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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR ESTIMATION OF RELUGOLIX IN API FORM AND MARKETED PHARMACEUTICAL DOSAGE FORM

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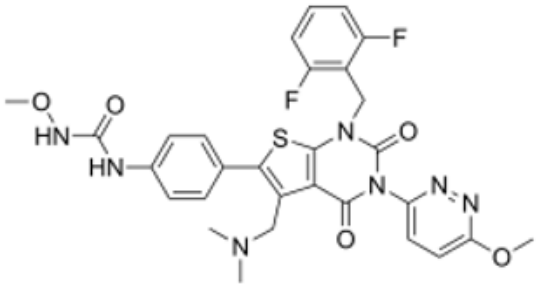
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Abstract: A new, simple, rapid, precise, accurate and reproducible RP-HPLC method for estimation of Relugolix in bulk form and marketed formulation. Separation of Relugolix was successfully achieved on a Symmetry ODS C18 (4.6 x 250mm, 5µm) column in an isocratic mode of separation utilizing Acetonitrile: Methanol in the ratio of 80:20% v/v at a flow rate of 1.0 mL/min and the detection was carried out at 272nm. The method was validated according to ICH guidelines for linearity, sensitivity, accuracy, precision, specificity and robustness. The response was found to be linear in the drug concentration range of 10-50mcg/mL for Relugolix. The correlation coefficient was found to be 0.999 for Relugolix. The LOD and LOQ for Relugolix were found to be 1.1µg/mL and 3.2µg/mL respectively. The proposed method was found to be good percentage recovery for Relugolix, which indicates that the proposed method is highly accurate. The specificity of the method shows good correlation between retention times of standard solution with the sample solution. Therefore, the proposed method specifically determines the analyte in the sample without interference from excipients of pharmaceutical dosage forms.

Keywords: Relugolix, RP-HPLC, Accuracy, Precision, Robustness, ICH Guidelines.

INTRODUCTION

Relugolix is an oral GnRH receptor antagonist for androgen deprivation therapy in the treatment of advanced prostate cancer. Relugolix is a gonadotropin-releasing hormone (GnRH) receptor antagonist used in the treatment of several hormone-responsive conditions1. It was first approved in Japan in 2019, under the brand name Relumina, for the symptomatic treatment of uterine fibroids, and more recently by the United States' FDA in 2020, under the brand name Orgovyx, for the treatment of advanced prostate cancer. This branded product was later approved by the European Commission on April 29, 2022. Relugolix has also been studied in the symptomatic treatment of endometriosis2. Relugolix is a gonadotropin-releasing hormone (GnRH) receptor antagonist used primarily to treat hormone-dependent conditions in both men and women. It works by blocking receptors that stimulate the production of testosterone and estrogen, thereby slowing the growth of specific cancers and improving painful, bleeding gynecological conditions. The IUPAC Name of Relugolix is 3-(7-amino-3-oxo-1H-isoindol-2-yl) piperidine-2, 6-dione3. The Chemical Structure of Relugolix is shown in follows



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Fig-1: Chemical Structure of Relugolix

EXPERIMENTAL

Instruments Used

Table-1: Instruments Used

S.No.	Instruments and Glass wares	Model
1	HPLC	WATERS Alliance 2695 separation module, Software: Empower 2, 996 PDA Detector.
2	pH meter	Labindia
3	Weighing machine	Sartorius
4	Volumetric flasks	Borosil
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil
7	Digital Ultra Sonicator	Labman

Chemicals Used:

Table-2: Chemicals Used

S.No.	Chemical	Providers
1	Relugolix (Pure)	Synpharma Research Lab, Hyderabad
2	Water and Methanol for HPLC	LICHROSOLV (MERCK)
3	Acetonitrile for HPLC	Merck

HPLC Method Development:

Preparation of Standard Solution:

Accurately weigh and transfer 10 mg of Relugolix working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol. Further pipette 0.3ml of the above Relugolix stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Preparation of Sample Solution:

Take average weight of the Powder and weight 10 mg equivalent weight of Relugolix sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Further pipette 0.3ml of the above Relugolix stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure:

Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines32-33.

