DEVELOPMENT OF BIO-ANALYTICAL METHOD OF LASMIDITAN IN SPIKED HUMAN PLASMA SAMPLES BY LIQUID CHROMATOGRAPHY AND MASS SPECTROSCOPY

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Abstract:

A sensitive, simple bio-analytical method using the Liquid Chromatography-Mass Spectroscopy (LC-MS) technique for the quantification of Lasmiditan (LST) in human plasma was developed and validated. The drug was extracted by using simple liquid-liquid extraction using a mixture of ethanol and diethyl ether in the ratio of 80:20 (v/v) for the sample preparation involved prior to LC-MS analysis. Separation of analytes and eletriptan (ETN) internal standard were chromatographed on a Phenomenex Luna C18 (100×4.6mm, 5µ) column. The mobile phase Methanol, Acetonitrile, and 0.8% Triethylamine (TEA) mixture in the ratio of 55:30:15 (v/v) with pH 5.6 was eluted using a gradient elution mode a with a flow rate of 0.6 mL/min. Quantification of the drug in plasma was performed in (MRM)multiple-reaction-monitoring mode with the ion transitions m/z 378 → 97 for LST, m/z 383 → 84 for ETN.The method was fully validated with linearity, precision, accuracy,matrix effects, recovery, and stability. The method results showed linearity in the range of 0.1–300 ng/mL (r2 = 0.999) and the stability study confirms that the method was found to be stable. The method showed good precision (RSD% values between 0.59- and 1.03%) and accuracy (90.3 -98.1 %). The present study could be readily applicable for therapeutic monitoring of the Lasmiditandrug in patients'blood.

Keywords: Lasmiditan, LC-MS, Bioanalytical methods, Method validation.

INTRODUCTION

Lasmiditan (LST) is a highly selective agonist of the serotonin 1F [5HT1F] receptor belonging to the triptan class drug. It isindicated for the acute treatment ofmigraine (active by short term) with or without aura (a sensory phenomenon or visual disturbance) in adults[1]. The drug is approved by US-FDA (United States Food and drug administration) in 2019[2-4]. LST drug belongs to the 4-halo benzoic acids class organic compounds and their derivatives. The chemical structure of the LST is presented in figure1. The drugworks by inhibiting the ion of neuronal firing more than the vasoconstriction of cerebral arteries[5-8]. LST is an oral medication and is available in a tablet dosage form. The risk of driving impairment while taking Lasmiditan includes dizziness, central nervous system (CNS) depression,tiredness, numbness, and sedation.

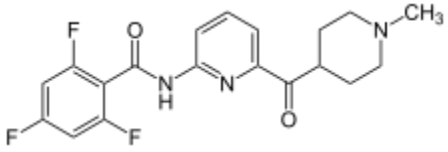

Fig.1:Chemical Structure of Lasmiditan

The present work describes the bioanalytical method development and validation of LST by LC-MS for quantitative analysis in plasma with Eletriptan (ETP) as an internal standard.Liquid chromatography (LC) and Mass spectrometry (MS) are widely used indrug analysis because of the detection of low and high-molecular-weight analytes efficiently.Bio-analytical method validation and regulated bioanalysis are an integral part of technological platforms and regulations [9-10].Eletriptan (ETP) also belongs to the triptans class used for the treatment of migraines[11-14]. Various pharmacokinetic and Safety, efficiency, and durability analyses[15-21] are reported with the LST, and some of the authors mentioned the usage of LC-MS analysis for the determination of LST in biological samples. There is no clear mention of method details to evaluate the selectivity, specificity, or stability of the method. Only one HPLC method[22] is reported with the LST for analysis of the drug in the formulation. Hence the present study is a novel approach to the development of the LC-MS method in spiked human plasma.

MATERIALS AND METHODS

Materials

The solvent acetonitrile, water, and methanol of HPLCgrade quality were procured fromMerck chemicals, Mumbai. Analytical grade triethylamine was obtained from Merck (Darmstadt, Germany). Human plasma in K_3EDTA was obtained from diagnostic laboratories in Guntur and was stored at -20 °C until use.

Instrumentation

The LC-MS system is equipped with the Water Alliance 2695 HPLC system and Waters ZQ (LAA 1369) Mass spectrophotometer. Alliance HPLC (Waters Corporation, Molford, MA, USA) consists of a quaternary gradient pump with an online degasser, automatic sampler, and temperature-controlled column compartment connected with a waters UV 487 detector. The HPLC system is coupled with a Water ZQ mass detector with a triple quadrupole analyzer. Chromatograms were recorded through a computer and treated with the aid of the software MassLynx 4.0 from Waters. Analytical columns of Symmetry (Waters) C18 (150 x 4.6 mm i.dx5), HypersilC18 (100 x 4.6 mm i.dx5 mm), Phenomenex Luna C18 (100×4.6mm, 5µ) were used for separation. Single pan Analytical balance (Make-Sortorious AG, Germany, Model-CP225D) is used for weighing the standards and samples and to increase the solubility, Ultrasonicator (Make-GT professional ultrasonic cleaner, China, Model-D_3) was used.

Preparation of mobile phase

The solvent mixtures in various combinations used for method development used as a mobile phase were prepared for one liter, sonicatedfor 10 mins to ensure the homogeneous solution using an ultrasonicator, and filtered by vacuum filtrationthrough 0.45 µ nylon membrane filter.

Preparation of stock solution

Individual stock solutions of LSTstandard and ETN internal standard solution with a concentration of 1000 mg/L were preparedby dissolving 100 mg drug and IS in 100 mL of methanol separately in a 100 ml volumetric flask.The ETN IS a standard solution of 10 µg/mL prepared separately from the stock solution.LST working solutions were then spiked into the human plasma to make calibration standards and QC samples.LST quality control (QC) samples from the primary prepared stock solution were prepared on seven levels (1, 10, 25, 50, 100, 200, and 300µg/ml). Calibration curve standards of LST standard and 10 µg/mL of ETN IS were spiked to human plasma. Both drugs were extracted by using the liquid-liquid extraction

method.All calibration standards and QC samples were prepared freshly daily. Among the concentrations of calibration solution, 25 ng/mL, 100 ng/mL, and 300 ng/mL concentrations were considered as LQC(low-quality control), MQC(middle-quality control), and HQC(high-quality control) standard solutions respectively.

Sample preparation

A simple liquid–liquid extractionmethod was followed for the extraction of LST from plasma. Various solvents like methanol, ethanol, acetonitrile, chloroform, and dichloromethane were tested for effective drug extraction. Among all studied solvents, ethanol, and diethyl ether in the ratio of 80:20 (v/v) was proved to be the most efficient extracting solvent. The extraction solvent was prepared by dissolving the 20 mL of ethanol in the 80 mL of diethyl ether and sonicating it to dissolve properly.Liquid-liquid extraction method was used to isolate the LST drug from the plasma. The extraction procedurewas validated by spiking 1 mL of human plasma with a knownconcentration drug and IS solution (50 µL) into polypropylene tubes and vortexed for 5 minutes. About 100 µl of extraction solution i.e ethanol and diethyl ether in the ratio of 80:20 (v/v) were added and vortexed for 10 minutes. This mixture was centrifuged at 4000 rpm for 5 minutes at room temperature. The upper organic layer was transferred intoa clear polypropylene tube and placed into the low-volume evaporator to dry under a nitrogen stream at 40°C. Thedried residue was reconstituted using methanol for the preparation of quality control samples. The blank plasma solution was prepared by following the same above procedure without the addition of any drug. an aliquot of 10 µl wasinjected into the LCMS system.

