

Determination of Ozanimod in Human Plasma by Using Liquid Chromatography-Tandem Mass Spectrometry

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Abstract

A rapid, sensitive and reliable liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed and validated for the quantitative determination of ozanimod in human plasma. Plasma samples were prepared using protein precipitation with acetonitrile followed by evaporation and reconstitution. Chromatographic separation was achieved on an InertSustain AQ-C18 column (3 μ m, 4.6 $\times$ 75 mm) using gradient elution at a flow rate of 1.2 mL/min. Detection was performed using electrospray ionization in positive-ion mode with multiple reaction monitoring (MRM). The monitored transitions were m/z 405.2 $\rightarrow$ 146.0 for ozanimod and m/z 411.1 $\rightarrow$ 147.05 for ozanimod-D6, used as the internal standard. The total chromatographic run time was 4.5 min, with mass spectrometric data acquisition performed between 2.1 and 3.51 min. The method exhibited linearity over the concentration range of 5 - 600 pg/mL using a weighted (1/C²) linear regression model. The method was validated with respect to selectivity, linearity, accuracy, precision, recovery, matrix effect and stability in accordance with applicable bioanalytical validation principles. The developed method provides a simple and rapid analytical approach for the sensitive quantification of ozanimod in human plasma and is suitable for application in pharmacokinetic and bioequivalence studies.

Keywords

Ozanimod, Ozanimod-D6, Human Plasma, LC-MS/MS, Bioanalytical Method Validation

1. Introduction

Ozanimod is an orally administered sphingosine-1-phosphate (S1P) receptor mod-

ulator that selectively targets the S1P₁ and S1P₅ receptor subtypes. Through modulation of S1P₁ receptor signaling, ozanimod promotes the retention of lymphocytes within lymphoid tissues, thereby reducing lymphocyte circulation in peripheral blood. This mechanism contributes to the therapeutic efficacy of ozanimod in immune-mediated inflammatory diseases. Ozanimod has been approved for the treatment of relapsing forms of multiple sclerosis and moderately to severely active ulcerative colitis [1] [2].

Following oral administration, ozanimod undergoes extensive metabolism and forms several circulating metabolites, including the major active metabolites CC112273 and CC1084037. The U.S. Food and Drug Administration (FDA) has identified ozanimod, CC112273 and CC1084037 as the major active moieties contributing to the pharmacological activity of ozanimod. According to the FDA clinical pharmacology assessment, approximately 6%, 73% and 15% of circulating total active drug exposure is represented by ozanimod, CC112273 and CC1084037, respectively [2]. These findings demonstrate the substantial contribution of the active metabolites to systemic exposure and highlight their importance in the overall pharmacokinetic and pharmacological profile of ozanimod. The pharmacokinetic profile of ozanimod following oral administration has been characterized in clinical studies [3]. Furthermore, ozanimod has a considerably shorter terminal half-life than its major active metabolites, which exhibit prolonged systemic persistence following repeated administration. The prolonged half-lives of these metabolites may contribute to their accumulation and sustained contribution to overall active systemic exposure [2] [4]. Nevertheless, direct quantification of the parent drug remains relevant for the characterization of ozanimod exposure and for analytical applications specifically focused on the administered parent compound. Therefore, sensitive and reliable bioanalytical methods for the determination of ozanimod in human plasma remain important in pharmacokinetic, clinical pharmacology and bioequivalence studies [3] [4].

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been employed for the quantitative determination of ozanimod in human plasma owing to its high sensitivity and selectivity. In the first-in-human study of ozanimod, plasma concentrations were determined using a validated LC-MS/MS method following protein precipitation with acetonitrile containing 0.1% formic acid [3]. In subsequent pharmacokinetic studies, validated LC-MS/MS methods were developed for the determination of ozanimod and its major active metabolites using different sample preparation approaches, including supported liquid extraction [5]. Although these methods enabled sensitive and reliable quantification, simplified sample preparation and reduced analysis time may offer practical advantages for bioanalytical studies involving large numbers of plasma samples.

Sample preparation is an important component of bioanalytical method development because it can influence analyte recovery, matrix effects, sensitivity and overall analytical reproducibility. Protein precipitation using acetonitrile is a simple and rapid approach for plasma sample preparation and requires fewer pro-

cessing steps than extraction techniques such as solid-phase extraction or support-liquid extraction. When combined with appropriate chromatographic and mass spectrometric conditions, protein precipitation can provide an efficient and practical approach for the routine analysis of pharmaceutical compounds in biological matrices.

The selection of an appropriate internal standard is also critical for reliable quantitative analysis by LC-MS/MS. Stable isotope-labeled internal standards are particularly advantageous because their close structural similarity to the analyte enables them to closely track the analyte during sample preparation, chromatographic separation, and mass spectrometric analysis. This approach can help compensate for variability associated with sample handling, matrix effects and instrumental response, thereby improving the reliability and reproducibility of quantitative measurements [6] [7]. In the present study, ozanimod-D6 was selected as the internal standard. Its close structural similarity to ozanimod allows comparable behavior during sample preparation and chromatographic separation, while the mass difference enables selective discrimination by tandem mass spectrometry.

