DEVELOPMENT AND VALIDATION OF A RELIABLE AND RAPID LC-MS/MS METHOD FOR QUANTIFICATION OF SIPONIMOD IN RAT PLASMA AND ITS APPLICATION TO A PHARMACOKINETIC STUDY

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Abstract

Siponimod is novel alkoxyimino derivative indicated for the treatment of adult patients with secondary progressive multiple sclerosis. LC-MS/MS method developed for the estimation of siponimod in rat plasma using fingolimod as internal standard (IS) with wide range of linearity from 0.050 to 1000 ng/mL. Protein precipitation technique was used to extract both siponimod and fingolimod by adding 200 µL of acetonitrile to 100 µL plasma. 0.1% formic acid in Milli-Q water used as mobile phase-A and 0.1% formic acid in acetonitrile used as mobile phase-B in gradient mode to achieve good baseline. Kinetex C_{18} column used to achieve the separation with in short chromatographic run of 1.50 minutes. Positive electrospray ionization mode used to quantify the both siponimod and internal standard in multiple reaction monitoring (MRM) mode. The developed method is rapid and selective for estimation of siponimod with good sensitivity at lower limit of quantification of 0.050 ng/mL. The standard curve is linear in the range of 0.050 to 1000 ng/mL, with $r^2 > 0.9997$. The intra- and inter-day precision and accuracy results met acceptance criteria as per Food and Drug Administration (FDA) guidelines. Bench top, auto sampler, long-term storage and freeze thaw cycle stability established to know the stability of analyte and IS. The developed method was fully validated and can be applied to pharmacokinetic studies.

Keywords: Siponimod, LC-MS/MS.

Abbreviations used: IS, Internal standard; MRM, Multiple reaction monitoring; QC, Quality control

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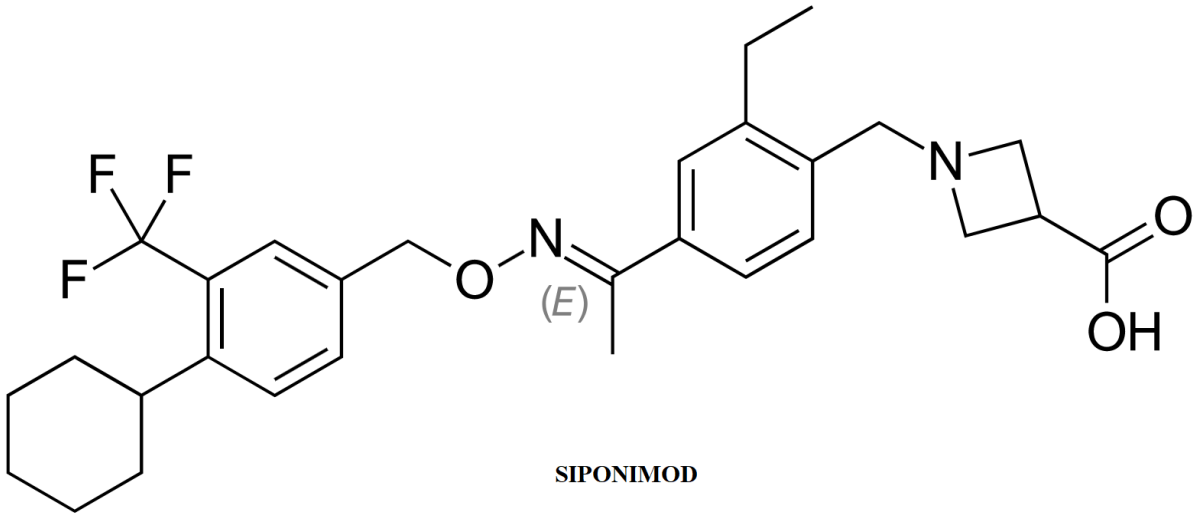
Introduction

Multiple Sclerosis (MS) is a chronic, immune-mediated inflammatory condition that causes neuro-axonal injury in the Central Nervous System (CNS) leading to permanent and severe neurological impairment and disability (1). The disease is the leading cause of non-traumatic disability in young adults, with a mean age at diagnosis of ≈ 30 years and an estimated ≈ 2.2 million individuals affected worldwide in 2016 (2). The disease has a significant impact on quality of life and imposes a considerable burden from a societal and health payer perspective (3, 4). About two-thirds of patients who suffer from a RRMS (relapsing-remitting MS) will within a median time of 15 to 20 years from onset, develop a secondary progressive multiple sclerosis (SPMS) characterized by sustained disability with or without superimposed relapses (5). To date Multiple sclerosis is treatable but no cure is available. However, management of progressive MS, including SPMS, remains challenging, with limited options available for treating these forms of the disease (6, 7). Hence, there is huge scope for new drug development in this disease area. To fill this gap siponimod is invented to treat multiple sclerosis.

