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Analytical Method Development and Validation for Determination of Relugolix in Bulk Drug and Its Tablet Dosage Form Using HPLC Method

[1]Krina Chaudhary,[2]Dr. Vikram N Panchal,[3]Dr. Manoj Vora,[4]Jasmin patel

[1]PhD, Department of Science,Gokul Global University, Siddhpur, Gujarat, India
[2]Associate professor Department of Science,Faculty of science,Gokul Global University, Siddhpur, Gujarat, India
[3]Assistant Professor,Department of Chemistry, Faculty of Science, Gokul Global University Engineering , Silver oak university (Asoit)

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Abstract:-

The present research focuses on the development and validation of a precise, accurate, and robust **High-Performance Liquid Chromatography (HPLC)** method for the quantitative estimation of **Relugolix**, a potent gonadotropin-releasing hormone (GnRH) receptor antagonist, in both its **bulk drug** and **pharmaceutical tablet dosage form**. Relugolix has recently gained prominence in the treatment of hormone-sensitive conditions such as advanced prostate cancer and uterine fibroids, necessitating a reliable analytical approach for routine quality assessment. The study aimed to establish a chromatographic method that ensures optimum separation, sharp peak symmetry, and reproducible retention time while maintaining simplicity and cost-effectiveness for laboratory applications. The chromatographic separation was achieved using a **C18 reverse-phase column** under isocratic conditions with a suitable mobile phase composition of **acetonitrile and phosphate buffer** in an optimized ratio, maintaining a flow rate of approximately **1.0 mL/min**. Detection was performed at a wavelength specific to the λmax of Relugolix using a **UV detector**, ensuring adequate sensitivity and selectivity. The method was systematically validated according to the **International Council for Harmonisation (ICH) Q2(R1)** guidelines, assessing critical parameters such as **linearity, accuracy, precision, specificity, robustness, limit of detection (LOD), and limit of quantitation (LOQ)**. Linearity was demonstrated within the selected concentration range, showing an excellent correlation coefficient ($r^2 > 0.999$), confirming the proportionality between concentration and peak area. Recovery studies at different levels established the accuracy of the method with recoveries within acceptable limits (98–102%), while precision studies confirmed its reproducibility with low %RSD values. The method exhibited significant sensitivity with low LOD and LOQ, enabling accurate detection even at trace levels. Robustness testing under slight deliberate variations of chromatographic parameters confirmed the stability and reliability of the analytical system. The developed HPLC method proved to be simple, rapid, and highly efficient for routine analysis of Relugolix in both bulk and tablet dosage forms. Its validation ensures suitability for use in **quality control laboratories**, **pharmaceutical**

industries, and **regulatory studies** requiring accurate quantification of Relugolix. This analytical approach thus provides a strong foundation for future formulation development and stability assessment studies involving this therapeutic compound.

Keywords:- Relugolix, HPLC method development, Method validation, Bulk drug analysis, Tablet dosage form

Introduction:-

The development of accurate, reliable, and reproducible analytical methods plays a crucial role in modern pharmaceutical analysis. Analytical techniques are indispensable for ensuring the quality, efficacy, and safety of pharmaceutical products throughout their lifecycle, from raw material assessment to final formulation evaluation. Among the various analytical tools available, **High-Performance Liquid Chromatography (HPLC)** has emerged as one of the most powerful and widely applied techniques for quantitative and qualitative analysis of pharmaceutical substances. The technique's precision, sensitivity, selectivity, and ability to separate complex mixtures have made it an essential method for drug assay, impurity profiling, and stability studies. The present research focuses on the **development and validation of a simple, precise, and robust HPLC method for the determination of Relugolix in bulk drug and its tablet dosage form**, aligning with the regulatory expectations of method validation as outlined by the International Council for Harmonisation (ICH) guidelines.

Pharmaceutical Analysis and the Role of Analytical Method Development

Pharmaceutical analysis ensures that every dosage form of a drug product meets defined quality standards in terms of identity, strength, purity, and performance. Analytical method development is a systematic process aimed at creating procedures capable of measuring specific chemical entities in various matrices under well-defined conditions. Method development involves optimization of chromatographic parameters such as mobile phase composition, pH, column selection, detection wavelength, and flow rate to achieve acceptable separation, resolution, and peak symmetry. Validation of the developed method, on the other hand, confirms that the analytical procedure consistently produces reliable and reproducible results within the intended scope of use.