In the present study, a rapid, sensitive and practical LC-MS/MS method was developed and validated for the quantitative determination of ozanimod in human plasma. A simple acetonitrile-based protein precipitation procedure was employed using a limited plasma volume, with ozanimod-D6 as the stable isotope-labeled internal standard. Chromatographic separation was achieved on an Inert-Sustain AQ-C18 column using a short gradient program and detection was performed by electrospray ionization in positive-ion mode with multiple reaction monitoring (MRM). The method provided quantitative determination over the concentration range of 5 - 600 pg/mL using a weighted ($1/C^2$) calibration model, with a total chromatographic run time of 4.5 min. The developed method was validated in accordance with applicable bioanalytical validation criteria and demonstrated to be applicable to the analysis of human plasma samples in bioequivalence studies.

2. Experimental Section

2.1. Chemicals

Ozanimod HCl (purity 99.60%) and ozanimod D6 (purity 99.77%), used as the internal standard (IS), were supplied by SimSon Pharma Limited (India). Methanol, acetonitrile and formic acid (HPLC-grade) were purchased from Merck (Darmstadt, Germany), while dimethyl sulfoxide (DMSO) was obtained from Carlo Erba (Italy). Paracetamol and ibuprofen were purchased from BioOrganics & Applied Materials Pvt. Ltd. (Bangalore, India). Blank human plasma containing K_2EDTA was obtained from Bioivt (UK). Ultrapure water used for the preparation of mobile phases and solutions was obtained using a Milli-Q water purification system (Millipore, USA).

2.2. Stock Solutions, Calibration Standards and QCs

Ozanimod (OZA) and ozanimod-D6 (OZA-D6, used as the internal standard [IS]) were used for the preparation of calibration standards and quality-control (QC) samples. Ozanimod was supplied as the hydrochloride salt (ozanimod HCl; purity, 99.60%). The primary OZA stock solution was prepared at a nominal concentration of 1 mg/mL, expressed as ozanimod free-base equivalent. The required amount of ozanimod HCl reference standard was accurately weighed, with correction for both the molecular-weight difference between ozanimod HCl and ozanimod free base and the assigned reference-standard purity. The weighed reference standard was dissolved in 1 mL of dimethyl sulfoxide (DMSO) and diluted to volume with methanol. The resulting stock solution corresponded to 1 mg/mL of ozanimod free-base equivalent.

A primary ozanimod-D6 (OZA-D6) stock solution was prepared at a nominal concentration of 0.2 mg/mL, with correction for the assigned reference-standard purity (99.77%). The required amount of OZA-D6 reference standard was accurately weighed, dissolved in 0.5 mL of dimethyl sulfoxide (DMSO) and diluted with methanol to the required final volume. The resulting stock solution corresponded to a nominal concentration of 0.2 mg/mL after correction for reference-standard purity. The OZA-D6 stock solution was stored at -20°C when not in use.

All stock and working solutions were protected from light and stored at -20°C when not in use. All OZA calibration-standard and QC concentrations reported throughout the manuscript represent ozanimod free-base-equivalent concentrations.

Calibration standards were prepared by spiking blank human K_2EDTA plasma with appropriate volumes of the OZA calibration working solutions to obtain concentrations of 5, 10, 40, 80, 120, 240, 420 and 600 pg/mL. The QC samples were prepared independently at 5 pg/mL (LLOQ), 15 pg/mL (LQC), 50 pg/mL (MQC 1), 180 pg/mL (MQC 2) and 480 pg/mL (HQC). In addition, a dilution QC sample was prepared at 1020 pg/mL to assess the accuracy and precision of diluted samples. Calibration standards and QC samples were prepared freshly as required and stored at -70°C until analysis. For stability assessments, aliquots of the LQC and HQC samples were prepared separately and stored at -70°C and -20°C until analysis.

2.3. Instrumentation

Quantitative analysis of ozanimod was performed using a Shimadzu LCMS-8060 triple quadrupole mass spectrometer coupled to an LC-40D XR liquid chromatography system equipped with a DGU-403 degasser, SIL-40C XR autosampler, CTO-40S column oven and FCV-0206 valve unit. Instrument control, data acquisition and processing were performed using Shimadzu LabSolutions software.

Chromatographic separation was achieved on a GL Sciences InertSustain AQ-C18 analytical column ($3\text{ }\mu\text{m}$, $4.6 \times 75\text{ mm}$) maintained at 40°C . The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (acetonitrile).

The initial mobile phase composition was 60% A and 40% B, with a flow rate of 1.2 mL/min. The mobile phase composition was then changed to 0% A/100% B at 3.51 min. The column was subsequently flushed with 100% B until 4.30 min, after which the initial mobile-phase composition was restored at 4.31 min. The total chromatographic run time was 4.50 min. The autosampler temperature was maintained at 10°C. Ozanimod and ozanimod-D6 were detected using electrospray ionization in positive-ion mode (ESI+) and multiple reaction monitoring (MRM). MRM acquisition was performed over the 2.10 - 3.51 min interval for both analytes. The optimized MRM transitions were m/z 405.20 $\rightarrow$ 146.00 for ozanimod and m/z 411.10 $\rightarrow$ 147.05 for ozanimod-D6. The optimized chromatographic and mass spectrometric conditions are summarized in **Table 1** and **Table 2**.

Table 1. Mass spectrometric conditions for the determination of ozanimod and ozanimod-D6.

