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Original Article

Optimization of LC-MS/MS Analytical Method for Trace Level Quantification of Potential Genotoxic Impurities in Siponimod Pure Drug and Formulations

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Abstract

The generation of single or multiple genotoxic impurities during synthesis of siponimod should be avoided for production of safe formulation. Technically, complete elimination of genotoxic impurities was not possible and hence there is a need to propose an accurate method for trace level detection of genotoxic impurities.Method optimization studies were conducted by analysis standard solution in various method parameters. The results noticed in every varied method condition were tabulated for finalizing the appropriate conditions for analyzing siponimod. The optimized method consists of waters C18 (150 × 4.6 mm; 5 µm) column, ammonium acetate (0.02M) at pH 4.2 (fixed with 1 % formic acid) and methanol in 45:55 (v/v) at 0.5 mL/min flow rate. The mass analyser was operated in multiple reaction positive ion mode with characteristic mass transition at m/z of 517 (parent ion)and 213 (product ion) for siponimod, 434(parent ion) and 173 (product ion)for alcohol and 432(parent ion) and172 (product ion)for aldehyde impurity. No impurity or unwanted compounds detected in both LC chromatograms and mass spectra, confirming the method specificity.Validation of method for parameters including linearity, precision, recovery, ruggedness, and robustness yielded acceptable results. The method is suitable for assessing potential genotoxic impurities during the synthesis of siponimod and the manufacturing of pharmaceutical products.

Keywords: *Alcohol impurity, Aldehyde impurity, Genotoxic impurities, LC-MS analysis, Pharmaceutical formulation*

Introduction

The medical drug siponimod belongs to the sphingosine l-phosphate receptor modulators drug used for the treatment of secondary progressive multiple sclerosis which is the progressive neurological deterioration of multiple sclerosis which is an autoimmune disease of the central nervous system (Kappos *et al.* 2004) It decreases the risk of multiple sclerosis and disability. Side effects such as liver function abnormalities, high blood pressure and headaches are possible during the use of siponimod (Ghasemi *et al.* 2017). It has a molecular formula of $C_{29}H_{35}F_3N_2O_3$ with a molecular mass of 516.605 g/mol and its structure is shown in figure 1.

Commercially, siponimod was synthesized in a multistep chemical reaction process involving various chemical reactions such as hydroxylation, the Suzuki-Miyaura reaction, hydrogenation, sequence – oxidation, condensation and substitution (Pan *et al.* 2013). During the process of

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synthesizingsiponimod, there is a possibility for the formation of various impurities. Those impurities are the unwanted substances that remain in the pure drug or formulation. These may arise from reactants, starting materials, reagents, solvents, catalysts, intermediates or may be as intermediate compounds (Reddy *et al.* 2015). The presence of these impurities in pure drug or drug product may influence the quality and effectiveness of the drug and its products. The regulatory agencies such as the United States Food and Drug Administration (US FDA), European Medicines Agencyetc., are issuing guidelines and limitation for these impurities in pharmaceutical products for the safety of patients. The ICH and US FDA issued guidelines for the detection and quantification of impurities in formulation dosages (Menz *et al.* 2023; Raman *et al.* 2011).

