LC-MS/MS Identification of Ozanimod Degradation Products and Development of a Green HPLC Method for Impurity Quantification

Rajesh Varma B[†], Usha Rani N[‡], Prasada Rao P.T.S.R.K[§], Rekha K[II], Sreenivasa Rao B[†]*

[†] Department of Chemistry, GITAM Institute of Science, GITAM (Deemed to be University), Visakhapatnam, 530 045, A.P., India; *e-mail: sbattula2@gitam.edu
[‡] Department of FED, PVP Siddhartha Institute of Technology, Vijayawada, Andhra Pradesh, PIN: 520007, India;
[§] Department of Chemistry, P.B.Siddhartha College of Arts and Science, Vijayawada, Andhra Pradesh, India;
[II] Lecturer in Biotechnology, Dr.V.S Krishna Govt. Degree College, Visakhapatnam,530 022, A.P., India;

Received: October 17, 2024; Accepted: December 16, 2024
DOI: 10.17721/moca.2025.65-71

A green high-performance liquid chromatography (HPLC) method was developed and validated for the quantification of Ozanimod and its impurities, addressing the lack of environmentally friendly approaches in the literature. The method utilizes ethanol and aqueous trifluoroacetic acid (TFA) (70:30 v/v) as the mobile phase, with a Waters XBridge C18 column (150 × 4.6 mm, 5 µm), a 0.7 mL/min flow rate, and 270 nm detection. Method validation demonstrated acceptable linearity, accuracy, precision, and sensitivity for Ozanimod and its impurities. A forced degradation study identified three degradation products (DPs) under acidic and basic stress conditions, designated as DP 1, DP 2, and DP 3. Structural elucidation using LC-MS/MS and MS^n fragmentation analysis confirmed their identities. The green assessment of the method was conducted using GAPI (Green Analytical Procedure Index) and AGREE (Analytical GREEnness) tools, confirming a significant reduction in hazardous solvent usage while maintaining excellent chromatographic performance. This study introduces a validated, eco-friendly HPLC method for the quantification of Ozanimod and its impurities, along with structural characterization of its DPs, making it a sustainable and reliable approach for routine pharmaceutical analysis and stability studies.

Keywords: ozanimod, impurities, LC-MS, degradation products, green analytical method

Introduction

Ozanimod is an immunomodulatory medication used to treat relapsing multiple sclerosis and ulcerative colitis [1]. The compound functions as an agonist for sphingosine-1-phosphate receptors, meaning it activates these specific receptors [2]. This activation leads to the retention of lymphocytes, which are white blood cells crucial for the immune response, within peripheral lymphoid organs [3]. Ozanimod is sold under the brand name Zeposia at a 0.92 mg dosage. In 2020, the FDA approved Ozanimod, and it was later approved by the European Commission [4].

Severe side effects of Ozanimod include breathing problems, macular edema, heart issues, liver damage, and an increased risk of skin cancer with long-term use. Common side effects include headache, back pain, diarrhea, hypertension, and fatigue [5].

The scientific name of Ozanimod is 5-(3-{(1S)-1-[(2-Hydroxyethyl)amino]-2,3-dihydro-1H-inden-4-yl}-1,2,4-oxadiazol-5-yl)-2-isopropoxybenzonitrile, with a molecular weight of 404.470 g/mol and a molecular formula of $C_{23}H_{24}N_4O_3$. Its structure is represented in Figure S1A.

A literature survey revealed that no analytical methods have been proposed for the estimation of Ozanimod and its impurities. Although a few methods have been reported for its pharmacokinetics and quantum analysis [7-11], no green analytical methods have been developed for the quantification of Ozanimod and its impurities.

Green analytical methods align with green chemistry principles, minimizing hazardous chemical usage, reducing waste generation, and conserving resources. Additionally, these methods enhance cost savings and efficiency, making them an attractive option for the accurate and reliable quantification of pharmaceutical impurities. Considering these aspects, this research aims to propose a green HPLC-based analytical method for the quantification of Ozanimod and its impurities while also evaluating its degradation behavior using LC-MS/MS.

The selected Ozanimod impurities for this study include the bromo impurity and the hydroxy amide impurity. The bromo impurity, with the scientific name (S)-2-((4-(5-(3-Bromo-4-isopropoxyphenyl)-1,2,4-oxadiazol-3-yl)-2,3-dihydro-1H-inden-1-yl)amino) ethan-1-ol, has a molecular formula of $C_{22}H_{24}BrN_3O_3$ and a molecular weight of 458.4 g/mol (Figure S1B). The hydroxy amide impurity, scientifically known as 3-Cyano-N-(2-hydroxyethyl)-4-isopropoxybenzamide, has a molecular formula of $C_{13}H_{16}N_2O_3$ and a molecular weight of 248.3 g/mol (Figure S1C).

