Bioanalytical Method For Development, Validation and Sensitive Quantification of Elagolix Sodium Using RP – HPLC Method

Jonee Panwar[1]*, Garima Garg[1], Hasan Ali[2]

[1] Department of Pharmaceutical Sciences / Department of Pharmaceutics, IIMT College of Medical Sciences, IIMT University, Meerut-250406, Uttar Pradesh, India.

[2] Department of Pharmacy, Meerut Institute of Technology, Baral Partapur, Meerut-250103, Uttar Pradesh, India.

Corresponding Author:

Jonee Panwar[1]*

Email: dr.joneepanwar@gmail.com

Abstract: Background: Nearly 190 million women worldwide suffer with endometriosis, a chronic gynecological disorder that is dependent on estrogen and causes pain, infertility, and a decline in quality of life. Elagolix sodium is a gonadotropin-releasing hormone (GnRH) antagonist that suppresses gonadotropins quickly, making it a promising therapeutic treatment.

Objective: Finding specifics about absorption, distribution, metabolism and excretion (ADME) profiles in the context of female Sprague–Dawley (SD) rats is critical to translational research and therapeutic optimization, which necessitates further steroid pharmacokinetic profile studies utilizing preclinical models.

Method: Sensitive quantification of drug levels in plasma is paramount for defining exposure–response relationships and optimal efficacy. In the present work, a RP-HPLC bioanalytical method for quantification of elagolix sodium plasma samples has been developed and validated.

Result: Validation was carried out as per ICH M10 and FDA guidelines to the linear range (R^2 = 0.998); LLOQ (103.4=ng/mL), HQC (4300=ng/mL), intraday accuracy range from 98.16% to 103.32% & precision varies from 0.51% to 5.26% and interday accuracy range from 97.41% to 105.06% & precision falling between 0.64% to 5.64%, recovery for elagolix sodium varied from 47.73% to 59.33%.

Conclusion: For large preliminary sample cohorts, this RP HPLC approach provides an economical and reliable substitute for LC MS/MS by combining simplicity with international standards. This validated method enables the translational research in therapeutic endometriosis, facilitates optimizing doses to achieve the desired effect and signals the importance of readily available analytical platforms for the advancement of women's health worldwide

Keywords: Endometriosis, Elagolix sodium, RP-HPLC, Bioanalytical method, Method validation.

Introduction

Endometriosis is a chronic estrogen-dependent gynecological disorder defined by the ectopic implantation of endometrial tissue, most commonly on the rectovaginal septum ovaries and pelvic peritoneum. It is estimated that worldwide, nearly 190 million women of reproductive age have endometriosis, with a prevalence of approximately 10% among these patients1,2. Endometriosis causes a large socioeconomic burden; recent data indicate that the disability- adjusted life years (DALYs) due to endometriosis will exceed 2.2 million by 20503. While endometriosis in itself has low mortality, it is associated with increased risk of ovarian cancer, cardiovascular diseases, and autoimmune disorders, making the global health implications even more significant4,5. Endometriosis-related pain, infertility,

and psychological distress collectively contribute to lower productivity and quality of life and are thus an important public health issue globally1.

Pharmacological treatment has evolved from traditional hormonal therapies to the utilization of GnRH antagonists such as elagolix sodium. Elagolix sodium rapidly achieves gonadotropin suppression without the flare impact observed when using GnRH agonists and is a potential therapy for estrogen-dependent diseases[4]. Clinical trials showed its effectiveness for management of endometriosis associated pain, however preclinical pharmacokinetic studies in female Sprague-Dawley (SD) rats are still a must to investigate the ADME profiles of elagolix sodium[6]. Thus, sensitive quantification of drug concentration in plasma is essential for establishing exposure–response relationships, optimizing dosage regimens and effective therapeutic function[7].

Bioanalysis, the quantitative measurement of drugs and metabolites or endogenous biomarkers in a biological matrix is crucial to pharmacokinetic and toxicological assessment[8]. RP-HPLC is the most commonly used technique due in part to its robustness, reproducibility, and availability[9]. Although LC-MS/MS methods are more sensitive and specific, they require complex and expensive instrumentation that hinders their use in the routine analysis of a large number of samples across many laboratories[10]. RP-HPLC with UV detection offers a low-cost method for sensitive analysis of elagolix sodium in plasma. RP- HPLC methods also offer the potential for selectivity optimization through variation of mobile phase type, column type and detection wavelength, which is crucial since biological matrices can potentially interfere with quantitation[11].