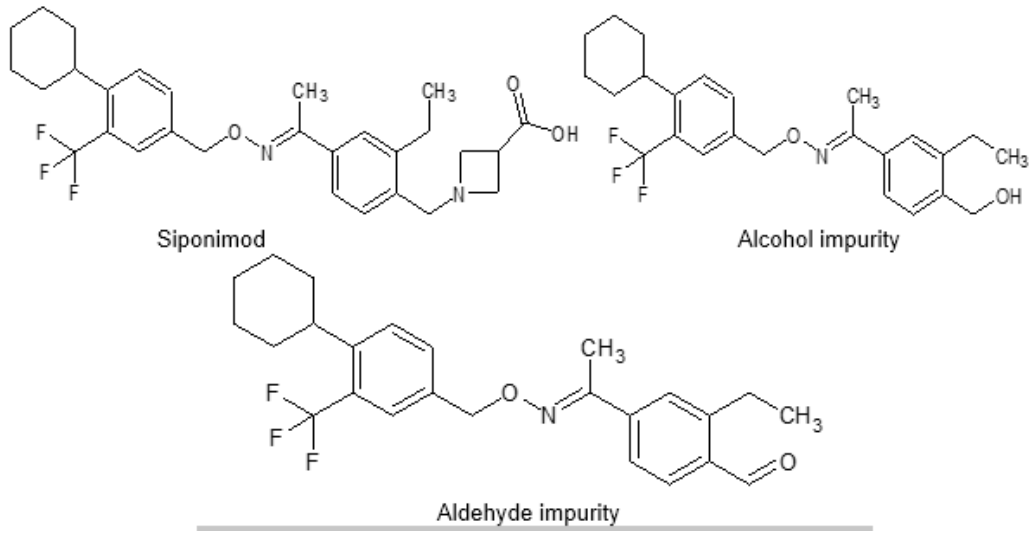

Figure1: Molecular structure of Siponimod and its impurities

Genotoxic impurities in the pharmaceutical context refer to substances that have the potential to cause damage to the genetic material within cells. This damage can lead to mutations, and chromosomal abnormalities, ultimately increasing the risk of cancer. In the pharmaceutical industry, ensuring the safety of drugs is of paramount importance, and controlling genotoxic impurities is a crucial aspect of this safety assurance. Hence there is a significant need to control genotoxic impurities to protect the health and safety of patients. Cutting-edge analytical tools such as LC-MS and LC-MS/MS are essential for the identification and quantification of trace-level genotoxic impurities (Wang *et al.* 2022).

The review of the available literature proved that only one analytical method was reported for the quantification of siponimod in combination with ponesimod in dosage forms using UPLC (Kethipalli & Ramachandran 2022). One LC-MS/MS method was reported for evaluating the safety, tolerance and pharmacokinetics of siponimod in subjects with various levels of hepatic impairment (Shakeri *et al.* 2017). One LC-MS/MS method was reported for the quantification of siponimod in human urine samples (Li *et al.* 2010). No method is available for quantifying the potential genotoxic impurities of siponimod. Hence, this study plans to propose a sensitive LC-MS method for quantification of potential genotoxic impurities of siponimod. The impurities such as aldehyde and alcohol impurity of siponimod were selected based on their availability for developing analytical method for quantifying of these genotoxic impurities in siponimod bulk drugs and formulations.

Materials and Methods

The research Instrumentation and Chemicals:

The Siponimod genotoxic impurities were quantified on an LC-MS system (Alliance 2695 model, Waters, Japan) connected with an optima ZQ mass analyser (Waters, Japan) and masslynx4.2 software. The siponimod (99.32 %),and its studied potential genotoxic impurities such as alcohol and aldehyde impurity were obtained from Novartis Pharmaceuticals, Hyderabad, Telangana. The 2 mg

pharmaceutical tablet formulation of siponimod with the brand Mayzent® was obtained from the pharmacy. LiChropur™ grade chemicals such as trifluoroacetic acid, ammonium acetate and HPLC grade solvents methanol and acetonitrile, along with 0.2 µ filters were brought from Merck Chemicals, Mumbai.

Stock and standard solution preparation:

The stock solution and subsequent dilutions of siponimod and its genotoxic impurities ware prepared independently with the same diluent. Initially an appropriate quantity of siponimod and impurities was dissolved separately in diluent to achieve a 0.1 mg/mL solution. Then a series of dilutions were made to achieve 0.025 to 1.0 µg/mL separately and an equal volume of same level of siponimod and impurities was mixed to obtain the calibration curve concentration.

Formulation solution preparation:

Mayzent® tablets of siponimod were utilized to evaluate the method's efficiency for the quantification of genotoxic impurities. Mayzent® tablets were fine powdered and an appropriate quantity of fine powder was dissolved in 100 mL solvent to achieve a 0.1 mg/mL concentration of siponimod. The undissolved tablet particles were removed by filtration through a 0.2 µ filter and diluted to a precision-level concentration.

Method development:

The separation, qualitative and quantitative evaluation of potential genotoxic impurities in siponimod was followed by guidelines outlined by ICH (ICH Q14. 2022). The method optimization process utilizes 0.5µg/mL of siponimod and its impurities. A lot of changes were made to the method conditions, and after each change, the peak area response, symmetry, suitability, and mass pattern were checked to make sure the method worked. Optimization of method parameters, such as the composition, pH, and flow rate of the mobile phase, as well as column configuration and temperature were performed. The conditions that yielded acceptable results were deemed suitable and subsequently advanced for further validation.

Method validation:

The optimized method underwent comprehensive validation, including assessments for sensitivity, analysis range, ruggedness, robustness, and in accordance with ICH guidelines (ICH Q2(R2). 2022) and relevant literature sources (Varma *et al.* 2022; Varma *et al.* 2023; Bhupatiraju *et al.* 2022; Bhupatiraju *et al.* 2023). Additionally, the developed method was scrutinized for its suitability for evaluating genotoxic impurities in formulations.