Experimental Procedure

Chemicals and reagents

The Ozanimod standard drug (purity > 98%), along with its bromo and hydroxy amide impurities, as well as the Zeposia® (0.92 mg) commercial formulation, were generously provided by Avet Lifesciences Private Limited, Hyderabad, India. All chemicals used in this study were of analytical grade and sourced from Merck Chemicals, Mumbai. Additionally, high-purity solvents required for HPLC analysis, including ethanol, methanol, acetonitrile, and water, were obtained from SD Fine Chemicals, Mumbai.

Instrumentation

The analytical experiments were conducted using an Agilent 2695 series HPLC-UV system (USA) equipped with a quaternary pump, degasser, autosampler, and temperature-controlled column compartment, ensuring consistent analytical conditions. Data integration was performed using Empower 3 software.

For detailed characterization of degradation products (DPs), an Agilent 1290 series LC-MS/MS system (USA) with a Q-TOF mass detector was utilized. Electrospray Ionization (ESI) in positive mode was employed, with an optimized capillary voltage of 3.5 kV. Nitrogen was used as the nebulizer gas at 40 psi, ensuring efficient ionization. The desolvation temperature was maintained at 350°C for effective solvent evaporation, and a cone voltage of 30 V was applied to enhance stable ion transfer while minimizing noise interference. Data analysis was performed using Mass Hunter Workstation software.

The pH of the samples was precisely measured using a Mettler Toledo pH meter (Switzerland). Prior to analysis, samples were fully dissolved with the aid of an ultrasonic bath (Oscar Ultrasonic Pvt Ltd, India) to ensure homogeneity.

Standard solution preparation

For the preparation of the stock solution, exactly 10 mg of the standard substance was accurately weighed and transferred into a 10 mL volumetric flask. 5 mL of ethanol was added, and the mixture was sonicated to ensure complete solubility of the standard in ethanol. After confirming full dissolution, the solution was filtered through a 0.22 µm nylon membrane filter to remove any impurities. The filtered solution was then diluted to the 10 mL mark with additional ethanol, achieving a final concentration of 1000 µg/mL. The prepared stock solution was stored appropriately for future use as a standard solution.

Forced degradation study

The stress study was conducted following ICH Q1A (R2) guidelines [12] and relevant literature [13-15].

Acid degradation study. Accurately weighed 100 mg of the drug was transferred into a 100 mL volumetric flask, containing 50 mL of methanol as a diluent. 5 mL of 2N HCl was added, and the solution was refluxed at 80°C for 8 hours. After 3 days, the solution was neutralized with 2N NaOH and diluted to the 100 mL mark. 5 mL of this solution was further diluted to 20 mL using the diluent and filtered through a 0.22 µm membrane filter. The filtered solution was then diluted to 100% concentration and used for analysis.

Base degradation study. For alkaline degradation, 100 mg of the drug was transferred into a 100 mL volumetric flask containing 5 mL of 3N NaOH and 45 mL of diluent. The solution was refluxed at 80°C for 8 hours, then neutralized with 3N HCl and diluted to the 100 mL mark. 5 mL of this solution was further diluted to 20 mL using the diluent, filtered through a 0.22 µm membrane filter, and diluted to 100% concentration before analysis.

Oxidative degradation study. For oxidative degradation, 100 mg of the drug was accurately weighed and transferred into a 50 mL flask containing diluent and 5 mL of 30% H_2O_2. The solution was kept in the dark at room temperature for 3 days, then filtered through a 0.22 µm membrane filter and diluted to 100% concentration before analysis.

Thermal and photolytic stress studies. The standard drug was exposed to thermal stress by placing it in an air oven at 80°C for 2 days. For photolytic stress, it was kept in a UV chamber (200 W h/m²) at room temperature for 3 days. The stress-exposed drug was then diluted to 100% concentration and analyzed using the proposed method.

