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DEVELOPMENT AND VALIDATION OF A METHOD FOR QUANTIFICATION OF SIPONIMOD IN SPIKED RAT PLASMA

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Keywords:

Development, Validation, Siponimod, rat plasma, Stability studies, LC–MS/MS

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ABSTRACT: The creation of reliable bioanalytical LC-MS (liquid chromatography-mass spectroscopy) methods is crucial for the discovery, development, and eventual marketing approval of new drugs. A brand-new medication called siponimod was developed to treat Multiple Sclerosis (MS). This medication is a sphingosine-1-phosphate (S1P) receptor modulator and is hypothesised to contribute to the reduction of MS-related inflammation of the central nervous system. Since the last several decades, the usage of ant sclerosis agents has grown dramatically, however there is currently no bio analytical approach available for quantifying siponimod in biological matrix investigations employing LC-MS/MS. To create a novel, quick, and accurate LC-MS/MS approach for simultaneously measuring siponimod in rat plasma utilising the liquid-liquid extraction method (LLE) and deuterated siponimod (SId6). Method: Chromatographic separation was performed in isocratic mode using acetonitrile and OPA buffer at a flow rate of 1 ml/min on a reverse phase Phenyl C18 (150 mm x 4.6 mm, 3.5 m) column. Utilising a positive-mode electrospray ion interface and multiple reaction monitoring (MRM) procedures, quantification was accomplished. Over the concentration range of 5.00–100.00pg/mL, the technique demonstrated good linearity. For siponimod at HQC and LQC, the intra-batch and inter-batch precision (%CV) was 4.3%, and the matrix effect (%CV) was 0.021% and 0.13%, respectively. The method's ease of use makes it suitable for use in laboratories and offers a useful tool for pharmacokinetic investigations. The specific test has been expertly used in research with rats.

INTRODUCTION: Multiple Sclerosis (MS) is a chronic, inflammatory autoimmune disease of the central nervous system that interferes with brain and body connection. The majority of people with this condition present with their first symptoms between the ages of 20 and 40, which are frequently the most productive years of life.

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Fatigue, altered gait, bowel or bladder problems, aberrant muscle twitching, visual distortion, and depressive or mood swings are among the symptoms that may occur. One of the most prevalent neurological disabilities among young people is MS, which affects women more commonly than males.[1]

Within six hours following the initial dosage, siponimod reduces the peripheral blood lymphocyte count in a dose-dependent manner. This is brought about by the reversible buildup of lymphocytes in lymphoid tissues as a result of a lack of lymphocyte release. As a result, the inflammation linked to multiple sclerosis is

reduced. 90% of patients' lymphocyte counts revert to normal within 10 days following the end of treatment [2]. The first signs of MS damage are inflammation of the white and grey matter tissues in the central nervous system brought on by localised immune cell infiltration and associated cytokines. Other components in the pathophysiology of MS include B cells and their cytokines. Inflammation is promoted by the lymphotoxin [or transforming growth factor beta TGF and TNF- generated by these cells. The central nervous system has the S1P receptor, a significant receptor involved in lymphocyte function. Lymphocyte egress and recirculation are two of the many physiological processes that S1P receptor (S1PR) signalling is connected [3-5]. According to a literature review, there are no bioanalytical chromatographic techniques for siponimod estimation using the LC-MS/MS approach [6-12]. Therefore, the primary goal of the current study was to create a straightforward bioanalytical technique for siponimod estimation from rat plasma and to apply it to stability tests while taking into account precision, sensitivity, rapidity, selectivity, and stability studies according to US-FDA requirements.