Parameter	Ozanimod	Ozanimod-D6
Ionization mode	ESI+	ESI+
MRM acquisition time (min)	2.10 - 3.51	2.10 - 3.51
MRM transition (m/z)	405.20 $\rightarrow$ 146.00	411.10 $\rightarrow$ 147.05
Dwell time (ms)	100	100
Q1 pre-bias (V)	-30.0	-30.0
Collision energy (V)	-27.0	-32.0
Q3 pre-bias (V)	-15.0	-14.0
Common source parameters		
Nebulizing gas flow	3 L/min	
Heating gas flow	10 L/min	
Drying gas flow	10 L/min	
Interface temperature	300°C	
Desolvation temperature	400°C	
Heat Block temperature	526°C	
DL temperature	250°C	

Table 2. Chromatographic conditions for ozanimod analysis.

Parameter	Condition
LC system	Shimadzu LC-40D XR
Analytical column	GL Sciences InertSustain AQ-C18, 3 μ m, 4.6 $\times$ 75 mm
Column temperature	40°C
Autosampler temperature	10°C
Mobile phase A	0.1% formic acid in water
Mobile phase B	Acetonitrile
Flow rate	1.2 mL/min
Injection volume	20 μ L

Continued

Total run time	4.50 min	
Initial composition	60% A/40% B	
Divert valve to MS	2.00 - 3.50 min	
Gradient program		
Time (min)	A (%)	B (%)
0.00	60	40
3.50	60	40
3.51	0	100
4.30	0	100
4.31	60	40

2.4. Sample Preparation

Plasma samples were prepared using protein precipitation followed by evaporation and reconstitution. Briefly, 200 μ L of blank plasma, calibration standards and QC samples were transferred into appropriately labeled polypropylene tubes. Subsequently, 50 μ L of the ozanimod-D6 internal standard working solution (0.5 ng/mL) was added to all samples except matrix blanks, to which 50 μ L of methanol was added. The samples were vortex-mixed for 5 s, followed by the addition of 300 μ L of acetonitrile for protein precipitation. After vortex mixing, the samples were centrifuged at 5500 rpm for 10 min. Following centrifugation, the supernatant was transferred to clean tubes and evaporated to dryness at 50°C under a gentle stream of nitrogen. The dried extracts were reconstituted with 200 μ L of a 50:50 (v/v) mixture of 0.1% formic acid in water and acetonitrile and vortex-mixed for 60 s. The resulting extracts were transferred to an appropriate collection plate and 20 μ L was injected into the LC-MS/MS system for analysis.

3. Results and Discussion**3.1. Method Validation**

The developed LC-MS/MS method was validated in human plasma to demonstrate its suitability for the quantitative determination of ozanimod. The validation procedure included the assessment of selectivity, linearity, sensitivity, accuracy, precision, recovery, matrix effect, dilution integrity, carry-over and stability. Stability was investigated under bench-top, freeze-thaw, autosampler, long-term, whole-blood, stock solution and working solution conditions. The method demonstrated acceptable performance for all evaluated validation parameters and met the pre-defined acceptance criteria.

3.1.1. Specificity and Selectivity

Selectivity was evaluated using blank human plasma obtained from eight independent sources, including haemolysed and hyperlipidemic plasma. The blank plasma samples were processed and analyzed to assess potential interference at the reten-

tion times of ozanimod and ozanimod-D6. No relevant interfering signals were observed in the evaluated blank plasma samples.

Potential interference between ozanimod, ozanimod-D6, paracetamol and ibuprofen was investigated using separately prepared samples. Ozanimod was evaluated at the QC4 concentration (480 pg/mL) without the internal standard, while ozanimod-D6 was evaluated separately at 0.5 ng/mL without ozanimod. Paracetamol and ibuprofen were each evaluated at 10 µg/mL in plasma. LLOQ samples and the interference test samples were prepared separately and analyzed in triplicate ($n = 3$).

The retention times of ozanimod and ozanimod-D6 were approximately 2.91 and 2.87 min, respectively. For the evaluation of blank plasma interference, the response at the retention time of ozanimod was required to be below 20% of the ozanimod response at the LLOQ, while the response at the retention time of ozanimod-D6 was required to be below 5% of the corresponding internal standard response. For the interference assessment involving the analyte, internal standard and selected concomitant medications, no relevant responses were observed at the respective retention times. All results complied with the predefined acceptance criteria. Representative MRM chromatograms of blank human plasma and plasma spiked with ozanimod at the LLOQ are presented in **Figure 1**.