Mobile Phase Optimization:

Initially the mobile phase tried was methanol: Water and ACN: Water with varying proportions. Finally, the mobile phase was optimized to ACN: Methanol 80:20% v/v) respectively.

Optimization of Column:

The method was performed with various C18 columns like Symmetry, Zodiac and Xterra. Symmetry ODS C18 (4.6 x 250mm, 5µm) Column was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

Preparation of Mobile Phase:

Accurately measured 800 ml (80%) of HPLC Acetonitrile and 200 ml of Methanol (20%) were mixed and degassed in a digital ultra sonicater for 15 minutes and then filtered through 0.45 µ filter under vacuum filtration4.

Diluent Preparation:

The Mobile phase was used as the diluent.

Method Validation Parameters

System Suitability

Accurately weigh and transfer 10 mg of Relugolix working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.3ml of the above Relugolix stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure:

The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits5.

Specificity Study of Drug:

Preparation of Standard Solution:

Accurately weigh and transfer 10 mg of Relugolix working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 0.3ml of the above Relugolix stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Preparation of Sample Solution:

Take average weight of the Powder and weight 10 mg equivalent weight of Relugolix sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette 0.3ml of the above Relugolix stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure:

Inject the five replicate injections of standard and inject the three replicate injections sample solutions and calculate the assay by using formula6:

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

Preparation of Drug Solutions for Linearity:

Accurately weigh and transfer 10 mg of Relugolix working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I (10ppm of Relugolix):

Take 0.1ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

Preparation of Level – II (20ppm of Relugolix):

Take 0.2ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent7.

Preparation of Level – III (30ppm of Relugolix):

Take 0.3ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

Preparation of Level – IV (40ppm of Relugolix):

Take 0.4ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

Preparation of Level – V (50ppm of Relugolix):

Take 0.5ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

Procedure:

Inject each level into the chromatographic system and measure the peak area8.

Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient9.

Precision

Repeatability

Preparation of Relugolix Product Solution for Precision:

Accurately weigh and transfer 10 mg of Relugolix working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Take 0.3ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent. The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits10.

Intermediate Precision:

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days by maintaining same conditions.

Procedure:

Analyst 1:

The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Analyst 2:

The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits11.

Accuracy:

For Preparation of 50% Standard Stock Solution:

Accurately weigh and transfer 10 mg of Relugolix working standard into a 10ml of clean dry volumetric flasks add about

7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Take 0.15ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

For Preparation of 100% Standard Stock Solution:

Accurately weigh and transfer 10 mg of Relugolix working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Take 0.3ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

For Preparation of 150% Standard Stock Solution:

Accurately weigh and transfer 10 mg of Relugolix working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Take 0.45ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

Procedure:

Inject the Three replicate injections of individual concentrations (50%, 100%, 150%) were made under the optimized conditions. Recorded the chromatograms and measured the peak responses. Calculate the Amount found and Amount added for Relugolix and calculate the individual recovery and mean recovery values12.

Robustness:

The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results. .

For Preparation of Standard Solution:

Accurately weigh and transfer 10 mg of Relugolix working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Take 0.3ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

Effect of Variation of Flow Conditions:

The sample was analyzed at 0.9ml/min and 1.1ml/min instead of 1ml/min, remaining conditions are same. 20µl of the above sample was injected and chromatograms were recorded13.

Effect of Variation of Mobile Phase Organic Composition:

The sample was analyzed by variation of mobile phase i.e. ACN: Methanol was taken in the ratio and 75:25, 85:15 instead of 80:20, remaining conditions are same. 20µl of the above sample was injected and chromatograms were recorded.

