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Original Article

BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF MARALIXIBAT IN RAT PLASMA BY LC-MS/MS DETECTION AND ITS APPLICATION TO A PHARMACOKINETIC STUDY

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ABSTRACT

Objective: To quantify maralixibat in rat plasma utilizing liquid-liquid extraction (LLE) approach, a practical, efficient, and accurate LC-MS/MS approach was devised.

Methods: As an internal standard (IS), Elobixibat was adopted. Utilizing an Agilent eclipse C18, 150 mm x 4.6 mm, 3.5 µm column, the drug separation was accomplished using an isocratic mobile phase entailing acetonitrile (ACN) and buffer (1 ml Tri fluoro acetic acid into 1liter water and stir well. Filtered through 0.22µ membrane filter paper) composition of 70:30 (v/v), dispensed at 1.0 ml/min.

Results: Multiple reaction monitoring (MRM) positive mode allowed for the simultaneous detection of maralixibat and elobixibat exhibiting proton adducts around m/z 676.0278-290.3625 and m/z 696.8541-480.6328, correspondingly. The correlation coefficient (r2) of the approach was ≥0.99977 across a linearity concentration spanning between 5.00–100.00 ng/ml. This technique achieved intra-day accuracy and precision between 99.31-100.93% and 0.22-6.55%, correspondingly. Across 3 freeze-thaw sessions, bench top testings, and postoperative stability investigations, maralixibat was shown to be stable.

Conclusion: Through intravenous injection, this approach was effectively utilized in rats for studying the drug's pharmacokinetics.

Keywords: Maralixibat, Elobixibat, LC-MS/MS, Development and validation, Rat plasma, USFDA

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INTRODUCTION

Alagille-Watson syndrome (ALGS), Byler disease, and biliary cirrhosis are three uncommon but severe liver illnesses that can be treated with maralixibat, an oral ileal bile acid transporter (IBAT) antagonist manufactured by mirum pharmaceuticals [1, 2]. In ALGS patients, surgical blockage of enterohepatic circulation has been suggested to reduce itchiness. However, the pharmaceutical blockage of IBAT was proposed as a potential alternative to surgical treatment since it plays a critical role in bile acid reuptake [3, 4]. Maralixibat works by reducing the reuptake of bile acids in the liver, thereby decreasing the buildup of toxic bile acids in the liver and alleviating symptoms such as itching and fatigue. In 2021, the USFDA approved maralixibat, marketed under the brand name Livmarli™, to treat biliary pruritus in individuals with ALGS over the age of one [5]. The drug has since received approval in several other countries and is currently undergoing further evaluation for ALGS treatment in European nations [6, 7]. The recommended dose of maralixibat is 380 µg/kg/day, administered orally in the form of a solution 30 min before the first meal of the day [1, 8]. The initial prescribed dose is 190 µg/kg/day, which can be increased to 380 µg/kg/day after one week, depending on tolerability. The maximum allowable dosage of the drug is 28.5 mg [1]. Maralixibat is also being evaluated for its therapeutic potential in treating other liver illnesses, such as Byler disease and biliary cirrhosis. Both the USFDA and the European Medicines Agency (EMA) have designated Maralixibat as an orphan drug for the treatment of ALGS, Byler disease, and biliary cirrhosis, indicating the need for alternative treatment options for these rare and often debilitating conditions [9]. While maralixibat has shown promise in clinical trials, it can cause side effects such as diarrhea, nausea, and abdominal pain [8, 10, 11]. As with any medication, it is important for patients to discuss the potential benefits and risks with their healthcare provider before starting treatment. However, the approval of maralixibat represents a significant step forward in the treatment of rare liver diseases, offering new hope to patients and their families.