Bioanalytical methods should be validated rigorously following international regulatory guidelines, such as ICH M10 and FDA recommendations[12,13] to ensure their reliability. In addition to that, analytical method validation justifies that the method is accurate, precise, and selective in the range of application with the required sensitivity. Important features are the linearity of calibration curves[11], recovery, stability, LOQ, LOD, and reproducibility in a variety of conditions. Defining these criteria in the context of a biochemical analysis ensures that the assay method is suitable for its intended use, allowing for reliable PK characterization and further facilitating regulatory submissions. Additionally, validated bioanalytical methods are critical to translate preclinical data into clinical practice to ensure translation of therapeutics is based on robust globally accepted analytical assays[13].

In the current work, we describe an innovative saturated RP-HPLC bioanalytical method for quantifying elagolix sodium in female SD rat plasma with high sensitivity and reliability. This approach combined methodological simplicity with regulatory compliance to yield reproducible pharmacokinetic data that ultimately enhanced translational research and supported optimized endometriosis treatment. It underscored the challenge in conducting research and supporting innovations with validated, reproducible methods that can be distributed globally, whilst improving access to analytical platforms for drug development in chronic gynaecological disorders. Thus, these methods provided both an enabling technology for pharmacokinetic profiling as well as a pathway towards improved global women's health.

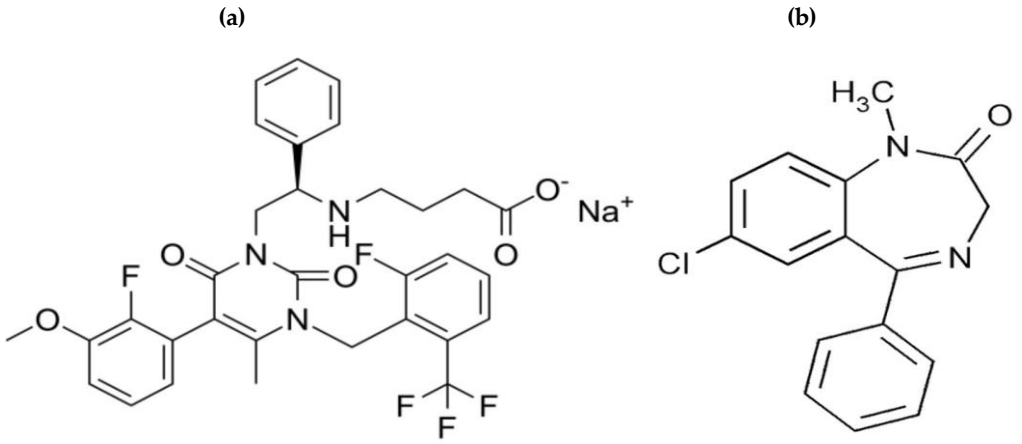

Figure 1: Chemical structure of (a) elagolix sodium, (b) Diazepam

MATERIALS AND METHODS

Chemicals and Reagents

Elagolix sodium was obtained in pure form as gift sample from Alkem Laboratory, Mumbai and Diazepam (as internal standard) was received as a kind gift sample from Unicure India Limited. Acetonitrile was procured from Thermo Fisher Scientific, Mumbai and methanol was purchased from Sigma-Aldrich (Merck), Germany. MSB Chemical Limited, India provided ethanol. All other chemical reagents and solvents were of the highest purity and analytical grade to ensure the reproducibility and accuracy of results.

Method Development and Validation

Chromatographic system and conditions

Chromatographic separation was performed on an Agilent HPLC system (1525 series, Waters Corporation, USA) using the column of BEH C18 Acquity (2.1mm × 100 mm, 1.7µm). The system was equipped with a binary pump A of HPLC connected to Waters 2489 UV/Vis detector for analyte quantification at 275 nm. A caplugs injector (RC-11, USA) was used for the manual introduction of samples. Oasis HLB cartridges were obtained from Waters Corporation, USA. All data acquisition (Table 1), peak integration and statistical assessment was performed using Empower software, Version 3 and SPSS® software version 22 (SPSS Inc. USA). Before analysis, systems were standardized with certified reference standards and performance qualification was confirmed based on retention time precision and resolution criteria. The mobile phases were freshly prepared at the start of each day, filtered through 0.22µm membranes and degassed to ensure baseline stability. The temperature of the column was kept constant to reduce variability and standardized before each set of samples, which were validated for linearity, sensitivity, precision and accuracy.