Method Development:

At initial development, methanol was chosen as the major solvent as the standard LST and IS are found highly dissolved in methanol. Different compositions of the mobile phase, with different solvents methanol, acetonitrile, and water in varying combination was tried as mobile phase, and a low response was observed. The pH modifiers were added to enhance the response of separation and sensitivity of the method. The separation was carried on columns with different configurations, and eluents were recorded using a UV detector coupled with a mass spectrophotometer.

Mass spectroscopic conditions for the detection of LST and IS are developed by optimizing the modes of electrospray ions, the voltage of cone, extractor, capillary, and temperature of source

and flow rate of the nitrogen gas. The mass instrument is optimized to obtain sensitivity and signal stability during the infusion of the analyte. The LST drug and IS have shown more response in the positive ion mode than in the negative ion mode. Electrospray ionization (ESI) provided a maximum response over atmospheric pressure chemical ionization (APCI) mode.

Method validation:

Validation of the proposed bio-analytical method for analysis of LST standard and ETN internal standard in human plasma was performed for selectivity, specificity, precision, linearity, recovery, LOD, LOQ, ruggedness, and stability as per USFDA guidelines. The blank plasma was prepared without the addition of a drug injected. A system suitability sample was prepared by spiking 100 µL plasma with 100 µL of aqueous standards of LST standard and ETN IS. The peak area ratio for LST and IS obtained from multiple reaction monitoring was calculated.These requirements are described in ICH guidelines.

RESULTS AND DISCUSSION

Method Development and Optimization

The main objective of this work was to develop a novel, simpleand sensitive method for the determination of LMB in humanplasma using liquid chromatography coupled with massspectrometry (LC-MS). Sample pre-treatment is to remove the interference of endogenousplasma constituents with a high relative extract recoveryof the analyte. Variousorganic solvents were investigated for liquid–liquid extraction and compared.Protein precipitation using ethanol and diethyl ether in the ratio of 80:20 (v/v) gave high extraction recovery and fewer interferencesfrom endogenous substances in plasma. Hence, LLE with ethanol and diethyl ether in the ratio of 80:20 (v/v) was applied to extract LST along with eletriptan(ETN) internal standardfrom plasma. Optimization of chromatographic conditions is intended to consider the various goals of themethod development and to weigh each goal (resolutions, run time, sensitivity, peak symmetry, etc) accurately,according to the requirements of LC-MS can be used for the estimation of LST in plasma samples.

The development of the LC-MSmethod followed systematic changes in the chromatographicfactors. The process involved the selection of appropriate conditionsand their

optimization. These conditions included thetype of column packing, column dimensions, mobile phasecomposition with flow rate, oven temperature, and sampleamount.Mobile phases with different combinations of acetonitrile, water and methanolin combination with different buffers in different pH ranges (3.5-6.5) were studied. To obtain a suitable stationary phase, a lot of commercially availablecolumns were assessed including Phenomenex Luna C8 (100×4.6mm, 5µ) column, Hypersil Gold C18 (50/100 mm × 3.0 mm, 5 µm)and Phenomenex Luna C18 (100×4.6mm, 5µ) column were tested in order optimize the LC separation.

The compositionof mobile phases was also investigated at initial methanol: acetonitrile in the ratios of 80:20, 20:80, and 60:40 (v/v) corresponding pH values are 5.8, 5.2 & 5.5 with Phenomenex Luna C8 (100×4.6mm, 5µ) column was tested for separation and determination of LST and IS. Peaks are responses, the shape of the peaks and resolution were not acceptable at initial trial conditions (Fig. 2-4).

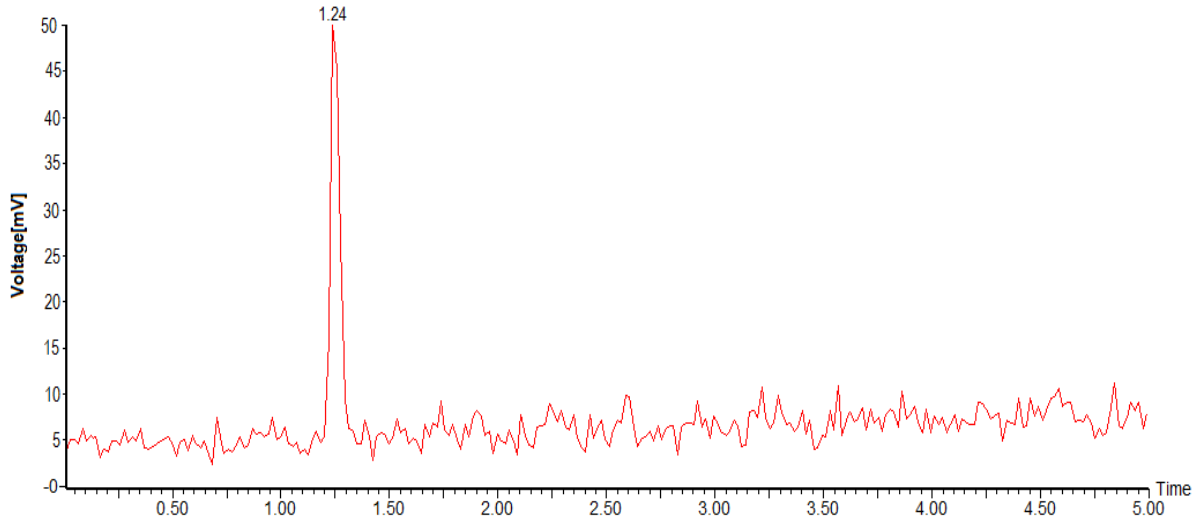

Fig.2: Chromatogram obtained from (Methanol: Acetonitrile (80:20 v/v:) Mobile Phase

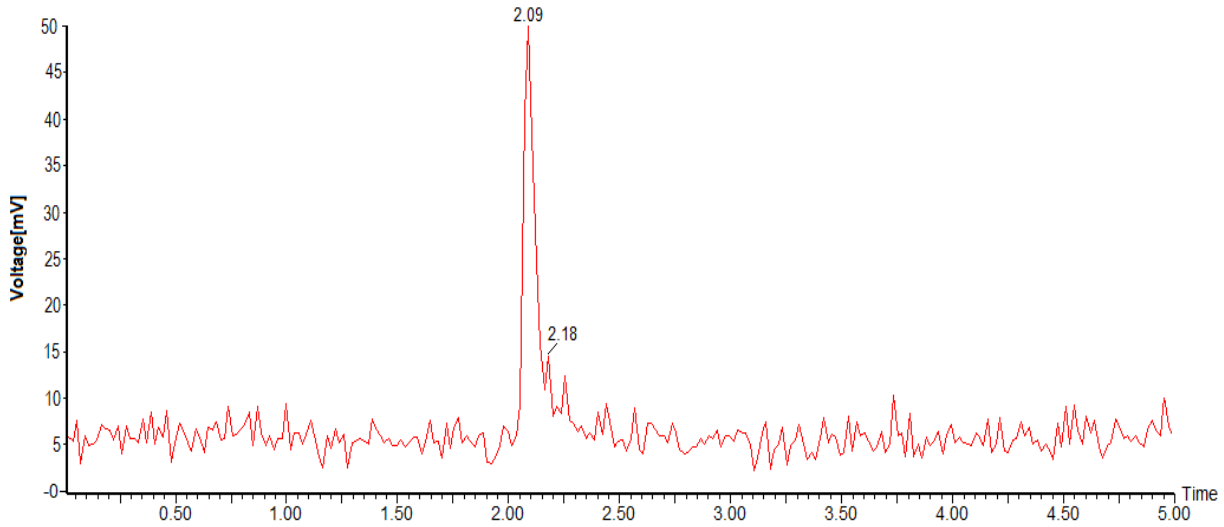

Fig.3: Chromatogram obtained from (Methanol: Acetonitrile (20:80 v/v:) Mobile Phase