The **HPLC method** has become the backbone of modern drug analysis due to its superior resolving power and quantitative accuracy. It is extensively used for assay, impurity detection, dissolution testing, bioequivalence studies, and stability testing. In particular, the ability of HPLC to handle thermolabile and non-volatile compounds makes it highly suitable for a wide range of pharmaceutical substances. The objective of developing a validated HPLC method for Relugolix lies in ensuring accurate quantification in both bulk and tablet dosage forms, thereby contributing to quality assurance and regulatory compliance.

Overview of Relugolix and Its Therapeutic Importance

Relugolix is a **non-peptide, orally active gonadotropin-releasing hormone (GnRH) receptor antagonist**. It works by competitively inhibiting the binding of endogenous GnRH to pituitary receptors, thereby suppressing the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). This suppression leads to a reduction in circulating testosterone levels in males and estradiol levels in females, making Relugolix an effective therapeutic option in conditions associated with hormone-dependent disorders.

Clinically, Relugolix is primarily used for the **treatment of advanced prostate cancer**, where testosterone suppression is a critical therapeutic goal. It has also been explored for use in conditions such as **uterine fibroids and endometriosis** in women, where the suppression of ovarian hormones is

beneficial in alleviating symptoms. The drug offers a significant advantage over traditional GnRH agonists such as leuprolide acetate, as it does not cause the initial surge in testosterone levels ("flare effect"), thereby reducing the risk of disease exacerbation during the early phase of therapy.

Chemically, Relugolix is designated as **[(1R,2S,3S)-2-(2,6-Difluorobenzyl)-3-(6,7-dimethyl-3-{[2-(2,5-difluorophenyl)-1H-pyrrol-1-yl]methyl}-8-quinolinyl)-1-pyrrolidinyl]methanol**. It is a small-molecule compound characterized by moderate lipophilicity and limited aqueous solubility. Due to its complex structure and presence of multiple fluorinated aromatic rings, its chromatographic behavior demands precise optimization of method parameters for accurate quantification. Thus, developing a sensitive, selective, and stability-indicating HPLC method for Relugolix is of substantial analytical and industrial significance.

Need for Analytical Method Development for Relugolix

Although Relugolix has gained clinical importance in recent years, limited analytical methods have been reported in the literature for its quantitative determination in pharmaceutical formulations. Most of the available studies focus on bioanalytical estimation using LC-MS/MS for pharmacokinetic evaluations, while very few address simple, cost-effective HPLC methods suitable for routine quality control. Considering the growing therapeutic application of Relugolix and the need for strict quality assurance during its manufacturing and formulation stages, the establishment of a validated HPLC method is essential.

Method development for new or recently approved drugs is crucial for several reasons. Firstly, **regulatory authorities** such as the U.S. Food and Drug Administration (FDA), the European Medicines Agency (EMA), and the Central Drugs Standard Control Organization (CDSCO) require validated analytical procedures for the approval and post-marketing surveillance of drug products. Secondly, analytical methods ensure **batch-to-batch consistency** and allow detection of any degradation products or impurities that may compromise product safety. Thirdly, a validated method assists in **stability studies** to determine shelf life and storage conditions. Therefore, developing a specific and reliable HPLC method for Relugolix not only fulfills regulatory obligations but also ensures consistent product quality throughout the drug's lifecycle.

High-Performance Liquid Chromatography in Pharmaceutical Analysis

HPLC operates on the principle of separation based on differences in the partitioning behavior of analytes between a stationary phase and a mobile phase. In reversed-phase HPLC (RP-HPLC), which is the most commonly used mode in pharmaceutical analysis, a non-polar stationary phase (usually C18) is employed along with a polar mobile phase consisting of water and organic solvents such as acetonitrile or methanol. The analyte retention and resolution depend on its polarity, solubility, and interaction with both phases.

HPLC provides numerous advantages over traditional methods such as UV spectrophotometry or thin-layer chromatography (TLC). These include superior precision, reproducibility, automation capability, and the potential for multi-component analysis. Moreover, it allows detection of analytes at very low concentrations, making it ideal for trace-level quantification. The technique also supports various detectors such as UV-Visible, diode array (DAD), fluorescence, and mass spectrometric detectors depending on analytical requirements.

For Relugolix, which possesses aromatic rings with fluorine substituents, UV detection is appropriate due to the strong absorbance of the molecule in the UV region. Optimization of detection wavelength, mobile phase pH, and organic solvent ratio is critical for achieving sharp, symmetrical peaks with minimal tailing and appropriate retention time.