Table 1: System suitability parameters of the optimized chromatographic conditions

S. No.	Condition	Details
1	Column	Acquity BEH C18 column (2.1 mm × 100 mm, 1.7 μm)
2	Mobile phase	Acetonitrile and 0.1% formic acid in water (gradient elution)
3	Flow rate	0.5 mL/min.
4	Volume of injection	20 μL
5	Detection wavelength	275 nm
6	Column temperature	28°C
7	Theoretical plate	2300
8	Retention time	6.5
9	Run time	10 minutes
10	Tailing factor	1.29

Stock and standard solution preparation

Elagolix sodium (ELAG) primary stock solution was prepared by dissolving 10 mg of ELAG in a volumetric flask and adding methanol to get the volume up to 10 mL (concentration of 1 mg/mL). ELAG plasma standards were prepared from 100 to 5000 ng/mL. Then, four QC samples were prepared at LLOQ=103.4 ng/mL, LQC=305 ng/mL, MQC=2430 ng/mL and HQC=4300 ng/mL levels. These bio-samples were prepared and performed as outlined section on the preparation of sample and examined using the suggested technique utilizing an internal standard (1ppm) of diazepam. All the tested samples were kept at 5 °C.

Analyte separation from plasma

The analyte in the plasma sample was extracted using solid-phase extraction (SPE). In this research, Oasis HLB (Hydrophilic Lipophilic Balance) cartridges were used. One millilitre of methanol was used for the conditioning of cartridges, and one millilitre of deionized water was used to equilibrate them. The prepared sample (500 μL) was mixed with 250 μL of mobile phase and 150 μL of 5% v/v formic acid. The sample was put onto the cartridges after being vortexed for five minutes. Methanol (1 mL) was used to elute the cartridges after they had been cleaned with a 5% v/v methanolic aqueous solution. The eluates were dried by evaporation. After that, 500 μL of the mobile phase was used to reconstitute the residues (Figure 3).

Validation of the method

Selectivity

The experiment of the selectivity test was performed with six independent batches prepared from the same blank rat plasma using the SPE methods. The results were examined to ascertain the degree to which endogenous biochemicals might be contributing to the resulting interferences for the

analyte. The ELAG-containing plasma sample and the blank plasma sample were compared at the LLOQ. To guarantee the accuracy of the sample analysis results, the selectivity research is conducted using blank plasma samples. At a retention time of the analyte, the area of any interfering chromatogram peak must not be more than 20% (for a ratio of 0.2) or 5% (for a ratio from connection point to signal receptor)[14,15].

Linearity and range

To evaluate the linearity, different concentrations of standard solutions containing 1 ppm of ISTD were prepared in the range of 103.4–5000 ng/mL. The calibration curve was constructed from six non-zero standards, which provided evidence of direct proportionality between peak area and analyte concentration of ELAG and ISTD[16,17]. Linear regression analysis gives an R^2 of 0.998, which suggests there is a robust linearity and wide reproducibility between inputs and outputs. The validated range effectively encompassed anticipated plasma concentrations, enabling precise quantification of the ELAG at both low and high levels[18].

Accuracy and precision

A known concentration of ELAG was compared to the experimental result to evaluate the accuracy of the analytical method. Samples of QC (LLOQC, LQC, HQC, and MQC) were examined to evaluate the precision and accuracy of the analytical procedure in each sample. The six replicates of each QC sample were defined after processing. Precision was determined with the percentage coefficient of variation (% CV), while accuracy was reported as percentage bias. Apart from LLOQC, not exceeding a deviation from the nominal concentrations of ± 20.0%, and the precision for acceptability at each QC level must be less than ±15.0% [19].

Sensitivity

To test the accuracy of this analytical method, a known concentration of ELAG was compared with the experimental value. By Samples of QC (LLOQC, LQC, HQC, and MQC) method was performed to estimate precision and % accuracy of the analytical data from each sample. The QC samples were processed and then estimated in six replicates. Except for LLOQC, where the deviation from nominal concentrations cannot exceed ±20.0%, the acceptability threshold for each QC level's precision is less than ±15.0%.[19].