RESULTS AND DISCUSSION

Development of Analytical Method:

Optimization of Method:

The chromatographic conditions were optimized to achieve effective separation, good peak resolution, and reproducible results during the quantitative estimation of the drug using the RP-HPLC method. The analysis was performed using a Symmetry ODS C18 column (250 mm × 4.6 mm i.d., 5 µm particle size) as the stationary phase. The mobile phase consisted of acetonitrile and methanol in the ratio of 80:20% v/v. The flow rate was maintained at 1.0 mL/min, with UV detection at a wavelength of 272 nm. An injection volume of 20 µL was selected to ensure reproducible sample introduction, while the column temperature was maintained at ambient conditions. The total chromatographic run time was fixed at 8 minutes. These optimized chromatographic conditions were selected to obtain satisfactory separation and reliable quantitative estimation of the drug in bulk and marketed pharmaceutical dosage forms.

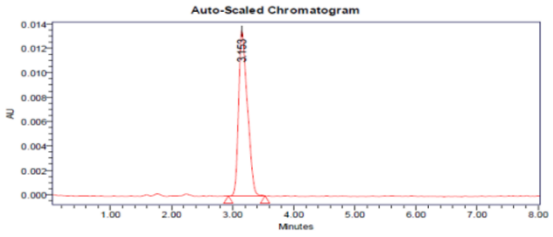


Fig-2: Optimized Chromatographic Condition

Analytical Method Validation:

The developed method was validated as per ICH guidelines in terms of specificity, linearity, accuracy, precision, limits of detection (LOD) and quantification (LOQ) and system suitability32-33.

System Suitability:

Table-3: Results of System Suitability for Relugolix

S. No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Relugolix	3.192	225645	20584	6286	1.38
2	Relugolix	3.146	225847	20965	6358	1.39
3	Relugolix	3.123	228656	20758	6285	1.41
4	Relugolix	3.167	228547	20859	6278	1.40
5	Relugolix	3.158	229658	20968	6395	1.42
Mean			227670.6			
Std. Dev.			1810.899			
% RSD			0.795403			

Specificity

The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components14-16. Analytical method was tested for specificity to measure accurately quantitates Relugolix in drug product.

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

$$= 99.24\%$$

The % purity of Relugolix in pharmaceutical dosage form was found to be 99.24%.

Linearity

Chromatographic Data for Linearity Study:

Table-4: Data for Linearity

Concentration µg/ml	Average Peak Area
10	78683
20	146545
30	213584
40	279895
50	346568

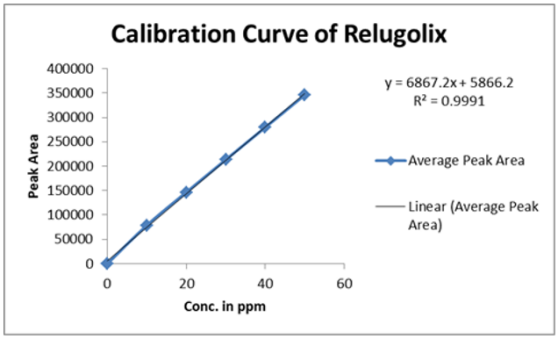


Fig-3: Calibration Curve of Relugolix

Linearity Plot: The plot of Concentration (x) versus the Average Peak Area (y) data of Relugolix is a straight line.

Y = mx + c

Slope (m) =6867

Intercept (c) = 5866

Correlation Coefficient (r) = 0.99

Validation Criteria: The response linearity is verified if the Correlation Coefficient is 0.99 or greater17.

Conclusion: Correlation Coefficient (r) is 0.99, and the intercept is 5866. These values meet the validation criteria.

Precision:

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions18-19.

Repeatability: Obtained Six (6) replicates of 100% accuracy solution as per experimental conditions. Recorded the peak areas and calculated % RSD.

