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DEVELOPED AND VALIDATED STABILITY INDICATING METHOD USING RP-HPLC TO QUANTITATIVELY DETERMINE RELUGOLIX IN TABLETS

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ABSTRACT

A quantitative approach for determining and validating the dosage form of Relugolix tablets was developed utilising RP-HPLC. The equipment utilised includes HPLC systems equipped with Photodiode Array (PDA) and Ultraviolet (UV) detectors, specifically from Waters or Agilent manufacturers. A reversed-phase Octadecyl HPLC column (*YMC Triart C18*) having a particle size of 5 microns with dimensions of 150mm x 4.6mm (inside diameter) has been employed for routine analysis (Part number: TA12S05-1546WT). Two mobile phases were used: A & B. Mobile phase A is a 70:30% v/v ratio of 10 millimolar mono potassium phosphate buffer (pH 8.30) and acetonitrile. On the other hand, mobile phase B is of 100% methanol. Gradient program operates at a flow rate of 1.0 mL/min, with an injection volume of 20 µL, with the column oven temperature of 30°C, temperature 5°C, and detection wavelength of 236 nm. A diluent comprising 90% trifluoroacetic acid (0.1%) in methanol and 10% water by volume was utilised for sample extraction. The established stability-indicating method determined a concentration range of 50-150 µg/mL and an intraclass correlation coefficient of 1.00. Spectral purity is validated using forced degradation tests. The specificity (placebo and degradation interference), recovery, precision, linearity, ruggedness, robustness, filter validation, and solution stability have been conducted in accordance with ICH Q2 and Q14 guidelines. This method has been successfully developed and assessed to quantitatively determine Relugolix in tablet formulations for release and stability analysis in a quality control laboratory.

Keywords: Relugolix Solid Formulations, Stability-Indicating Method, Analytical Method Development, Validation, Forced Degradation, ICH Q2 & Q14.

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INTRODUCTION

Relugolix, a medication employed in the therapy of prostate cancer in the advanced state, is the first drug approved by the FDA for the relief of symptoms related to uterine fibroids. It is the first oral gonadotropin-releasing hormone (GRH) receptor antagonist approved for treating prostate cancer, hence providing an accessible choice for patients; it is also apparently utilised in the management of endometriosis.[1] Relugolix is a non-peptide, small-molecule compound that is orally bioavailable. Relative to leuprolide, another androgen deprivation therapy for prostate cancer, Relugolix is more efficacious in reducing testosterone levels and is also comparatively user-friendly. Initially approved in 2019 under the brand name Relumina in Japan, it is indicated for the symptomatic treatment of uterine fibroids.[2] In 2020, the FDA in the United States approved it for treating advanced prostate cancer under the brand name Rexigo. In 2021, the FDA approved Myfembree, the inaugural combination medication administered once daily, for perimenopausal women experiencing excessive menstrual bleeding linked to uterine fibroids. The formulation comprises Relugolix, Oestradiol, and Norethindrone. On April 29, 2022, the European Commission subsequently approved this branded product.[3,4] While forced degradation studies are not part of official stability testing, they are crucial for elucidating the degradation pathways of active pharmaceutical ingredients and their dosage form, as well as for validating the stability-indicating

properties of analytical methods.[5] The aforementioned tests involve subjecting the drug component to more severe circumstances than those utilised in accelerated testing, including elevated temperatures, extreme pH levels (both acidic and basic hydrolysis), oxidation, and light exposure. This study developed and validated a quantitative method for determining Relugolix in oral solid dosage forms utilising the widely recognised analytical methodology of reverse phase HPLC, alongside a stability-indicating assay based on a proven forced degradation study[6]. To know the spectral purity of Relugolix in tablet form. A concise summary was provided about the chemical stability of the dosage form and the characteristics of Relugolix in the presence of excipients under various stress conditions. In compliance with ICH guidelines[7], a simple, sensitive, reliable and robust analytical method was established for analysing Relugolix in tablet form. The literature presents a limited number of analytical methodologies and various deficiencies in elucidating the significance of the stability-indicating method, the results obtained, and the validation research in accordance with guidelines for the Relugolix tablet dosage form. Thus, the present study supports 3rd goal- Good Health and Well-Being, as it involves developing and validating a reliable RP-HPLC method to ensure the quality, safety, and stability of Relugolix tablets. Accurate pharmaceutical analysis helps maintain effective drug therapy for diseases such as prostate cancer and uterine fibroids, thereby contributing to improved patient health outcomes.