Results

The isocratic 0.5 mL/min flow of 0.02M ammonium acetate at pH 4.2 (adjusted with 1% formic acid) and methanol in 45:55 (v/v) was finalized to be appropriate for the resolution of alcohol and aldehyde impurities along with siponimod. Optimizing a suitable mass detector condition was crucial for effective and sensitive detection of impurities. The electrospray ionization (ESI) source at positive and negative ionization modes was evaluated for effective detection of analytes. In the mass spectra, the intensity of fragments in positive ionization mode was significantly higher than in negative mode and hence positive ionization mode was finalized as appropriate for sensitive detection of impurities. Optimal ion source parameters were fine-tuned to achieve the desired response, favourable peak shapes, and precise quantitation (Table 1).

Table 1: optimized mass operating conditions for analyzing genotoxic impurities of Siponimod

Sl No	Compound	Parent ion (m/z)	Production (m/z)	Fragmentor (V)	Electron Multiplier Voltage (V)	Collision energy (eV)	MS1 RES
1	Siponimod	517	213	155	700	35	Wide
2	Alcohol impurity	434	173	138	700	25	Wide
3	Aldehyde impurity	432	172	135	700	20	Wide

The specificity of the fine-tuned method was assessed by analyzing a standard solution containing a concentration of 0.5 µg/mL of siponimod and its genotoxic impurities, along with the diluent as a blank. In the chromatogram for the blank (figure 2), no peaks were observed in the entire runtime. Conversely, the standard chromatogram displayed well-resolved and symmetric peaks representing siponimod, alcohol and aldehyde impurities in this study.

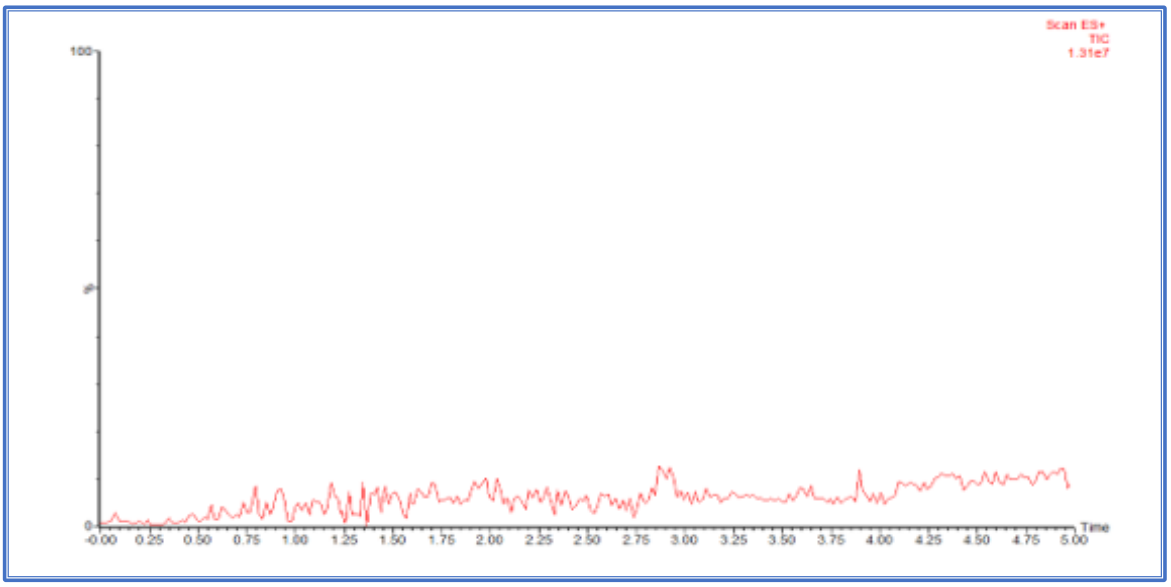

Figure 2: Unspiked chromatogram in the optimized method

The individual injection chromatograms of siponimod, alcohol and aldehyde impurities were compared with a combined standard solution injection chromatogram for identifying the retention time (tR) of analytes in the standard sample. Siponimod, alcohol, and aldehyde impurities exhibited retention times (tR) of 1.09 min, 1.96 min, and 2.81 min, respectively. The tR values for the analytes found in the mixed standard solution were the same as those found in separate tests of siponimod and its genotoxic impurities. Figure 4 illustrates individual analysis chromatograms whereas figure 3 illustrates the combined solution analysis chromatogram. The individual as well as combined solution chromatographic results confirm that the chromatogram confirms method specificity and doesn't visualize any additional detections or impurities throughout the runtime. This confirms the method's specificity for the analysis of siponimod and its genotoxic impurities.