Siponimod chemically{1-({4-[(1E)-N-{[4-cyclohexyl-3-(trifluoro) phenyl] methoxy} ethanimidoyl] -2-ethylphenyl}methyl) azetidine-3-carboxylic acid} is novel alkoxyimino derivative indicated for the treatment of adult patients with secondary progressive multiple sclerosis (SPMS) with active disease evidenced by relapses or imaging features of inflammatory activity. Figure 1 represents the structure of siponimod. Siponimod is a highly selective $S1PR_1$ and $S1PR_5$ modulator, with both receptors (mainly $S1PR_1$) pivotal in pathways regulating lymphocyte egress from lymph nodes, and $S1PR_1$ (on astrocytes) and $S1PR_5$ (on oligodendrocytes) playing a key role in myelination and CNS repair (9). Siponimod is designed not to target the S1P3 and S1P4 receptors at pharmacological doses, in contrast to fingolimod (which targets S1P1, S1P3, S1P4, and S1P5 receptors) (10).



SIPONIMOD

Figure 1. Chemical structures of siponimod

Siponimod is exists as a co-crystal with fumaric acid and observed the polymorphism. Siponimod fumaric acid is a BCS class II compound with good absorption characteristics but practically insoluble in aqueous media, although solubility increases slightly at low pH or above pH 6.8. It is not very soluble in many organic solvents (10). Siponimod was highly plasma protein bound (>99%) in all species.

Literature survey reveals that no validated method have been reported till date for the quantitative determination of Siponimod in biological matrix by using LC-MS/MS. One method was reported by Anita et al., 2022 (13) for simultaneous estimation of siponimod and ponesimod by UPLC, which is useful for assay purpose in bulk dosage form. The other method captured by Ulrike et al., 2018 (14) was not a validated method with lack of sensitivity and has long chromatographic run time which is not useful for analyzing more samples per day. Hence, authors attempted to develop a sensitive, rapid and cost effective LC-MS/MS method for quantification of siponimod, which helps the researchers for therapeutic drug monitoring and pharmacokinetics (11, 12).

The objective of this work is, to develop a simple, selective and sensitive method, which employs low cost protein precipitation technique for

sample preparation and liquid chromatography with electrospray ionisation-tandem mass spectrometry for quantitation of siponimod in rat plasma. The proposed method has significant advantages like very short run time, wider linearity range with a more sensitivity and cost effective. The present method has been validated as per the current US FDA guidelines (15).

Experimental

Materials and reagents

Siponimod reference standard purchased from Beijing Mesochem Technology Co., Ltd (Mainland, China). Sigma-Aldrich (Hyderabad, India) selected to procure reference standard of internal standard (IS; Fingolimod). HPLC grade acetonitrile purchased from J.T Baker (Phillipsburg, USA). HPLC grade dimethyl sulfoxide (DMSO) and formic acid procured from Sigma-Aldrich (Hyderabad, India). Milli-Q water (18.2 mΩ and TOC≤50 ppb) from Milli-Q purification system, (Millipore, Bangalore, India) was used throughout the study. Drug-free rat plasma purchased from Biochemed (Winchester, USA).

Chromatographic and mass spectrometric conditions

Binary solvent manager, sample manager and column manger from waters Acquity UPLC system (Waters Corporation, Milford, USA) used for solvent and sample delivery. 0.1% formic acid in Milli-Q water used as mobile phase-A and 0.1% formic acid in acetonitrile used as mobile phase-B. Gradient elution program as follows (minutes, % mobile phase B) (0.01, 20) (0.20, 20) (0.90, 80) (1.10, 80) (1.20, 20) (1.50, 20) to separate Siponimod and IS. Flow rate of 0.6 mL/min and Kinetex C_{18} column (50 mm×3.0 mm, 1.9 µm; Phenomenex, India) maintained at 40°C used to achieve good peak shape. The auto-sampler maintained at 5°C and injection volume was 10 µL. 1.50 min is the total chromatographic run time. The analyte and IS were detected using a Waters XEVO TQ mass spectrometer (Waters corporation, Milford, USA) equipped with Z spray source. Positive ion electrospray ionization (ESI) with MRM mode used to quantify the Siponimod and IS. Both cone gas and desolvation gas used nitrogen with a flow rate of 50 L/Hr and 800 L/Hr respectively. The mass parameters capillary voltage, extractor voltage, source temperature and desolvation temperature were set at 3.50 KV, 3 V, 150°C and 400°C respectively. The MRM transitions along with the cone voltage and collision energy for both siponimod and IS were as follows: Siponimod m/z 517.2→416.3, 32 V, 25 eV; Fingolimod m/z 308.2→255.2, 40 V, 21 eV with dwell time 100 ms. Masslynx software, version 4.2 used for data acquisition and calculations.