Analytical method validation

Method validation was performed in accordance with ICH Q2 (R1) guidelines [16] and relevant literature [17-21]. System suitability was assessed by repeatedly injecting a known-strength Ozanimod solution spiked with 1% impurities. Chromatographic parameters, including resolution, tailing factor, theoretical plate count, and %RSD of the area, were evaluated to ensure method performance. Linearity was determined by analyzing six different concentrations of Ozanimod with 1% impurities, plotting the area response against concentration. A correlation coefficient (R^2) > 0.999 was considered acceptable. To assess accuracy, Ozanimod and its impurities were spiked at levels ranging from 50% to 150% of the target concentration. The results were correlated with the calibration data to confirm method reliability.

Precision and ruggedness were evaluated by analyzing a 100% level solution under various conditions, including same-day, different-day, and different-analyst testing. The %RSD of the area response confirmed the method's repeatability and reproducibility.

Robustness was assessed by introducing minor, intentional variations in method parameters, such as detector wavelength, mobile phase composition, and flow rate. The impact on % area response and system performance was observed to ensure the method's reliability under varied conditions.

The limit of detection (LOD) and limit of quantification (LOQ) for impurities were determined

using the slope and standard error from the calibration curve, ensuring adequate sensitivity.

Characterization of degradation products

Stressed solutions were analyzed using an HPLC-MS/MS system under the proposed optimized conditions. The method enabled the detection of peaks corresponding to Ozanimod, its impurities, and degradation products (DPs). The mass range covered during the analysis was 50 to 500 m/z. In LC-MS analysis, 25% of the column eluents were directed to the mass spectrometer detector through a splitter positioned between the column and the detector, ensuring efficient detection while maintaining system stability.

Evaluation of method greenness

The proposed quantitative method was evaluated for greenness using GAPI and AGREE metric tools [22]. Green evaluation plays a crucial role in assessing various parameters to ensure drug safety while minimizing health and environmental impact. This evaluation considers multiple factors, including sample collection, analytical method, solvent and reagent usage, energy consumption, waste disposal, and other environmental aspects.

Among these tools, GAPI (Green Analytical Procedure Index) is highly effective in assessing environmental sustainability. In this evaluation, a color-coded figure (green, red, and yellow) was generated, divided into six sections, each representing key factors such as sample origin, processing method, sample preparation, reagent use, instrumentation, and quantitative analysis type (denoted by the letter "O").

The AGREE metric tool was utilized to further assess environmental sustainability. This approach is based on the twelve principles of green analytical chemistry, providing a quantitative measure of method sustainability.

Results and discussions

The optimization started with a Zodiac C18 column. Many organic modifiers have been tried to get the desired asymmetric peak shape, resolution, and minimum tailing. However, the first set of conditions did not provide an acceptable asymmetric peak shape. Given the above, changes were introduced into the mobile phase, and the pH was altered to attempt to yield better results but did not produce permissible results. Hence, a Waters XBridge C18 column with dimensions of 150 × 4.6 mm and particle size 5 µm was employed. A mobile phase of ethanol: 0.01% aq. Trifluoroacetic acid in the ratio of 70:30 (v/v) was utilized, which provided adequate resolution with an asymmetric peak shape of low tailing. Therefore, these conditions were optimized in view of the development of the method for the analysis of Ozanimod and its impurities. Under such optimized conditions, the wavelength maximum was set at 270 nm, based on the iso-absorption wavelength of Ozanimod and its impurities.

Degradation Study of Ozanimod

Ozanimod's degradation behavior was investigated under various stress conditions using HPLC. The stress conditions applied and the corresponding observed results are summarized in Table S1.

Table S1 presents the data obtained from the stress study, indicating that no degradation was observed for Ozanimod under oxidative, thermal, and photolytic conditions. However, the drug underwent degradation in acidic and basic environments (Figure S2). During these sensitivity tests, three new peaks were detected in the chromatograms at retention times of 1.1 min, 3.1 min, and 7.0 min, in addition to the known impurities. These peaks were identified as degradation products (DPs) and were designated as DP 1, DP 2, and DP 3, based on their elution order. Further structural characterization of these DPs was performed using the LC-MS/MS technique.

LC–MS/MS studies of DPs

The exact mass determination was based on the investigation of high-resolution mass fragmentation patterns. The exact mass prediction of Ozanimod and its degradation products (DPs) was obtained using RDB (Ring Double Bond) measurements while adhering to the nitrogen rule. The protonated elemental composition of the DPs and their corresponding product ions are summarized in Table 2, while Figures 3 to 5 illustrate the fragmentation pathways of DP 1, DP 2, and DP 3, respectively.