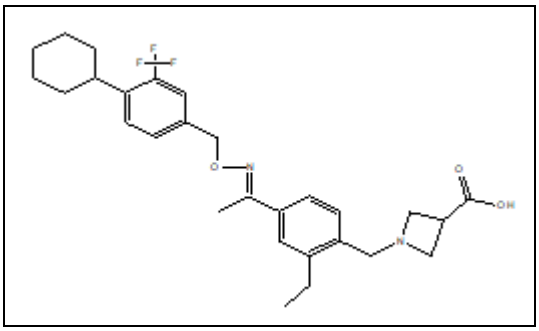

FIG. 1: CHEMICAL STRUCTURE OF SIPONIMOD

MATERIALS AND METHODS:

Chemicals and Reagents: As gift samples, the medications siponimod and siponimod-d6 were purchased. Mass spectra show the preferred mass transitions, m/z 569.7/516.4 and 569.2/516.7, which were used to quantify siponimod and siponimod-D6, respectively **Fig. 3** and **4**. We bought our acetonitrile, methanol, and ethyl acetate from the Merck Chemical Division in Mumbai. Throughout the experiment, milli-Q-system (Millipore) ultra-pure water was employed.

Equipments: The Waters, Alliance e2695-HPLC system is connected to a SCIEX QTRAP 5500 quadrupole linear ion trap tandem mass spectrometer with an ESI ion source, and software empower 2.0.

EXPERIMENTAL:

Optimized Chromatographic Conditions: At room temperature, the analyte was chromatographically separated using a Phenyl C18 (150mm x 4.6mm, 3.5m) column. As the mobile phase, acetonitrile and OPA buffer were combined at a 50:50 v/v ratio. With a 10 mL injection volume, the flow rate is 1.0 mL/min. a triple quadruple mass spectrometer that uses positive ionisation and electro spray ionisation to locate and measure analyte and internal standards. Declustering potential (40V), entry potential (10V), exit potential (15V), and collision energy (15V) for siponimod is the intensification of the source and compound characteristics. The source conditions were optimised as follows: source temperature: 500°C, ion spray voltage: 5500V

Preparation of Standard Solutions:

Siponimod and Siponimod-D6 Standard Stock Solution (200ng/ml): Weigh 10mg of siponimod standard & siponimod D6 into a 100ml volumetric flask diluted volume with diluent. Further diluted 0.1ml to 100ml with diluent.

Preparation of Linearity & Quality Control Solution: Prepare the linearity solutions for concentrations of siponimod that range from 5ng/mL to 100ng/mL. 2.5 ng/mL (LLOQ), 5.0 ng/mL, 12.5 ng/Ml, 25.0 ng/mL (LQC), 37.50 ng/mL, 50.00 ng/mL (MQC), 62.50 ng/mL, 75.00 ng/mL (HQC), and 100 ng/mL. Centrifuge for 15 to 20 minutes @ 4000 RPM. Obtain the supernatant liquid. Inject the solution into the chromatographic system after filtering it using a 0.45 nylon syringe filter.

Extraction Procedure: Label the plasma samples that have been centrifuged and given siponimod at a concentration of 50ng/ml in accordance with the time intervals. 300 l of diluent should be added to 200 l of plasma and thoroughly mixed. To further precipitate all the proteins and blend in the vortex cyclo mixture, add 300 L of methanol. Centrifuge for 15 to 20 minutes @ 4000 RPM. Collect the supernatant solution, filter it with a 0.45-nylon

syringe, and then inject it into the chromatographic apparatus.

Buffer Preparation: 1000 cc of water and 1 cc of triethanolamine were combined. Orthophosphoric acid was used to adjust the pH to 2.5 via 0.22 millimeter membrane filter paper.

Methodology for Analysis: Fill the chromatograph with sample solutions, linearity solutions, and blank (mobile phase) solutions before recording the chromatograms. Calculate the peak area because of the Siponimod peak. Using the equation derived from the linearity curve, determine the amount of siponimod contained in the plasma sample.

Bioanalytical Method Validation: The method was validated in accordance with US Food and Drug Administration bioanalytical method validation guidelines. Studies were conducted to demonstrate the capability of the proposed method, including system suitability, selectivity and specificity, LOQ (limit of quantification or sensitivity), injector carryover, linearity, precision and accuracy, recovery, matrix effect, dilution integrity, re-injection reproducibility, ruggedness (analyst and column), and sample stability [13-16].