In addition to its use in treating liver diseases, maralixibat has also shown potential in treating other conditions, such as nonalcoholic steatohepatitis (NASH), a type of liver inflammation caused by a buildup of fat in the liver [12]. In preclinical studies, maralixibat has been shown to reduce liver inflammation and improve insulin sensitivity, suggesting it may have therapeutic potential for NASH and related conditions. The development of maralixibat represents a significant advance in the field of liver disease treatment, particularly for rare and difficult-to-treat conditions. Prior to its approval, patients with ALGS and other related diseases had limited treatment options, often requiring surgical interventions or relying on symptomatic relief [9]. With maralixibat, patients now have access to a targeted and effective medication that can alleviate their symptoms and improve their quality of life. Furthermore, the development of maralixibat highlights the potential of targeted therapies and precision medicine in treating rare diseases. By understanding the underlying mechanisms of these conditions and developing drugs that target specific pathways, researchers and clinicians can provide more effective and personalized treatment options for patients with rare and complex diseases. There are currently no available methods for the bioanalysis of maralixibat in any type of biological matrix [13]. To address this gap, a recent study has reported the development of a novel bioanalytical method for the quantification of maralixibat in biological samples.

The method involves the use of liquid chromatography-tandem mass spectrometry (LC-MS/MS) to separate and quantify maralixibat in plasma samples. The method has been validated and found to be accurate, precise, and sensitive. The development of this bioanalytical method for maralixibat has significant implications for both clinical practice and research. Clinicians can use this method to measure drug levels in patients and optimize dosing regimens, which can lead to improved treatment outcomes for patients with rare liver diseases. In addition, researchers can use this method to better understand the pharmacokinetics of maralixibat and to investigate potential drug-drug interactions or other factors that may affect the drug's efficacy and safety. The development of this bioanalytical method also highlights the importance of ongoing research and innovation in the field of bioanalysis, particularly for rare and orphan drugs. With the increasing number of targeted

therapies being developed for rare diseases, the need for accurate and sensitive bioanalytical methods is more important than ever. This method for maralixibat may serve as a valuable tool for the development and optimization of other targeted therapies in the future.

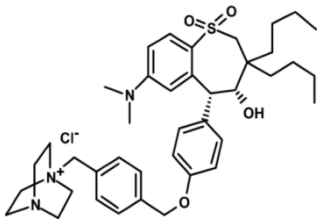

Fig. 1: Structure of maralixibat

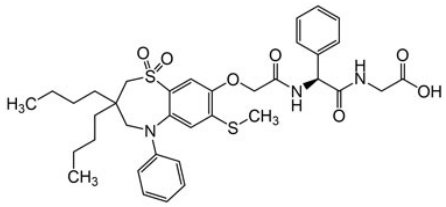

Fig. 2: Structure of elobixibat

MATERIALS AND METHODS

Chemicals and reagents

Maralixibat and elobixibat, with a purity level of 99.8% were acquired from Zydus cadila, which is located in Ahmedabad, India. The essential solvents required for the study, including acetonitrile (ACN), tri-fluoro-acetic acid (TFA), methanol (MEOH), and water (Milli Q or equivalent), were obtained from Merck in Mumbai, India. Manisha Laboratories, which is located in Mumbai, India, provided the rats and rat plasma.

LC-MS/MS instrument and conditions

The experiment was conducted using a Waters, Alliance e-2695 version HPLC entailing a column oven, degasser, and autosampler. A mass spectrophotometer (SCIEX QTRAP 5500), having an ESI interface, was coupled with the HPLC device. Chromatographic findings were construed with SCIEX software. By using the Turbo Ion Spray (ESI) positive mode and Unit Resolution, the detection was carried out. For maralixibat and IS, components at m/z 290.3625 and m/z 480.6328, correspondingly, were selected as the production, with the MH+(m/z 676.02) and (m/z 696.8541) being studied as the precursor ion. The following mass characteristics were chosen: nitrogen for the collision gas, drying gas maintained at 120 to 250 °C and supplied at 5 ml/min, and source temperature of 550 °C. Entrance, exit, and declustering potentials were 10V, 7V, and 40V; correspondingly, for both drug and IS, the dwell period was 1 sec.

Chromatographic conditions

As the analytical column, an Agilent eclipse C_{18}, 150 mm x 4.6 mm, 3.5 µm was used. ACN and buffer (1 ml Tri fluoro acetic acid into 1-liter water and stir well. Filtered through 0.22µ membrane filter paper) were the two components of the mobile phase, and their proportion was 70:30 (v/v). The mobile phase was delivered at 1.0 ml/min. At a cumulative duration of 6 min, maralixibat's retention time (RT) was discovered to be around 2.182 min.