Table-5: Results of Method Precision for Relugolix:

S. No.	Peak name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Relugolix	3.165	225645	20562	6125	1.36
2	Relugolix	3.163	225847	20645	6129	1.36
3	Relugolix	3.158	226542	20534	6135	1.35
4	Relugolix	3.167	226598	20564	6189	1.36
5	Relugolix	3.171	226584	20549	6138	1.35
6	Relugolix	3.181	226859	20685	6179	1.37
Mean			226345.8			
Std. Dev			482.1068			
%RSD			0.212996			

Intermediate Precision:

Analyst 1:

Table-6: Results of Ruggedness for Relugolix

S. No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Relugolix	3.165	226534	20653	6235	1.35
2	Relugolix	3.163	226542	20598	6198	1.36
3	Relugolix	30158	225989	20653	6254	1.36
4	Relugolix	3.167	226512	20548	6281	1.35
5	Relugolix	3.171	226531	20653	6199	1.36
6	Relugolix	3.171	225898	20658	6253	1.35
Mean			226334.3			
Std. Dev.			304.2622			
% RSD			0.13443			

Analyst 2:

Table-7: Results of Intermediate Precision Analyst 2 for Relugolix

S. No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Relugolix	3.173	225487	20542	6253	1.35
2	Relugolix	3.134	225484	20532	6098	1.36
3	Relugolix	3.161	225364	20541	6254	1.35
4	Relugolix	3.174	226513	20534	6235	1.36
5	Relugolix	3.199	225487	20549	6199	1.36
6	Relugolix	3.199	226532	20451	6235	1.35
Mean			225811.2			
Std. Dev.			553.0524			
% RSD			0.244918			

Accuracy:

Accuracy at different concentrations (50%, 100%, and 150%) was prepared and the % recovery was calculated20-22.

Table-8: The Accuracy Results for Relugolix

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	109283.3	15	15.060	100.40%	100.42%
100%	212732	30	30.124	100.413%	
150%	316263.3	45	45.201	100.446%	

Limit of Detection for Relugolix

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value23.

LOD = 3.3 × σ / s

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

Result:

= 0.597µg/ml

Limit of Quantitation for Relugolix

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined24.

LOQ = 10×σ/S

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

Result:

= 1.811µg/ml

Table-9: Results of LOD and LOQ

SE of Intercept	556.1832432
SD of Intercept	1243.66354
LOD	0.597636545
LOQ	1.811019833

Robustness

The robustness was performed for the flow rate variations from 0.9 ml/min to 1.1ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for Relugolix25-27. The method is robust only in less flow condition and the method is robust even by change in the Mobile phase ±5%. The standard and samples of Relugolix were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count28-31.

Table-10: Results for Robustness

Parameter used for Sample	Peak Area	Retention	Theoretical	Tailing Factor
Actual Flow rate of 1.0 mL/min	225645	3.155	6125	1.36
Less Flow rate of 0.9 mL/min	236586	3.488	6452	1.38
More Flow rate of 1.1 mL/min	219865	2.877	6098	1.42
Less organic phase	235848	4.705	6126	1.43
More organic phase	241245	2.090	6324	1.39

SUMMARY AND CONCLUSION

The analytical method was developed by studying different parameters. First of all, maximum absorbance was found to be at 272nm and the peak purity was excellent. Injection volume was selected to be 20µl which gave a good peak area. The column used for study was Symmetry ODS C18 (4.6 x 250mm, 5µm) because it was giving good peak. Ambient temperature was found to be suitable for the nature of drug solution. The flow rate was fixed at 1.0ml/min because of good peak area and satisfactory retention time. Mobile phase is Acetonitrile: Methanol (80:20% v/v) was fixed due to good symmetrical peak. So, this mobile phase was used for the proposed study. Run time was selected to be 8.0min because analyze gave peak around 3.155 and also to reduce the total run time. The percent recovery was found to be 98.0-102 was linear and precise over the same range. Both system and method precision were found to be accurate and well within range. The analytical method

was found linearity over the range of 10-50µg/ml of the Relugolix target concentration. The analytical passed both robustness and ruggedness tests. On both cases, relative standard deviation was well satisfactory. The method was validated in accordance with ICH guidelines. The method is robust enough to reproduce accurate and precise results under different chromatographic conditions. Hence the proposed RP-HPLC method proved to be simple, accurate and reproducible for the determination of Relugolix in a reasonable run time. The method was validated showing satisfactory data for all the method validation parameters tested. The developed method can be conveniently used by quality control laboratories.

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