Linearity: The results are shown in **Table 1** and **Fig. 2**. Calibration standards were created to reach a linearity range of 2.5, 12.50, 25.00, 37.50, 50.00, 62.50, 75.00, and 100.00ng/mL of siponimod tested. Using the linear regression model = axe + b, the developed standard curve for siponimod exhibits correlation coefficient (R2) greater than 0.999 with linearity range of 2.50-100.00 ng/mL; where, y= Peak area ratio of analyte, X = Concentration (ng/mL) of analyte in plasma, a = Slope, b = Intercept, and R2= Correlation coefficient.

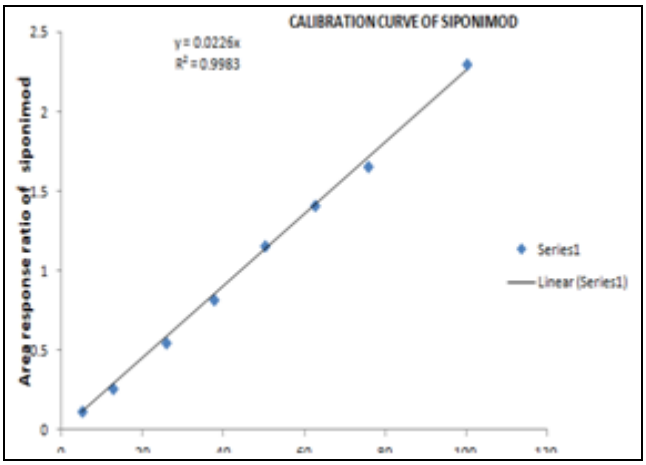

FIG. 2: SIPONIMOD CALIBRATION CURVE DATA

TABLE 1: SIPONIMOD CALIBRATION CURVE

S. no.	Conc of siponimod (ng/ml)	Area response ratio of siponimod
1	5.00	0.121
2	12.50	0.258
3	25.00	0.553
4	37.50	0.817
5	50.00	1.161
6	62.50	1.410
7	75.00	1.658
8	100.00	2.296
Slope	0.02226	
Intercept	0.00675	
R^2 Value	0.9983	