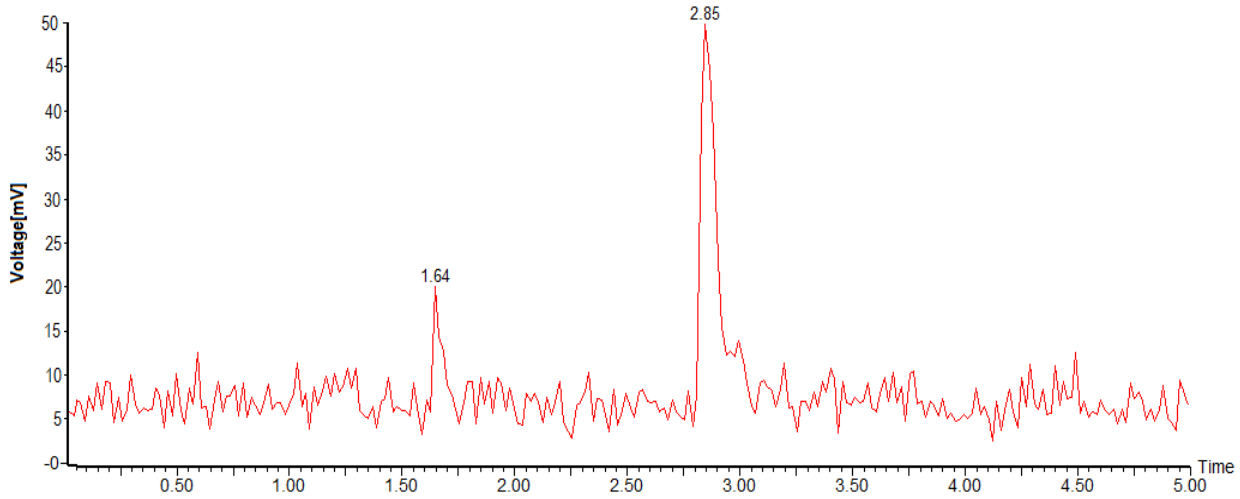

Fig.4: Chromatogram obtained from (Methanol: Acetonitrile (60:40 v/v))Mobile Phase

After several changes in the composition of the mobile phase, the methanol and acetonitrile combination helped in providing sharp peaks with higher sensitivity.The mobile phase combination of methanol, acetonitrile, and 0.8% Triethylamine in the ratio of 55:40:5 and 55:35:10 (v/v) corresponding pH values 5.7 and 5.7. These two conditions baseline drift, broad peaks, and less than two resolution and low plate counts are observed. Hence these trials are not acceptable (Fig. (5-6)).

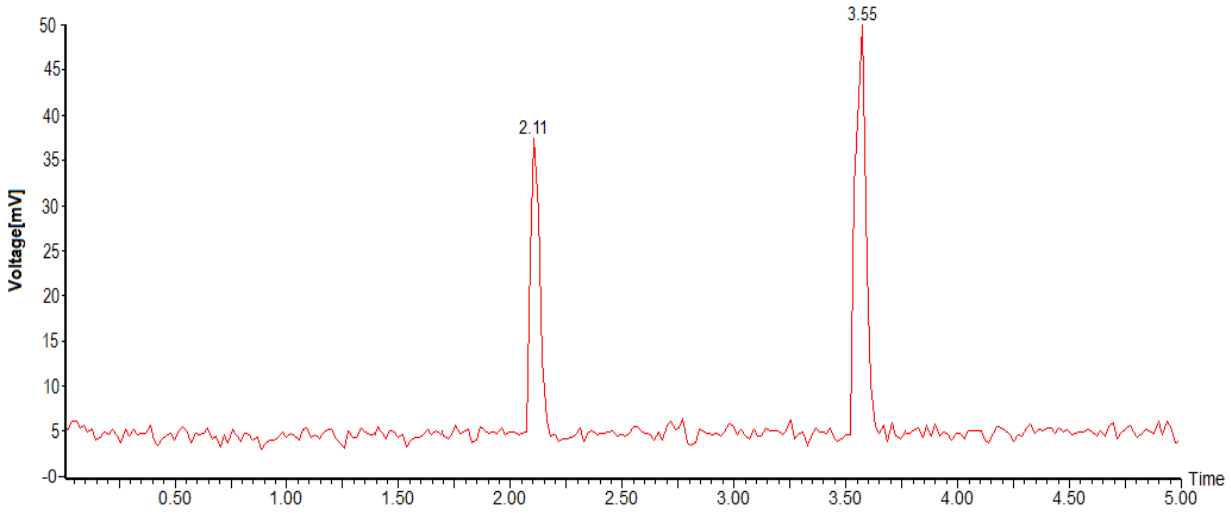

Fig.5: Chromatogram obtained from (Methanol: Acetonitrile: 0.8% Triethylamine (55:40:5 v/v/v)) Mobile Phase

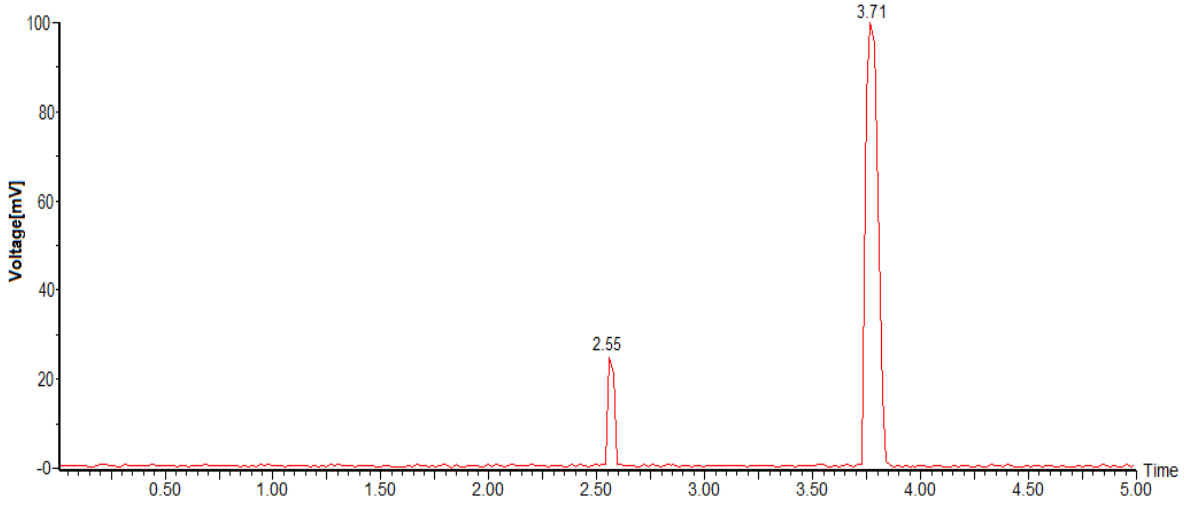

Fig.6: Chromatogram obtained from (Methanol: Acetonitrile: 0.8% Triethylamine (50:40:10 v/v/v)) Mobile Phase