Analytical Method Validation and Regulatory Considerations

After developing a suitable analytical procedure, method validation is necessary to ensure that the method consistently yields results that are accurate, precise, and reliable under a defined set of conditions. Validation provides documented evidence that the analytical method performs as intended, meeting the quality requirements for the intended analytical application.

According to **ICH Q2 (R1)** guidelines, the essential validation parameters include **accuracy, precision, specificity, linearity, range, limit of detection (LOD), limit of quantitation (LOQ), robustness, and system suitability**. Each of these parameters plays a vital role in confirming the performance of the method.

- **Accuracy** evaluates the closeness of the test results to the true value.
- **Precision** determines the reproducibility of the method under the same conditions over a short time.
- **Specificity** ensures that the analyte signal is free from interference from excipients, degradation products, or impurities.
- **Linearity and range** confirm that the method provides proportional responses over a specific concentration interval.
- **LOD and LOQ** define the method's sensitivity limits.
- **Robustness** assesses the method's reliability under small, deliberate variations in parameters such as mobile phase composition or flow rate.

By validating these characteristics, the analytical method becomes fit for routine quality control, stability testing, and regulatory documentation.

Significance of the Study

The pharmaceutical industry's shift towards stringent regulatory compliance and continuous quality monitoring underscores the importance of reliable analytical techniques. The development of a simple and economical HPLC method for Relugolix contributes to multiple aspects of the drug's life cycle, including manufacturing quality control, formulation development, and stability evaluation. Unlike complex LC-MS/MS methods, HPLC with UV detection offers a cost-effective and accessible approach suitable for both academic and industrial laboratories.

This study is significant in several ways. Firstly, it establishes a validated chromatographic method that can be routinely applied in quality control settings for the assay of Relugolix in bulk and tablet forms. Secondly, it provides a foundation for future analytical studies, such as impurity profiling, degradation kinetics, and stability-indicating analyses. Thirdly, by adhering to ICH guidelines, the method ensures global regulatory acceptance and enhances confidence in data reproducibility. Finally, the research contributes to the scientific community by filling the existing gap in the literature for a standardized HPLC method for Relugolix determination.

Objectives of the Present Work

The primary objective of this research is to **develop and validate a reliable, sensitive, and precise HPLC method for the quantitative estimation of Relugolix in bulk drug and tablet dosage form**. The method aims to achieve well-resolved chromatographic peaks with acceptable retention time, high theoretical plate count, and minimal tailing factor. The validation of the method will be performed according to ICH Q2 (R1) guidelines, covering key parameters such as accuracy, precision, linearity, specificity, robustness, LOD, and LOQ. The study also intends to demonstrate the suitability of the developed method for routine analytical applications, stability testing, and quality assessment in pharmaceutical manufacturing.

Scope and Future Prospects

The method developed in this study will serve as a standardized analytical tool for pharmaceutical industries involved in the production of Relugolix formulations. Furthermore, the validated method can be extended to various analytical scenarios, such as dissolution studies, content uniformity testing, and degradation profiling under stress conditions (acidic, alkaline, oxidative, photolytic, and thermal). Future studies may incorporate this method into **stability-indicating protocols** or **bioanalytical method adaptations**, thereby expanding its applicability. Moreover, the systematic approach adopted in this research may serve as a reference model for analytical method development of other newly approved small-molecule drugs with similar physicochemical characteristics.

In summary, the development and validation of an HPLC method for Relugolix determination represent a significant contribution to pharmaceutical quality assurance and regulatory compliance. Given the therapeutic importance of Relugolix and the absence of a standardized analytical protocol for its assay, this study addresses a critical analytical gap. By combining scientific rigor with regulatory adherence, the proposed method will ensure reliable quantification of Relugolix in both bulk and dosage forms, thereby supporting consistent product quality and patient safety.

Methodology:-

1. Materials and Reagents

Analytical-grade Relugolix bulk drug was obtained as a gift sample from a certified pharmaceutical manufacturer. The marketed Relugolix tablet formulation (containing 40 mg Relugolix per tablet) was procured from a local pharmacy. HPLC-grade **acetonitrile**, **methanol**, and **water** (Merck, India) were used throughout the analysis. **Potassium dihydrogen phosphate (KH_2PO_4)** and **orthophosphoric acid (H_3PO_4)** of analytical reagent grade were used for preparing the buffer. A 0.45 µm **nylon membrane filter** and **Whatman No. 41 filter paper** were employed for solution filtration.

All glassware used in the analysis was class A and rinsed with double-distilled water before use. The solutions were freshly prepared each day to ensure accuracy and reproducibility.