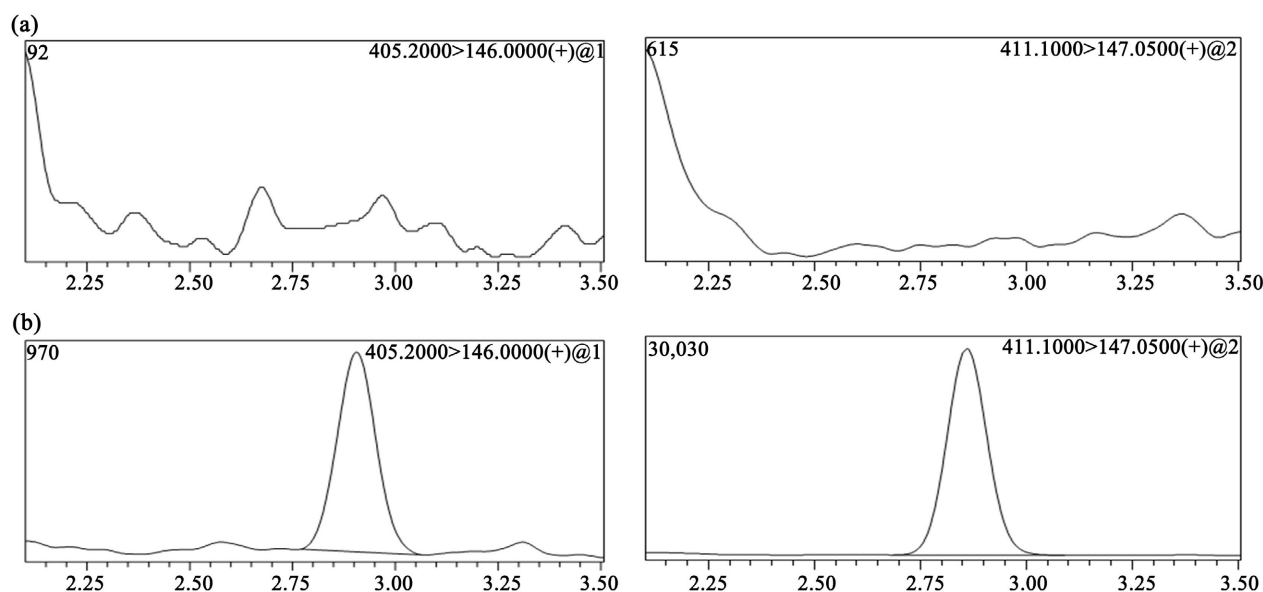


Figure 1. MRM chromatograms of human blank plasma (a), 5 pg/mL ozanimod spiked with internal standard (b).

3.1.2. Carry-Over

Carry-over was evaluated within each validation batch by analyzing a processed blank plasma sample immediately after the ULOQ calibration standard. The blank sample was examined for residual responses at the retention times of ozanimod and ozanimod-D6. No relevant residual response was detected for either the analyte or the internal standard following injection of the ULOQ sample. The observed responses in the post-ULOQ blank samples remained below 20% of the ozanimod

response at the LLOQ and below 5% of the corresponding ozanimod-D6 response. The predefined acceptance criteria were therefore met, indicating that carry-over did not affect the quantitative analysis.

3.1.3. Linearity

The linearity of the developed LC-MS/MS method was evaluated over the concentration range of 5 - 600 pg/mL using eight calibration levels: 5, 10, 40, 80, 120, 240, 420 and 600 pg/mL. Calibration standards were prepared in human plasma and analyzed in each validation batch. Quantification was based on the peak-area ratio of ozanimod to the internal standard, ozanimod-D6, plotted against the nominal concentration of ozanimod.

A weighted linear regression model with a weighting factor of $1/C^2$ was used for the calibration curve. The suitability of the calibration model was assessed by comparing the back-calculated concentrations with the nominal concentrations and by evaluating the residuals across the calibration range.

The calibration standards met the predefined acceptance criteria in all validation batches. The selected calibration model showed an appropriate linear relationship between the nominal and back-calculated concentrations over the validated range of 5 - 600 pg/mL.

The results of the calibration standards are presented in **Table 3** and a representative calibration curve is shown in **Figure 2**. Based on these results, the calibration model was considered suitable for the quantitative determination of ozanimod in human plasma over the concentration range of 5 - 600 pg/mL.

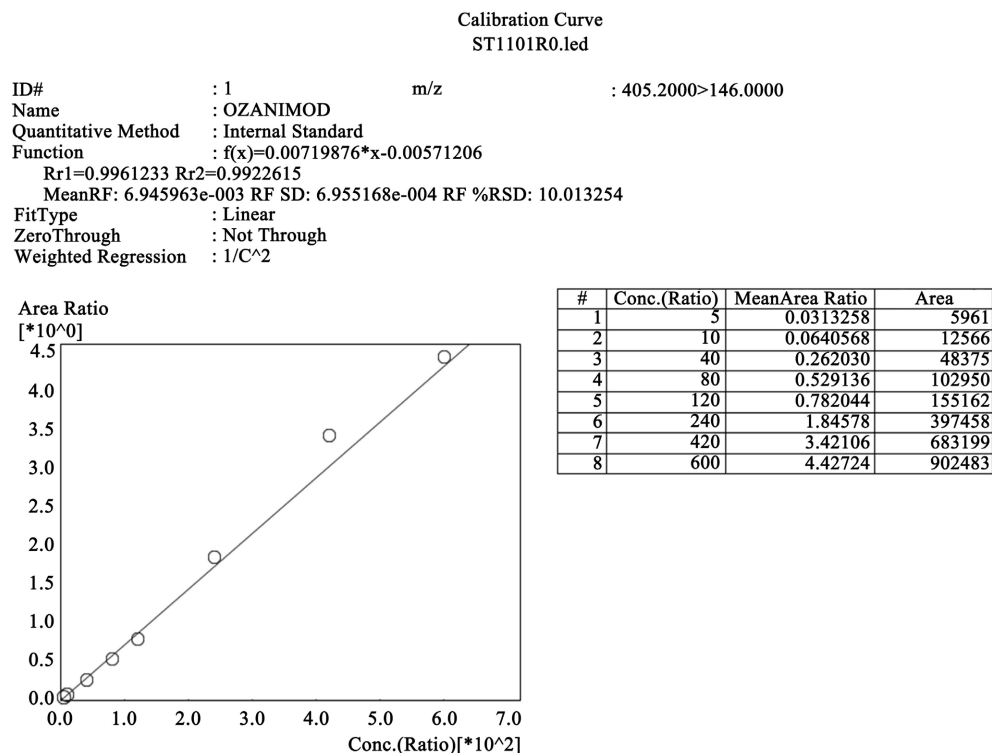


Figure 2. Calibration curve for ozanimod.