Preparation of calibration standards and quality control samples

1mg/mL concentration of Siponimod stock solution was prepared by dissolving requisite amount in DMSO. Working stock solutions at the concentrations in the range of 1 to 20000 ng/mL were prepared by further diluting the Siponimod stock solution in acetonitrile: water (20:80, %v/v). Requisite volume of working stock solution spiked in drug free rat plasma to get nine-point calibration curve standard solutions of siponimod at concentrations of 0.05, 0.10, 0.50, 2.50, 10.00, 100.00, 400.00, 800.00 and 1000.00 ng/mL. The quality control (QC) samples were similarly prepared in drug free rat plasma by a separate weighing of standards at concentration of 750 ng/mL (high quality control, HQC), 375 ng/mL (middle quality control, MQC), 0.150 ng/mL (low quality control, LQC) and 0.050 ng/mL (lower limit of quantification quality control, LLOQ QC). 1 mg/mL concentration of IS (Fingolimod) stock solution was prepared in DMSO. Final IS solution at the concentration of 800 ng/mL was prepared by further diluting the IS stock solution with acetonitrile. All the prepared plasma samples were stored at −70 °C.

Sample preparation

For the extraction of siponimod and IS from rat plasma, a simple protein precipitation technique was established. Aliquot of 100µL calibration standards and QC samples retrieved from deep freezer and thawed at ambient temperature. To acidify the retrieved samples, 5 µL of formic acid added and vortexed to mix, further added 5 µL of IS (800 ng/mL of fingolimod). 200 µL of acetonitrile was added to sample for extraction of both siponimod and IS. Added 100 µL blank plasma, 5 µL of formic acid, 5 µL of acetonitrile and 200 µL of acetonitrile to prepare blank sample. Briefly vortexed these samples for 10 min and then centrifuged at 13200 rpm for 10 min on refrigerated centrifuge (Eppendorff, USA) at 4°C. Separate the supernatant and transfer into HPLC vial and inject 10 µL of aliquot on the LC-MS/MS system for analysis.

Method validation

The developed bioanalytical method thoroughly validated to evaluate the parameters like