Preparation of standards and quality control (QC) samples

Both the IS (200.00 mg/ml) parent stock solutions, as well as the standard stock solutions containing maralixibat (200.00 mg/ml), were made using MeOH. Prior to quantification, both solutions were kept in the freezer at 2–8 °C. For generating QC concentrations spanning between 5.00-100.00 ng/ml for analytical standards and 5.00-75.00 ng/ml for QC standards, standard stock solutions were incorporated into pure rat plasma. Prior to an assessment, the solutions were kept in the refrigerator at-30 °C.

Sample preparation

For extracting Maralixibat from rat plasma, the LLE technique was adopted. In this method, plasma samples (200 µl, at the optimum dosage) were incorporated into pre-labeled tubes and refluxed shortly. Next, 500 microliters of each standard stock and IS stock were poured, and they were mixed thoroughly for about 10 min before being centrifugation at 4000 rpm at 20 °C. All sample's supernate was deposited in a separate tube with a label and vaporized at 40 °C till dry. The samples were rapidly refluxed post-dilution using 300 microliters of ACN and 500 µl of dilutants, and then they were relocated to autosampler vials before injecting.

Method validation

Selectivity and specificity

Six distinct blank plasma samples were employed to assess the technique's selectivity for intrinsic chemicals that co-elute with the drug and IS. The samples were pre-treated and examined to look for any apparent interferences. Depending on respective retention durations and MRM outputs, the elution peaks of the drug and IS were discovered. It is recommended that the peak area of Maralixibat in blank solution at the appropriate retention time must not exceed 20% of the average peak area of maralixibat's lower limit of quantification (LLOQ). Comparable to this, elobixibat's peak area at the appropriate retention period in blank solution shouldn't be greater than 5% of the average peak area of LLOQ maralixibat.

Matrix effect

The matrix factor is the ratio of the peak response ratio in the presence of extracted matrix and the Peak response ratio in neat standards (Aqueous standards). By calculating the matrix factor, the matrix effect was measured to anticipate the variance of the matrix effect observed for samples derived from distinct subjects. 6 sets of blank biological matrices were eluted in three replications with the pure standard at the low-quality control (LQC), and high-quality control (HQC), and alternative doses were made to contrast the pure standards at the exact dosage. The coefficient of variation (CV %), which measures the matrix factor's total precision, must be below 15%.

Linearity

For maralixibat concentrations between 5.00 and 100.00 ng/ml, the calibration curves exhibited linearity. It was 0.99977 for the average correlation coefficient. By correlating the peak areas of the drug to that of IS, samples were measured. Plots of peak area ratios vs plasma levels were made.

Precision and accuracy

In the case of back-calculated concentrations, precision and accuracy should be below 15% and±15% of their actual concentrations, correspondingly. Nevertheless, for the LLOQ samples, precision and accuracy should be below 20% and±20% of their actual concentrations, correspondingly.

Stability

Following 3 freeze-thaw sessions, samples (LQC and HQC; n=6) were taken from the fridge or freezer in accordance with the prescribed clinical procedures. To assess stability during freeze-thaw, make LQC and HQC samples (all requiring 6 solutions) and preserve them at-28±5 °C for up to 6h before injecting them into an LCMS device. For stability of the autosampler: Make LQC and HQC samples, and then pump them into the LCMS network hourly for a maximum of 24 h. Formulate LQC and HQC samples (that all have 6 solutions) for short-term stability, and then introduce the samples into the LCMS device following seven days of storage at 5±3 °C. Long-term stability: Formulate LQC and HQC samples (all having six solutions) and maintain at-20±3 °C before injecting into the LCMS device every seven to twenty-eight days while keeping it at 5±3 °C. The stability samples' precision and accuracy should be below 15% and±15% of their actual concentrations, correspondingly.

Recovery

Assessment of QC samples allowed researchers to assess the drug and IS extracted efficiency from rat plasma. By correlating peak areas derived from samples with those reported for standard solution injected alongside the blank plasma residues, recoveries at various concentrations (25, 50, and 75 ng/ml) were calculated. In order to acquire the necessary sensitivity, a recovery exceeding 50% was deemed sufficient.