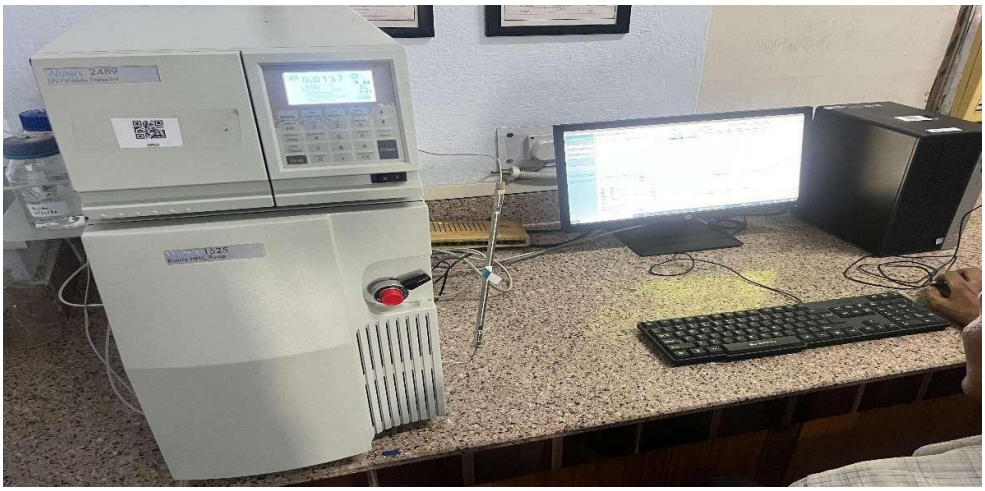

Figure 2: RP-HPLC Instrumentation Setup for Quantification of Elagolix Sodium

RESULTS AND DISCUSSION

The goal of this study was to develop and test a bioanalytical technique using RP-HPLC for measuring elagolix sodium in the plasma of female SD rats following oral administration of the optimized ELAG-loaded SNEDDS. The following headings have been used to discuss the current work.

Method Development

For a particular analyte measurement, optimization of the components including the preparation of the sample and the separation of chromatographic are essential to achieve highly reproducible quantification in biological fluids. The composition of the mobile phase and rate of flow were adjusted during the bioanalytical method development process for ELAG. Acetonitrile (ACN) (50, 60, and 70%) was investigated in conjunction of ammonium acetate buffers (pH 3–7) and PBS (pH 3–7). Finally, the composition of the mobile phase and the flow rate chosen using the peak characteristics and sensitivity criteria. Phosphate buffer was chosen for method development because it produced the peak with the best symmetry and resolution among the buffers that were investigated.

Increasing the buffer concentration (>25 mM) causes peak tailing, which ultimately results in low resolution. On the other hand, in this situation, adopting a lower buffer concentration (<25 mM) reduces sensitivity. It was discovered that a phosphate buffer concentration of about 25 mM produced greater resolution and increased sensitivity. Additionally, a changing pH effect was noted. The stationary phase gained a negative charge if the pH of the mobile phase was increased, which reduced retention of ELAG and accelerated elution. Conversely, lowering the pH will result in a decrease the negative charge on the stationary phase, which will lower the elution of the analyte. After numerous trials and errors, a pH of 4 was determined to be appropriate. Gradient elution was ultimately discovered to be the best mobile phase. Diazepam (ISTD) and ELAG were eluted first in this experiment, and with times of retention of 3.4 and 6.5 minutes, respectively, for a roughly 10 min runtime. The flow rate of this experiment was 0.5 mL/min, and the absorbance was measured at 275 nm. Enrichment of the analyte involved solid phase extraction (SPE).

HPLC columns come in a variety of internal diameters and length combinations, as well as different particle sizes. The length and internal diameter of an HPLC column typically have an impact on sensitivity, analytical speed, and ultimately efficiency. Taking dimension into account, the application determines how many analytes can be in a single analyte, which applies to analytical preparation. Therefore, it is possible to choose the column with the necessary size to accomplish an effective, sensitive, and quick examination[20].