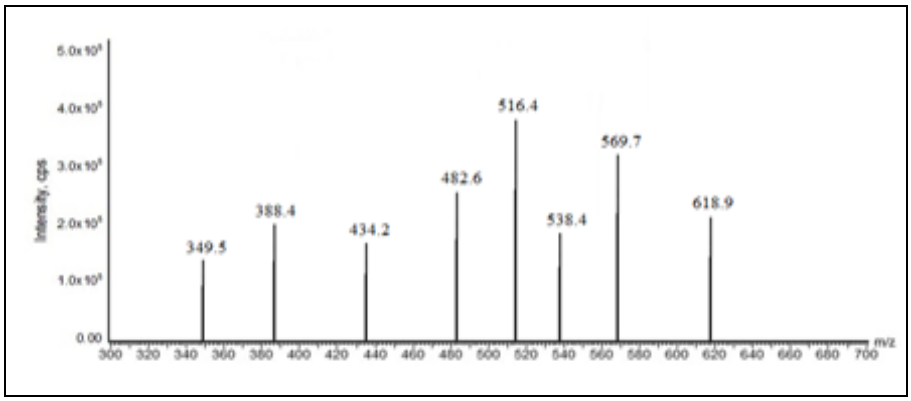

FIG. 3: MASS SPECTRA OF SIPONIMOD

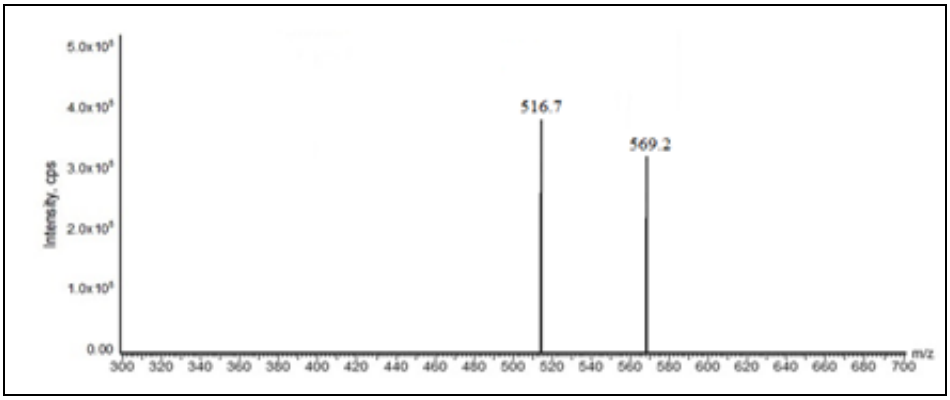

FIG. 4: MASS SPECTRA OF SIPONIMOD-D6

Accuracy and Precision: Six duplicates containing Siponimod were examined at six different QC levels in order to evaluate the intra-assay precision and accuracy. The analysis of the four levels of QC samples from four independent runs was used to calculate the inter-assay precision. Except for LLQC, where it should be within 80-120% for accuracy and 20% of RSD, the requirements for acceptable data include accuracy within 85-115% from the actual values and a precision of within 15% relative standard deviation (RSD). The outcomes were shown in **Table 2.**

TABLE 2: ACCURACY AND PRECISION DATA OF SIPONIMOD (INTER BATCH)

QC sample	Spiked concentration (ng/ml)	Mean (ng/ml)	SD	Accuracy (%)	RSD (%)
LLOQ	2.5298	2.529	0.001	96.32	0.03
LQC	25.209	25.178	0.0137	98.28	1.72
MQC	50.113	50.229	0.106	100.05	0.21
HQC	75.385	75.381	0.005	99.89	0.01

Recovery: At low, medium, and high quality control concentration levels, the recovery of medication and IS was assessed. By comparing its reaction in repeated samples with that of clean standard solution responses, recovery was estimated. The extraction efficiency of an analyte from a sample matrix is measured by comparing the analytical response to the amount of analyte added to the value obtained from the sample matrix. Acetonitrile solvent was used for extraction due to the fundamental characteristics of siponimod. Analyte recoveries ranged from 95.1% to 99.8% in experiments using spiking chemicals, and for IS, they were 94.25%. **Table 3** of the report provided the results.

TABLE 3: RECOVERY OF ANALYTE DATA OF SIPONIMOD

Replicate Number	HQC		MQC		LQC	
	Extracted Response	Un Extracted Response	Extracted Response	Un Extracted Response	Extracted Response	Un Extracted Response
Mean (n=6)	$3.3932X10^5$	$3.6890X10^5$	$2.1303X10^5$	$2.6342X10^5$	$1.2340X10^5$	$1.650\ X10^5$
SD	0.0312	0.0449	0.0390	0.0785	0.0234	0.0778
%RSD	0.04	0.06	0.08	0.15	0.09	0.29
%Mean Recovery	98.12	99.89%	99.12%	100.18%	96.54%	99.63%
% Recovery	98.99%					
Overall SD	1.1604					
Overall %RSD	1.23					

Specificity and Selectivity: In six independent randomly selected blank rat plasma samples, neither Siponimod nor ISTD had any conflicting peaks. As can be seen from the chromatogram below in **Fig. 8**, the entire run duration was 10 min and the drug and IS retention times were approximately 5.795 min and 5.791 min, respectively. There were no interfering peaks around the siponimod and IS peaks in the plasma chromatogram of blank plasma. The chromatogram of blank plasma spiked with IS shows the same thing. The findings were shown in **Fig. 5, 6** and **7.**