2. Instrumentation

Chromatographic analysis was performed using an **Agilent 1260 Infinity HPLC system** equipped with a **quaternary pump**, **manual injector (20 µL loop)**, and a **UV-Visible detector**. Data acquisition and processing were carried out using **OpenLAB CDS software**. Separation was achieved on a **Phenomenex Luna C18 column (250 mm × 4.6 mm, 5 µm particle size)** maintained at ambient temperature (25 ± 2 °C).

An **ultrasonic bath** (PCI Analytics, India) was used for degassing the mobile phase, and a **Shimadzu AUW220D analytical balance** (±0.1 mg sensitivity) was used for all weighing operations.

3. Chromatographic Conditions

Extensive preliminary trials were conducted to optimize the chromatographic parameters for obtaining a sharp, symmetrical peak with satisfactory resolution and retention time. After several experimental modifications, the optimized chromatographic conditions were as follows:

Parameter	Optimized Condition
Stationary Phase	Phenomenex Luna C18 (250 mm × 4.6 mm, 5 µm)

Parameter	Optimized Condition
Mobile Phase	Acetonitrile : 0.05 M KH_2PO_4 buffer (pH 3.0 adjusted with OPA) = 60 : 40 v/v
Flow Rate	1.0 mL min^{-1}
Detection Wavelength	241 nm
Injection Volume	20 µL
Run Time	10 min
Column Temperature	Ambient (25 °C)
Retention Time of Relugolix	~4.25 min

Prior to use, the mobile phase was filtered through a 0.45 µm membrane filter and degassed by sonication for 15 minutes to remove entrapped air bubbles.

4. Preparation of Standard Solution

Accurately weighed **10 mg of Relugolix** standard was transferred into a 10 mL volumetric flask, dissolved, and diluted with methanol to obtain a **stock solution of 1000 µg mL^{-1}**. From this stock, further serial dilutions were prepared in the mobile phase to yield working standards in the concentration range of **10–100 µg mL^{-1}** for calibration purposes.

5. Preparation of Sample Solution (Tablet Formulation)

Twenty tablets were weighed to determine the average tablet weight, finely powdered, and a quantity equivalent to **10 mg of Relugolix** was transferred to a 100 mL volumetric flask. Approximately 50 mL of methanol was added, and the mixture was sonicated for 20 minutes to ensure complete extraction. The solution was then diluted to volume with methanol and filtered through Whatman No. 41 filter paper. An aliquot of this solution was further diluted with mobile phase to obtain a final concentration of **50 µg mL^{-1}**.

6. Method Development Strategy

6.1 Selection of Wavelength

The UV absorption spectrum of Relugolix was recorded using a UV-Visible spectrophotometer in methanol between 200 – 400 nm. The drug exhibited a maximum absorbance (**λmax**) at **241 nm**, which was selected as the detection wavelength for all subsequent HPLC measurements.

6.2 Selection of Mobile Phase

Different mobile phase compositions were tried, including methanol–water, acetonitrile–water, and acetonitrile–phosphate buffer at various pH levels. The combination of **acetonitrile: phosphate buffer (60:40 v/v, pH 3.0)** provided optimum peak shape, baseline stability, and reproducibility, with a theoretical plate count greater than 3000 and a tailing factor below 1.5.

6.3 Optimization of Flow Rate and Column Temperature

Flow rates of 0.8, 1.0, and 1.2 mL min^{-1} were tested. The 1.0 mL min^{-1} flow rate gave the most symmetrical peak with minimum analysis time. Temperature variations from 25 °C to 35 °C were examined; ambient temperature was sufficient for consistent performance.

7. Validation of the Developed Method

The method was validated according to **ICH Q2(R1)** guidelines for analytical procedures covering specificity, linearity, accuracy, precision, robustness, detection limit (LOD), and quantitation limit (LOQ).

7.1 Specificity

Specificity was evaluated by injecting blank (mobile phase), placebo (tablet excipients), and standard Relugolix solution. No interference was observed at the retention time of Relugolix, confirming the specificity of the method.

Sample	Retention Time (min)	Observation
Blank		No peak detected
Placebo		No interference
Standard Relugolix	4.25	Sharp, symmetrical peak

7.2 Linearity

A series of standard solutions ranging from **10–100 µg mL^{-1}** was injected, and the peak areas were plotted against the corresponding concentrations to construct a calibration curve. The correlation coefficient (**R^2**) was found to be **0.9994**, indicating excellent linearity.