selectivity, linearity, precision and accuracy, recovery, matrix effect, dilution integrity and stability, as per industrial guidance for the bioanalytical method validation (US DHHS et al., 2018). Selectivity experiment executed to differentiate and quantify the analytes in the presence of any interferences from plasma. To evaluate this, eight different blank rat plasma (which includes one hemolyzed, one lipemic and six normal lots) chromatograms compared with corresponding spiked plasma at LLOQ QC level. Interferences from plasma at retention time of the analyte should be less than 20% of the peak area of LLOQ response. Interferences from plasma at retention time of the IS should be less than 5% of the mean response of internal standard in LLOQ samples. At concentrations ranging from 0.05 to 1000 ng/mL for siponimod, assessed the linearity by constructing calibration curves. Linearity of five standard calibration curves containing at least nine non-zero standards determined by constructing a weighed ($1/x^2$) least squares linear regression method through the measurement of the peak area ratio of analyte to IS. In addition, to confirm the absence of direct interferences analyzed the blank and blank+IS samples. To construct calibration curves, blank and blank+IS data were not included. The acceptance criteria of accuracy for each of the back calculated concentrations were ±15% except for LLOQ, where it was ±20%. At least 75% of the standards, including the LLOQ and ULOQ should met the acceptance criteria to accept the calibration run, otherwise the calibration curve should be rejected. Two different precision and accuracy batches analysed same day to evaluate the Intra-day precision and accuracy. Each precision and accuracy batch covers CC standards and six replicates of each quality control (LLOQ, LQC, MQC and HQC) samples. Five different precision and accuracy batches analyzed in different days during validation period to evaluate the Inter-day precision and accuracy. The acceptance values included for accuracy within ±15% deviation from the original values, except for the LLOQ where it should be ±20% and a precision of ±15% coefficient of variance (%CV), except for LLOQ, where it should be ±20%. To evaluate the extraction efficiency of developed method, recovery experiment planned. At three different QC levels (six replicates of each LQC, MQC and HQC) recovery of analyte estimated by comparing the peak area response of extracted analytes with respect to unextracted analytes (extracted blank sample spiked with the analytes) and that represent 100% recovery. In similar way recovery of IS was calculated by comparing the mean peak area of extracted QC samples (n=18) with mean peak area of unextracted QC samples. Recovery of the analytes and IS should be precise and reproducible at all QC levels and need not be 100%. To ensure that sensitivity of the method and it not compromising by matrix, matrix effect experiment should be performed. By comparing the peak area ratios (analyte/IS) obtained from post extraction spiking [5 µL of formic acid was added to 100 µL of blank plasma and it was extracted with 200 µL of acetonitrile, followed by adding 5 µL of IS (800 ng/mL of fingolimod) and 5 µL of standard solution containing siponimod at concentrations of 0.150, 375, 750 ng/mL] with respect to mean peak area ratio (analyte/IS) obtained at the same concentration of neat solution, matrix effect will be evaluated. At all three QC levels using six replicates at each level for analyte matrix effect will be calculated, where IS was determined at a single concentration of 800 ng/mL. Dilution integrity to be accomplished to extend the upper concentration limits with acceptable precision and accuracy. By analysing six replicate samples at concentration of two times the ULOQ concentration was prepared and diluted to 2- and 4-fold with blank plasma and evaluated the dilution integrity of analyte. Stability tests were conducted to evaluate the analyte stability in stock solutions and in plasma samples under different conditions. By comparing the area response of the analyte (stability samples) with the response of the sample prepared from fresh stock solution evaluated the stock solution stability at room temperature and refrigerated conditions (2–8°C). Bench-top stability (8 h), processed sample stability (autosampler stability for 24 h), freeze thaw stability (three cycles) and long-term stability (60 days) were verified at LQC and HQC levels using six replicates at each level. Samples were considered stable if assay values were within the acceptable limits of accuracy (±15%) and precision (±15% CV).

Pharmacokinetic study

Male sprague dawley rats (n = 6) were used to carry out the pharmacokinetic study. The animals had free access to drinking water during the experimental period and fasted overnight. Oral formulation of siponimod was prepared in gravimetric dilution pattern. It is prepared by triturating accurately weighed amount of powdered compound in methylcellulose solution (0.5%, w/v water) as suspension. Rats were fasted overnight (12 h) and administered Siponimod (5mg/kg) using an Oral gavage at 2 mL/Kg

volume. About 0.25 mL of blood sample was collected by retro-orbital bleeding into prefilled eppendorff tubes having potassium EDTA at pre-dose and 0.25, 0.50, 1, 2, 4, 6, 8, 12 and 24 h. To separate the plasma samples were centrifuged using micro17R refrigerated centrifuge (Thermo scientific, Germany) at 9600rpm for 5 minutes and then stored at −80 °C till further analysis.

Discussion

Method development and optimization

To attain the maximum stable response of the precursor ions and the major product ions of the Siponimod, positive electro spray ionization mode was selected. For developing a selective and sensitive method, MRM mode selected for quantification of analytes. In tuning, verified the Siponimod response in both positive ion and negative ion mode and selected positive ion mode for getting maximum response, which is helpful to attain lower limit of quantification. $[M+H]^+$ ion is the prominent ion for Siponimod in the full scan of Q1 spectrum and it was used as the precursor ion to obtain Q3 product ion spectra. Optimized the cone voltage and collision energy to get highest intensity for precursor ion and product ion respectively. The mass transition ion pair selected as m/z 517.2→416.3 for siponimod. The product ion mass spectra of siponimod presented in Figure 2.