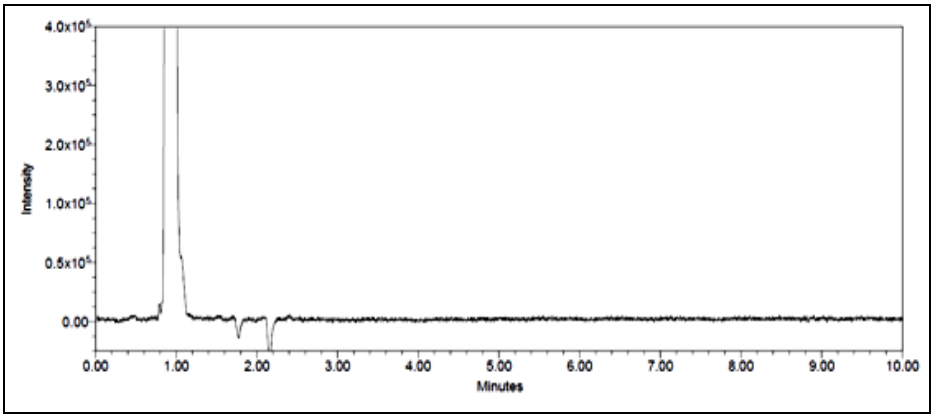

FIG. 5: CHROMATOGRAM OF BLANK PLASMA

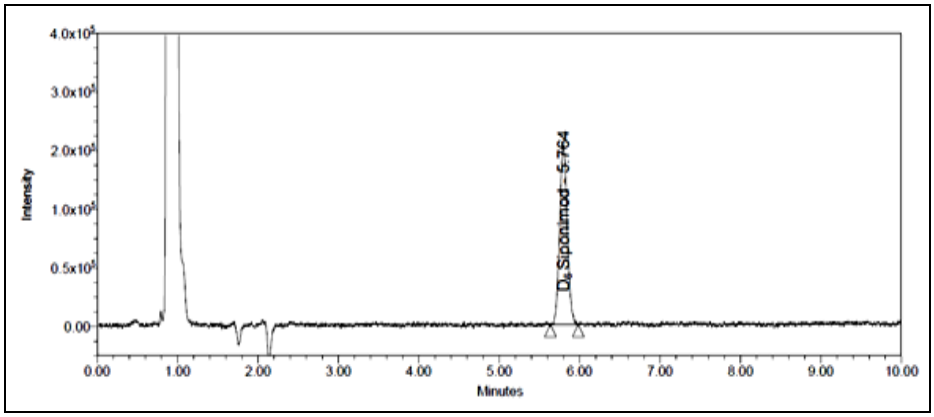

FIG. 6: BLANK RAT PLASMA SPIKED WITH IS

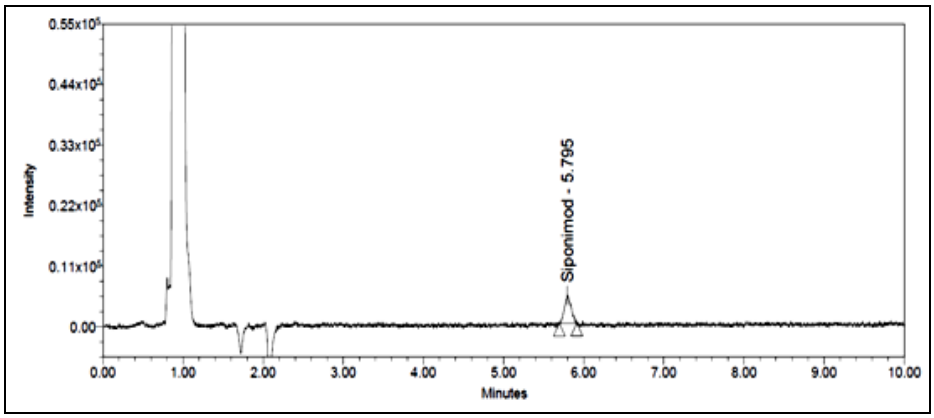

FIG. 7: BLANK RAT PLASMA SPIKED WITH ANALYTE AT LLOQ AND IS

Sensitivity: Siponimod and the internal standard area ratio's respective RSDs were determined to be 1.18% and 98.89%. It so passed the sensitivity test. The LLOQ's typical S/N ratio is around 5. **Table 4** presented the outcomes.