DP 1. Protonated DP 1 was formed during the acid degradation study, as shown in Figure S3. The total molecular weight of DP 1 is 338.3294 g/mol, with a molecular formula of $C_{18}H_{15}N_3O_4$. It was eluted at 1.10 min in the acid degradation study. During fragmentation, DP 1 undergoes the loss of an -NH_2 group, resulting in a product ion with a molecular formula of $C_{18}H_{13}N_2O_4$ and a molecular weight of 321.3062 g/mol. This product ion further fragments into two major product ions: one due to the loss of a -CHN group, forming a fragment with a molecular formula of $C_{17}H_{14}NO_2$ and a molecular weight of 265.2980 g/mol, and another due to the loss of a C_9H_4 moiety, resulting in a fragment with a molecular formula of $C_9H_9N_2O_4$ and a molecular weight of 210.1782 g/mol. Further fragmentation of $C_{17}H_{14}NO_2$ leads to the formation of two additional product ions: $C_{11}H_{12}N$ and $C_8H_8NO_4$. Similarly, the $C_9H_9N_2O_4$ fragment also forms $C_8H_8NO_4$ due to the loss of a -CHN group. The degradation pathway of DP 1 is illustrated in Figure S3, and its mass spectrum is presented in Figure S4. The final molecular name of DP 1 is 5-[3-(1-amino-2,3-dihydro-1H-inden-4-yl)-1,2,4-oxadiazol-5-yl]-2-hydroxybenzoic acid.

DP 2. DP 2 was exclusively formed during the base degradation study, with a molecular formula of $C_{21}H_{22}N_4O_3$ and a molar mass of 379.4243 g/mol. Under these stress conditions, the peak was eluted at 3.13 min. The loss of a C_3H_5 group from DP 2 resulted in the formation of a fragmented product ion with a molecular formula of $C_{18}H_{17}N_4O_3$ and a mass of 338.3520 m/z,

identified as 4-[(5E)-5-{5-[amino(hydroxy)methyl]-4-oxocyclohex-2-en-1-ylidene}-4,5-dihydro-1,2,4-oxadiazol-3-yl]-1H-inden-1-iminium. This fragmented ion further loses a CH_5NO group, forming another product ion with a molecular formula of $C_{17}H_{14}N_3O_2$ and a molar mass of 293.3114 m/z. The name of this formed product ion is (4-[(5E)-5-(4-oxocyclohex-2-en-1-ylidene)-4,5-dihydro-1,2,4-oxadiazol-3-yl]-1H-inden-1-iminium).Further fragmentation involves the elimination of ethenone (C_2H_2O), yielding a product ion with a molecular formula of $C_{15}H_{12}N_3O$ and a molar mass of 251.2747 m/z, identified as (4Z)-4-[5-(buta-1,3-diyn-1-yl)-1,2,4-oxadiazol-3(2H)-ylidene]-3a,4-dihydro-1H-inden-1-aminium. From this compound, oxygen and five carbon atoms are separated, producing another fragmented ion ($C_{10}H_{12}N_3$) with a mass of 175.2218 m/z, named amino(1-amino-1H-inden-4-yl) methaniminium. Further fragmentation results in another product ion (C_7H_7) with a molar mass of 92.1299 m/z. The mass spectrum of DP 2 is presented in Figure S5, and the proposed degradation pathway is illustrated in Figure S6. Based on this pattern, the proposed name for DP 2 is 5-[3-(1-amino-2,3-dihydro-1H-inden-4-yl)-1,2,4-oxadiazol-5-yl]-2-(propan-2-yloxy)benzamide.

DP 3. DP 3 was exclusively formed during the acid degradation study, with a molecular formula of $C_{21}H_{20}N_4O_2$ and a molar mass of 361.4091 g/mol. Under these stress conditions, the peak was eluted at 7.03 min. The loss of a C_3H_5 group from DP 3 resulted in the formation of a fragmented product ion with a molecular formula of $C_{18}H_{15}N_4O_2$ and a mass of 320.3367 m/z, identified as 4-{(5Z)-5-[3-(aminomethyl)-4-oxocyclohexa-2,5-dien-1-ylidene]-2,5-dihydro-1,2,4-oxadiazol-3-yl}-1H-inden-1-iminium. This fragmented ion further loses a C_3HNO group, forming another product ion with a molecular formula of $C_{15}H_{14}N_3O$ and a molar mass of 253.2906 m/z, identified as 4-[(5E)-5-(but-3-en-2-ylidene)-2,5-dihydro-1,2,4-oxadiazol-3-yl]-1H-inden-1-iminium. Further fragmentation involves the elimination of cyclopentadienone (C_5H_2O), yielding a product ion with a molecular formula of $C_{10}H_{12}N_3$ and a molar mass of 175.2218 m/z, identified as amino(1-amino-1H-inden-4-yl) methaniminium. From this fragment, $C_3H_5N_3$ is separated, producing another fragmented ion (C_7H_7) with a mass of 92.1299 m/z, identified as phenylmethylium. The proposed degradation pathway of DP 3 is illustrated in Figure S7, and its mass spectrum is presented in Figure S8. Based on this pattern, the proposed name for DP 3 is 5-[3-(1-amino-2,3-dihydro-1H-inden-4-yl)-1,2,4-oxadiazol-5-yl]-2-(propan-2-yloxy)benzonitrile.