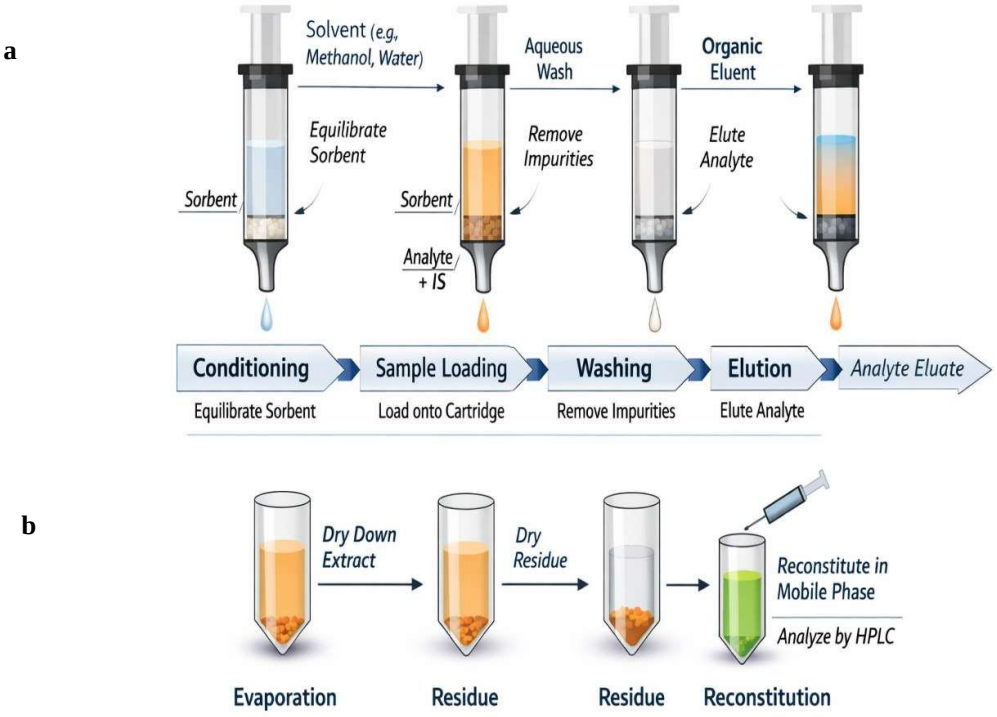

Figure 3: (a) Schematic representation of various steps of solid phase extraction, and (b) Reconstitution of sample ready for analysis

The effectiveness of chromatographic separation is significantly impacted by column length. Efficiency rises as the length of the column grows. The improvement in resolution is a factor of about 1.4 when the column length is doubled. There are few applications for the benefit of small column length, which will result in short run durations with reduced backpressure. Longer columns typically show better resolution, but this comes at a higher cost due to longer analytical periods and excessive use of the solvent-based mobile phase[21]. In this study 15 cm column demonstrated good resolution and reduced retention time; the total run time was 10 minutes in this method. When choosing the stationary phase, particle size is crucial. The column's efficiency rises as the particle size decreases. Large particles minimize the effectiveness of the column, which leads to inadequate separation. In this case, 1.7 µm particle size column was used to improve efficiency by minimizing backpressure problems[21]. Small internal diameter columns exhibit high sensitivity, but they also have higher back pressure. Conversely, larger diameter columns necessitate greater flow rates, which leads to the usage of larger mobile phase volumes. A column with an internal diameter of 2.1 mm was used in order to improve sensitivity and troubleshoot the issue of back-pressure and solvent waste. The wavelength of 275 nm yielded the maximum sensitivity and least interference for ELAG in our study.

Method Validation

The bioanalytical technique for measuring ELAG in plasma samples was developed and validated. Determining a bioanalytical method's selectivity is crucial for identifying and estimating the analyte in biological samples that contain other potentially interfering components, such as drugs, metabolites, endogenous materials, and other compounds associated with xenobiotics. Therefore, verification is required to ensure that the product being measured is the anticipated molecule[14,18] and

analyses of blank plasma samples were carried out for this aim. The LLOQ was used to estimate the selectivity of the samples. ELAG and ISTD retention times in different plasma samples showed no discernible interference. At the ELAG and ISTD retention times, no endogenous component displayed any interference peaks Figure 4 (a) and (b). Hence, the method of selectivity against endogenous substances was demonstrated by the absence of a response peak in the blank plasma sample at the analyte retention time and ISTD, which largely follows USFDA and EMEA guidelines (generally, no interference components are allowed where response is <20% of LLOQ for 5% for the internal standard and the analyte at their respective retention time). Comparison of chromatograms of blank plasma samples and spiked (LLOQ) Figure 4 (a) & (b) showed the selectivity of this method, respectively; there was no interference by any plasma component at the retention time. Accordingly, the approach employed is selective in this work for measuring ELAG levels in rat plasma.