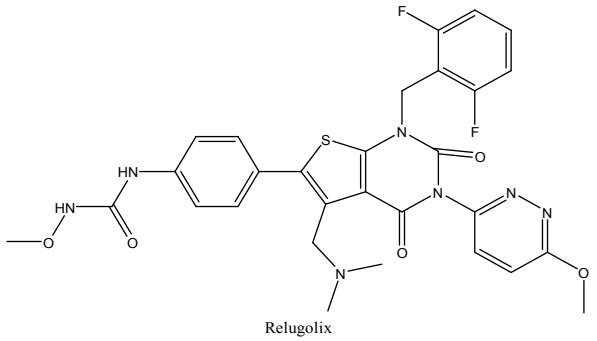

Fig.-1: Molecular Structure of Relugolix

EXPERIMENTAL

Chemicals and Reagents

Sodium Hydroxide, Potassium Dihydrogen Phosphate, Potassium Hydroxide, Hydrogen Peroxide 30%, Potassium Nitrate (AR grade) from Merck chemicals. Acetonitrile, Methanol (HPLC grade), from Standard chemicals. Trifluoroacetic Acid (LC-MS grade), Citric Acid, Sodium Citrate from Sigma-Aldrich. Hydrochloric Acid (AR grade) from Rankem, all these chemicals were utilized in this study. The working standard of Relugolix was gifted by Dr Reddy's laboratory SEZ PU1. Relugolix tablets (120mg strength) purchased (Manufactured and Marketed by Zydus Cadila) Brand name: Rexigo.

Instrumentation

An analytical balance (Make Sartorius) is used for weighing the chemicals. HPLC made by Agilent/Waters with PDA/UV detectors were utilized for determining the Relugolix with Empower-3 software. Centrifuge (Make Hermle) was used for the separation of excipient residue from the active solution. Sonication (Make Hwashin) were utilized for solubilization purposes. 0.45-micron membrane filters (Make Merck) are utilized for the filtration of the mobile phase. A pH meter (Make Thermo Scientific) was used for determining the pH. Water purifier system (Milli-Q). Photo stability chamber (MACK) was used for the photolytic degradation (200watt hour/sq.m for UV & < 1.2 million Lux hours for Visible Light Stressed)

General Procedure for Preparation of Solutions

Preparation of Buffer

Weighed 1.36g of monopotassium phosphate buffer in 1 litre of ultrapure water produced in the laboratory and adjusted the pH to 8.30 by using diluted Potassium hydroxide, and nylon filter paper of 0.45 μm was used for filtration.

Mobile Phase-A Preparation
70% of pH 8.30 buffer and 30% of Acetonitrile were mixed, and air bubbles were removed for 10 minutes using a sonicator.

Second Mobile Phase-B is Methanol (100%).
Diluent Preparation
1 ml of Trifluoroacetic acid was transferred into 1000 ml of methanol and was mixed well, and later, 90% of 0.1% trifluoroacetic acid in methanol and 10% of water volume by volume were mixed and sonicated for 10 min to degas the diluent.

Preparation of Standard Solution
Accurately measured 50 milligrams of Relugolix was transferred to a clean 50 mL flask. 35 mL diluent was transferred to the mixture, followed by sonication (5 minutes). The diluent was added to prepare a 1000 ppm stock solution. Carefully transfer 2 ml of the stock solution into a dried 20 ml volumetric flask, then adjust the volume with the diluent to achieve a final concentration of 100 ppm.