Table 3. Results and regression parameters of the calibration curve for linearity.

Concentration (pg/mL)	Batch 1		Batch 2		Batch 3		Concentration (mean $\pm$ SD; pg/mL)	CV (%)	RE (%)
	Conc. (pg/mL)	RE (%)	Conc. (pg/mL)	RE (%)	Conc. (pg/mL)	RE (%)			
5	5.145	2.901	5.032	0.647	4.965	-0.695	5.048 $\pm$ 0.091	1.800	0.951
10	9.692	-3.082	10.179	1.792	10.502	5.017	10.124 $\pm$ 0.408	4.027	1.242
40	37.193	-7.018	36.983	-7.543	36.275	-9.312	36.817 $\pm$ 0.481	1.306	-7.958
80	74.297	-7.128	72.931	-8.837	71.302	-10.872	72.843 $\pm$ 1.500	2.059	-8.946
120	109.430	-8.809	108.828	-9.310	110.835	-7.638	109.697 $\pm$ 1.030	0.939	-8.586
240	257.196	7.165	261.659	9.024	264.359	10.150	261.071 $\pm$ 3.618	1.386	8.780
420	476.023	13.339	459.647	9.440	450.470	7.255	462.047 $\pm$ 12.945	2.802	10.011
600	615.794	2.632	628.721	4.787	636.578	6.096	627.031 $\pm$ 10.495	1.674	4.505

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

3.1.4. Accuracy and Precision

Accuracy and precision were evaluated using QC samples at five concentration levels (5, 15, 50, 180 and 480 pg/mL), with six replicates analyzed in each of three independent validation batches. Quantification was performed using a weighted ($1/C^2$) linear regression model based on the peak-area ratio of ozanimod to the internal standard, ozanimod-D6.

Table 4. Within-batch and between-batch precision and accuracy of the method for determining ozanimod in plasma samples.

Nominal concentration (pg/mL)		Intra-batch (n = 6)			Sample	Inter-batch (n = 18)		
		Concentration (mean $\pm$ SD; pg/mL)	RE (%)	CV (%)		Concentration (mean $\pm$ SD; pg/mL)	RE (%)	CV (%)
5	Batch01	5.172 $\pm$ 0.142	3.45	2.75	LLOQ	5.169 $\pm$ 0.242	3.37	4.69
	Batch02	5.280 $\pm$ 0.295	5.59	5.58				
	Batch03	5.054 $\pm$ 0.249	1.07	4.94				
15	Batch01	15.339 $\pm$ 0.525	2.26	3.42	LQC	15.192 $\pm$ 0.524	1.28	3.45
	Batch02	15.510 $\pm$ 0.325	3.40	2.10				
	Batch03	14.726 $\pm$ 0.381	-1.83	2.59				
50	Batch01	48.794 $\pm$ 0.585	-2.41	1.20	QCM 1	49.201 $\pm$ 0.879	-1.60	1.79
	Batch02	50.087 $\pm$ 0.601	0.17	1.20				
	Batch03	48.723 $\pm$ 0.713	-2.55	1.46				
180	Batch01	179.085 $\pm$ 2.368	-0.51	1.32	QCM 2	179.340 $\pm$ 1.976	-0.37	1.10
	Batch02	179.858 $\pm$ 1.522	-0.08	0.85				
	Batch03	179.080 $\pm$ 2.207	-0.51	1.23				
480	Batch01	447.020 $\pm$ 7.607	-6.87	1.70	HQC	447.144 $\pm$ 7.655	-6.85	1.71
	Batch02	441.831 $\pm$ 7.0852	-7.95	1.60				
	Batch03	452.579 $\pm$ 4.685	-5.71	1.03				

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

The accuracy and precision results are presented in **Table 4**. Across the three validation batches, the RE values ranged from -7.95% to 5.59% , while the CV values ranged from 1.03% to 5.58% . When data from all three batches were combined, the overall RE% ranged from -6.85% to 3.37% , and the CV% ranged from 1.10% to 4.69% across the tested concentration levels.

All QC samples met the predefined acceptance criteria for accuracy and precision, confirming the suitability of the method for the quantification of ozanimod in human plasma.

3.1.5. Matrix Effect and Plasma Matrix Variability

The potential impact of different plasma matrix sources on the quantitative determination of ozanimod was evaluated in accordance with the requirements of ICH M10. Eight independent plasma matrix batches were investigated. Low-quality control (LQC) and high-quality control (HQC) samples at nominal concentrations of 15 and 480 pg/mL, respectively, were prepared using each matrix batch and analyzed in quadruplicate.

At the LQC level (15 pg/mL), the mean calculated concentrations across the individual matrix batches ranged from 14.920 to 16.556 pg/mL. The corresponding relative error (RE) values ranged from -0.531% to 10.376% , while the coefficient of variation (CV) values ranged from 1.576% to 11.035% .

At the HQC level (480 pg/mL), the mean calculated concentrations across the individual matrix batches ranged from 445.892 to 456.094 pg/mL. The corresponding RE values ranged from -7.106% to -4.980% , while the CV values ranged from 0.539% to 1.771% .