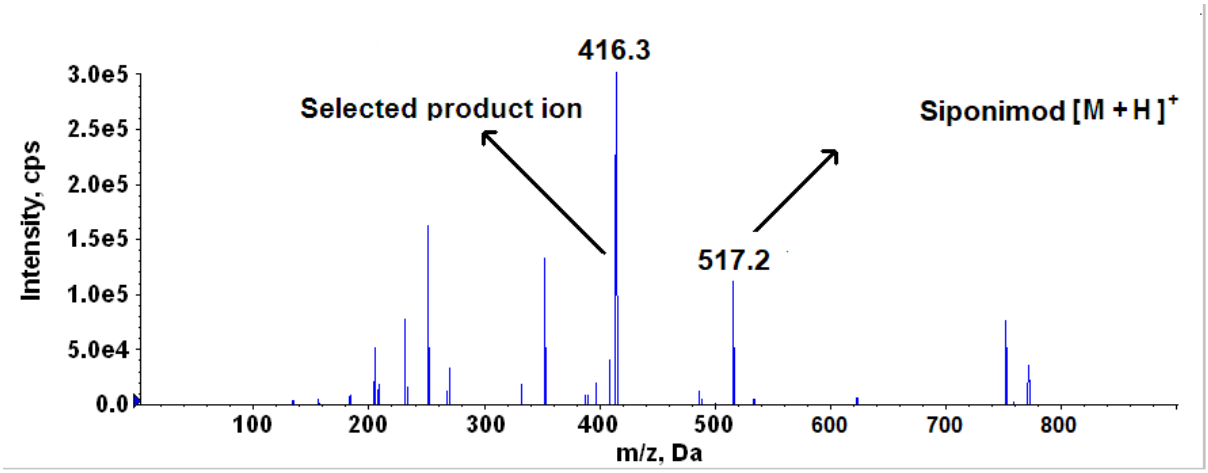


Figure 2. Product ion mass spectra of $[M+H]^+$ of Siponimod

To achieve a good chromatographic resolution and symmetric analyte peak shapes optimized the composition of mobile phase and column. Shorter column was selected to reduce the chromatographic run time. To achieve complete chromatographic resolution of analyte and IS from interfering biological matrix, verified the feasibility of various mixtures of solvents such as methanol and acetonitrile with different buffers such as ammonium acetate, ammonia solution with altered flow rates on different types of columns such as C_{18} and C_8. After conducting several trails, mobile phase system consisting of 0.1% formic acid in Milli-Q water – 0.1% formic acid in acetonitrile (minutes, % mobile phase B) in gradient mode (0.01, 20) (0.20, 20) (0.90, 80) (1.10, 80) (1.20, 20) (1.50, 20) with a flow rate of 0.6 mL/min on a Kinetex C_{18} column achieved the good chromatographic separation of the analyte and IS with desired response. The retention times of siponimod and IS was 0.91 and 1.01 min respectively. Several compounds tested to find a suitable IS and finally fingolimod found to be compatible with targeted analyte in terms of chromatographic behavior, ionization yield and extraction efficiency.

Prior to LC-MS/MS analysis, sample pre treatment needed to remove protein and potential interferences from plasma. Protein precipitation technique used for sample preparation in this study. Protein precipitation technique helps in getting a neat chromatogram of a blank sample and consistent recovery of siponimod from the plasma. This extraction procedure provide good extraction efficiency, hence it helps in lower the limit of quantification. This extraction procedure requires less time to perform the assay, which gives high throughput sample analysis and decreases overall cost of the assay.

Results

Selectivity

Blank plasma sample and blank plasma spiked with LLOQ standard of siponimod and IS chromatograms represented in Figure 3 (A and B). Verified the blank plasma sample at the retention time of analyte and IS, no significant endogenous

interferences observed in the respective MRM channel.