Sample Solution Preparation
Five tablets were ground up with a dry mortar and pestle, and about 125mg of the powdered sample (which is the same as 50mg of Relugolix) was put into a 100 mL volumetric flask. After adding 50 mL of the diluent, sonication is done for 20 min while shaking it occasionally; the volume was filled with diluent and thoroughly mixed. A fraction of the sample was centrifuged for five minutes at 4000 rpm. To reach the concentration 100 ppm, 5 mL of the supernatant was pipetted out and put into a 25 mL volumetric flask that had been well cleaned and dried. The volume was then adjusted with diluent. After passing the sample through a 0.45-micrometre PVDF filter, 1st three millilitres were thrown away. The aliquot was then put into an HPLC and examined.

Preparation of Placebo Solution
125 mg of the powdered placebo (which is the same as 50mg of Relugolix) was put into a 100 mL volumetric flask. After adding 50 mL of the diluent, sonication is done for 20 min while shaking it occasionally; the volume was filled up to the mark of the diluent and thoroughly mixed. A fraction of the placebo was centrifuged for five minutes (4000 rpm). To reach a final concentration of 100 ppm, 5 mL of the supernatant was pipetted out and put into a 25 mL volumetric flask that had been well cleaned and dried. The volume was then adjusted with diluent. After passing the sample solution through a 0.45-micrometre PVDF filter, the first 3 millilitres were thrown away. The aliquot was then put into an HPLC and examined.

Detection Method Chromatographic Conditions
Chromatographic separation and quantification were completed by using a Reversed phase Octadecyl HPLC Column (YMC triart C18) with material particle size 5-micron, column dimensions 150-millimetre x 4.6-millimetre (inner diameter). First mobile phase-A constitutes 10 millimolar(mM) potassium dihydrogen phosphate with pH 8.30 and acetonitrile in a ratio of 70:30%v/v, and mobile phase-B is methanol 100% with gradient flow rate, volume of injection is twenty microliters, column oven temperature 30℃, sampler temperature 5℃, detection wavelength 236nm. 0.1%TFA in methanol (10mL): water (90mL) ratio as a diluent for sample extraction.

Gradient Program

Gradient Time (min)	Line-A	Line-B
0	50	50
6	50	50
7	0	100
12	0	100
13	50	50
18	50	50

Forced Degradation Study

Preparation of 1N HCl Solution

4.13ml of concentrated HCl was added to a 50ml volumetric flask containing 25ml water and filled with water up to the mark.

Preparation of 1N NaOH Solution

Weighed accurately 2g of sodium hydroxide pellets was transferred to a volumetric flask of 50ml containing approximately 25ml of milli-Q, sonicated until dissolved and the volume was filled with water up to the mark.

Preparation of 3% H_2O_2

5ml of 30% H2O2 was transferred to a volumetric flask(50ml) and the volume was filled with water up to the mark.

Preparation of Humidity Chamber

12g of Potassium nitrate was weighed accurately and added into 300ml water and dissolved. This solution was transferred into a desiccator, and a data logger was placed for humidity reading, then the desiccator was sealed with Aluminum foil to achieve 90%RH. A forced degradation study was conducted by using below stress conditions for Relugolix tablets dosage forms as per the proposed method. Results summarized in Table-1.

Table-1: Stressed Conditions

S. No.	Stress conditions
1	Unstressed conditions
2	Humidity Stressed (about 90% RH for 7days at Room temperature)
3	Acid Stressed (1ml of 1N HCl for 24hrs at Room temperature)
4	Base Stressed (1ml of 1N NaOH for 30min at Room temperature)
5	Peroxide Stressed (1ml of 30% H2O2 at Room temperature for 48hrs)
6	UV Light Stressed (200 watt-hour/sq.m) for 7days
7	Visible Light Stressed (NLT 1.2 million Lux hours) for 7days

Method Validation

Validation of the analytical method for the assay and its dissolution process using HPLC was conducted following the guidelines set out in ICH Q2(R2) recommendations[7] and USP 1225. This validation encompasses various parameters, including system evaluation, specificity, accuracy, linearity, precision and robustness.