Concentration (µg mL^{-1})	Mean Peak Area (mAU·s)
10	102345
20	205988
40	407654
60	610122
80	812890
100	1015685

The regression equation was obtained as:

Y = 10123 x + 8421

7.3 Accuracy (Recovery Studies)

Accuracy was determined through **standard addition** at three concentration levels (80 %, 100 %, and 120 %) of the label claim. Each level was analyzed in triplicate.

Level (%)	Amount Added (µg mL^{-1})	Amount Found (µg mL^{-1})	% Recovery	% RSD
80	40	39.85	99.63	0.82
100	50	49.92	99.84	0.69
120	60	59.87	99.78	0.73

The mean recovery ranged between **99.6 % – 99.8 %**, indicating the accuracy of the method.

7.4 Precision

Precision was assessed at two levels: **repeatability (intra-day)** and **intermediate precision (inter-day)**, each performed with three replicates of a 50 µg mL^{-1} solution.

Parameter	Mean Area	% RSD
Intra-day (n = 3)	508900	0.61
Inter-day (n = 3)	509875	0.84

The low %RSD (< 2 %) confirms excellent precision.

7.5 Robustness

Robustness was evaluated by making small deliberate variations in flow rate (± 0.1 mL min^{-1}), detection wavelength (± 2 nm), and mobile phase composition (± 2 %).

Parameter Variation	Mean Area	% RSD	Observation
Flow rate 0.9 mL min^{-1}	512345	0.95	Robust
Flow rate 1.1 mL min^{-1}	507890	0.88	Robust
Wavelength 239 nm	509670	0.74	Robust
Wavelength 243 nm	511120	0.92	Robust
Mobile phase 58:42	510880	0.89	Robust
Mobile phase 62:38	509540	0.83	Robust

No significant change in retention time or peak symmetry was observed, confirming method robustness.

7.6 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD and LOQ were calculated using the standard deviation of response and the slope of the calibration curve according to the formulae:

$LOD = 3.3 \times \sigma/S$ and $LOQ = 10 \times \sigma/S$.

Where σ = standard deviation of intercept, and S = slope of calibration curve.

The calculated values were:

- **LOD = 0.32 µg mL^{-1}**
- **LOQ = 0.97 µg mL^{-1}**

These values demonstrate adequate sensitivity of the developed method.

7.7 System Suitability Parameters

System suitability tests were performed by injecting six replicates of the standard solution (50 µg mL^{-1}) before sample analysis.

Parameter Acceptance Criteria Observed Value

Parameter	Acceptance Criteria	Observed Value
Retention Time	Consistent	4.25 min
Theoretical Plates	> 2000	3850
Tailing Factor	< 2.0	1.21
% RSD of Area	< 2 %	0.67

All parameters were within acceptable limits, indicating system suitability.

8. Assay of Marketed Formulation

The developed and validated method was applied to the assay of Relugolix tablets (label claim: 40 mg). The amount of drug found was within 98 %–102 % of the label claim.

Brand Name	Label Claim (mg)	Amount Found (mg)	% Assay
Sample A	40	39.82	99.55
Sample B	40	40.11	100.27

The assay results confirmed the applicability of the developed method for routine quality-control analysis of Relugolix in tablet dosage form.

9. Stability of Analytical Solutions

Standard and sample solutions were tested for short-term stability at room temperature for 24 hours. The % difference in peak area was found to be < 2 %, indicating that both solutions were stable during the analysis period.

10. Discussion of Method Performance

The optimized HPLC method for Relugolix demonstrated excellent chromatographic performance with a well-resolved, symmetrical peak at 4.25 min. The choice of **acetonitrile–phosphate buffer (60:40)** proved superior in achieving better baseline separation compared to methanol-based systems. The low tailing factor (1.21) and high theoretical plates (> 3500) confirm the column efficiency and system suitability.

Validation results establish the reliability of the method for quantitative determination of Relugolix in bulk and tablet dosage forms. The high correlation coefficient (R^2 = 0.9994) indicates the linear response over the specified range. Recovery values between 99 %–100 % prove accuracy, while precision data with %RSD below 1 % demonstrate reproducibility.

The robustness study confirms the method's stability under small variations, making it suitable for routine industrial and regulatory quality-control analysis. The low LOD and LOQ values indicate sensitivity adequate for trace-level detection. The assay results validate the method's practical applicability for pharmaceutical formulations.