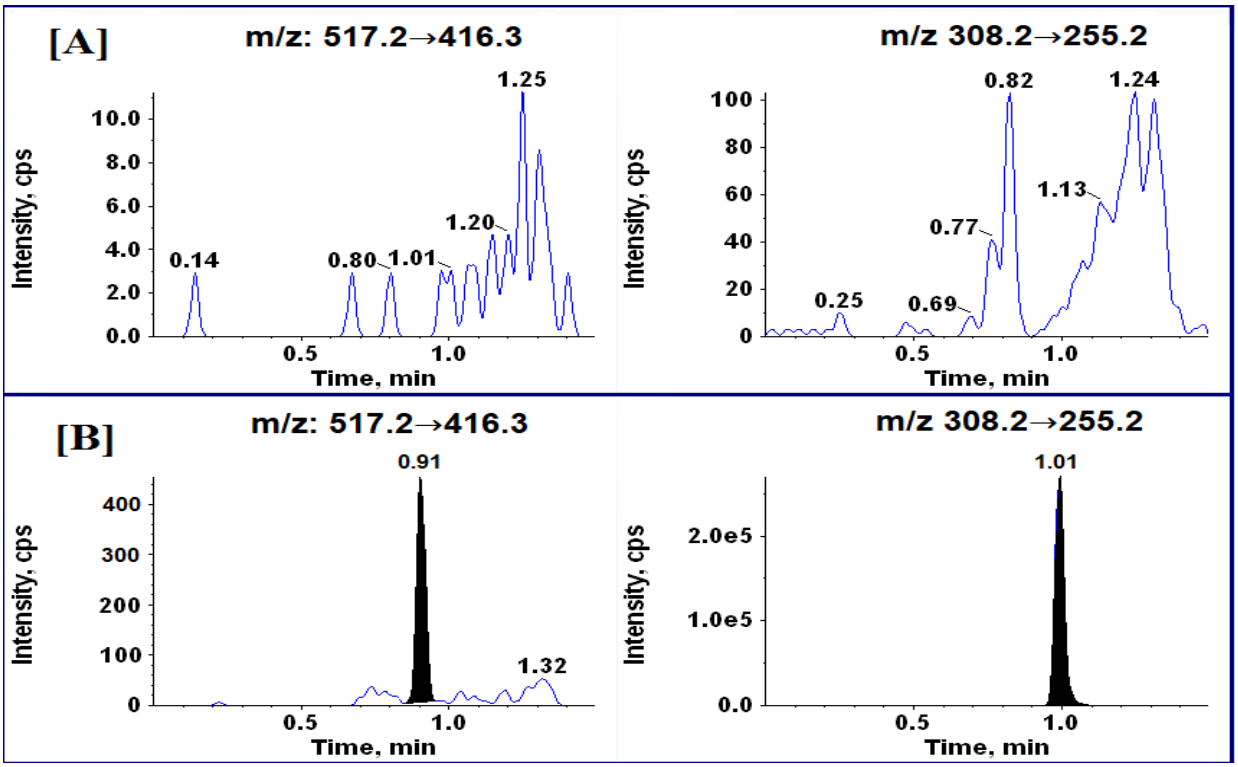


Figure 3. Typical multiple reaction monitoring mode chromatograms [A] Blank chromatograms of siponimod and IS, and [B] Rat plasma spiked with siponimod at LLOQ level and IS.

Linearity

By plotting the peak area ratio of analyte to IS (y) versus the nominal concentration(x) of the calibration points, linearity of each calibration curve was determined and fitted to the y = mx+c using a regression factor ($1/x^2$). At the concentration range of 0.050 to 1000 ng/mL, the nine-point calibration curve was found to be linear. Correlation coefficients were in the range $0.980<r^2<0.995$. The percentage accuracy values ranged from 100.95% to 102.50%, while the precision (%CV) values ranged from 2.68 to 8.46. Calibration curve results for Siponimod summarizes in Table I.

Table I. Precision and accuracy data for back - calculated concentrations of calibration standards

Concentration added (ng/mL)	Siponimod		
	Mean (n=5)	CV (%)	Accuracy (%)
0.050	0.051	7.86	102.40
0.100	0.102	8.46	101.60
0.500	0.508	3.37	101.64
2.500	2.532	4.94	101.29
10.000	10.095	4.49	100.95
100.000	102.505	6.47	102.50
400.000	406.546	3.83	101.64
800.000	818.620	2.68	102.33
1000.000	1020.667	4.92	102.07

Precision and Accuracy

Table II summarizes the intra- and inter-day precision and accuracy values for siponimod and they are within the acceptance limit. The intra-day accuracy ranged between 100.56% and 101.91% with a precision of 5.54% to 8.60%, the inter-day accuracy between 99.13% and 102.51% with a precision of 5.27% to 8.19%.

Table II. Intra -day and inter -day accuracy and precision of siponimod

Concentration added (ng/mL)	Siponimod			
	LLOQ 0.050	**LQC 0.150**	**MQC 375.000**	**HQC 750.000**
Intra-day (n=12)				
Mean	0.051	0.151	382.159	761.602
CV (%)	8.60	5.54	7.03	6.02
Accuracy (%)	101.33	100.56	101.91	101.55
Inter-day (n=30)				
Mean	0.050	0.149	384.415	743.537
CV (%)	8.19	5.27	6.54	6.80
Accuracy (%)	99.40	99.13	102.51	99.14

Recovery

Table III summarizes the mean overall recoveries (with the precision) and extraction recoveries of siponimod and IS were good and reproducible.