RESULTS AND DISCUSSION

Stability-Indicating Method Development Followed by Optimization

The study focused on stability-indicating method development was to effectively separate all potential degradants from the main drug component, Relugolix, with good resolution. [8] After evaluating several chromatographic conditions, the reversed-phase Octadecyl HPLC Column (YMC triart C18) with a material particle size of 5 microns and column dimensions were found to be suitable for achieving the desired separation. Relugolix was dissolved in trifluoroacetic acid -TFA (*0.1%*) in methanol in water *(90:10 v/v)*. Multiple trials were conducted using acetonitrile as the organic phase and different buffer solutions as the aqueous phase, under both isocratic and gradient elution modes. Following extensive experimentation, the gradient elution program outlined was identified as the most effective for achieving a clear separation between Relugolix and its degradation products. The method parameters were optimized accordingly, and the final analytical conditions are also presented in the materials and methods section. An essential step in the method was the selection of an optimal UV detection wavelength. Using an HPLC system equipped with a PDA (*photodiode array*) detector, a 100 µg/mL solution of Relugolix was analyzed. The UV absorption maximum was found to be at 247 nm for the main analyte. However, the detection wavelength was finalized at 236 nm due to proper baseline stabilization, without baseline noise, and optimum response was observed for Relugolix and its degradants. Relugolix, being soluble in organic solvents, shows a strong affinity for non-polar stationary phases. Therefore, a C18 column was chosen for its compatibility with the drug's properties. With a mobile phase of buffer pH 8.30 and

acetonitrile, a well-resolved chromatographic peak with minimal tailing was obtained. Additionally, satisfactory chromatographic performance was observed when methanol was used as the organic component of the mobile phase. In summary, the finalized analytical conditions and HPLC gradient program successfully enabled the separation of Relugolix from its degradants with reliable resolution and peak integrity.

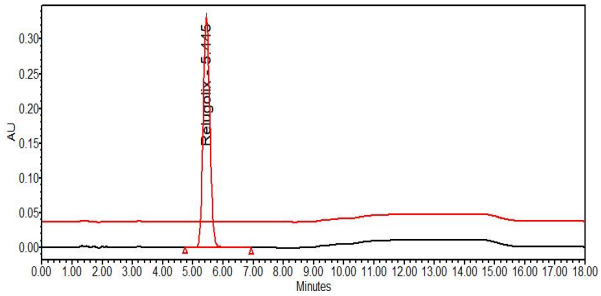

Fig.-2: Blank and Standard Overlaid Chromatograms

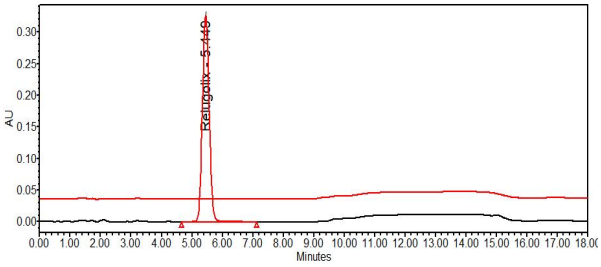

Fig.-3: Placebo and Sample Overlaid Chromatograms

Forced Degradation Study

A study was performed[9,10], as mentioned Table-1 to illustrate the successful separation of degradants from Relugolix. Different stress conditions were exposed to both the drug formulation and the placebo to promote degradation. The stressed samples were then analyzed using the HPLC system equipped with a PDA detector, adhering to specified test conditions. Degradation peaks have been distinctly separated in all conditions from Relugolix. Cchromatograms for degraded samples were analyzed for the peak purity of Relugolix using Waters (*Empower-3*) software. Without exception, the purity angle in the forced degradation samples was lower than the purity threshold; therefore, there is no interference from degradants when quantifying the Relugolix in Relugolix Tablets. Hence, this method is regarded as stability-indicating.

Acceptance Criteria

- At least one stress condition % of net degradation should be between 5% and 20%.
- The main analyte peak, the spectral purity angle, should be below the Purity Threshold.