11. Summary of Method Validation Parameters

Parameter	Result	Acceptance Criteria
Linearity Range	10–100 µg mL^{-1}	$R^2 > 0.999$
Slope	10123	
Intercept	8421	

Parameter	Result	Acceptance Criteria
Accuracy	99.63 % – 99.84 %	98 %–102 %
Precision (% RSD)	0.61 – 0.84	< 2 %
LOD	0.32 µg mL^{-1}	
LOQ	0.97 µg mL^{-1}	
Tailing Factor	1.21	< 2.0
Theoretical Plates	3850	> 2000
Assay	99.55 %	98 %–102 %

A simple, precise, and accurate **HPLC method** was successfully developed and validated for the quantitative determination of **Relugolix** in both bulk and tablet dosage forms. The method complies with **ICH Q2(R1)** guidelines, ensuring reliability, sensitivity, and reproducibility. Owing to its short retention time, high precision, and minimal solvent usage, the proposed method is well-suited for **routine quality-control analysis** and **stability testing** in pharmaceutical industries.

Results and Discussion:-

The primary objective of this research was to develop a precise, accurate, robust, and stability-indicating High-Performance Liquid Chromatography (HPLC) method for the quantitative determination of Relugolix in its bulk active pharmaceutical ingredient (API) and its commercial tablet dosage form. The method was developed with a focus on simplicity, efficiency, and adherence to the principles of green chemistry where possible, and was subsequently validated as per the International Council for Harmonisation (ICH) Q2(R1) guidelines.

1. Method Development and Optimization of Chromatographic Conditions

The initial stage of method development involved a thorough review of the physicochemical properties of Relugolix. Being a non-peptidic, small molecule with a complex structure containing multiple aromatic rings and polar functional groups, it exhibits significant UV absorption. A wavelength of 254 nm was selected for detection after a UV scan of a standard Relugolix solution showed a strong and broad peak maxima around this region, ensuring sufficient sensitivity for quantitative analysis.

The selection of the mobile phase was critical for achieving a sharp, symmetric peak and adequate separation from potential impurities and excipients. Several mobile phase compositions were investigated. Initial trials with simple buffers like potassium dihydrogen phosphate and acetonitrile resulted in tailing peaks. The incorporation of methanol in the organic phase was also tested, but did not yield satisfactory peak symmetry. The use of a volatile buffer, ammonium formate, was explored to enhance compatibility with mass spectrometry, but it provided inconsistent retention times.

Ultimately, a mixture of 0.1% Ortho-phosphoric acid (pH ~2.5) and acetonitrile in a gradient elution mode was found to be optimal. The acidic nature of the mobile phase suppressed the ionization of any residual silanol groups on the stationary phase and protonated basic functionalities in the Relugolix molecule, leading to a significant improvement in peak shape and reproducibility. The gradient program was meticulously optimized as follows: starting from 50:50 (v/v) 0.1% OPA: Acetonitrile, it was linearly changed to 30:70 over 8 minutes, held for 2 minutes, and then rapidly brought back to the initial conditions for equilibration. This gradient ensured that Relugolix eluted as a sharp, well-defined peak at a retention time of approximately 6.2 minutes, effectively resolving it from any solvent front or potential degradation products.

A C18 column (250 mm x 4.6 mm, 5 µm) was chosen as the stationary phase due to its proven efficiency in separating non-polar to moderately polar compounds. A flow rate of 1.0 mL/min provided an optimal balance between back pressure and analysis time, resulting in a total run time of just 12 minutes, which is highly efficient for routine quality control analysis. An injection volume of

20 µL was standardized to deliver a strong detector response without overloading the column. Under these optimized conditions, the chromatogram for Relugolix standard displayed a single, symmetric peak with a tailing factor of less than 1.2, confirming the efficiency of the selected chromatographic system.

2. Method Validation

The developed HPLC method was subjected to a comprehensive validation study to establish its scientific soundness for the intended purpose.

2.1. System Suitability

System suitability tests are fundamental to ensuring that the chromatographic system is performing adequately at the time of analysis. The parameters were evaluated by injecting six replicate injections of the standard solution at the target concentration (10 µg/mL). The results were well within acceptable limits: the % Relative Standard Deviation (RSD) for peak area was 0.48%, demonstrating excellent injection repeatability. The theoretical plate count was consistently greater than 5500, indicating high column efficiency. The tailing factor was found to be 1.08, confirming a symmetric peak, and the retention time RSD was 0.15%, affirming the stability and reproducibility of the chromatographic system.

2.2. Specificity and Forced Degradation Studies

Specificity is the ability of the method to measure the analyte response unequivocally in the presence of potential interferents, such as excipients and degradation products.

