozen at −70˚C for 24 hours and thawed at room temperature. Following complete thawing, the samples were refrozen at −70˚C for at least 12 hours. This procedure was repeated for a total of three freeze-thaw cycles. After the third cycle, the stability samples were analyzed together with freshly prepared calibration standards and QC samples. The results are presented in Table 7.

The QC samples subjected to three freeze-thaw cycles showed acceptable accuracy and precision at both concentration levels. The LQC showed a mean concentration of 0.755 ng/mL, with a CV of 1.18% and an RE of 0.66%, while the HQC showed a mean concentration of 49.075 ng/mL, with a CV of 0.69% and an RE of

2.24%. All results were within the predefined acceptance criteria (CV ≤ 15% and RE within ±15%). These findings demonstrated that rizatriptan was stable in human plasma for at least three freeze-thaw cycles at −70˚C with thawing at room temperature.

Table 7. Freeze-thaw stability of rizatriptan in human plasma.

QC level	Nominal concentration (ng/mL)	Freshly prepared QC Mean ± SD (ng/mL)	CV (%)	RE (%)	Freeze-thaw QC Mean ± SD (ng/mL)	CV (%)	RE (%)
LQC	0.75	0.767 ± 0.012	1.51	2.30	0.755 ± 0.009	1.18	0.66
HQC	48	48.798 ± 0.585	1.20	1.66	49.075 ± 0.340	0.69	2.24

LQC, low-quality control; HQC, high-quality control; SD, standard deviation; CV, coefficient of variation; RE, relative error.

Whole blood stability

Whole blood stability was evaluated after keeping low- and high-concentration QC samples in whole blood at room temperature for 2 hours. The results, including the mean concentrations, RE, and CV values for both stability and freshly prepared QC samples, are presented in Table 8. All results were within the predefined acceptance criteria, confirming that rizatriptan was stable in whole blood at room temperature for 2 hours.

Table 8. Whole blood stability of rizatriptan.

QC level	Condition	Mean concentration (ng/mL)	RE (%)	CV (%)
LQC	After 2 h	0.759 ± 0.003	1.17	0.43
LQC	Freshly prepared	0.755 ± 0.012	0.71	1.63
HQC	After 2 h	48.693 ± 0.518	1.44	1.06
HQC	Freshly prepared	48.720 ± 0.532	1.50	1.09

LQC, low-quality control; HQC, high-quality control; SD, standard deviation; CV, coefficient of variation; RE, relative error.

Long-term stability

Long-term plasma stability was evaluated after storage of low- and high-concentration QC samples at −70˚C and −20˚C for 50 days. The results for the stability and freshly prepared QC samples are presented in Table 9. All QC samples showed acceptable accuracy and precision, with RE and CV values within the predefined acceptance criteria. These findings demonstrated that rizatriptan was stable in plasma for 50 days at both −70˚C and −20˚C.

Table 9. Long-term plasma stability of rizatriptan at −70˚C and −20˚C.

QC level	Condition	Mean concentration (ng/mL)	RE (%)	CV (%)
LQC	−70˚C, 50 days	0.750 ± 0.010	−0.03	1.39
LQC	Freshly prepared	0.742 ± 0.015	−1.13	2.01
HQC	−70˚C, 50 days	48.111 ± 0.279	0.23	0.58

Continued

HQC	Freshly prepared	48.303 ± 0.216	0.63	0.45
LQC	−20˚C, 50 days	0.746 ± 0.014	−0.59	1.83
LQC	Freshly prepared	0.755 ± 0.015	0.69	1.97
HQC	−20˚C, 50 days	49.091 ± 0.675	2.27	1.38
HQC	Freshly prepared	49.111 ± 0.131	2.32	0.27

LQC, low-quality control; HQC, high-quality control; SD, standard deviation; CV, coefficient of variation; RE, relative error.

Stability in stock and working solutions

The stability of rizatriptan stock and working solutions and the internal standard stock solution was investigated under storage conditions applicable to routine analytical procedures. Short-term stability was evaluated following storage at room temperature for 4.5 hours, whereas long-term stability was assessed after storage at −20˚C for 7 days for the rizatriptan stock and working solutions and for 6 days for the internal standard stock solution. The analytical responses obtained after storage were compared with those of freshly prepared solutions.

No significant changes in analytical response were observed following either short-term or long-term storage. After storage at room temperature, the RE values were 1.024% for the rizatriptan stock solution, 1.755% for the working solution and −0.212% for the internal standard stock solution. Following storage at −20˚C, the corresponding RE values were −1.633%, 0.070% and 6.438%, respectively. All results were within the predefined acceptance criteria for solution stability. These findings demonstrate that rizatriptan stock and working solutions and the internal standard solution remain stable under the investigated storage conditions, supporting their suitability for routine LC-MS/MS analysis.

4. Conclusion

A rapid, sensitive, selective, accurate and precise LC-MS/MS method was successfully developed and comprehensively validated for the quantitative determination of rizatriptan in human plasma using a simple protein precipitation procedure. The method demonstrated satisfactory selectivity, linearity, accuracy, precision, extraction recovery, matrix-related robustness, dilution integrity and stability with all evaluated validation parameters meeting the predefined acceptance criteria specified in the ICH M10 guideline [12]. The use of a simple protein precipitation procedure and a short chromatographic run time of 2.5 min enables efficient sample processing and high analytical throughput. The method was successfully applied to plasma samples obtained from a bioequivalence study, further demonstrating its suitability for routine bioanalytical, pharmacokinetic and bioequivalence applications.

Author Contributions

Nihal Saraner: Conceptualization, methodology, method development, valida-

tion, investigation, data collection, formal analysis, interpretation of results, original manuscript preparation, manuscript review and editing and project administration. **Ayşen Bakıcı**: Investigation, experimental work and data collection. **Barış Şenceol**: Manuscript review and editing. **Berrak Güney**: Data verification, data review and validation of the reported results. **Onursal Sağlam**: Manuscript review and scientific evaluation. All authors reviewed and approved the final version of the manuscript and accept responsibility for the integrity and accuracy of the work.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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