Pharmacokinetic evaluation of maralixibat in plasma

It was possible to measure the levels of maralixibat in rat plasma using the established technique. The work followed existing GCP criteria and received approval from the Mumbai, Maharashtra-based animal ethics committee. Manisha laboratories in Mumbai, Maharashtra, provided male sprague-dawley rats. Rats received a single dosage of maralixibat injection (9.5 mg/ml), and samples were obtained at intervals of 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, and 1.75 h after the treatment. At every interval, 5 ml blood samples were drawn into K2 EDTA vacutainer as a fraction. A pre-dose sample was also taken to check for plasma interferences that could have been evident. For extracting plasma samples, they were centrifuged and then preserved at-70 °C} 10 °C. Four quantities of the IS were injected into plasma samples before processing them with QC samples. WinNonlin (Version 5.2) software was adopted for studying the pharmacokinetics of maralixibat.

Research ethics

This investigation was conducted in strict line with the Committee for Control and Supervision of Experiments on Animals (CPCSEA) recommendations and regulations. The CPCSEA is a statutory agency created by the Indian government that regulates animal research. This has allowed the pharmacokinetic performance methods at the Animal House Facility of Manisha Laboratories (City: Mumbai, State: Maharashtra, Country: India) under registration number 1074/PO/Re/S/05/CPCSEA for experimental investigations on small animals for educational purposes. All rat tests were conducted at Manisha Laboratories' Animal House Facility with protocol No: CPCSEA/MS Lab/PK/75345 as part of this research work. Tests conducted without anesthetic, with all required precautions taken to guarantee that animals are not subjected to needless pain or suffering before, during, or after experiments.

RESULTS AND DISCUSSION

Method development

Because of its specificity, accuracy, and repeatability, LC-MS/MS has been regarded as being among the utmost effective analysis approach. The objective of our research was to design and evaluate a quick, efficient, and reliable assessment technique for the accurate assessment of maralixibat in rat plasma samples. Maralixibat and elobixibat were extracted from the plasma samples using an easy extraction method. To improve clarity and boost the signal of maralixibat and elobixibat, separation conditions, particularly the make-up and characteristics of the mobile phase, were finalized after several tests. By directly injecting solutions of maralixibat and elobixibat into the MS-ESI pump, the MS optimization process was carried out. A superior spray pattern was achieved by optimizing additional factors, including the nebulizer and the heat gases, which improved ionizing as well as the droplet drying process. In the current study, the protonated ionic maralixibat and elobixibat molecules. maralixibat and elobixibat's product ion band produced high-abundance fragmentation ions with m/z values of 676.0278-290.3625 and 696.8541-480.6328. A rather aqueous mobile phase was used instead of an organic one when the MRM channels had been modified to provide a quick and accurate LC technique. ACN and buffer were used as the mobile phase, supplied at 1.0 ml/min with an injection volume of 10 µl, for efficiently separating and eluting the samples.