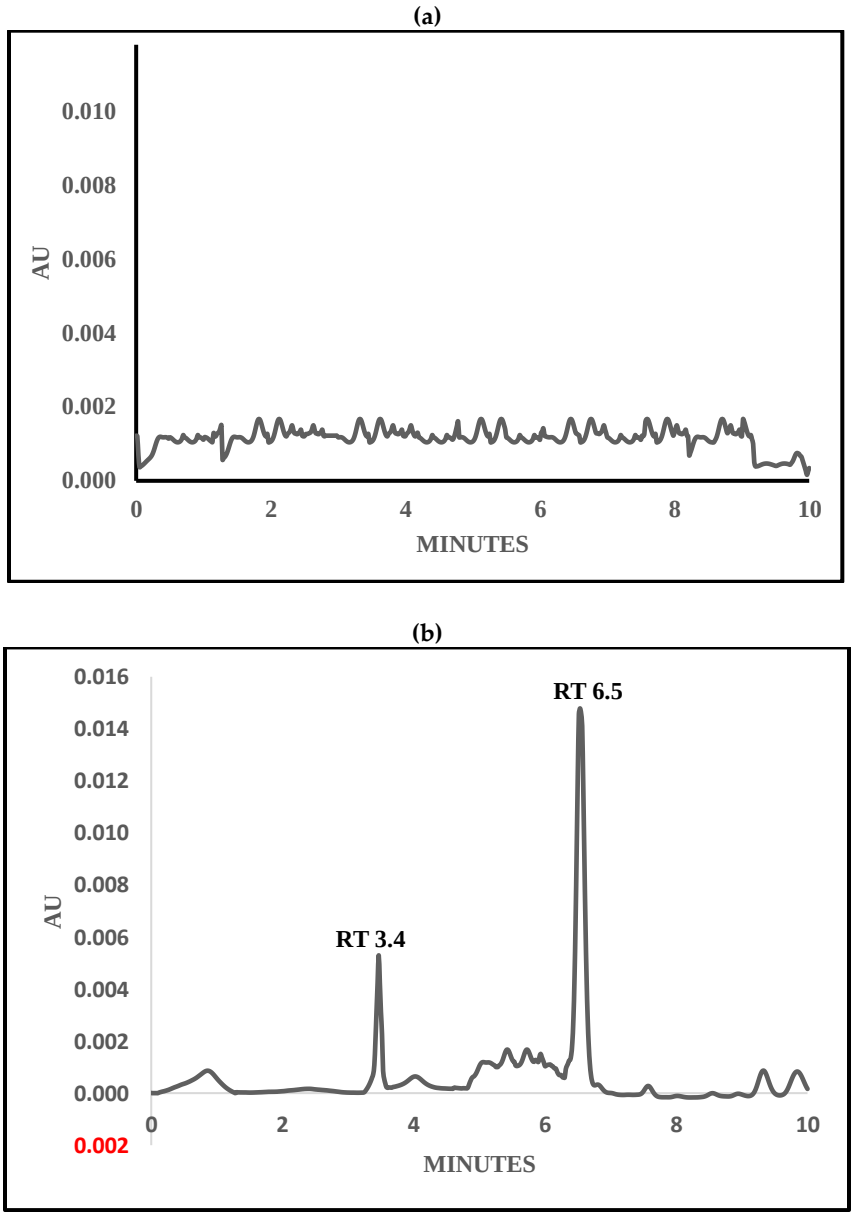

Figure 4: Representative chromatograms of (a) blank plasma sample, (b) elagolix sodium and ISTD spiked at LLOQ in plasma

Plotting the ELAG concentration versus the peak area ratio of ELAG to ISTD, resulted in a standard curve that ranged from 103.4 ng/mL to 5000 ng/mL. The linear equation, y = 0.841x + 0.144, with correlation coefficient R^2=0.998, represented the standard curve (Figure 5). The developed method's (LLOQ was 103.4 ng/mL. LQC, LLOQC, MQC, and HQC are four QC levels. ELAG's intraday accuracy ranges from 98.16% to 103.32%, while its precision varies from 0.51% to 5.26% (Table 2). Additionally, ELAG's interday accuracy ranged from 97.41% to 105.06%, with better accuracy, at the same QC levels, precision falling between 0.64% and 5.64% (Table 2). All evaluated plasma sample findings exhibit good agreement and fall within the USFDA and EMEA-defined acceptable bioanalytical technique validation ranges of accuracy and precision. The average concentration for the QC samples should be lower than 15% of the nominal values according to EMEA and USFDA recommendations for bioanalytical method validation, except for the LLOQ, which must be less than 20% of the nominal values (EMEA 2011, FDA 2013). The obtained LLOQ of 103.4 ng/mL. To get the clean plasma samples and prevent any interferences, a variety of plasma extraction techniques were evaluated. Although the protein precipitation process provides a quick and easy way to obtain clean plasma, its high noise levels make it significantly unacceptable[16,17].