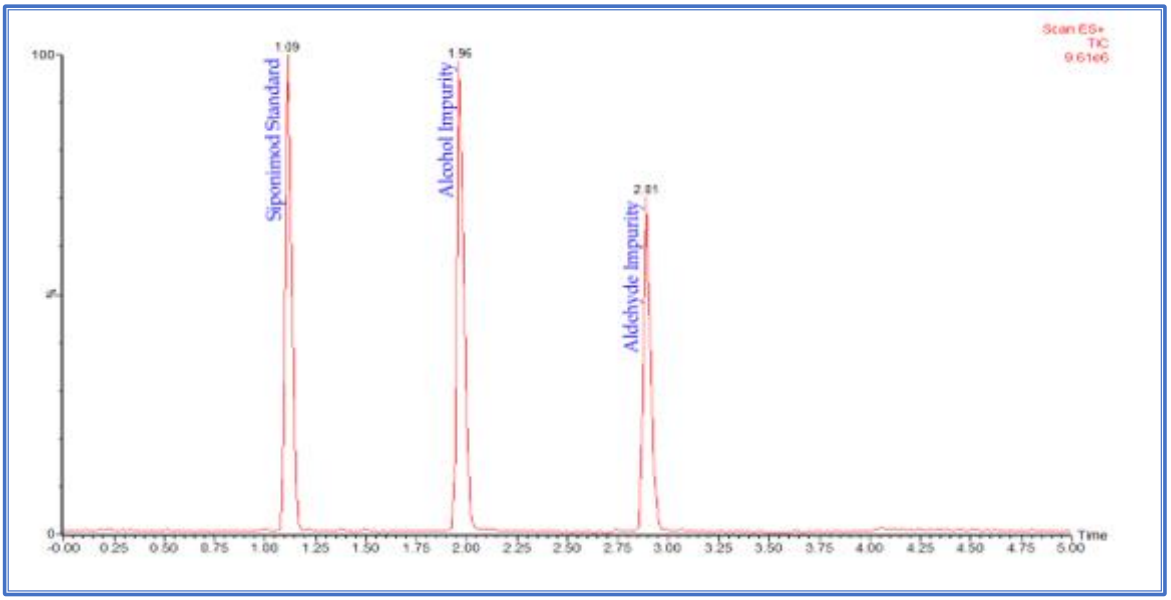

Figure 3: System suitability chromatogram obtained in the optimized method

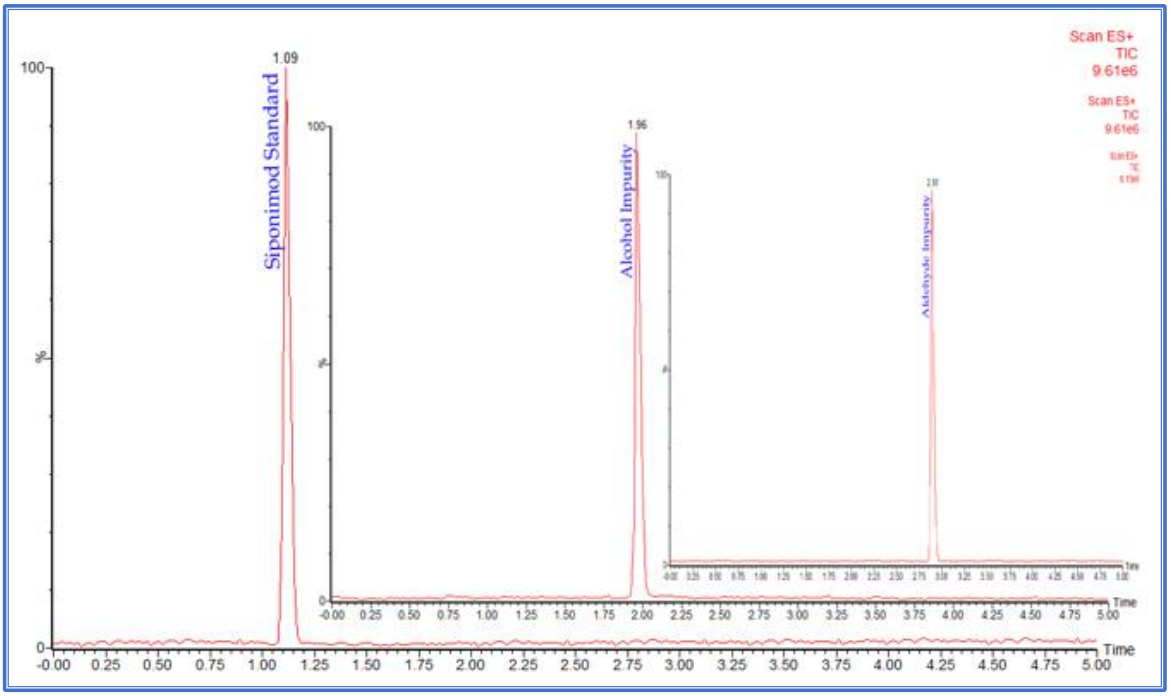

Figure 4: Individual analysis chromatograms of siponimod and its genotoxic impurities in the optimized method