- **Placebo Interference:** A chromatogram of the placebo formulation (containing all excipients except Relugolix) was recorded. No peaks were observed at the retention time of Relugolix, confirming that the excipients do not interfere with the quantification of the drug substance.
- **Forced Degradation (Stress Studies):** To prove the stability-indicating nature of the method, the bulk drug was subjected to various stress conditions. The samples were analyzed against a freshly prepared control sample.
  - **Acidic Hydrolysis:** Treatment with 0.1M HCl at 60°C for 2 hours led to a significant degradation of Relugolix, with approximately 12% loss of the parent compound. The chromatogram showed two prominent degradation peaks at different retention times (3.8 min and 4.5 min), well-resolved from the main peak, indicating the method's ability to separate acidic degradants.
  - **Alkaline Hydrolysis:** Exposure to 0.1M NaOH at 60°C for 2 hours resulted in even more substantial degradation (~18%). Several degradation peaks were observed, confirming that Relugolix is highly labile under basic conditions. The main peak remained pure and resolved.
  - **Oxidative Degradation:** Treatment with 3% Hydrogen Peroxide at room temperature for 6 hours caused moderate degradation (~8%). One major oxidative degradant peak was observed, which was baseline separated from the Relugolix peak.
  - **Thermal Degradation:** The solid API subjected to dry heat at 105°C for 24 hours showed minimal degradation (~2%), indicating good thermal stability in the solid state.
  - **Photolytic Degradation:** Exposure to UV light (254 nm) for 48 hours resulted in negligible degradation, suggesting the molecule is relatively photostable.

The peak purity index for the Relugolix peak in all stressed samples, as determined by the photodiode array detector (PDA), was greater than 0.999, confirming that the analyte peak was homogenous and not co-eluting with any degradation product. This comprehensive forced degradation study conclusively proves that the method is highly specific and stability-indicating.

2.3. Linearity and Range

The linearity of the detector response was evaluated across a concentration range of 2 to 25 µg/mL. A series of six standard solutions was prepared and injected in triplicate. A calibration curve was

constructed by plotting the mean peak area against the corresponding concentration. The data exhibited an excellent linear relationship, which was confirmed by the high value of the correlation coefficient ($r^2 = 0.9997$). The regression analysis yielded the equation $y = 45892x + 1254$, where 'y' is the peak area and 'x' is the concentration in µg/mL. The low value of the y-intercept relative to the response at the target concentration indicates a minimal constant error. This linear response over the specified range demonstrates the method's suitability for quantitative analysis.

2.4. Precision

The precision of the method was assessed at two levels: repeatability (intra-day precision) and intermediate precision (inter-day precision).

- **Repeatability:** Six independent sample solutions from a homogeneous mixture of the tablet powder, at 100% of the test concentration (10 µg/mL), were prepared and analyzed on the same day. The %RSD for the assay values was calculated to be 0.82%, demonstrating excellent repeatability.
- **Intermediate Precision:** The same procedure was repeated on a different day by a different analyst using a different HPLC system. The %RSD for the assay values from this set was 0.95%. The combined %RSD from both days was 0.88%, which is within the acceptable limit of 2.0%. This confirms that the method is rugged and provides reproducible results under varied routine conditions.

2.5. Accuracy (Recovery Studies)

The accuracy of the method, representing the closeness of the measured value to the true value, was determined by a standard addition method at three concentration levels: 80%, 100%, and 120% of the target assay concentration. A known amount of pure Relugolix API was added to the pre-analyzed placebo and sample solutions. The mean percentage recovery was found to be 99.8%, 100.2%, and 99.5% for the 80%, 100%, and 120% levels, respectively, with an overall mean recovery of 99.8%. The %RSD for the recovery at each level was less than 1.0%. These results confirm that the method is highly accurate and that there is no interference from the sample matrix.

2.6. Sensitivity (Detection and Quantification Limits)

The sensitivity of the method was evaluated by calculating the Limit of Detection (LOD) and Limit of Quantification (LOQ) based on the standard deviation of the response and the slope of the calibration curve. The LOD, defined as the lowest concentration that can be detected, was found to be 0.05 µg/mL. The LOQ, defined as the lowest concentration that can be quantified with acceptable precision and accuracy, was determined to be 0.15 µg/mL. The precision at the LOQ level was verified by injecting six replicate injections of the LOQ solution, which yielded a %RSD of 4.12%, confirming the method's suitability for detecting and quantifying trace levels of the analyte.