TABLE 4: SENSITIVITY RESULTS OF SIPONIMOD

Replicate Number	LLOQ
	Nominal Concentration(ng/ml)
	2.5420
	Nominal Concentration Range(ng/ml)
	(2.5285-2.5896)
	Calculated Concentration(ng/ml)
Mean (n=6)	2.5420
SD	0.03006
%RSD	1.18
% Mean Accuracy	98.89%

LOD and LOQ: The calibration curve approach was used to individually estimate LOD and LOQ. By injecting steadily smaller quantities of standard solutions, the LOD and LOQ of the chemical were discovered using a newly designed LCMS approach. For siponimod, the LOD concentrations are 0.5 ng/ml and the s/n values are 4. Siponimod limit of quantification is 5.1ng/ml. 24 is their s/n value. The outcomes were shown in **Table 5.**

TABLE 5: LOD & LOQ DATA FOR SIPONIMOD

Name	LOD		LOQ	
	Concentration (ng/ml)	s/n	Concentration (ng/ml)	s/n
Siponimod	0.5	4	5.1	24

Matrix Effect: By contrasting the responses of post-extracted plasma standard QC samples (n = 6) with the responses of the analyte from neat samples at comparable concentrations, the matrix of plasma components over the ionisation of the analyte was identified. Rat plasma that has been chromatographically screened was used to evaluate the matrix effect planned approach. For siponimod at HQC and LQC, the precision (%CV) is 0.15% and 0.61%, respectively.

TABLE 6: MATRIX EFFECT RESULTS OF SIPONIMOD

S. no.	Plasma Lot No.	HQC	LQC
		Nominal Concentration(ng/ml)	
		75.4718	25.5097
		Nominal Concentration Range(ng/ml)	
		(75.298-75.655)	(25.206-25.734)
		Calculated Concentration(ng/ml)	
Mean (n=18)		75.4718	25.5079
SD		0.11682	0.15656
%CV		0.15	0.61
% Mean Accuracy		100.14%	99.84%
No. of QC Failed		0	0

Stability Study:

Auto Sampler Stability: By contrasting the extracted plasma samples that were injected right away (time 0 h) with the samples that were reinjected after being kept in the autosampler at -20°C for 24.0 hr, the sample stability of the autosampler was assessed. Comparing the extracted plasma samples that were immediately injected (time 0.0hr) with the samples that were reinjected after being stored in the autosampler at 20°C for 24.0hr at each LQC and HQC concentration level (LQC 25.00ng/mL and HQC 75.00ng/mL of siponimod) allowed us to assess the reinjection reproducibility. Siponimod %CV and mean accuracy were discovered to be 0.13% and 0.68%, respectively. So, on day one, it passed the Stability test. The outcomes were shown in Table 7.

TABLE 7: AUTO SAMPLER STABILITY DATA OF SIPONIMOD

Replicate No.	HQC	LQC
	Nominal Concentration(ng/ml)	
	75.3168	25.4691
	Nominal Concentration Range(ng/ml)	
	(75.206-75.871)	(25.101-25.691)
	Back Calculated Concentration(ng/ml)	
Mean (n=6)	75.3168	25.4698
SD	0.0992	0.1723
% RSD	0.13	0.68
% Mean Accuracy	98.36%	97.68%

Freeze thaw Stability: The freeze-thaw stability was tested by contrasting newly spiked quality control samples with stability samples that had been frozen at -205°C and thawed three times. The mean accuracy and the %RSD for siponimod were determined to be 0.098% and 0.25%, respectively, for six aliquots of each LQC and HQC concentration level.

So, at -80°C, it passed the Freeze thaw test. A range of 85–115% was determined to contain the mean percent accuracy. **Table 8** presented the results.