Table-2: Results of Percentage Assay, Net degradation of stressed samples

S. No.	Stress condition	%Assay	%Net degradation
1	Unstressed sample	99.5	NA
2	Humidity Stressed (about 90% RH for 7days at RT)	99.55	0.05
3	Photolytic stressed (200watt hour/sq.m & NLT 1.2 million Lux hours)	99.3	0.2
4	Acid Stressed (1ml of 1N HCl at RT for 24hrs)	98.3	1.2
5	Base Stressed (1ml of 1N NaOH at RT for 30min)	93.0	6.5
6	Peroxide Stressed (1ml of 30% H_2O_2 at RT for 48hrs)	98.0	1.5

Table-3: Outcome of Peak Purity of Relugolix in Stressed Samples

Sample	Purity Angle	Threshold	Purity	Retention Time
Standard	0.08	0.249	Pass	5.4
Unstressed sample	0.084	0.25	Pass	5.31
Acid stressed	0.03	0.246	Pass	5.48

Base Stressed	0.036	0.253	Pass	5.44
Peroxide Stressed	0.034	0.247	Pass	5.47
Photolytic	0.086	0.253	Pass	5.45
Humidity	0.1	0.258	Pass	5.45

Table-4: Interference in Stressed Placebo

Sample	Interference (Yes /No)
Control Placebo	No
Acid stressed	No
Base Stressed	No
Peroxide Stressed	No
Photolytic	No
Humidity	No

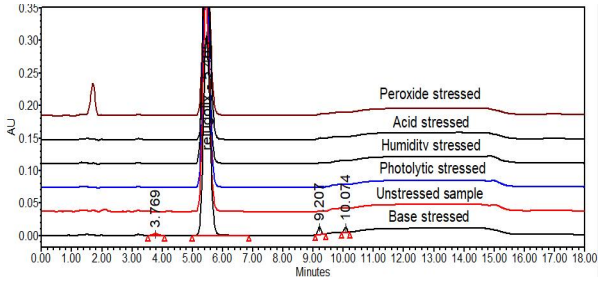

Fig.-4: Overlaid for Sample Chromatogram of all Stressed Conditions

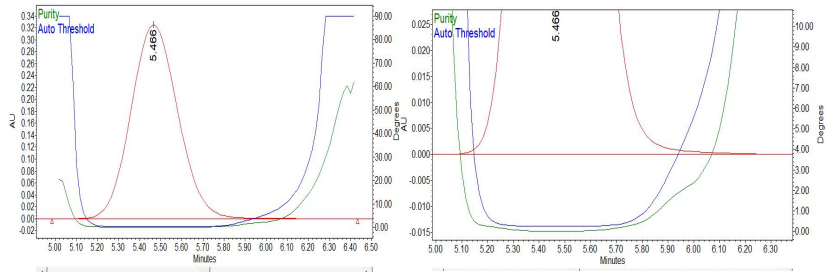

Fig.-5: Spectral purity of Unstressed Sample (Unzoomed & Zoomed)

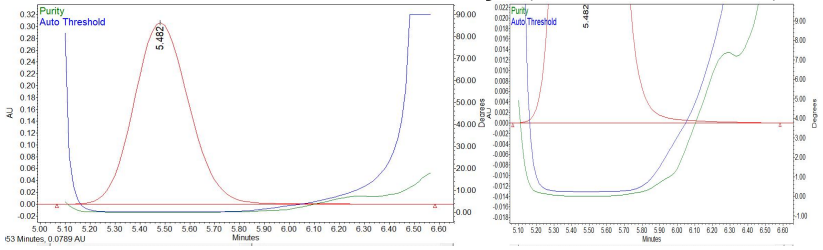

Fig.-6: Spectral purity of Base-stressed Sample (Unzoomed & Zoomed)

Method Validation[11,12]

System Suitability

Relugolix standard solution was of 100parts per million, was prepared and injected. System suitability parameters of the active were evaluated and tabulated in Table-5. System suitability parameters had met the acceptance criteria as per USP 621. The system suitability was ensured before every analysis.

Acceptance Criteria

- No Interference greater than 0.1% shall be observed at the retention time of Relugolix, the symmetry factor should be NMT 2.0, and the USP plate efficiency should be NLT 2000.
- The percentage of relative standard deviation(%RSD) for the Relugolix area response of the 6 multiple injections of the Standard preparation-A should be NMT 2.0.
- The standard solution-A&B Similarity factor should be between 0.98 and 1.02, wherever applicable.