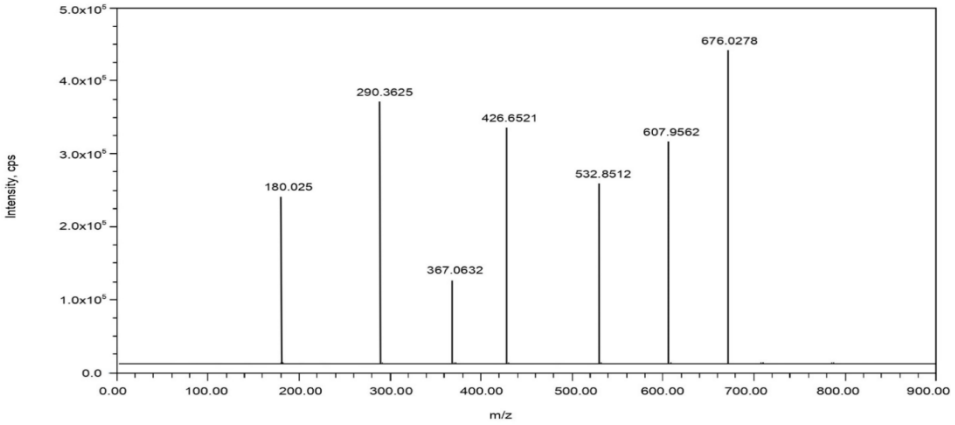

Fig. 3: Mass spectra of parent ion and daughter ion of Maralixibat

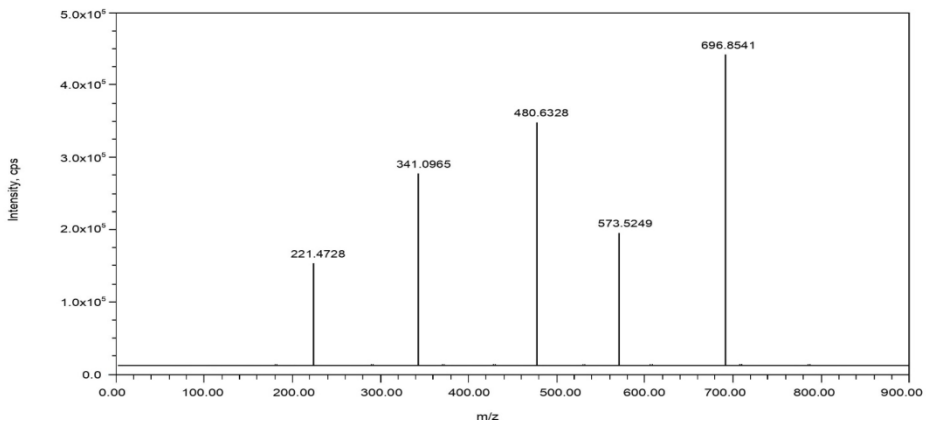

Fig. 4: Mass spectra of parent ion and daughter ion of Elobixibat

Method validation

The proposed approach was shown effective throughout the linearity concentration across 5.00–100.00 ng/ml. According to accepted standards, the validating assessments were performed, covering precision, selectivity specificity, linearity, matrix effect, accuracy, stability, and recovery [14-16].

Selectivity and specificity

When maralixibat and elobixibat were analyzed utilizing the MRM technique, no interfering substances were present. Six separate batches of rat plasma were used to test for specificity. Here, just one empty plasma interference was displayed (fig. 5). Fig. 6 and fig. 7 are chromatograms of maralixibat and elobixibat.

Matrix effect

For maralixibat, the total accuracy of the matrix parameter was found to be 1.49 at LQC and 0.50 at HQC.

Linearity

The peak area ratio against maralixibat concentration was used to construct a calibration graph [17-19]. The findings were discovered to be linear across the 5.00–100.00 ng/ml concentration levels. For every curve, the r2 values were higher than 0.99977 (table 1).