For all eight individual plasma matrix batches, the accuracy results at both QC levels were within the predefined acceptance criterion of $\pm 15\%$ and the precision results were within the acceptance criterion of $\leq 15\%$ CV (**Table 5**). No individual matrix batch showed an unacceptable effect on the accuracy or precision of ozanimod quantification.

Table 5. Accuracy and precision of ozanimod in different human plasma matrix sources for matrix-effect evaluation.

Matrix source	LQC mean (pg/mL)	LQC RE (%)	LQC CV (%)	HQC mean (pg/mL)	HQC RE (%)	HQC CV (%)
Haemolysed plasma	15.030	0.197	1.576	452.576	-5.713	0.949
Lipaemic plasma	14.920	-0.531	2.372	456.094	-4.980	1.771
Plasma source 1	15.420	2.803	1.669	449.841	-6.283	0.683
Plasma source 2	16.556	10.376	11.035	447.186	-6.836	1.315
Plasma source 3	15.175	1.165	2.608	455.097	-5.188	1.646
Plasma source 4	15.220	1.466	3.303	449.956	-6.259	0.865
Plasma source 5	15.572	3.810	3.353	454.425	-5.328	0.539
Plasma source 6	15.360	2.398	5.164	445.892	-7.106	1.021

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

Overall, the results demonstrated that variability among the investigated plasma matrix batches did not adversely affect the accuracy or precision of ozanimod determination. These results indicated that the investigated variability among plasma matrix sources did not adversely affect the accuracy and precision of ozanimod determination.

3.1.6. Recovery

Recovery was evaluated using quality control (QC) samples at three concentration levels (15, 180 and 480 pg/mL), with six replicates at each concentration level ($n = 6$). Recovery was calculated by comparing the analytical response of pre-extraction spiked QC samples with that of corresponding post-extraction spiked reference samples. The pre-extraction samples were prepared by spiking ozanimod into blank human plasma prior to extraction, whereas the post-extraction reference samples were prepared by adding ozanimod to blank plasma extracts after completion of the extraction procedure. The mean recovery rates of ozanimod were 101.085%, 101.128% and 98.441% at 15, 180 and 480 pg/mL, respectively. The overall mean recovery across the three QC concentration levels was 100.218%, with a standard deviation (SD) of 1.539% and a coefficient of variation (CV) of 1.535%. The mean recovery of ozanimod-D6 was 99.54%. The recovery results are presented in **Table 6**. The observed variability in recovery was within the predefined acceptance criterion of $\leq 15\%$. These results demonstrated consistent and reproducible recovery of ozanimod from human plasma over the investigated concentration range.

Table 6. Recovery of ozanimod at three QC concentration levels ($n = 6$).

QC concentration (pg/mL)	Mean recovery (%)
15	101.085
180	101.128
480	98.441
Overall mean $\pm$ SD (%)	100.218 $\pm$ 1.539
CV (%)	1.535
Internal standard (ozanimod-D6)	99.536

SD, standard deviation; CV, coefficient of variation.

3.1.7. Dilution Integrity

To verify the performance of the validated LC-MS/MS assay for biological samples with analyte concentrations exceeding the upper limit of quantification (ULOQ), dilution integrity was systematically evaluated using matrix-matched dilutions. Quality control samples spiked at a nominal concentration of 1020 pg/mL, which exceeded the calibration range, were subjected to 2-fold and 20-fold dilutions ($n = 6$) with blank human plasma prior to extraction. The back-calculated concentrations were corrected for the corresponding dilution factors to assess method accuracy and precision.

For the 20-fold dilution, the mean back-calculated concentration was 1121.18 pg/mL, corresponding to a relative error (RE) of 9.92% and a coefficient of variation (CV) of 2.59%. For the 2-fold dilution, the mean concentration was 1080.21 pg/mL, with an RE of 5.90% and a CV of 1.47%. Both dilution levels met the pre-defined bioanalytical acceptance criteria of $\pm 15\%$ for accuracy and precision. These results demonstrate that the method maintains acceptable accuracy and precision following dilution of plasma samples with blank human plasma.

3.1.8. Stability

The stability of ozanimod in stock solutions, working solutions, human plasma, whole blood and processed samples was evaluated under conditions representative of routine bioanalytical sample handling, processing and storage. The stability experiments included short-term and long-term stability of stock and working solutions, dry extract stability, autosampler stability, freeze-thaw stability, short-term (bench-top) stability, whole-blood stability and long-term plasma stability. All stability assessments were performed using freshly prepared calibration standards and quality-control (QC) samples, where applicable. The stability studies were conducted in accordance with the principles and recommendations of the ICH M10 Guideline on Bioanalytical Method Validation and Study Sample Analysis [8]. The acceptance criteria were predefined in accordance with bioanalytical validation requirements. Accuracy was assessed based on relative error (RE) within $\pm 15\%$, while precision was assessed based on a coefficient of variation (CV) not exceeding 15%; for the LLOQ, an RE within $\pm 20\%$ was accepted. For calibration standards, back-calculated concentrations were required to be within $\pm 15\%$ of the nominal values, except for the LLOQ, for which $\pm 20\%$ was accepted [8].