TABLE 8: FREEZE THAW STABILITY DATA OF SIPONIMOD

Replicate No.	HQC		LQC	
	Nominal Concentration (ng/ml)			
	75.5112	75.4856	25.4895	25.4243
	Nominal Concentration Range (ng/ml)			
	(75.426-75.569)	(75.358-75.548)	(25.368-25.561)	(25.357-25.545)
	Back Calculated Concentration (ng/ml)			
	Comparison samples	Stability samples	Comparison samples	Stability samples
Mean(n=6)	75.5112	75.4850	25.4895	25.4947
SD	0.0496	0.0652	0.0658	0.0723
% RSD	0.07	0.09	0.26	0.28
% Mean Accuracy	99.11%	99.05%	99.07%	98.33%
% Mean Stability	99.06%		98.80%	

Bench top Stability at Room Temperature: When compared to freshly prepared stock solutions at different LQC and HQC concentration levels, the stability of spiked rat plasma samples stored at room temperature bench top stability using standard stock solutions of Siponimod and Siponimod D6 (ST stability samples) was set aside on the bench for 9.5 hours. Siponimod %CV and mean accuracy were determined to be 0.22% and 0.34%, respectively, meaning that it passed the Bench top stability test. Table 9 presented the results.

TABLE 9: BENCH TOP STABILITY RESULTS OF SIPONIMOD

Replicate No.	HQC		LQC	
	Nominal Concentration (ng/ml)			
	75.454	75.476	25.381	25.430
	Nominal Concentration Range (ng/ml)			
	(75.236-75.698)	(75.157-75.754)	(25.258-25.489)	(25.149-25.602)
	Back Calculated Concentration (ng/ml)			
	Comparison samples	Stability samples	Comparison samples	Stability samples
Mean(N=6)	75.454	75.476	25.381	25.430
SD	0.11966	0.21280	0.08979	0.16040
% RSD	0.16	0.28	0.35	0.63
%Mean Accuracy	99.25%	99.17%	99.19%	98.48%
% Mean Stability	99.21%		99.34%	

Wet Extract: The mean accuracy and %RSD for siponimod were discovered to be 0.11% and 0.13%, respectively. It therefore passed the Wet extract at -2°C.

TABLE 10: WET EXTRACT OF SIPONIMOD

Replicate No.	HQC		LQC	
	Nominal Concentration (ng/ml)			
	75.5565	75.5442	25.5527	25.5820
	Nominal Concentration Range (ng/ml)			
	(75.418-75.698)	(75.457-75.526)	(25.512-25.577)	(25.501-25.651)
	Back Calculated Concentration (ng/ml)			
	Comparison samples	Stability samples	Comparison samples	Stability samples
Mean (n=6)	75.5565	75.5442	25.5527	25.5820
SD	0.1095	0.0592	0.0258	0.0424
% RSD	0.14	0.08	0.10	0.17
% Mean Accuracy	99.36%	99.28%	98.65%	98.41%
% Mean Stability	98.82%		98.53%	

RESULTS & DISCUSSION: Development of bio-analytical Method and Stability Indicating LCMS/MS Method Validation of the estimate of siponimod in pharmaceutical dose form and in bulk. Using a Waters Alliance HPLC system, a Quaternary Gradient Pump of the E2695 Series

with an Auto Sampler Injector and 50ng/ml of sample is injected, eluted with mobile phase containing 0.1% OPA and Acetonitrile in the ratio of 50:50 v/v, and detected by UV detector at 246nm. Siponimod peak was eluted after 5.7 minutes of retention time.

The chosen medicines in this suggested HPLC technique demonstrated high linearity. Results for the recovered medications were determined to be within acceptable ranges (98–102%). These show that the suggested approach was effective for the analysis.

CONCLUSION: Siponimod was the subject of the experiments in the study at hand. HPLC & LCMS/MS paired with a PDA detector is the apparatus utilized in these investigations. Due of its inexpensive cost, HPLC LCMS/MS is generally available in all analytical laboratories.

Although solid and protein precipitation extraction techniques were used in the published literature, we created liquid-liquid extraction for sample preparation that has higher sensitivity and longer column life than protein precipitation. Due to its high economic rate, the solid phase extraction technique was not used. These strategies' many parameters are chosen after careful rationale and several trials and errors. The siponimod in plasma measurement technique using HPLC is particularly sensitive and specific. The procedures created in our lab are quite straightforward and use a liquid-liquid extraction approach, making them high throughput for analysis. All validation data complied with USFDA guidelines for range approval.

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CONFLICT OF INTEREST: According to the authors, there are no conflicts of interest.

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