The peak corresponding to siponimod, identified at a retention time (tR) of 1.09 min, exhibited a parent ion at m/z 517 (m+1). The mass spectra revealed product fragments at m/z 414 and 213. For the alcohol impurities peak at tR of 1.96 min, the parent ion was observed at m/z 434, while the aldehyde impurity at tR of 2.81 min showed a parent ion at m/z 432. The mass spectra of alcohol impurity displayed product ion fragments at m/z 430 and 173, whereas the aldehyde impurities exhibited product ion fragments at m/z 428 and 172.

Mass fragmentation patterns of individual analyses of analytes were correlated with the combined analysis of analytes at the same retention time. No additional detections or fragments corresponding to impurities or unidentified fragments were observed in mass patterns of siponimod, aldehyde and alcohol impurities. The intensity of daughter fragments was noticed to be notably high in the mass spectrum. Therefore, this method is deemed precise and appropriate for analyzing Siponimod and its genotoxic impurities. The mass fragmentation spectrum of siponimod and its genotoxic impurities is depicted in figure 5. Table 2 represents the accurate mass results of siponimod and its genotoxic impurities in the study.

Table 2: mass accurate results of siponimod and its genotoxic impurities in the study

Sl No	Compound	Retention time	Molecular Formula	Calculated mass *m/z*	Observed mass *m/z*	Fragment ion *m/z*	Formula of fragment ion
1	Siponimod	1.09	$C_{29}H_{35}F_3N_2O_3$	516.5950	516.5489	413.4752 246.3117 212.2902	$C_{25}H_{26}F_3NO$ $C_{14}H_{21}F_3$ $C_{14}H_{16}N_2$
2	Alcohol impurity	1.96	$C_{25}H_{30}F_3NO_2$	433.5064	433.5013	429.4746 246.3117 173.2111	$C_{25}H_{26}F_3NO_2$ $C_{14}H_{21}F_3$ $C_{11}H_{11}NO$
3	Aldehyde impurity	2.81	$C_{25}H_{28}F_3NO_2$	431.4905	431.4534	427.4587 246.3117 171.1952	$C_{25}H_{24}F_3NO_2$ $C_{14}H_{21}F_3$ $C_{11}H_9NO$