Stability in Stock and Working Solutions

The stability of ozanimod stock and working solutions and the ozanimod-D6 internal standard solution was evaluated under storage conditions applicable to routine analytical procedures. Short-term stability was assessed following storage at room temperature for 5 hours, while long-term stability was evaluated after storage at -20°C for 9 days. 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 -0.318% for the ozanimod stock solution, 0.190% for the working solution and 0.338% for the ozanimod-D6 internal standard stock solution. Following storage at -20°C for 9 days, the corresponding RE values were -0.909% , -2.107% and -1.148% , respectively. All results were within the predefined acceptance criteria. These findings demonstrate that ozanimod stock and working solutions and the ozanimod-D6 internal standard solution remained stable under the investigated storage conditions.

Dry Extract Stability

The stability of ozanimod in dry plasma extracts was evaluated using LQC and HQC samples. The processed QC samples were stored as dry extracts at -70°C for

24.5 hours. Following the storage period, the samples were reconstituted and analyzed with freshly spiked calibration standards. The stability samples were evaluated in terms of accuracy and precision.

The mean RE values were 0.175% and 1.790% for the LQC and HQC samples, respectively, while the corresponding CV values were 3.903% and 1.995% (Table 7). All individual QC results and mean accuracy and precision values were within the predefined acceptance criteria. Therefore, ozanimod was considered stable in dry plasma extracts at -70°C for 24.5 hours.

Table 7. Accuracy and precision results of QC samples for dry extract stability.

QC level	Nominal concentration (pg/mL)	Condition	Mean concentration $\pm$ SD (pg/mL)	RE (%)	CV (%)
LQC	15	After 24.5 h	15.026 $\pm$ 0.586	0.175	3.903
HQC	480	After 24.5 h	488.593 $\pm$ 9.749	1.790	1.995

Bench-top (Short-term) Stability

Short-term stability of ozanimod in human plasma was evaluated at the LQC (15 pg/mL) and HQC (480 pg/mL) levels. Six replicates at each concentration level were allowed to thaw unassisted at room temperature and were maintained under these conditions for 5 h prior to sample preparation. Following the stability period, the samples were processed and analyzed in the same analytical run with freshly prepared calibration standards and QC samples.

After 5 h at room temperature, both LQC and HQC samples met the predefined acceptance criteria for accuracy and precision. The results were comparable to those obtained from freshly prepared QC samples, demonstrating that ozanimod was stable in human plasma for at least 5 h at room temperature (Table 8).

Table 8. Results of the short-term (bench-top) stability assessment of ozanimod in human plasma.

QC level	Nominal concentration (pg/mL)	Condition	Mean concentration $\pm$ SD (pg/mL)	RE (%)	CV (%)
LQC	15	After 5 h	15.971 $\pm$ 0.351	6.472	2.196
LQC	15	Freshly prepared	15.778 $\pm$ 0.302	5.184	1.916
HQC	480	After 5 h	436.432 $\pm$ 3.305	−9.077	0.757
HQC	480	Freshly prepared	429.664 $\pm$ 7.001	−10.487	1.630

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

Autosampler Stability

The stability of the analyte in plasma extracts was evaluated after storage in the autosampler at 10°C for 27 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 27 hours. At the end of the storage period, the autosampler-stored QC samples and freshly prepared QC samples were analyzed in the same analytical run using freshly prepared calibration standards. 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 9**.

The freshly prepared and autosampler-stored QC samples showed comparable results at both concentration levels. For the LQC, the RE and CV values were 3.68% and 4.24%, respectively, after 27 h in the autosampler. For the HQC, the corresponding values were -4.73% and 1.61% , respectively. The freshly prepared LQC and HQC samples showed RE values of -0.40% and -5.81% , and CV values of 2.93% and 1.21% , respectively.

All results were within the acceptance criteria of $\pm 15\%$ for RE and $\leq 15\%$ for CV. Therefore, the analyte was considered stable in plasma extracts for at least 27 h at 10°C .

Table 9. Autosampler stability of ozanimod in plasma extracts at 10°C for 27 h.

QC level	Nominal concentration (pg/mL)	Freshly prepared QC mean $\pm$ SD (pg/mL)	CV (%)	RE (%)	Autosampler-stored QC mean $\pm$ SD (pg/mL)	CV (%)	RE (%)
LQC	15	14.939 ± 0.438	2.93	-0.40	15.552 ± 0.659	4.24	3.68
HQC	480	452.124 ± 5.480	1.21	-5.81	457.290 ± 7.375	1.61	-4.73

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 ozanimod in human plasma was evaluated over four freeze-thaw cycles at the LQC (15 pg/mL) and HQC (480 pg/mL) levels. Six replicates at each concentration level were stored at -70°C for 24 hours and then allowed to thaw unassisted 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 four freeze-thaw cycles. After completion of the fourth freeze-thaw cycle, the stability samples were processed and analyzed in the same analytical run with freshly prepared calibration standards and freshly prepared QC samples. The results are summarized in **Table 10**.

The freeze-thaw and freshly prepared QC samples showed comparable results at both concentration levels. For the LQC, the freeze-thaw samples showed a mean concentration of 15.316 pg/mL , with a CV of 3.39% and an RE of 2.10% , compared with 15.244 pg/mL , 2.42% and 1.63% , respectively, for the freshly prepared QC samples. For the HQC, the corresponding values for the freeze-thaw samples were 426.741 pg/mL , 1.69% and -11.10% , whereas the freshly prepared QC samples showed a mean concentration of 425.976 pg/mL , a CV of 2.93% , and an RE of -11.26% .