Method validation

Linearity. In the linearity study, a linear regression method was used to evaluate the relationship between the concentration of Ozanimod and its impurities with their corresponding area measurement responses. A linear relationship was observed in the concentration range of 5 to 30 µg/mL for Ozanimod and 0.05 to 0.30 µg/mL for its impurities.

The regression equations obtained were:

Ozanimod: $y = 62758x + 6279$ ($R^2 = 0.999$)

Bromo impurity: $y = 715797x + 1974.9$ ($R^2 = 0.9996$)

Hydroxy amide impurity: $y = 2E+06x - 4168.7$ ($R^2 = 0.999$)

These results confirm that the adopted method is suitable for both qualitative and quantitative determination of Ozanimod and its impurities within the studied concentration ranges.

Precision and ruggedness. The precision and ruggedness assay values along with the corresponding % RSD for Ozanimod and its impurities demonstrated high precision and reproducibility (Table 1). The measured assay value for Ozanimod was 99.30%, with % RSD values of 0.89% for intraday precision, 0.65% for interday precision, 1.27% for combined intra- and interday precision, and 0.29% for ruggedness studies.

For Bromo Impurity, the assay value was 98.76%, with an RSD of 0.64% for intraday precision and 0.38% for ruggedness, confirming high measurement reliability. Similarly, the Hydroxy Amide Impurity had an assay value of 98.80%, with the lowest % RSD of 0.49% for intraday precision, indicating high repeatability in daily estimates.

These results confirm that the proposed method provides highly accurate and reliable quantification, as the measured values were close to 100%, ensuring the accurate estimation of analytes.

Robustness. The robustness results presented in Table S4 demonstrate that the analytical method used for testing Ozanimod and its impurities remains dependable under various conditions. When the mobile phase composition was adjusted to a 75:25 (v/v) ratio

Table 1. Precision and ruggedness results of Ozanimod and its impurities.

S No	Analyte	% Assay	% RSD in Intraday precision	% RSD in Interday precision		% RSD in Ruggedness
				Day 1	Day 2	
1	Ozanimod	99.30	0.89	0.65	1.27	0.29
2	Bromo Impurity	98.76	0.64	0.38	0.23	0.87
3	Hydroxy Amide Impurity	98.80	0.49	0.41	0.68	0.91

of ethanol and 0.01% aqueous trifluoroacetic acid, the resolution between Ozanimod and the Hydroxy Amide Impurity was 11.09, with an assay percentage of 98.04%.

Further modification of the mobile phase composition to 65:35 (v/v) improved the separation to 11.85 and increased the assay percentage to 99.76%. Similarly, the method maintained reliable results when flow rate and detector wavelength were altered.

These findings confirm that the proposed method consistently provides accurate resolution, peak shape, plate count, and assay percentages, ensuring robust performance under different analytical conditions.

Accuracy. Accuracy was evaluated at three different concentration levels: 50%, 100%, and 150%. At the 50% concentration level, 15 µg/mL of Ozanimod and 0.15 µg/mL of its impurities were used. At the 100% concentration level, 20 µg/mL of Ozanimod and 0.20 µg/mL of its impurities were analyzed. At the 150% concentration level, 25 µg/mL of Ozanimod and 0.25 µg/mL of its impurities were tested.

The recovery results for Ozanimod were 99.86% at 50%, 98.53% at 100%, and 99.12% at 150% accuracy, with %RSD values of 0.54, 0.99, and 0.86, respectively.

For the Bromo impurity, the recoveries were 100.65% at 50%, 99.89% at 100%, and 98.45% at 150% accuracy, with %RSD values of 0.67, 0.87, and 0.33, respectively.