Table III. Mean overall recoveries of siponimod and IS

Analyte name	Sample concentration (ng/mL)	Response unextracted (Mean ± CV (%))	Response extracted (Mean ± CV (%))	Recovery	Overall recovery (Mean ± CV (%))
	0.150	1529 ± 3.03	1421 ± 3.59	92.95	
Siponimod	375.000	3811499 ± 1.45	3667993 ± 4.88	96.23	96.45 ± 3.75
	750.000	7614018 ± 0.85	7626985 ± 2.10	100.17	
IS	800	8573226 ± 3.43	7836405 ± 1.62	91.41	

Matrix effect

There was no matrix effect observed from plasma in the measurement of siponimod and IS. The average matrix factor values ranged from 1.00 to 1.05, while the precision (%CV) values ranged from 1.10 to 1.84 for siponimod.

Dilution integrity

The upper limit of concentration was increased to 2000 ng/mL for siponimod by a half and quarter dilution with rat blank plasma. The mean back calculated concentrations for half and quarter dilution samples were within 85% to115% of nominal value, while precision values ranged from 4.43 and 4.89% respectively.

Stability studies

At concentration of 1000 ng/mL, stock solution stability was performed. 100% siponimod remains unchanged after storage for 24 days at 2-8°C and at room temperature for 6 h. Table IV summarizes the results of bench-top stability (8 h), processed sample stability (auto sampler stability for 24 h), freeze–thaw stability (three cycles) and long-term stability (60 days) and found to be within the acceptance limit.

Table IV. Stability data of siponimod in rat plasma

Stability	Siponimod	
	LQC	**HQC**
Bench top (27°C, 8 h)		
Mean (n=6)	0.158	773.399
CV (%)	3.26	3.37
Change (%)	5.67	-0.75
Auto sampler (4°C, 24 h)		
Mean (n=6)	0.154	792.525
CV (%)	3.79	0.89
Change (%)	7.68	3.01
Freeze-thaw (-80°C, After 3rd cycle)		
Mean (n=6)	0.154	792.536
CV (%)	3.67	2.07
Change (%)	0.87	1.61
Long term (-80°C, 60 days)		
Mean (n=6)	0.155	774.906
CV (%)	3.63	2.50
Change (%)	2.09	0.11

Application of the method in pharmacokinetic study

Rat plasma samples collected from sprague dawley rats (n=6) and applied the established LC-MS/MS method to calculate the siponimod concentration in plasma. Figure 4 shows the mean plasma concentrations vs time profiles of siponimod. In addition, Table V represents the pharmacokinetic parameters. At T_{max} 4.00 hours, Siponimod achieved the maximum concentration (C_{max}) in rat plasma (82.619 ng/mL). The area under curve from zero hour to last hour measurable concentration (AUC_{0-t}) and area under curve from zero hour to infinity ($AUC_{0-\alpha}$) 476.176 ng.h/mL and 567.179 ng.h/mL. The chromatograms of siponimod after oral administration represented in Figure 5.

Table V. Pharmacokinetic data of siponimod (n=6, Mean ± SD).

Parameters	Siponimod (5mg/kg)
C_{max} (ng/mL)	82.619±5.07
T_{max} (h)	4.00±0.00
$T_{1/2}$ (h)	5.56±0.30
AUC_{0-t} (ng.h/mL)	476.176±37.23
$AUC_{0-\alpha}$ (ng.h/mL)	567.179±35.43

C_{max}, the maximum plasma concentration; T_{max}, the time to reach C_{max}; $T_{1/2}$, elimination half-life; AUC_{0-t}, the area under the plasma concentration-time curve from zero to the last sampling time; $AUC_{0-\alpha}$, the area under the plasma concentration-time curve from time zero to infinity