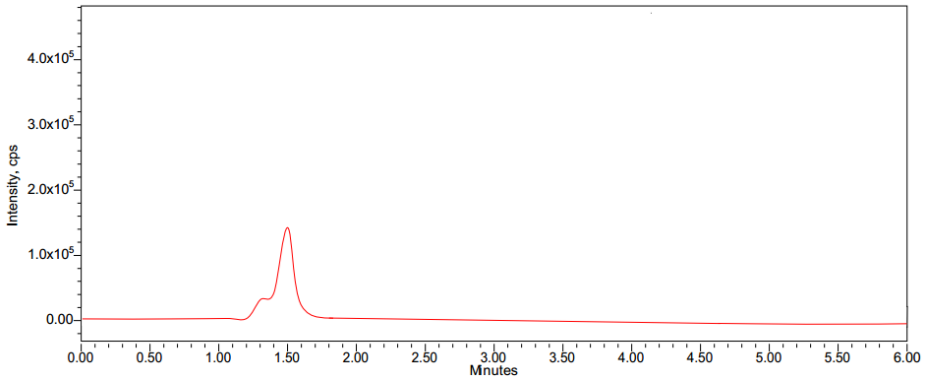

Fig. 5: Empty plasma interference

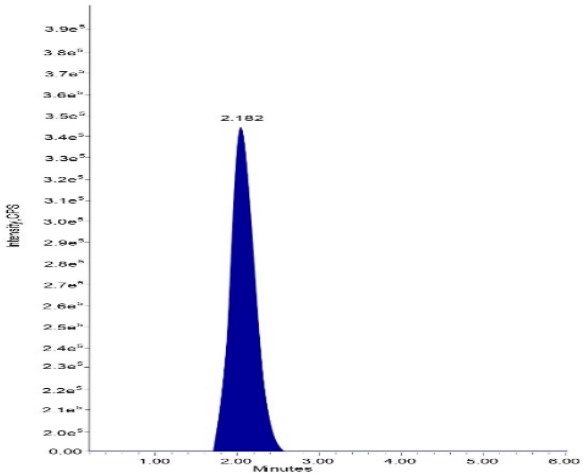

Fig. 6: Chromatogram of maralixibat

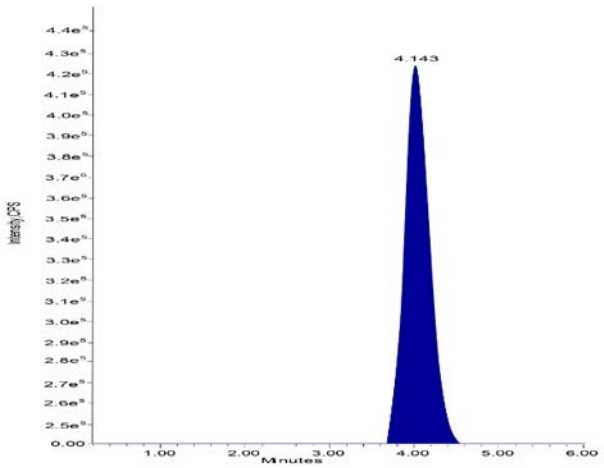

Fig. 7: Chromatogram of elobixibat

Table 1: Linearity outcomes of maralixibat

Final conc. (ng/ml)	Response	Area response ratio
0	0	0.0
5.00	0.381	0.090
12.50	0.852	0.202
25.00	1.754	0.416
37.50	2.601	0.616
50.00	3.457	0.819
62.50	4.305	1.020
75.00	5.211	1.233
100.00	6.721	1.592
Slope	0.0161	
Intercept	0.00734	
R^2	0.99977	

(Number of experiments, n= 1)

Precision and accuracy

Through the calculation of within and between batch fluctuations at 4 QC sample levels (5.00, 25.00, 50.00, and 75.00 ng/ml) in 6 repetitions, the accuracy and precision of this approach were maintained under control [20, 21]. Table 2 shows maralixibat's findings for accuracy and precision.

Stability

The stability of the drug was demonstrated by its estimation in rat plasma after three sessions of freezing and thawing (-30 °C-normal temperature) [14, 16, 18, 22]. The resulting quantities of maralixibat were around 102.72% and 101.18% of the experimental values, and no discernible deterioration of the drug was seen even following 6h of preservation inside the autosampler. Furthermore, the long-term stability of the drug was assessed in QC samples following preservation at 30 °C for 28 d. The concentrations fell within 86.87% and 96.57% of the experimental values (table 3).

Recovery

The recoveries after sample extraction by employing the LLE technique with ACN were measured at control concentrations of maralixibat and computed by correlating the peak area ratios of the drug in plasma samples with that of solvent samples. At 3 separate levels of 25.00, 50.00, and 75.00 ng/ml, the recovery of maralixibat was estimated and discovered to be 99.65%, 98.73%, and 100.75%, correspondingly [23-25]. Consequently, maralixibat and elobixibat recovered 98.73 and 106.48% of their corresponding doses, accordingly.

Pharmacokinetics and statistical analysis

Quantifying maralixibat levels in rat plasma samples has been accomplished using the devised approach. Table 4 represents the pharmacokinetics of maralixibat in rat plasma and fig. 8 is the maralixibat concentration vs time curve in plasma samples.