All individual QC results met the predefined acceptance criteria of $\text{CV} \leq 15\%$

and RE within $\pm 15\%$ and the RE of the mean concentration at each QC level was also within $\pm 15\%$. Therefore, ozanimod was considered stable in human plasma for at least four freeze-thaw cycles when stored at -70°C and thawed at room temperature.

Table 10. Freeze-thaw stability of ozanimod in human plasma.

QC level	Nominal concentration (pg/mL)	Freshly prepared QC mean $\pm$ SD (pg/mL)	CV (%)	RE (%)	Freeze-thaw QC mean $\pm$ SD (pg/mL)	CV (%)	RE (%)
LQC	15	15.244 $\pm$ 0.369	2.42	1.63	15.316 $\pm$ 0.520	3.39	2.10
HQC	480	425.976 $\pm$ 12.491	2.93	-11.26	426.741 $\pm$ 7.229	1.69	-11.10

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 to assess potential analyte instability during the period between blood collection and plasma separation. Whole blood stability of ozanimod was investigated at the LQC (15 pg/mL) and HQC (480 pg/mL) levels following exposure to room temperature for 2 h. Six replicates of each QC level were prepared by spiking ozanimod into whole blood and maintained under the specified conditions. At the end of the exposure period, the samples were centrifuged and the resulting plasma was processed according to the validated sample preparation procedure. For comparison, freshly prepared QC samples at the corresponding LQC and HQC concentrations were prepared independently in plasma and processed in parallel with the whole-blood stability samples. Stability samples and freshly prepared QC samples were analyzed within the same analytical run using freshly prepared calibration standards. The results are summarized in **Table 11**.

Table 11. Whole blood stability of ozanimod.

QC level	Condition	Mean concentration (pg/mL)	RE (%)	CV (%)
LQC	After 2 h	14.967 $\pm$ 0.538	-0.22	3.60
LQC	Freshly prepared	15.610 $\pm$ 0.468	4.06	3.00
HQC	After 2 h	442.081 $\pm$ 10.556	-7.90	2.39
HQC	Freshly prepared	449.255 $\pm$ 4.976	-6.41	1.11

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

The obtained results showed acceptable accuracy and precision for both QC levels after 2 h at room temperature. The RE and CV values were within the predefined acceptance limits and the results were comparable to those obtained for the freshly prepared QC samples. Under the conditions investigated, ozanimod was considered stable in whole blood for 2 h at room temperature.

Long-term Stability

Long-term stability of ozanimod in human plasma was evaluated at the LQC and HQC levels after storage at -70°C and -20°C for 40 days. Six replicates at each concentration level were stored under the specified conditions. At the end of the stability period, the stability samples were processed and analyzed in the same analytical run together with freshly prepared calibration standards and QC samples. The freshly prepared calibration standards were used to construct the calibration curves for quantification of both the stability and freshly prepared QC samples. The results are presented in **Table 12**.

The QC samples stored at -70°C and -20°C showed acceptable accuracy and precision at both concentration levels. The results obtained from the stability samples were comparable to those of the freshly prepared QC samples. All reported RE and CV values for the stability samples were within the predefined acceptance criteria of $\pm 15\%$ and $\leq 15\%$, respectively. Therefore, ozanimod was considered stable in human plasma for 40 days when stored at -70°C and -20°C .

Table 12. Long-term plasma stability of ozanimod at -70°C and -20°C .

QC level	Condition	Mean concentration (pg/mL)	RE (%)	CV (%)
LQC	-70°C , 40 days	15.851 ± 0.455	5.67	2.87
LQC	Freshly prepared	15.799 ± 0.401	5.33	2.54
HQC	-70°C , 40 days	454.282 ± 10.062	-5.36	2.21
HQC	Freshly prepared	435.895 ± 48.983	-9.19	11.24
LQC	-20°C , 40 days	15.611 ± 0.547	4.07	3.50
LQC	Freshly prepared	15.293 ± 0.497	1.96	3.25
HQC	-20°C , 40 days	437.506 ± 5.826	-8.85	1.33
HQC	Freshly prepared	438.814 ± 6.173	-8.58	1.41

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

4. Conclusions

A rapid, sensitive and reliable LC-MS/MS method was developed and validated for the quantitative determination of ozanimod in human plasma. The method employed a simple protein precipitation procedure using acetonitrile followed by evaporation and reconstitution and ozanimod-D6 was used as a stable isotope-labeled internal standard. Chromatographic separation and mass spectrometric detection were achieved within a short total run time of 4.5 min, providing an efficient approach for the analysis of plasma samples.

The method demonstrated acceptable selectivity, linearity, accuracy, precision, recovery, matrix effect, dilution integrity, carry-over and stability over the validated concentration range of 5 - 600 pg/mL. Ozanimod was also shown to be stable under the investigated sample handling and storage conditions, including long-term

storage at -70°C and -20°C for at least 40 days. These results demonstrate the robustness and reliability of the developed analytical procedure.

Overall, the developed LC-MS/MS method provides a simple, sensitive and efficient approach for the determination of ozanimod in human plasma and is suitable for application in pharmacokinetic and bioequivalence studies.

Author Contributions

Nihal Saraner: Conceptualization, methodology, method development, validation, investigation, data collection, formal analysis, interpretation of results, original manuscript preparation, manuscript review and editing and project administration. **İlayda Akgül:** Investigation, experimental work and data collection. **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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