Table-5: Results of Parameters for the Suitability of the System

NO.	Parameters for Suitability	Observed Value
1	Per cent of interference of Relugolix in Blank	Nil
2	%RSD for Relugolix peak areas response of the 6 replicate standard injection	0.3
3	Similarity Factor of Check standard solution	1.00
4	USP Tailing Factor	1.1
5	USP Plate Count	4059

Specificity

A study was carried out to confirm that the placebo does not interfere with the results. The assay was conducted on the placebo in duplicate, utilizing an equivalent amount of placebo found in the test preparation. The chromatogram for the placebo displayed no peak at the retention time of Relugolix. This demonstrates that the additives/ excipients present in the formulation had not interfered with the estimate of Relugolix in Relugolix tablets.

Acceptance Criteria

No Interference shall be observed at the retention time of the Relugolix peak.

Table-6: Results for the Interference of Placebos

S. No.	Sample Name	% Interference
1	Preparation of Placebo-1	Nil
2	Preparation of Placebo-2	Nil

Linearity

Linearity was established for Relugolix by preparing serial dilutions ranging in concentration from 50 µg/ml - 150 µg/ml (50 -150% of the test concentration). Concentration was plotted (X-axis) against the area of the peak (Y-axis). It was determined that the correlation coefficient was within the range.

Acceptance Criteria

1. R^2 should be not less than 0.999.
2 The response at 100% level of Y-Intercept should not be more than ±2%

Table-7: Results Peak Linearity of Relugolix

% Level	Concentration(ppm)	Mean Area
50	50	2636737
75	75	3919524
100	100	5216176
125	125	6524382
150	150	7820995
	Correlation Coefficient	1.000
	Slope:	51893.50
	Intercept:	34213.20
	Bias at 100% response	0.1%

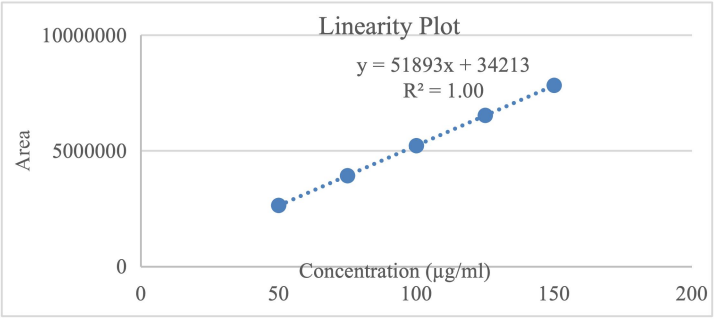

Fig.-7: Linearity Calibration Curve Plot

Precision

Six test preparations replicates were prepared at 100ppm level using API and Placebo and injected into the HPLC System. The percentage of assay in each replicate, the average of six replicates and the percentage of standard deviation were reported. All the results have met the acceptance criteria.

Acceptance Criteria

The six replicate preparations percentage of RSD of the assay shall be NMT 2.0. Each assay value shall be between 95.0% to 105.0%.

Table-8: Results of Method Precision of Assay

Sample. No	Retention Time (min)	%Recovery
1	5.55	100.8
2	5.556	100.2
3	5.548	99.2
4	5.548	100.5
5	5.553	100.1
6	5.561	100.8
Average	NA	100.4
%RSD	NA	0.78

Recovery

A Recovery study was conducted where samples were prepared in triplicate at 50, 100 and 150% levels and evaluated as per the test method. The percentage recovered and its mean were calculated. All the results met the acceptance criteria.

Acceptance Criteria

The individual % recovery for Relugolix for assay should be not less than 98.0 and not more than 102.0.

Table-9: Results of the Accuracy of Relugolix for assay

S. No.	Recovery Level	% Recovery	Mean Recovery (%)
1	50%	100.5	100.63
2		101	
3		100.4	
4	100%	100.5	100.47
5		100.1	
6		100.8	
7	150%	99.9	99.7
8		99.3	
9		99.9	

Solution Stability

Stability of standard and sample solutions was established at RT and refrigeration for a period of 5 days. As per the test procedure standard and sample solution (duplicate) was prepared, and final preparations of the standard and sample were stored at RT in a tightly closed glass container and evaluated after 24Hrs/2 days /5 days against a new standard solution each time.