Table 2: Precision and accuracy results of maralixibat

Acquisition number	HQC	MQC	LQC	LLOQ
	Nominal conc. (ng/ml)			
	75.0	50.0	25.0	5.0
	Drug peak area			
1	$5.209x10^5$	$3.421x10^5$	$1.704x10^5$	$0.342x10^5$
2	$5.213x10^5$	$3.427x10^5$	$1.721x10^5$	$0.346x10^5$
3	$5.222x10^5$	$3.430x10^5$	$1.729x10^5$	$0.382x10^5$
4	$5.227x10^5$	$3.434x10^5$	$1.735x10^5$	$0.371x10^5$
5	$5.231x10^5$	$3.444x10^5$	$1.739x10^5$	$0.322x10^5$
6	$5.239x10^5$	$3.449x10^5$	$1.748x10^5$	$0.333x10^5$
Number of experiments (n)	6	6	6	6
Mean response±SD	$5.224x10^5$±0.011	$3.434x10^5$±0.011	$1.729x10^5$±0.015	$0.349x10^5$±0.023
% CV	0.22	0.31	0.89	6.55
% Mean Accuracy	100.71%	99.31%	100.01%	100.93%

All values are mean±SD values (Number of experiments, n= 6)

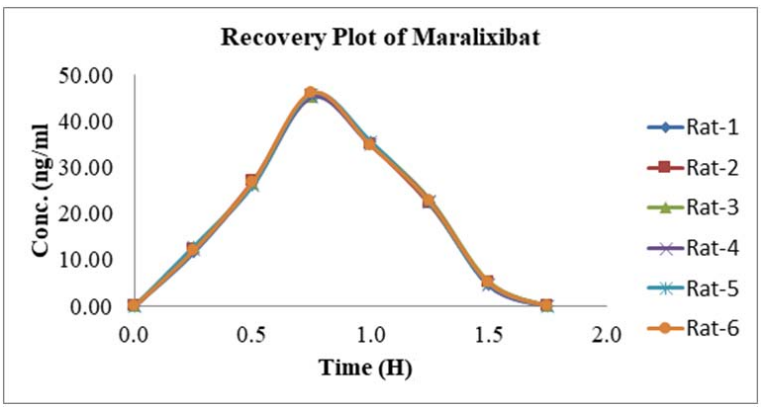

Fig. 8: Recovery plot of maralixibat in rat plasma

Table 3: Stability of maralixibat in plasma samples

Stability	Spiked plasma conc. (ng/ml)	Mean response±SD	RSD (%) (n=6)
Benchtop stability	25(ng/ml)	1.785 x10^5±0.005	0.28
	75(ng/ml)	5.229 x10^5±0.005	0.10
Autosampler stability		1.776 x10^5±0.014	0.77
		5.248 x10^5±0.018	0.35
Wet extract stability at 12 H		1.743x10^5±0.025	1.44
		5.224 x10^5±0.021	0.41
Wet extract stability at 18H		1.735x10^5±0.015	0.88
		5.232 x10^5±0.016	0.31
Dry extract stability at 12 H		1.752x10^5±0.010	0.55
		5.236 x10^5±0.024	0.45
Dry extract stability at 18H		1.734x10^5±0.016	0.92
		5.238 x10^5±0.019	0.36
Freeze-thaw		1.77 x10^5±0.005	0.30
		5.245 x10^5±0.024	0.46
Short-term		1.606 x10^5±0.003	0.20
		5.159 x10^5±0.010	0.19
Long-term (28 d)		1.502 x10^5±0.008	0.51
		5.009 x10^5±0.005	0.10

All values are mean±SD values (Number of experiments, n= 6)

Table 4: Pharmacokinetics of maralixibat

Time intervals (Hours)	Maralixibat (ng/ml)
0.25	12.327
0.5	26.541
0.75	45.633
1.0	35.183
1.25	22.615
1.5	4.907
1.75	0

(Number of experiments, n= 6)

CONCLUSION

With Elobixibat serving as the internal standard, our research aims to offer a concise, efficient, robust, and consistent approach for measuring Maralixibat through LCMS. The devised approach is more rapid and the RT for Maralixibat equals 2.182, while the total duration for the separation is 6.0 min. With r^2 value exceeding 0.99977, the approach is validated throughout a concentration ranging across 5.00–100.00 ng/ml for Maralixibat. Over 5 concentrations (LLOQ, LQC, MQC, HQC, and ULOQ), the within-batch, as well as between-batch accuracy (%CV), remained under 15%. In accordance with USFDA protocols, this may be verified.

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The author received no specific funding for this research work.

AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

All authors declare no conflict of interest.

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