Methanol, Acetonitrile, and 0.8% Triethylamine (TEA) in the ratio of 55:30:15 (v/v) at pH 5.6 with isocratic elution at a flow rate of 0.6 ml/min. Among studied chromatographic columns Phenomenex Luna C18 (100×4.6mm, 5µ) column at optimum conditions achieved with an adequate response, baseline separation within 2.0 min, symmetric peak shape, and resolution (resolution factor ≥2). Optimizedchromatograms are given in figure7.

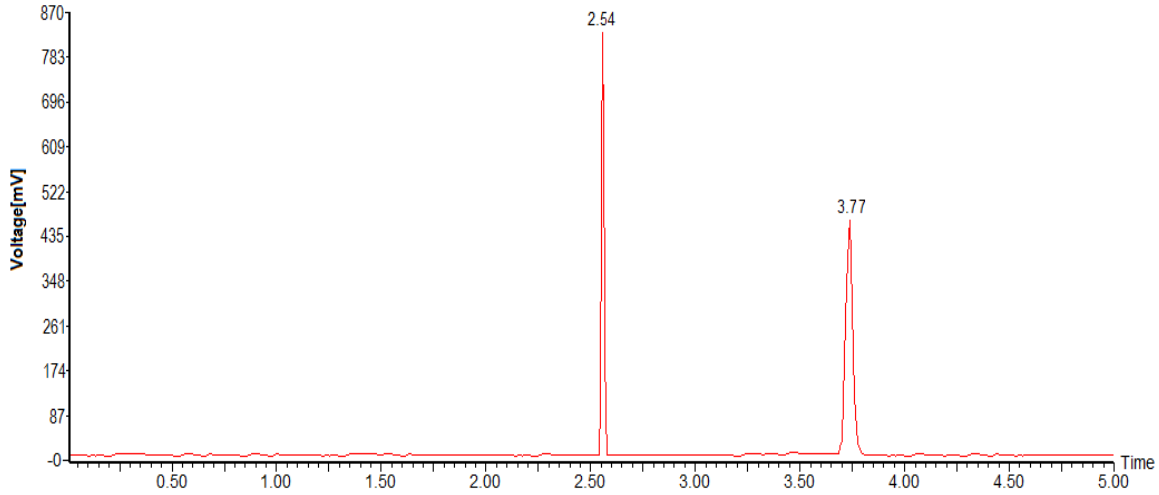

Fig.7: Chromatogram obtained from (Methanol: Acetonitrile: 0.8% Triethylamine (55:30:15 v/v/v)) Mobile Phase

The mass spectrometry parameters were optimized by directlyfusing the 1.0 lg/mL standard solution of LST into themass spectrometry. Considering the signal response,the positive mode was chosen to quantify LST. Optimized mass spectrometer conditions include nitrogen gas (320 psi) as a carrier gas with a flow rate of 5 L/min in mass spectral analysis with fixed MS tune temperature of 350°C, the capillary voltage was 3.5 KV, nebulizer pressure: 310 kPa,Cone voltage 50V, extractor voltage 3.0 V. For LST standard the mass resolution (2.5 amu) with the following m/z transitions: m/z 378 → 97 for LST eV, are used and for ETN IS, the m/z transition m/z 383 → 84 eVat a collision energy of 85 eV was used. The corresponding product ion mass spectrawere showed in figure (8-9).Theoptimized conditions (Table1) for estimation provided a well-defined separation between the drug, internal standard, andendogenous components. The blank plasma samples showed no interference at the retention time of the drugs andtheir internal standards. The optimized methods for the estimation of the drugs were precise as they showed a < 10 % coefficient ofvariation at all concentrations. Endogenous interferences were not detected at the retentiontime of LSTand internal standards. These observations show that thedeveloped assay method is specific and selective.

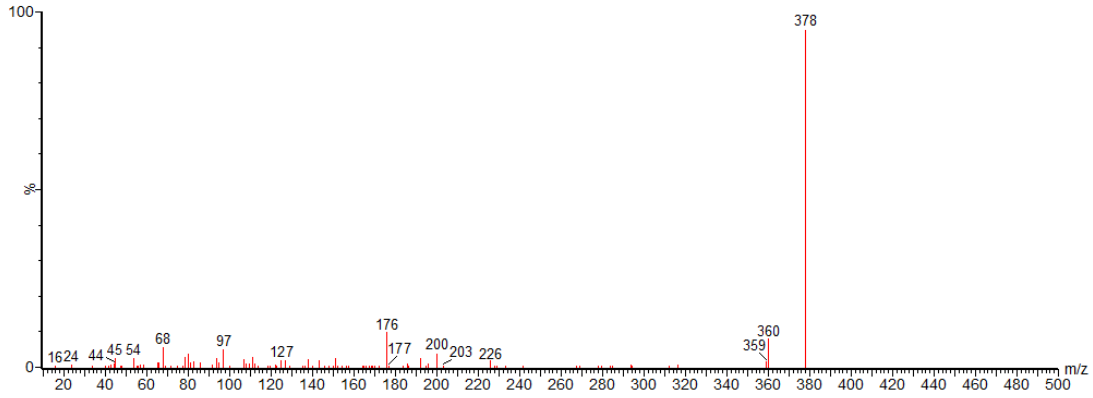

Fig.8:Lasmiditan mass fragmentation

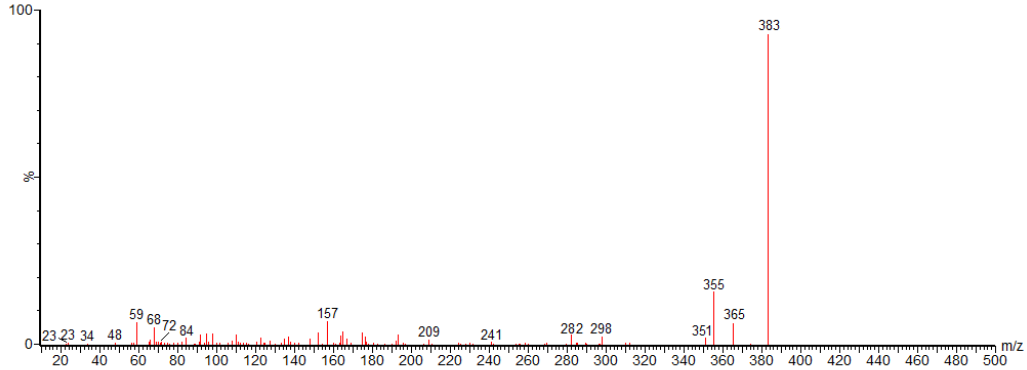

Fig. 9: Eletriptan(IS) mass fragmentation.