Similarly, the Hydroxy Amide Impurity showed recoveries of 98.32% at 50%, 100.24% at 100%, and 99.48% at 150% accuracy, with %RSD values of 0.41, 0.56, and 0.32, respectively

These results confirm that the recovery values fall within the acceptable range, demonstrating the accuracy and reliability of the proposed method. The detailed accuracy results are presented in Table 2.

LOD and LOQ. The sensitivity of the method for analyzing Ozanimod and its impurities is demonstrated by the LOD and LOQ results. The limit of detection (LOD) for impurities was 0.015 µg/mL, confirming the method's ability to detect trace amounts with high precision. The limit of quantification (LOQ) was 0.05 µg/mL, ensuring accurate quantification of low impurity levels. These low LOD and LOQ values validate the high sensitivity of the proposed method, enabling reliable detection and measurement of impurities in Ozanimod formulations.

Solution stability. To assess solution stability, a 100% standard solution of Ozanimod was stored at room temperature and tested at various time intervals. Stability was evaluated by comparing the percentage difference (% difference) between the injected samples and standards. The % difference observed was 0.13% for the sample and 0.27% for the standard, indicating minimal degradation and excellent stability of Ozanimod solutions over 48 hours. The detailed stability results are presented in Table S5.

Assessment of Green Analytical Chemistry

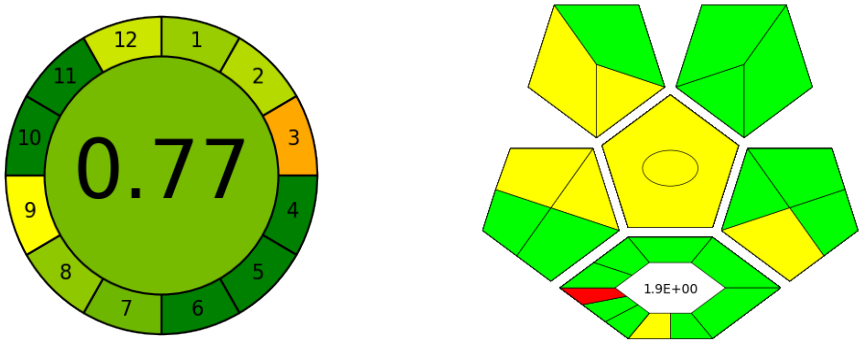
The current study focused on the green aspects of developing an analytical method for evaluating Ozanimod and its impurities. Unlike previous methods that relied on hazardous solvents such as acetonitrile and methanol, this study employed eco-friendly solvents like ethanol and water. These solvents were exclusively chosen for preparing the mobile phase and solutions to minimize environmental impact. Additionally, a 100 mm column was used to facilitate a shorter runtime, thereby reducing solvent consumption and energy usage.

The environmental assessment of the proposed method was conducted using two green evaluation tools: AGREE and GAPI. The AGREE software evaluated the 12 principles of green analytical chemistry on a 0.1 to 1.0 scale, yielding a total AGREE score of 0.77 (Figure 1A), confirming the method's high eco-friendliness and minimal environmental impact.

Similarly, the GAPI tool provided a visual representation of the method's environmental impact. This tool generates pictograms and pentagrams, aiding in the assessment of eco-friendliness. The analysis revealed minimal red pictograms, with most

Table 2. Accuracy results of Ozanimod and its impurities.

Target Amount in µg/mL	Spiked amount in µg/mL	Final amount in µg/mL	% recovery	%RSD
		Ozanimod		
10	5	15	99.86	0.54
10	10	20	98.53	0.99
10	15	25	99.12	0.86
		Bromo Impurity		
0.1	0.05	0.15	100.65	0.67
0.1	0.1	0.20	99.89	0.87
0.1	0.15	0.25	98.45	0.33
		Hydroxy Amide Impurity		
0.1	0.05	0.15	98.32	0.41
0.1	0.1	0.20	100.24	0.56
0.1	0.15	0.25	99.48	0.32



(A) AGREE **(B) GAPI**

Figure 1. Pictogram noticed in AGREE and GAPI green assessment tools.

areas marked green and yellow, indicating no major environmental concerns (Figure 1B). The GAPI rating of 1.9E+00 further confirmed that the method exerts low environmental pressure. The yellow pictograms corresponded to sample handling and preparation, suggesting potential areas for improvement in reducing environmental risks.