Acceptance Criteria

The standard solution-A&B Similarity factor should be between 0.97 and 1.03.For sample preparation, the difference in %Assay results from the initial time point to each time point is not more than ±2.0.

Table-10: Stability Results of Sample Solutions

Sample Solution Stability at RT				
Time intervals	%Assay		Deviation from Initial	
	Sample - One	Sample -Two	Sample - One	Sample - Two
Initial	99.7	101.4	Not applicable	Not applicable
Day-1	100.80	100.97	1.1	0.43
Day-2	100.2	102.4	0.5	1.0
Day-5	100.4	102.9	0.7	1.5

Sample solution stability at Refrigerator				
Initial	99.7	101.4	NA	NA
Day-1	100.1	102.1	0.4	0.7
Day-2	100.2	102.4	0.5	1.0
Day-5	99.9	102.6	0.2	1.2

Solution stability for Standard (Similarity factor)		
Time in days	Room temperature	Refrigerator
Initial	NA	NA
Day-1	1.01	1.02
Day-2	1.01	1.02
Day-5	1.02	1.02

Filter Validation

A study was performed to evaluate the effectiveness of two types of filters, specifically the *0.45µm* PVDF filter & *0.45µm* nylon filter. Assay results remained acceptably consistent between unfiltered samples and those filtered through either PVDF or Nylon. The acceptance criteria include the % assay difference of filtered solution to centrifuged solution was NMT ±2.0.

Table-11: Results of Filter Validation for Assay

Types of Filters	%Recovery	%Assay	The difference between centrifuged and filtered
Centrifuged	Sample-1	99.7	NA
	Sample-2	101.4	NA
0.45µm PVDF	Sample-1	100.15	0.4
	Sample-2	102.7	1.3
0.45µm Nylon	Sample-1	99.9	0.2
	Sample-2	102.1	0.7

Robustness

Robustness of the method proposed was confirmed through an evaluation of both the sample and the standard solution, which demonstrated only minor variations (Table-12). The system suitability criteria were satisfied under all robustness conditions, and the differences in % assay were no more than ±2.0(acceptance criteria). This indicates the method's robustness against planned analytical modifications.

Acceptance Criteria

Should meet these criteria of system suitability parameters as mentioned in system suitability.
Each assay value shall be between 95.0% to 105.0%.

Table-12: Conditions and Results of Robustness

Parameter	Variations	%Assay		%Difference		Tailing factor	Plate count	% RSD
		Standard	Sample	Standard	Sample			
As such	NA	100.2	101.4	NA	NA	1.1	2741	0.09
Flow rate	1.2 mL/min	99	100.5	1.2	0.9	1.0	2492	0.17
	0.8 mL/min	100	100.3	0.2	1.1	1.1	2666	0.21
Temperature	35℃	100.9	100.7	0.7	0.7	1.1	2868	0.29
	25℃	101.1	101.4	0.9	0	1.1	2786	0.57
pH	8.50	101	102.2	0.8	0.8	1.1	2695	0.40
	8.10	101.8	103.1	1.6	1.7	1.1	2577	0.10
Organic composition	70:29	101.1	102.1	0.9	0.7	1.1	2760	0.13
	70:31	100.7	102.1	0.5	0.7	1.1	2828	0.15

CONCLUSION

The present study provides an effective, developed, validated, and reliable RP-HPLC technique for determining Relugolix in oral tablet formulations quantitatively. The method developed provides its effectiveness in isolation of the medication from all associated contaminants and degradation products formed under forced degradation conditions, demonstrating a strong stability-indicating qualities developed by this analytical technique. This work bridges a critical gap in analytical methodology for

Relugolix and contributes significantly to improving quality assurance practices for its pharmaceutical dosage forms.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

The present work is related to *SDG-3: Good Health and Well-being*. All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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