Table1: Optimized chromatographic conditions of the method

S. No.	Parameter	Optimized Condition
1	Column	Phenomenex Luna C18 (100×4.6mm, 5µ) column
2	Mobile Phase	Methanol:Acetonitrile:0.8% Triethylamine 55:30:15 (v/v/v)
3	Mobile phase pH	5.6
4	Mobile phase flow rate (mL/min)	0.6
5	Elution	Isocratic
7	Sample volume	10 µL
8	Run time (min)	5

Method Validation

Validated the above-optimized bio-analytical method of Lasmiditan in spiked human plasma samples by liquid chromatography and mass spectroscopy as per the existing guidelines[23-25].

Table2: Key Parameters of Validation

S. No.	Parameter	Results observed
		Lasmiditan
1	API Concentration (µg mL^{-1})	300
2	Linearity (µg mL^{-1})	1-300
3	Method precision (% RSD)	99.4-101.2
4	Intermediate precision (% RSD)	99.4-101.7
5	% Recovery	90.3-98.1
6	Limit of quantitation (µg mL^{-1})	0.1
7	Limit of detection (µg mL^{-1})	0.25

System Suitability, System Precision, and Specificity

Substantiated the performance of the system from the obtained system suitability parameters and the corresponding tabulated parameters were satisfactory (table3). There is no significant interference from plasma found at retention times of LST. The retention time of LST was approximately 2.5 min. The obtained system suitability results are tabulated along with validation parameters in table2. Typical chromatograms of Placebo,system suitability, and system precision are shown in figure-(10-12). System precision was determined on six replicate injections of standard preparations and % RSD was evaluated.The results indicated that the methodexhibited good specificity with selectivity and was applied toplasma samples for the pharmacokinetic study.

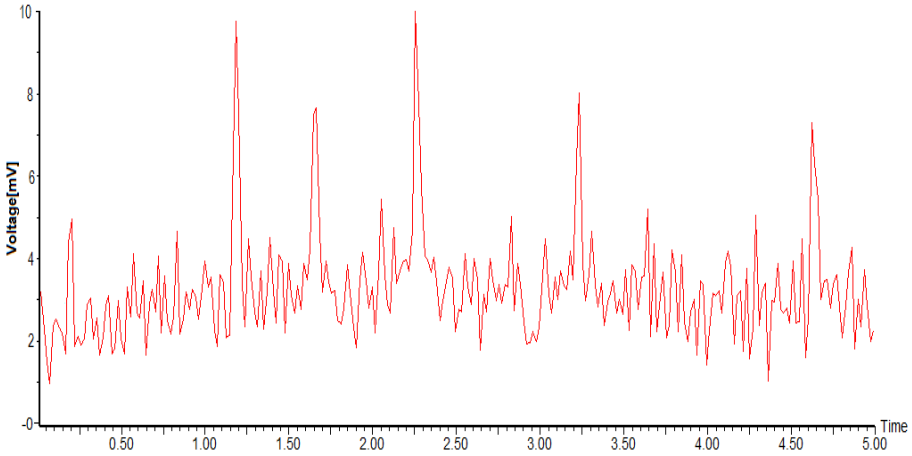

Fig. 10: Typical chromatogram of placebo and blank

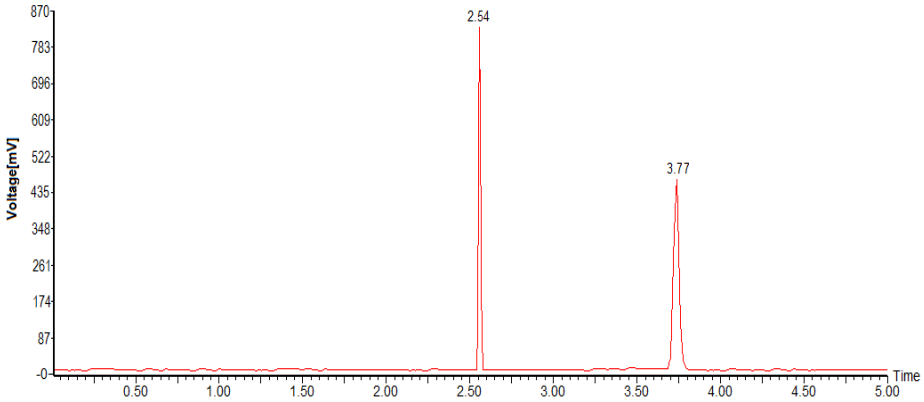

Fig. 11: Typical chromatogram of system suitability

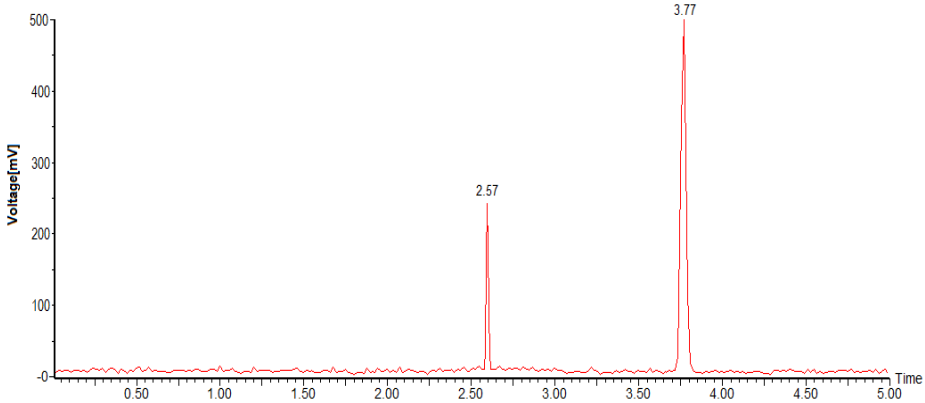

Fig.12:Typical chromatogram of standard in system precision

Table-3: Key System Suitability Parameters

System Suitability Parameter	Ruggedness	
	Lasmiditan	IS

USP resolution	---	6.59
USP tailing factor	0.95	1.06
USP plate count	4532	6320
Retention time (min)	2.54	3.77
Peak area	572315.8	189584.5
SD of area	1114.97	1142.75
% RSD of area	0.19	**0.61**
* from six standard injections at 300 µg mL^{-1} of Lasmiditan		

Stability Indicating Studies

The method stability of LST and IS in the method was evaluated by short-term, long term and freeze-thaw stability studies. The % stability or change in short-term stability was found to be 100.18, 99.56, and 100.55 for HQC, MQC, and LQC respectively. It was found that LST was stable in plasma after being stored at room temperature for 5 hours. The % stability or change in long-term stability was found to be 100.53, 98.80, and 100.75 for HQC, MQC, and LQC respectively. It was found that LST was stable in plasma after being stored at -70^0C for 3 months. The % stability or change in freeze-thaw stability was found to be 100.88, 99.36, and 100.32 for HQC, MQC, and LQC respectively. It was found that LST was stable in plasma after repeated three freeze-thaw cycles-70^0C for 3 months. The result of stability studies confirms that the method was found to be stable and suitable for the analysis of LST in biological samples. The stability study results are presented in table (4-12)respectively. The proposed LC-MS method for analysis of LST in plasma samples was found stable in both high and low concentrations under all tested conditions and time and no stability-related problems were observed. Hence it is expected no problems would occur during routine analysis of the samples for bioavailability, bioequivalence, and pharmacokinetic studies. Since there are no direct bio-analytical methods reported for LST analysis, the present study is a novel approach to the analysis of LST in biological samples.