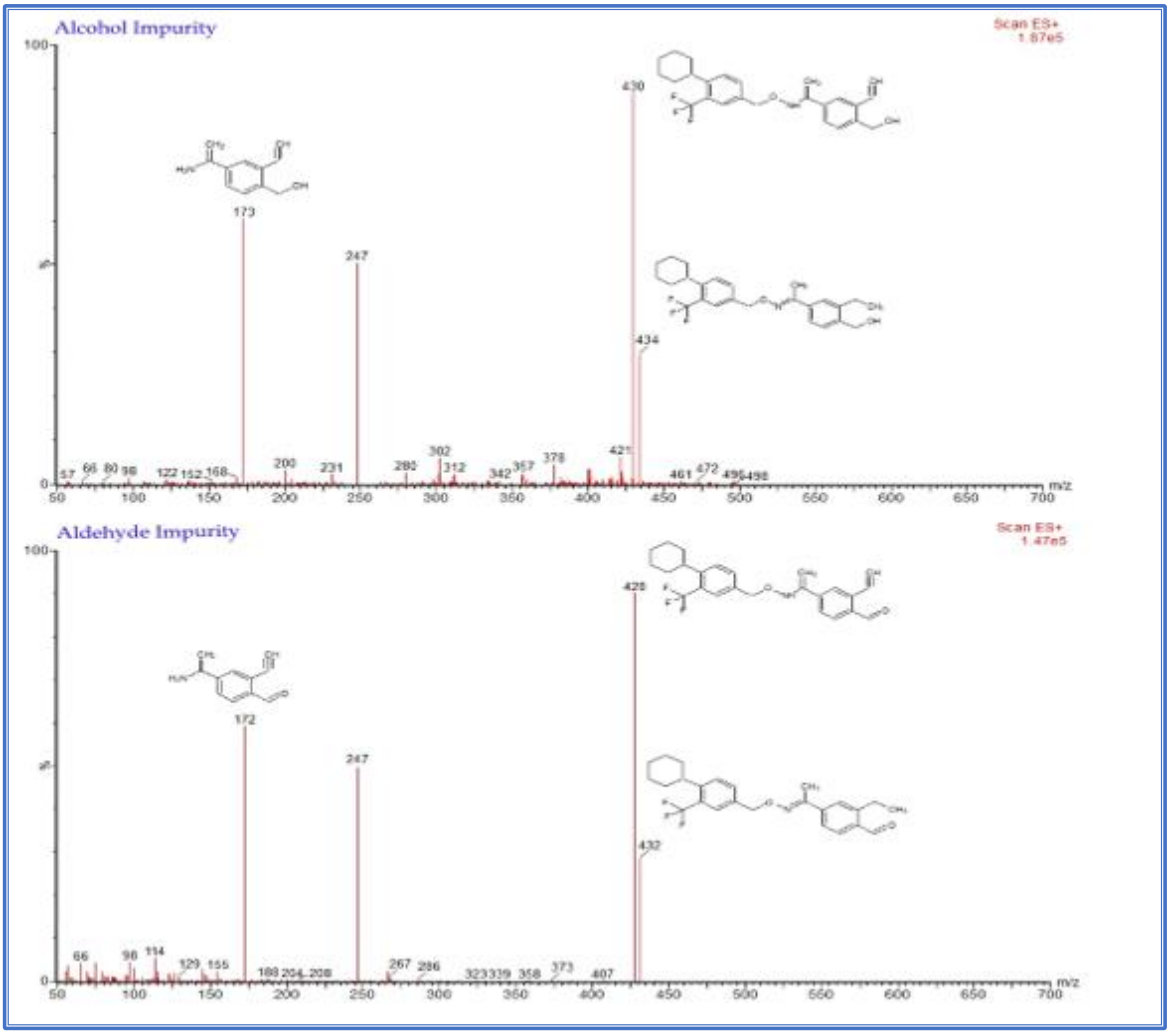

Figure 5: Fragmentation spectra of siponimod, alcohol and aldehyde impurities in the developed method

***Method Validation**:*

The developed method involved the analysis of various concentrations of siponimod and its genotoxic impurities. The calibration curve was established by correlating the area response of individual peaks with analyte strength. A highly correlated and accurately fitting linear curve was achieved with a concentration level of 0.025 µg/mL to 1.0 µg/Ml. The calibration parameters including the intercept, slope and regression equation ware evaluated by performing least-squares linear regression analysis.

The linear equations obtained were as follows:

For siponimod: $y = 845502x + 957.01$ ($R^2 = 0.9996$),
For alcohol impurity: $y = 954647x + 8095.6$ ($R^2 = 0.9997$)
For aldehyde impurity: $y = 786743x - 2525.7$ ($R^2 = 0.9995$)

These results demonstrating linearity are summarized in Table 3.

Table 3. Linearity results

Sl No	Concentration in µg/mL	Peak are response obtained		
		Siponimod	Alcohol Impurity	Aldehyde Impurity
1	0.025	39546.8	30215.8	24153.7
2	0.05	51125.3	43661.0	36912.5
3	0.1	95326.9	81504.5	69398.0
4	0.25	246925.8	212109.3	195318.3
5	0.5	491243.2	419030.4	392503.3
6	0.75	729856.3	626216.7	577316.3
7	1	956325.8	855680.2	791254.3

The recovery experiment was executed at concentration levels of 0.15 µg/mL, 0.20 µg/mL, and 0.25 µg/mL within the linearity range for siponimod, alcohol, and aldehyde impurities. The optimized method was used to analyze the recovery-level solution three times, and the peak area responses for each analyte were compared to their corresponding responses at the calibration level. The analyte strength equivalent to recovery was assessed in this proposed method. The chromatographic response of individual analyte was correlated with the equivalent level calibration curve response. The % recovery of siponimod, aldehyde and alcohol impurities in each injection was evaluated along with % RSD in every spiked level. According to guidelines, % recovery within the range of 98-102% and % RSD of < 2 were deemed acceptable. As indicated in Table 4, the % recovery fell within the range of 98.17 - 100.37, 98.37 - 100.91, and 98.25 - 100.58 for siponimod, alcohol, and aldehyde impurity, respectively. The % RSD at every recovery level was < 2 for siponimod, alcohol, and aldehyde impurities. The results, meeting the acceptable criteria, affirm the method as recoverable and accurate.