A review of the literature indicates that no analytical method has been previously reported for the evaluation of Ozanimod and its Bromo and Hydroxy Amide impurities. The method developed in this study enables the resolution and quantification of these impurities, representing a significant advancement in the analytical evaluation of Ozanimod. Additionally, by integrating green chemistry principles, this technique offers a more environmentally friendly alternative compared to conventional methods. Moreover, this study includes the detection and characterization of Ozanimod's degradation products (DPs), providing a comprehensive understanding of its stability. This novel approach enhances both the efficiency and sustainability of Ozanimod impurity profiling, ensuring accurate and reliable analysis.

Conclusion

This study presents a green analytical HPLC method for the quantification of Ozanimod and its impurities, aligning with green chemistry principles by reducing the use of hazardous chemicals and minimizing waste. Addressing a notable gap in the literature, this eco-friendly approach utilizes a Waters XBridge C18 column with a solvent mixture of ethanol and aqueous trifluoroacetic acid. The developed method demonstrated high precision and accuracy in quantifying Ozanimod and its impurities, meeting the required validation standards. Stress testing identified three distinct DPs under acidic and basic conditions, and their structures were confirmed through MS^n studies. The green assessment tools, GAPI and AGREE, validated that this method significantly reduces hazardous solvent usage while maintaining excellent chromatographic performance. Overall, this green HPLC method is well-suited for routine quality control and stability studies, offering a sustainable approach for the analysis of Ozanimod and its DPs in active pharmaceutical ingredients (APIs) and formulations.

References

1. Cohen J. A., Comi G., Arnold D. L., Bar-Or A., Selmaj K. W., Steinman L., Havrdova E. K., Cree B. A., Montalban X., Hartung H. P., Huang V., Frohna P., Skolnick B. E., Kappos L. Efficacy and safety of ozanimod in multiple sclerosis: Dose-blinded extension of a randomized phase II study. *Mult.Scler.* 2019, 25(9), 1255-1262.

2. Tran J. Q., Hartung J. P., Peach R. J., Boehm M. F., Rosen H., Smith H., Brooks J. L., Timony G. A., Olson A. D., Gujrathi S., Frohna P. A. Results from the First-in-Human Study with Ozanimod, a Novel, Selective Sphingosine-1-Phosphate Receptor Modulator. *J. Clin.Pharmacol.* 2017**,** 57(8), 988-996.

3. William J. S., Feagan G., Wolf C., HaensD., Severine Vermeire, Stephen B. H., Subrata Ghosh., Heather Smith., Matthew Cravets., Paul A. F., Richard Aranda., Sheila Gujrathi., Allan Olson.Ozanimod Induction and Maintenance Treatment for Ulcerative Colitis.*N. Engl. J. Med*. 2016**,** 374 (18), 1754–1762.

4. Gianna Melillo.FDA Approves Ozanimod for Patients With RMS, 2020**,** https://www.ajmc.com/view/fda-approves-zeposia-ozanimod-for-patients-with-rms

5. U.S. Food and Drug Administration Approves Bristol Myers Squibb's Zeposia (ozanimod), a New Oral Treatment for Relapsing Forms of Multiple Sclerosis". Bristol-Myers Squibb Company (Press release). March 26, 2020. Retrieved March 26, 2020.

6. Juif P. E., Kraehenbuehl S., Dingemanse J. Clinical pharmacology, efficacy, and safety aspects of sphingosine-1-phosphate receptor modulators. *Ex-*

pert.Opin.Drug.Metab.Toxicol. 2016**,** 12(8), 879–895.

7. Claudio Cianferotti., Giuseppe Barreca., VenkateshBollabathini., Luca Carcone., Damian Grainger., Samantha Staniland., Maurizio Taddei. Enantioselective Synthesis of Ozanimod, the Active Pharmaceutical Ingredient of a New Drug for Multiple Sclerosis.*Eur. J. Org. Chem.* 2021**,** 12,1924–1930.

8. Sekhar Surapaneni., Usha Yerramilli., April Bai., Deepak Dalvie., Jennifer Brooks., Xiaomin Wang., Julie SelkirkV., Yingzhuo Grace Yan., Peijin Zhang., Richard Hargreaves., Gondi Kumar., Maria Palmisano., Jonathan Q. T.Absorption, Metabolism, and Excretion, In Vitro Pharmacology, and Clinical Pharmacokinetics of Ozanimod, a Novel Sphingosine 1-Phosphate Receptor Modulators.*Drug.Metab. Dispos.* 2021**,** 49, 405–419.

9. Nabil Al-Zaqri., PooventhirancT., JagadeeswaraRaoD., Ali Alsalmea., Ismail Warade., Renjith Thomas.Structure, conformational dynamics, quantum mechanical studies and potential biological activity analysis of multiple sclerosis medicine ozanimod.*J. Mol. Struct.* 2021, 1227, 129685.