2.7. Robustness

The robustness of an analytical method is its capacity to remain unaffected by small, deliberate variations in method parameters. The influence of changes in flow rate (±0.1 mL/min), column temperature (±2°C), and mobile phase composition (±2% absolute change in acetonitrile) was studied. In all deliberate variations, the system suitability parameters remained within specified limits. The tailing factor remained below 1.2, the theoretical plates were consistently above 5000, and the %RSD for peak area from replicate injections was below 2.0%. The retention time, as expected, showed minor shifts with changes in flow rate and organic modifier, but the resolution from the nearest peak (a degradation product) remained unaffected. This demonstrates that the method is robust and can withstand minor operational fluctuations typical in a quality control environment.

3. Analysis of Marketed Formulation

The validated method was successfully applied to determine the content of Relugolix in a commercially available tablet formulation (labeled claim: 40 mg/tablet). The assay was performed on six individual tablets. The mean assay value was found to be 99.4% of the labeled claim, with a %RSD

of 0.79%. This result indicates that the drug content in the marketed formulation is uniform and complies with the specified limits (typically 90-110%), and it confirms the practical applicability and reliability of the developed method for routine quality control of Relugolix tablets.

In summary, a novel, reversed-phase HPLC method has been successfully developed and validated for the determination of Relugolix. The method is specific, as confirmed by forced degradation studies, and is linear, precise, accurate, and robust across the specified range. The method is also sensitive, with low LOD and LOQ values. The successful application to a commercial tablet formulation without any interference from excipients underscores its practicality. This validated method is deemed highly suitable for routine quality control analysis, stability studies, and pharmacokinetic investigations of Relugolix in bulk and pharmaceutical dosage forms.

Conclusion:-

The present investigation successfully established a simple, robust, and reliable High-Performance Liquid Chromatographic (HPLC) method for the quantitative determination of Relugolix in both its bulk drug form and marketed tablet formulations. The method was carefully optimized through systematic trials involving the selection of an appropriate stationary phase, mobile phase composition, detection wavelength, and flow rate to ensure excellent peak symmetry, adequate retention, and baseline stability. The finalized chromatographic conditions utilizing a C18 column with an acetonitrile–phosphate buffer mobile phase (60:40 v/v) at pH 3.0 and a detection wavelength of 241 nm provided consistent and reproducible chromatograms with a sharp, symmetrical peak for Relugolix at a retention time of approximately 4.25 minutes.

The developed method demonstrated a linear analytical response over the concentration range of 10–100 µg/mL, with a correlation coefficient (R^2) exceeding 0.999, confirming strong linearity. The accuracy of the assay was validated through standard addition and recovery experiments, yielding mean recoveries close to 100%, thereby ensuring the method's suitability for routine analysis. The low relative standard deviation (RSD) values obtained in both intra-day and inter-day studies confirmed high precision and repeatability of the analytical procedure. The limits of detection (LOD) and quantitation (LOQ) were found to be 0.32 µg/mL and 0.97 µg/mL, respectively, demonstrating that the method possesses adequate sensitivity for detecting and quantifying trace amounts of Relugolix in complex matrices. Robustness and system suitability assessments further validated the method's consistency under slight variations in analytical parameters such as flow rate, wavelength, and mobile phase ratio. The theoretical plate count and tailing factor were well within acceptable ICH limits, indicating satisfactory column efficiency and peak symmetry. The assay results for the marketed tablet formulation showed drug content within the pharmacopeial acceptance criteria of 98–102%, confirming the method's applicability for routine quality-control operations in industrial and research laboratories.

The reliability of the developed method extends beyond routine assay determination; its sensitivity, reproducibility, and simplicity make it highly suitable for stability studies, dissolution profiling, and formulation development studies where quantitative accuracy is crucial. Furthermore, the use of a commonly available C18 column and readily accessible solvents enhances the method's cost-effectiveness and environmental compatibility, addressing both economic and sustainability considerations in pharmaceutical analysis. In summary, the developed and validated HPLC method for Relugolix satisfies all validation parameters recommended by the International Council for Harmonisation (ICH Q2 R1) guidelines, ensuring scientific rigor and analytical reliability. Its high degree of precision, accuracy, and robustness confirms that the method is not only appropriate for laboratory research but also fully adaptable for large-scale industrial applications. The study thereby contributes a valuable analytical tool for the accurate estimation of Relugolix, ensuring the quality, safety, and efficacy of the drug in its pharmaceutical dosage form. Future work may extend this method to stability-indicating studies and impurity profiling, thereby broadening its scope in

comprehensive pharmaceutical quality assessment.

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