Table4: Short-term stability at HQC

S.no	Peak Area Obtained		The ratio of Peak area of standard/IS	Amount of Drug estimated	% Drug estimated
	Standard	IS			
1	555258.6	189065.6	2.937	291.837	97.279
2	569791.1	188312.1	3.026	300.674	100.225
3	568974.3	188043.1	3.026	300.672	100.224
4	570339.6	187855.9	3.036	301.694	100.565
5	569933.0	185727.4	3.069	304.934	101.645

6	571778.0	187281.4	3.053	303.383	101.128
SD	6154.14	1135.51	0.05	4.57	1.52
Average	567679.1	187714.2	3.02	300.53	100.18
%CV	1.08	0.60	1.52	1.52	1.52
Accuracy (%)	100.18				
* from six standard injections at 300 µg mL^{-1} of Lasmiditan					

Table5:Short-term stability at MQC

S.no	Peak Area Obtained		The ratio of Peak area of standard/IS	Amount of Drug estimated	% Drug estimated
	Standard	IS			
1	112807.4	189088.9	0.597	49.223	98.446
2	114089.0	187492.0	0.609	50.206	100.413
3	114224.8	189027.5	0.604	49.858	99.715
4	114164.6	188936.7	0.604	49.855	99.711
5	113829.7	187638.4	0.607	50.053	100.106
6	112793.0	188120.5	0.600	49.470	98.940
SD	672.99	726.30	0.00	0.37	0.73
Average	113651.4	188384.0	0.60	49.78	99.56
%CV	**0.59**	**0.39**	**0.74**	**0.74**	**0.74**
Accuracy (%)	**99.56**				
* from six standard injections at 50 µg mL^{-1} of Lasmiditan					

Table6: Short-term stability at LQC

S.no	Peak Area Obtained		The ratio of Peak area of standard/IS	Amount of Drug estimated	% Drug estimated
	Standard	IS			
1	15122.1	189466.5	0.080	0.998	99.768
2	15337.2	188917.5	0.081	1.015	101.481
3	15247.9	188837.1	0.081	1.009	100.933
4	14947.2	188292.6	0.079	0.992	99.229
5	15188.8	187883.9	0.081	1.011	101.052
6	15043.8	186496.0	0.081	1.008	100.832
SD	140.92	1044.65	0.00	0.01	0.86
Average	15147.9	188315.6	0.08	1.01	100.55
%CV	0.93	0.55	0.86	0.86	0.86
Accuracy (%)	100.55				
* from six standard injections at 1 µg mL^{-1} of Lasmiditan					

Table7:Long-term stability at HQC

S.no	Peak Area Obtained		The ratio of Peak area of standard/IS	Amount of Drug estimated	% Drug estimated
	Standard	IS			
1	569444.8	188876.6	3.015	299.593	99.864
2	567654.4	188048.5	3.019	299.966	99.989
3	564712.7	186877.2	3.022	300.282	100.094
4	569427.1	187066.9	3.044	302.482	100.827
5	567852.8	185133.0	3.067	304.796	101.599
6	569418.8	187094.1	3.043	302.433	100.811
SD	1846.13	1259.72	0.02	2.01	0.67
Average	568085.1	187182.7	3.04	301.59	100.53
%CV	0.32	0.67	0.67	0.67	0.67
Accuracy (%)	100.53				
* from six standard injections at 300 µg mL^{-1} of Lasmiditan					

Table8:Long-term stability at MQC

S.no	Peak Area Obtained		The ratio of Peak area of standard/IS	Amount of Drug estimated	% Drug estimated
	Standard	IS			
1	111337.4	188494.8	0.591	48.735	97.470
2	111947.0	186296.0	0.601	49.580	99.160
3	114223.6	188752.9	0.605	49.930	99.860
4	114002.4	188300.9	0.605	49.953	99.905
5	110431.3	186804.9	0.591	48.775	97.551
6	112324.2	187495.9	0.599	49.429	98.857
SD	1490.39	991.46	0.01	0.54	1.08
Average	112377.7	187690.9	0.60	49.40	98.80
%CV	1.33	0.53	1.09	1.09	1.09
Accuracy (%)	98.80				
* from six standard injections at 50 µg mL^{-1} of Lasmiditan					

Table 9:Long-term stability at LQC

S.no	Peak Area Obtained		The ratio of Peak area of standard/IS	Amount of Drug estimated	% Drug estimated
	Standard	IS			
1	15058.6	190074.6	0.079	0.990	99.031
2	15375.6	189153.6	0.081	1.016	101.608
3	15295.2	188638.8	0.081	1.014	101.352
4	14942.4	188255.0	0.079	0.992	99.217
5	15167.1	186476.6	0.081	1.017	101.669
6	15026.5	184882.8	0.081	1.016	101.595

SD	166.58	1902.67	0.00	0.01	1.26
Average	15144.2	187913.6	0.08	1.01	100.75
%CV	1.10	1.01	1.25	1.25	1.25
Accuracy (%)	100.75				
* from six standard injections at 1 µg mL^{-1} of Lasmiditan					

Table10:Freeze-Thaw stability at HQC

S.no	Peak Area Obtained		The ratio of Peak area of standard/IS	Amount of Drug estimated	% Drug estimated
	Standard	IS			
1	569740.1	186788.7	3.050	303.099	101.033
2	568287.5	186688.5	3.044	302.488	100.829
3	572435.1	189214.3	3.025	300.629	100.210
4	569331.3	188380.3	3.022	300.322	100.107
5	569480.0	185671.5	3.067	304.783	101.594
6	572240.7	186689.8	3.065	304.591	101.530
SD	1690.32	1301.47	0.02	1.90	0.63
Average	570252.5	187238.8	3.05	302.65	100.88
%CV	0.30	0.70	0.63	0.63	0.63
Accuracy (%)	100.88				
* from six standard injections at 300 µg mL^{-1} of Lasmiditan					

Table 11:Freeze-Thaw stability at MQC

S.NO	Peak Area Obtained		The ratio of Peak area of standard/IS	Amount of Drug estimated	% Drug estimated
	Standard	IS			
1	113880.7	189085.5	0.602	49.692	99.385
2	114141.0	187910.7	0.607	50.117	100.235
3	113580.9	188100.5	0.604	49.821	99.642
4	113561.6	189335.7	0.600	49.488	98.975
5	112858.9	187461.6	0.602	49.673	99.346
6	112042.1	187548.9	0.597	49.291	98.581
9	769.02	790.80	0.00	0.28	0.57
Average	113344.2	188240.5	0.60	49.68	99.36
%CV	0.68	0.42	0.57	0.57	0.57
Accuracy (%)	99.36				
* from six standard injections at 50 µg mL^{-1} of Lasmiditan					