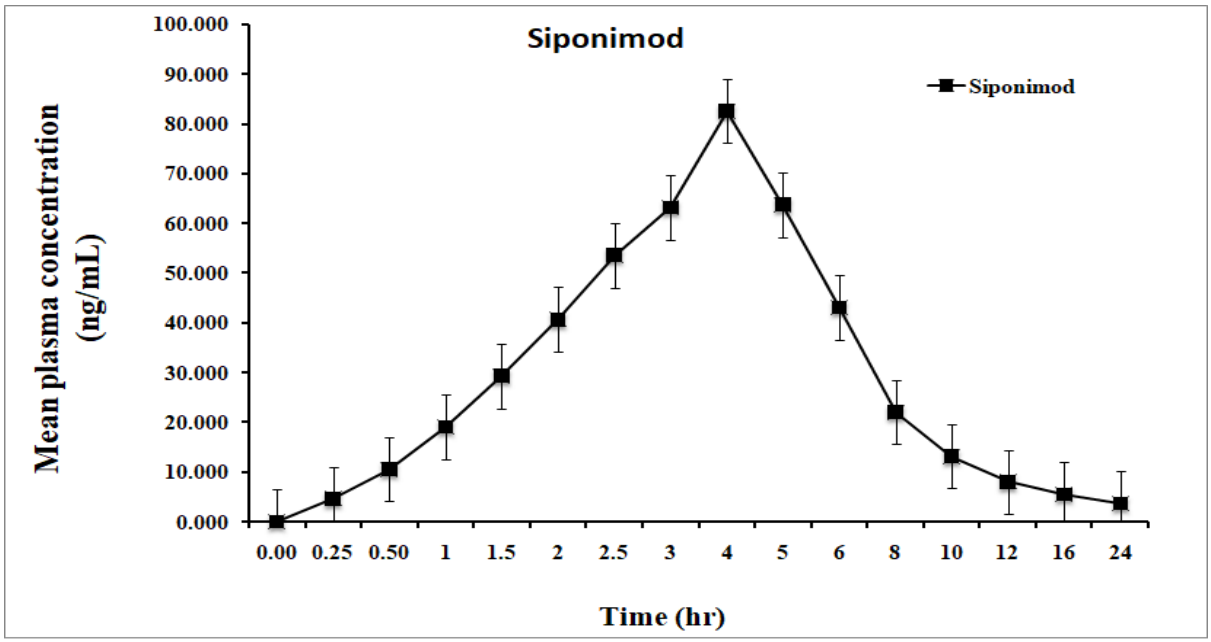


Figure 4. Mean plasma concentration vs time profile of Siponimod

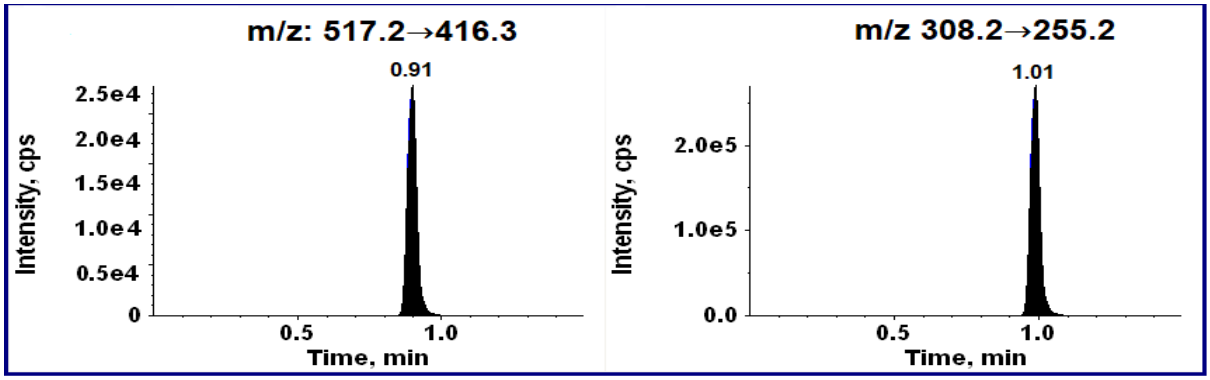


Figure 5. Typical multiple reaction monitoring mode chromatograms of siponimod and IS (right panel).

Conclusion

In summary selective, sensitive and rapid LC-MS/MS method was developed and validated for quantification of siponimod in rat plasma. Till date there is no LC-MS/MS method reported for quantification of siponimod in any biological matrix. Wide range of linearity, good extraction efficiency and shorter run time are the significant advantages of the developed method. Moreover, with the lower limit of quantification as low as 0.050 ng/mL, developed method shows good sensitivity for siponimod. Sample preparation technique and the shorter chromatographic runtime allows to analyse more samples per day. From the validation results, we can conclude that the present method can be helpful in determining the plasma concentrations of siponimod in samples obtained from pharmacokinetic studies with desired precision and accuracy.

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