Table 2: intermediate precision and accuracy for intra- day & inter-day of elagolix sodium in SD rat plasma

Levels	Spiked conc. (ng/mL)	Mean conc. found (ng/mL)*	Accuracy		Precision
			Accuracy (%)	% Bias	(% CV)
Intra-day					
LLOQC	103.4	106.83 (5.62)	103.32	3.32	5.26
LQC	305	308.76 (11.58)	101.23	1.23	3.75
MQC	2430	2385.39 (26.85)	98.16	-1.84	1.13
HQC	4300	4403.56 (22.51)	102.41	2.41	0.51
Inter-day					
LLOQC	103.4	108.63 (6.13)	105.06	5.06	5.64
LQC	305	297.11 (14.48)	97.41	-2.59	4.87
MQC	2430	2545.35 (23.45)	104.75	4.75	0.92
HQC	4300	4392.16 (27.93)	102.14	2.14	0.64

* All values given in the parentheses are expressed in mean ±S.D. (n=8)

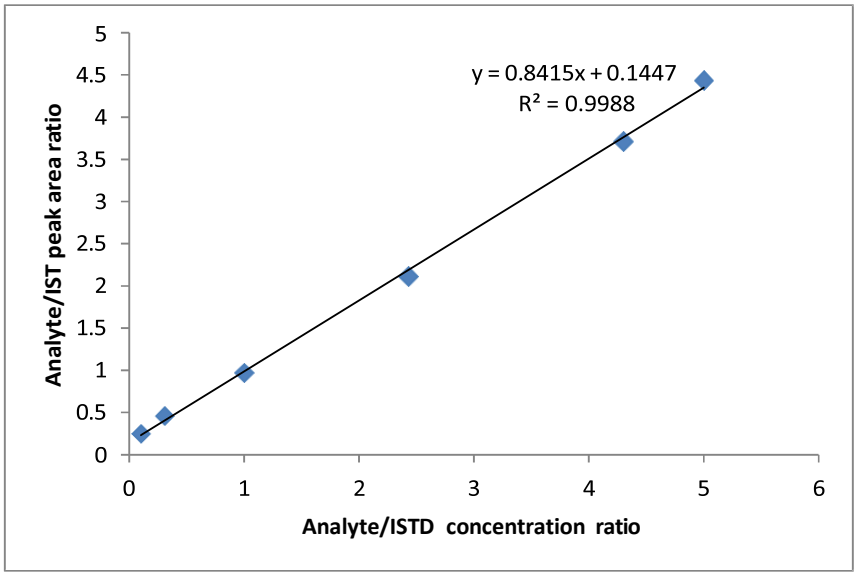

Figure 5: Standard curve of elagolix sodium

Nevertheless, the liquid-liquid extraction (LLE) methodology was a tedious and time-consuming process, performed through multiple steps but resulting in a purified sample with significantly less interference. In this study, liquid-liquid extraction (LLE) was not used because there were small amounts of plasma required for the research, since the plasma of female SD rats was utilized as a sample. The solid-phase extraction (SPE) technique was the method of choice for isolating the analyte in this experiment.

However, the solid-phase extraction (SPE) technique receives very low noise and much cleaner extracts with a very high sensitivity by minimizing highest volume plasma[17], when using Oasis HLB cartridges to extract plasma samples. In this cartridge, a hydrophilic lipophilic balanced water-wettable reversed-phase sorbent was used to have more options. It contains a defined ratio of hydrophilic N-vinyl pyrrolidone and lipophilic divinyl benzene, which endows higher reversed-phase capacity as well as neutral polar hook to improve the retention of polar analytes. More advantages include the utilization of various solvents and pH extremes with the HLB cartridge.

In summary, this SPE approach is more reliable and durable, eliminating the need for repeated preparation. Higher retention capacity, which allows for the retention of more analytes with fewer deviations, and an improved extraction method. According to USFDA rules, the recovery level of the analyte must be repeatable as well as uniform across all QC levels (LQC, MQC, and HQC), but it need not be 100%[19]. The extraction method described in this paper offers a quick way to extract the plasma sample's analytes.

Here, the suggested approach showed a ELAG was reliably recovered from the plasma sample of female SD rats. LQC, MQC, and HQC are the three QC levels at which the mean absolute recovery for ELAG varied from 47.73% to 59.33%, while the precision ranged from 4.15% to 9.83%. (Table 3).