Table12:Freeze-Thaw stability at LQC

S.NO	Peak Area Obtained		The ratio of Peak area of standard/IS	Amount of Drug estimated	% Drug estimated
	Standard	IS			
1	15011.0	189276.3	0.079	0.991	99.134
2	15311.6	189626.5	0.081	1.009	100.933
3	15198.1	189223.6	0.080	1.004	100.397
4	14923.6	188194.7	0.079	0.991	99.123
5	15085.5	186210.0	0.081	1.013	101.267
6	14931.1	184670.2	0.081	1.011	101.066
SD	154.23	1999.75	0.00	0.01	0.97
Average	15076.8	187866.9	0.08	1.00	100.32
%CV	1.02	1.06	0.96	0.96	0.96
Accuracy (%)	100.32				
* from six standard injections at 1 µg mL^{-1} of Lasmiditan					

Linearity:

The calibration curve for the proposed method was calculated by the peak area ratio of LST and IS against the LST concentration. The linearity range for LST was found to be a concentration range of 1.0-300 µg/mL (1, 10, 25, 50, 100, 200, and 300 µg/mL). The regression equation for calibration curves in plasma was (y = 0.0099x + 0.0951) for LST in the developed method was found with a correlation coefficient (r^2) of 0.999 (table13) indicating the good linearity of the method. The linearity chromatogram gives the results of the LST and the calibration curve graph of the proposed is presented in table14 and figure 12. The other validation parameters like precision and accuracy of the proposed method for LST were analyzed with LQC, MQC, and HQC levels obtained from the interpolation on their respective calibration curves.

Table13: Summary of regression parameters

S. No.	Parameter	Obtained Values (LNT)
1	Residual sum of squares	0.999
2	Slope	0.0099
3	Y-Intercept	0.0951

Table14:Results of linearity study for Lasmiditan

S No	Concentration in µg/ml	Peak Area observed for		The ratio of Standard/IS
		Lasmiditan - Standard	Eletriptan – IS	
1	1	15243.1	189576.2	0.080

2	10	36291.5	189005.3	0.192
3	25	59645.3	187485.9	0.318
4	50	114263.9	188695.7	0.606
5	100	210269.4	189157.6	1.112
6	200	396152.1	186361.9	2.126
7	300	572315.8	189584.5	3.019

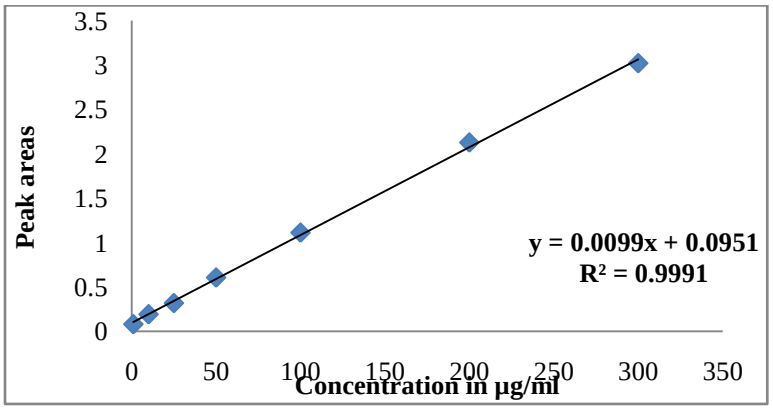

Fig. 12: Calibration graphs of Lasmiditan

Method Precision (M.P.) and Intermediate Precision (I.P.) (Ruggedness)

The Method Precision (M.P.)results were found to be 0.68%, 0.60 %, and 0.59 % for HQC, MQC, and LQC respectively and trueness ranged from 99.4-101.2 %. The Intermediate Precision (I.P.)results were found to be 1.03%, 0.78 %, and 0.86 % for HQC, MQC, and LQC respectively and trueness ranged from 99.4-101.7 %. The results of the precision study indicate that the assay method was sufficiently reliable and reproducible within the required analytical range. The results of the method and Intermediate precision studies are in table15-20.

Table15:Method precisionat HQC

S.NO	Peak Area Obtained		The ratio of Peak area of standard/IS	Amount of Drug estimated	% Drug estimated
	Standard	IS			
1	571535.9	189254.9	3.020	300.09	100.03
2	571935.9	188576.1	3.033	301.38	100.46
3	573268.1	189216.2	3.030	301.06	100.35
4	571253.6	188648.2	3.028	300.91	100.30
5	572020.9	186323.6	3.070	305.07	101.69
6	574146.9	187468.9	3.063	304.34	101.45
SD	1114.97	1142.75	0.02	2.04	0.68

Average	572360.2	188248.0	3.04	302.14	100.71
%CV	0.19	0.61	0.68	0.68	0.68
Accuracy (%)	100.71				
* from six standard injections at 300 µg mL^{-1} of Lasmiditan					

Table16: Method precision at MQC

S.NO	Peak Area Obtained		The ratio of Peak area of standard/IS	Amount of Drug estimated	% Drug estimated
	Standard	IS			
1	114296.8	189684.9	0.603	49.716	99.433
2	115242.6	188695.7	0.611	50.390	100.781
3	114225.9	189302.6	0.603	49.786	99.572
4	114326.9	189574.6	0.603	49.758	99.517
5	114229.5	188475.6	0.606	50.006	100.012
6	113263.7	188747.1	0.600	49.512	99.023
SD	627.05	506.73	0.00	0.30	0.61
Average	114264.2	189080.1	0.60	49.86	99.72
%CV	0.549	0.268	0.609	0.609	0.609
Accuracy (%)	99.72				
* from six standard injections at 50 µg mL^{-1} of Lasmiditan					

Table17: Method precision at LQC

S.NO	Peak Area Obtained		The ratio of Peak area of standard/IS	Amount of Drug estimated	% Drug estimated
	Standard	IS			
1	15193.5	189458.9	0.080	1.002	100.243
2	15348.1	189469.8	0.081	1.013	101.257
3	15264.9	190243.6	0.080	1.003	100.298
4	15002.1	188567.9	0.080	0.994	99.448
5	15326.9	190325.7	0.081	1.007	100.662
6	15174.8	189325.1	0.080	1.002	100.190
SD	126.54	649.81	0.00	0.01	0.60
Average	15218.4	189565.2	0.08	1.00	100.35
%CV	0.83	0.34	0.59	0.59	0.59
Accuracy (%)	100.35				
* from six standard injections at 1 µg mL^{-1} of Lasmiditan					

Table18:Intermediate precision at HQC

S.NO	Peak Area Obtained		The ratio of Peak area of standard/IS	Amount of Drug estimated	% Drug estimated
	Standard	IS			
1	570964.4	183864.9	3.105	308.580	102.860
2	571135.2	187868.9	3.040	302.094	100.698
3	569713.8	187799.0	3.034	301.454	100.485
4	568854.3	188346.4	3.020	300.125	100.042
5	570190.4	185643.5	3.071	305.210	101.737
6	573572.8	186695.4	3.072	305.290	101.763
SD	1621.22	1699.32	0.03	3.13	1.04
Average	570738.5	186703.0	3.06	303.79	101.26
%CV	0.28	0.91	1.03	1.03	1.03
Accuracy (%)	101.26				
* from six standard injections at 300 µg mL^{-1} of Lasmiditan					