10. Jonathan Q. T., Jeffrey P. H., Allan D. O., Boaz Mendzelevski., Gregg A. T., Marcus F. B., Robert J. P., Sheila Gujrathi., Paul A. F.Cardiac Safety of Ozanimod, a Novel Sphingosine-1-Phosphate Receptor Modulator: Results of a Thorough QT/QTc Study.*Clin. Pharmacol.Drug. Dev.* 2017**,** 7(3), 263-276.

11. Jonathan Q. T.,Peijin Zhang., Atalanta Ghosh.,Liangang Liu., Mary Syto., Xiaomin Wang., Maria Palmisano.Single-Dose Pharmacokinetics of Ozanimod and its Major Active Metabolites Alone and in Combination with Gemfibrozil, Itraconazole, or Rifampin in Healthy Subjects: A Randomized, Parallel-Group, Open-Label Study.*Adv. Ther.* 2020**,** 37, 4381–4395.

12. ICH Guideline: Stability testing of new drug substances and products, Q1A (R2), Current Step 4 version, 6 February 2003.

13. Rajesh V. B., Sreenivasa R. B., Maruthi V. N. R. K., Varaprasad R. M. Assessment of gas chromatography methodology approach for the trace evaluation of carcinogenic impurity. methyl chloride, in trimetazidine dihydrochloride. *Ann. Pharm. Fr.* 2023**,** 81, 64-73.

14. Varma B. H. R., Rao B. S.Gas Chromatography-Head Space-Mass Spectrometry Sensor based Quality Control of Dobutamine Hydrochloride Bulk Material for a mutagenic impurity, 2-bromopropane. *Res. J. Chem. Environ.* 2023, 27, 54-61.

15. Rajesh V. B., Battula S. R., Kapavarapu M. V. N. R., Mandapati V. R.A novel Rivaroxaban degradation impurity detection by RP-HPLC extraction by preparative chromatography, and characterization by LC-MS, NMR and FT-IR: Analysis of novel impurity in batch samples and tablets of Rivaroxaban. *Rasayan. J. Chem.* 2022**,** 15, 2373-2381.

16. ICH Guideline. Validation of analytical procedures: text and methodology Q2 (R1), Current Step 4 Version, 2005.

17. Murali Krishnam RajuP., Shyamala P., Venkata NarayanaB., DantuluriH. S. N. R., BhupatirajuR. V.A fast, validated UPLC method coupled with PDA-QDa detectors for impurity profiling in betamethasone acetate and betamethasone phosphate injectable suspension and isolation, identification, characterization of two thermal impurities.*Ann. Pharm. Fr.* 2022, 80(6), 837-852.

18. Rajesh Varma Bhupatiraju, Battula Sreenivasa Rao.Gas Chromatography-Head Space-Flame Ionization Sensor based assessment of four residuary solvents in rivaroxaban bulk medication. *Res. J. Pharm. Technol.* 2022, 15(11), 5158-5163.

19. Rajesh Varma Bhupatiraju., Srinivasa Kumar B., Pavani Peddi., Venkata Swamy Tangeti. An effective HPLC method for evaluation of process related impurities of Letermovir and LC-MS/MS characterization of forced degradation compounds. *J. Chem. Metrol*. 2023, 2(2), 181-198.

20. Rajesh VarmaBhupatiraju., Bikshal Babu Kasimala., LavanyaNagamalla., Fathima Sayed.Structural evaluation of degradation products of Loteprednol using LC-MS/MS: Development of an HPLC method for analyzing process-related impurities of Loteprednol.*Anal. Sci. Technol.* 2024, 37(2), 98-113.

21. Rajesh VarmaBhupatiraju., Srinivasa KumarB., VenkataSwamyTangeti., Kandula Rekha., Fathima Sayed.LC and LC-MS/MS Studies for Identification and Characterisation of Related Substances and Degradation Products of Abrocitinib.*Toxicol. Int.* 2024, 31(2), 321-334.

22. Rajesh VarmaBhupatiraju., Pavani Peddi., Subhashini Edla., Kandula Rekha., Bikshal Babu Kasimala.Green Analytical Approach for HPLC Method Development for Quantification of Sorafenib and Its Pharmacopeia Impurities: LC–MS/MS Characterization and Toxicity Prediction of Stress Degradation Products.*Sep. Sci. Plus.* 2024, 7(9),202400106.