Table 3: Recovery of elagolix sodium extraction in rat plasma

Recovery from extraction		
Levels	**% Avg. recovery***	**% CV**
LQC	55.92 (2.32)	4.15
MQC	47.73 (4.69)	9.83
HQC	59.33 (5.29)	8.92

Table 4: Recovery of freeze-thaw stability of elagolix sodium in rat plasma

Stability of freeze-thaw cycles								
Levels	Spiked conc. (ng/mL)	Initial mean conc. found (ng/mL)*	% CV	% Accuracy	Final mean conc. found (ng/mL)*	% CV	% Accuracy	% Absolute stability
LQC	305	307.43 9.48	3.08	100.8	298.89 11.32	3.79	98	97.22
MQC	2430	2523.72 12.72	0.5	103.86	2351.45 29.48	1.25	96.77	93.17
HQC	4300	4211.33 18.38	0.44	97.94	4123.83 32.14	0.78	95.9	97.92

* All values given in the parentheses are expressed in mean ±S.D. (n=8)

Analyte stability is an important parameter in plasma samples that should be evaluated using the established method of validation. The analysis conditions that could arise during sample preparation and handling must be replicated[16,17].

Table 4 displays the ELAG's absolute stability during freeze/thaw conditions at LQC, MQC, and HQC. Following three cycles of freezing and thawing, the ELAG spiked in SD rat plasma demonstrated stability throughout the time. The newly prepared QC samples demonstrated the acceptable precision

between 3.08% and 0.44% and accuracy between 97.94% and 103.86% (Table 4). The accuracy ranges from 95.90% to 98.00%, and the precision ranges from 3.79% to 0.78%, After cycles of freezing and thawing, the percentage of absolute stability fell between 93.17% and 97.92%. This technique was shown to be accurate and repeatable for the measurement of ELAG in SD rat plasma.

CONCLUSION

In this research, RP-HPLC was used to develop a straightforward and sensitive bioanalytical technique for measuring elagolix sodium. Solid phase extraction of biological materials allowed for the successful determination of plasma concentration of elagolix sodium in female Sprague-Dawley rats. The internal standard was diazepam, which ensured repeatability and dependable peak resolution. With a correlation coefficient (R^2) of 0.998, the method demonstrated outstanding linearity throughout the concentration range of 103.4–5000 ng/mL. Overall, the process demonstrated sensitivity and selectivity, making it appropriate for standard bioanalytical applications. Also, the validated method can be beneficial for pharmacokinetics and bioequivalence studies as a low-cost alternative to complex hyphenated techniques. The versatility of the tracker reinforces its importance for future applications in drug design and therapeutic monitoring.

ABBREVIATIONS

ELAG: Elagolix Sodium, **SNEDDS:** Self-Nanoemulsifying Drug Delivery Systems, **ISTD:** Internal Standard, **UV:** Ultraviolet, **RP-HPLC:** Reversed Phase High-Performance Liquid Chromatography, **LOD:** Limit of Detection, **LOQ:** Limit of Quantitation, **RSD:** Relative Standard Deviation, **ICH:** International Council for Harmonisation, **FDA:** Food and Drug Administration, **LC MS:** Liquid Chromatography–Mass Spectrometry, **LLOQ:** Lower Limit of Quantitation, **LQC:** Low Quality Control, **MQC:** Medium Quality Control, **HQC:** High Quality Control, **CV:** Coefficient of Variation, **ACN:** Acetonitrile, **SPE:** Solid-phase extraction, **LLE:** Liquid-liquid extraction, **USFDA:** United States Food and Drug Administration, **EMEA:** European Medicines Evaluation Agency.

AUTHOR'S CONTRIBUTIONS

Each author contributed significantly to the study's conceptualization, design, data collection, analysis, and interpretation. Each author approved the final version for submission, contributed to the manuscript's drafting or critical revision for important intellectual content, and took full responsibility for the work's correctness and integrity.

FUNDING

This research was carried out without funding.

DATA AVAILABILITY

All data generated and evaluated are contained within this research article.

CONFLICT OF INTEREST

The work presented in this publication was not influenced by any conflicts of interest or personal ties, according to the authors.

ETHICS DECLARATIONS

Ethical Approval

Institutional Animal Ethics Committee of IIMT College of Medical Sciences, IIMT University, Meerut, Uttar Pradesh, India, approved this study prior to the commencement of the animal experiments (Approval No. IAEC/2024-25/0010/CPCSEA, Date: 13/09/2024).

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CONSENT FOR PUBLICATION

All authors have given their approval to the final content and submission of the work..

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