Table19:Intermediate precision at MQC

S.NO	Peak Area Obtained		The ratio of Peak area of standard/IS	Amount of Drug estimated	% Drug estimated
	Standard	IS			
1	113937.7	187213.1	0.609	50.214	100.429
2	114507.5	186806.9	0.613	50.575	101.151
3	114059.9	189300.7	0.603	49.714	99.428
4	113942.2	189305.4	0.602	49.661	99.323
5	113722.1	187815.9	0.605	49.959	99.917
6	112887.7	187962.7	0.601	49.553	99.107
SD	535.66	1043.71	0.00	0.39	0.78
Average	113842.8	188067.4	0.61	49.95	99.89
%CV	0.47	0.55	0.78	0.78	0.78
Accuracy (%)	99.89				
* from six standard injections at 50 µg mL^{-1} of Lasmiditan					

Table20: Intermediate precision at LQC

S.NO	Peak Area Obtained		The ratio of Peak area of standard/IS	Amount of Drug estimated	% Drug estimated
	Standard	IS			
1	15129.7	190067.1	0.080	0.995	99.502
2	15386.5	189706.6	0.081	1.014	101.383
3	15312.2	190043.8	0.081	1.007	100.715
4	14997.3	188530.2	0.080	0.994	99.436
5	15305.0	188900.2	0.081	1.013	101.277
6	15157.3	187687.4	0.081	1.009	100.948
SD	144.88	952.39	0.00	0.01	0.87
Average	15214.7	189155.9	0.08	1.01	100.54
%CV	0.952	0.503	0.861	0.861	0.861
Accuracy (%)	100.54				
* from six standard injections at 1 µg mL^{-1} of Lasmiditan					

Robustness

The robustness of the methods was studied by changing the experimental conditions. Upon variation of the retention time shift of LST remained statistically not significant. No significant changes in the chromatographic parameters were observed when changing the experimental conditions (mobile phase pH, flow rate, composition, etc). Robustness at MQC level results is given in table21.

Table21: Robustness at MQC

	Lasmiditan						
Conditions			**RT**	**Peak area**	**Theo plate**	**Tail factor**	**Resolution**
Mobile phase	55:40:5	60:35:5	2.50	114301.8	4921	0.93	-----
		50:45:5	2.52	115246.6	4830	0.92	-----
pH	5.7	5.7	2.53	114232.9	4749	0.94	-----
		5.5	2.51	114333.9	4658	0.95	-----
Flow rate ml/min	0.5	0.7	2.53	114238.7	4846	0.93	-----
		0.5	2.52	113269.2	4921	0.95	-----
Eletriptan							
Mobile phase	55:40:5	60:35:5	3.79	189687.9	6419	1.07	6.71
		50:45:5	3.73	188702.2	6355	1.08	6.55
pH	5.7	5.8	3.73	189308.1	6315	1.09	6.68
		5.9	3.72	189582.1	6327	1.08	6.68
Flow rate ml/min	0.5	0.6	3.71	189582.1	6588	1.09	6.71
		0.4	3.72	188756.2	6305	1.10	6.70

Accuracy

In this study, the matrix effect was evaluated by analyzing the low (1.0 µg/mL), middle (50.0 µg/mL), and high (300.0 µg/mL) QC samples. The results of the recovery study are presented in Table 22-24. The results of the recovery study ranged between 94.45-96.14 % for HQC, 96.4 – 98.1 % for MQC, and 90.3-93.6 % for LQC respectively. These results showed no significant differences at different concentrations. The matrix effect on the ionization of the analyte was not obvious under these conditions. It is, therefore, derived that the developed methods are accurate and reliable.

Table22: Recovery of Lasmiditan at HQC

S.NO	Peak Area Obtained			The ratio of Peak area of standard/IS	% Drug estimated
	Aqueous	Extracted	IS		
1	595871.2	570781.7	189398.3	3.014	95.79
2	596328.5	568759.3	188681.6	3.014	95.38
3	596638.7	567343.8	189452.2	2.995	95.09
4	598475.2	565242.1	187370.5	3.017	94.45
5	597746.3	567835.5	189539.7	2.996	95.00
6	595748.2	572752.5	189054.4	3.030	96.14
SD	1088.03	2655.53	821.18	0.01	0.60
Average	596801.4	568785.8	188916.1	3.01	95.31
% CV	0.18	0.47	0.43	0.44	0.63
Accuracy (%)	95.31				
* from six standard injections at 300 µg mL^{-1} of Lasmiditan					

Table23: Recovery of Lasmiditan at MQC

S.NO	Peak Area Obtained			The ratio of Peak area of standard/IS	% Drug estimated
	Aqueous	Extracted	IS		
1	116683.5	112771.3	189337.7	0.596	96.65
2	116748.5	113614.4	187896.7	0.605	97.32
3	116572.5	113749.6	188664.1	0.603	97.58
4	115642.5	113439.6	186180.7	0.609	98.10
5	117647.5	113359.6	188756.9	0.601	96.36
6	116368.4	112631.7	188784.0	0.597	96.79
SD	647.58	456.31	1122.59	0.01	0.65
Average	116610.5	113261.0	188270.0	0.60	97.13

%CV	**0.56**	**0.40**	**0.60**	**0.85**	**0.67**
Accuracy (%)	**97.13**				
* from six standard injections at 50 µg mL^{-1} of Lasmiditan					

Table24: Recovery of Lasmiditan at LQC

S.NO	**Peak Area Obtained**			**The ratio of Peak area of standard/IS**	**% Drug estimated**
	Aqueous	**Extracted**	**IS**		
1	16254.9	15117.3	189215.8	0.080	93.00
2	16325.4	15273.4	188837.6	0.081	93.56
3	16425.8	15184.5	188436.4	0.081	92.44
4	16442.5	14852.3	186999.7	0.079	90.33
5	16357.1	15126.1	185440.9	0.082	92.47
6	16328.9	15022.3	184406.1	0.081	92.00
SD	69.64	145.25	1960.15	0.00	1.10
Average	16355.8	15096.0	187222.8	0.08	92.30
%CV	**0.43**	**0.96**	**1.05**	**1.06**	**1.20**
Accuracy (%)	**92.30**				
* from six standard injections at 1 µg mL^{-1} of Lasmiditan					

LOD and LOQ

LOD and LOQ values were found 0.25 and 0.1µg/mL which is the lowest concentration of linearity. The method was found to be sufficiently sensitive for the determination of the pharmacokinetic analysis of LST in plasma. These values, however, may be affected by the separation conditions (e.g., column, reagents, and instrumentation and data systems), instrumental changes (e.g., pumping systems and detectors), and use of non-HPLC grade solvents and may result in changes in signal to noise ratios.

CONCLUSION

The proposed LC-MS method for the estimation of LST in human plasma is selective, sensitive, and wide analysis range. The main advantage of the method here is simplicity in the preparation of the sample, the fast, reproducible bioanalytical method shows good consistent recovery with accepted accuracy and precision. The drug was extracted by using simple liquid-liquid extraction using a mixture of ethanol and diethyl ether in the ratio of 80:20 (v/v) for the sample preparation

involved before LC-MS analysis. The method was fully validated with linearity, precision, accuracy, matrix effects, recovery, and stability. The method results showed linearity in the range of 0.1–300 ng/mL (r^2 = 0.999) and the stability study confirms that the method was found to be stable. The method showed good precision (RSD% values between 0.59- and 1.03%) and accuracy (90.3-98.1 %). The present study could be readily applicable for therapeutic monitoring of the Lasmiditan drug in patients' blood. Hence the method is suitable for plasma-level monitoring of LST and can be used in bioavailability, bioequivalence, and pharmacokinetic studies.

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