Table 4. Accuracy results

S. No	Compound	Level	Concentration in µg/mL	Recovered in µg/mL Mean±SD	% Recovery	% RSD
1	Siponimod	50%	0.15	0.246±0.001	98.493±0.407	0.41
2		100%	0.2	0.495±0.004	98.993±0.717	0.72
3		150%	0.25	0.747±0.008	99.660±1.127	1.13
4	Alcohol Impurity	50%	0.15	0.247±0.001	98.877±0.455	0.46
5		100%	0.2	0.497±0.002	99.383±0.345	0.35
6		150%	0.25	0.750±0.009	99.980±1.153	1.15
7	Aldehyde Impurity	50%	0.15	0.247±0.001	98.783±0.556	0.56
8		100%	0.2	0.497±0.002	99.310±0.478	0.48
9		150%	0.25	0.750±0.004	100.063±0.553	0.55

The assessment of the repeatability and reproducibility of the developed method involved using a standard solution with a concentration of 0.5 µg/mL for siponimod, alcohol, and aldehyde impurities. The solution underwent six analyses within a day for intraday precision and six analyses over three consecutive days for intraday precision. Additionally, three different analysts analyzed precision level solution in one day (n=6) to evaluate the methods ruggedness. The peak responses of siponimod, alcohol, and aldehyde impurities were documented, and % RSD was calculated. Results, summarized in Table 2, revealed % RSD values below 2, confirming the precision and reproducibility of the method.

Nominal deviations in proposed conditions, like mobile phase composition (without pH variation) and intentional changes in pH, were introduced to evaluate method robustness. In each altered condition, the standard solution with a concentration of 0.5 µg/mL for siponimod, alcohol, and aldehyde impurities was analysed. The chromatographic response of individual analyte in every varied

condition was correlated with the equivalent level calibration curve response. Table 4 shows that the % change values for siponimod and its genotoxic impurities were less than 2. This means that the method was robust and didn't show any big changes when small things changed.

Method sensitivity was determined by evaluating the detection limit (LOD) and quantification limit (LOQ) by following the signal-to-noise approach. The established LOD as 0.004 µg/mL and LOQ as 0.013 µg/mL respectively confirm the method's high sensitivity for detecting analytes at very low concentrations.

Stability testing involves incubating the standard solution for siponimod and its genotoxic impurities in an auto-sampler at 25°C for 48 hours. Analyses were conducted every 6 hours, and % stability was evaluated by correlating area response with calibration curve response at same level. Method stability was proved by observing more than 99 % of the assays for 24 h.

The developed method was successfully applied for the identification and quantification of potential genotoxic impurities in formulations. A formulation solution at a concentration of 250 µg/mL, prepared using the Mayzent® formulation of siponimod, was analyzed. The genotoxic impurities spiked formulation was also analyzed revealing distinct peaks corresponding to impurities alongside siponimod. In contrast, unspiked sample did not show any peaks at the retention time of genotoxic impurities. This showed that the method worked for finding and measuring impurities in formulations. This means that it can be used to measure alcohol and aldehyde impurities in siponimod bulk drug and formulation dosages. The summarized results of the method validation are presented in Table 5.

Table 5. Method validation summary results for siponimod and impurities

Sl No	Parameter	Results observed		
		Siponimod	**Alcohol impurity**	**Aldehyde impurity**
1	Linearity range	0.025 - 1.0 µg/mL	0.025 - 1.0 µg/mL	0.025 - 1.0 µg/mL
2	% RSD Intraday Precision (n=6)	0.11	0.39	0.48
3	% RSD Inteaday Precision (n=6)	0.82	0.99	0.55
4	% RSD Ruggedness (n=6)	0.90	0.59	0.97
5	% Accuracy in 50% spiked level (n=3)	98.493	98.877	98.783
6	% RSD in 50% spiked level (n=3)	0.41	0.46	0.56
7	% Accuracy in 100% spiked level (n=3)	98.993	99.383	99.310
8	% RSD in 100% spiked level (n=3)	0.72	0.35	0.48
9	% Accuracy in 150% spiked level (n=3)	99.660	99.980	100.063
10	% RSD in 150% spiked level (n=3)	1.13	1.15	0.55
11	% Change Robustness			
	+ change mobile phase composition	0.35	0.39	0.29
	- change in mobile phase composition	0.66	0.60	0.89
	+ change in pH of mobile phase	0.27	0.78	0.01
	- change in pH of mobile phase	0.70	0.14	0.53
	+ change in column temperature	0.23	0.16	0.76
	- change in column temperature	0.53	0.57	0.82
12	% stability at 48 h (n=6)	97.58	98.13	98.25
13	LOD	0.004 µg/mL	0.004 µg/mL	0.004 µg/mL
14	LOQ	0.013 µg/mL	0.013 µg/mL	0.013 µg/mL
15	% assay in formulation	98.63	Not detected	Not detected

Discussion

The preparation of samples plays a crucial role in genotoxic impurity analysis, as matrix effects can be amplified, leading to issues like reduced sensitivity, abnormal recovery, and analyte instability. Various diluents were assessed for their extraction efficiency and impact on chromatography. Methanol, acetonitrile, isopropanol alone and combination with each other demonstrated good solubility for genotoxic impurities (Shaikh *et al*. 2024). The use of isopropanol as a diluent is deemed unsuitable due to the low peak response as well as the poor symmetry of the analytes. The use of

methanol and acetonitrile individually as diluent produce poor peak shape and poor recoveries respectively (Kundu & Halder 2024). Moreover, the equal combination of methanol and acetonitrile yields best peak symmetry with significantly high recoveries suggesting that this solvent composition was utilized as a diluent throughout the analysis.

In the process of method development, three columns of different configurations including the Zorbax SB (100 mm) columns, waters C18 (150 mm) column and YMC-Triart (150 mm) C18 column were tested for producing the best resolution with high peak symmetry for genotoxic impurities. The waters C18 (150 × 4.6mm; 5 µm pore size) column at 30 °C performs best among the other columns studied in terms of resolution, symmetry and hence was selected as appropriate for the study. Literature also confirms that, this column was very efficient for resolution of various compounds and its impurities (Piponski *et al*. 2022; Khalil *et al*. 2023). An appropriate mobile phase was finalized by optimizing different composition of mobile phase including ammonium acetate, acetic acid, aqueous ammonia at various strengths. Before finalizing the mobile phase, the elution modes such as isocratic and gradient along with flow rate were tested to achieve best resolution of genotoxic impurities (Patel *et al*. 2021).

The genotoxic impurities along with standard siponimod were resolved on waters C18 (150 mm) column using 0.02M ammonium acetate at pH 4.2 (adjusted with 1 % formic acid) and methanol in 45:55 (v/v) at 0.5 mL/min as mobile phase. The method produces sensitive detection limit with characteristic mass fragmentation. This facilitates the trace level detection and quantification of genotoxic impurities of siponimod.

Calibration curves exhibited excellent linearity, enabling accurate quantitation of analytes across a concentration range. Recovery experiments demonstrated acceptable % recovery and % RSD, confirming the method's accuracy and recoverability. Precision and reproducibility were established through intraday and interday analyses, along with ruggedness testing across different conditions. The method exhibited robustness with minimal % change under altered conditions. Sensitivity assessment revealed low LOD and LOQ, indicating high sensitivity for detecting impurities at low concentrations. Stability testing demonstrated method reliability over a 48-hour period (Reddy *et al.* 2023). Application of the method to formulations successfully identified and quantified genotoxic impurities, showcasing its suitability for quality control purposes. Overall, the developed method offers a reliable and sensitive approach for the analysis of alcohol and aldehyde impurities alongside siponimod, ensuring product safety and quality in pharmaceutical formulations (Dong *et al*. 2022).

The findings obtained in this study was correlated with the literature and observed that no method reported for quantification of genotoxic impurities of siponimod. This study proposed method can efficiently resolve the siponimod impurities studied suggest that the method appropriate for resolution, identification and quantification of genotoxic impurities in siponimod bulk and formulated samples.

Conclusion

It is very hard and time-consuming to figure out how to safely measure genotoxic impurities and what levels of impurities are acceptable in large batches and their mixtures. Hence, this study proposes a sensitive LC-MS/MS method for trace-level quantification of aldehyde and alcohol impurities in siponimod formulations. The method has the advantage of sensitive detection; it can detect up to 0.004 µg/mL of impurities and exhibit a sensitive calibration range of 0.025 µg/mL to 1.0 µg/mL. In this method, the ESI source was safeguarded, and favorable analytical conditions were ensured by diverting mobile phase entry into the mass detector, and a nominal quantity of mobile phase was entered into the detector. This method exhibits the capability of identifying and quantifying potential genotoxic impurities; additionally, it can be applied to in-process monitoring during the siponimod manufacturing process. The attained results confirmed that this study contributes to ensuring safe use of siponimod during formulation production.

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Conflict of interest

No conflict of interests.

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