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Development and Validation of a Novel HPTLC Method for Quantitative Determination of Relugolix in Pharmaceutical Dosage Forms

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ABSTRACT

The present study describes the steps to take and the results of an HPTLC approach that is simple, sensitive, specific, and stable that may be used to quantify the GnRH receptor antagonist Relugolix, which is used to treat prostate cancer. The desired separation was accomplished by using a mobile phase consisting of acetonitrile, methanol, and orthophosphoric acid in a ratio of 6:3:1, v/v/v, on silica gel 60 F_{254} aluminum plates. An Rf value of 0.45 ± 0.02 was obtained from the densitometric detection at 254 nm, where Relugolix showed a clear and well separated peak. An outstanding linear response was shown by a correlation coefficient (R^2) of 0.998, which was determined to be linear throughout the concentration range of 100-500 ng/spot. With an intra-day and inter-day %RSD < 1%, the method demonstrated excellent accuracy, and recovery rates ranged from 99.28% to 99.38%. We found that the limit of detection (LOD) is 18 µL and the limit of quantification (LOQ) is 84 µL, indicating that it is very sensitive. There was no interference from excipients, as shown by specificity testing, and consistent performance was shown by robustness tests when subjected to small variations in chromatographic settings. This HPTLC technique is a reliable option for regular quality control, stability testing, and quantitative estimate of Relugolix in bulk medication and pharmaceutical dosage forms since it meets all the validation standards outlined in the ICH Q2(R1) recommendations.

KEYWORDS: *Relugolix, HPTLC, Method Validation, Linearity, Pharmaceutical Analysis.*

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1. INTRODUCTION

The development and validation of analytical techniques are fundamental components of modern pharmaceutical research, ensuring the identification, purity, potency, and quality of active pharmaceutical ingredients (APIs) and medicinal products [1]. The increasing need for precise, efficient, and rapid analytical procedures has catalyzed the continuous advancement of chromatographic techniques such as High-Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), and High-Performance Thin-Layer Chromatography (HPTLC) [2]. Among them, HPTLC has been acknowledged as a versatile, economical, and successful technique for the qualitative and quantitative analysis of pharmaceuticals [3]. It allows for concurrent analysis of several samples, reduces solvent use, and minimizes sample handling, making it very suitable for routine quality control and stability assessments [4]. HPTLC, an advanced iteration of conventional TLC, integrates automated sample introduction, temperature-regulated development, densitometric scanning, and computerized data analysis to enhance accuracy and repeatability [5]. The approach provides enhanced sensitivity, selectivity, and resolution, along with the advantage of visual and densitometric analysis on a single plate [6]. Owing to its simplicity and adaptability, HPTLC is increasingly used for drug identification, impurity profiling, assay determination, and stability-indicating research [7]. Pharmacopoeial experts recommend it for assessments of herbal, bulk, and dosage forms because of its confirmed repeatability and accuracy [8].

Relugolix is a potent, non-peptide, oral antagonist of the gonadotropin-releasing harmone (GnRH) receptor, recently authorized for the treatment of advanced prostate cancer and uterine fibroids. It functions by competitively blocking GnRH receptors on the anterior pituitary, so obstructing the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), resulting in decreased testosterone levels in males and estrogen suppression in women. Relugolix has advantages such as oral administration, rapid start of testosterone suppression, and reversible pharmacological effects following cessation of the medication, in contrast to injectable equivalents such leuprolide and degarelix [11]. The molecular designation of Relugolix is "(1R,5S,6R,7R). -N-(1-(3-chloro-4-fluorobenzoyl)-5-(2,6-difluorobenzyl) -6-(methoxymethyl) -3,6 Diazabicyclo[3.1.1] (heptan-7-yl)-6-methyl -4-oxo-1,4-dihydroquinoline -3-carboxamide, has a chemical formula of C29H27ClF2N4O6 and a molecular weight of 623.00 g/mol [12]. It is a white powder that has modest solubility in methanol and acetonitrile, and is fully soluble in dimethyl sulfoxide [13].

The analytical determination of relugolix in pharmaceutical formulations is essential for dose consistency, quality control, and pharmacokinetic evaluation. Various approaches have been recorded for its examination, including UV-visible spectrophotometry, HPLC, and LC–MS/MS procedures [14]. Despite their great sensitivity, these approaches often require extensive sample preparation, prolonged analysis time, and significant operational costs [15]. HPTLC offers a faster, more economical, and environmentally sustainable method, capable of analyzing several samples simultaneously with little solvent use [16]. Moreover, HPTLC enables visual sample comparison, which is very beneficial during the first phases of formulation development and impurity profiling [17]. The International Council for Harmonisation (ICH) standards Q2(R1) emphasize the validation of all analytical processes used in drug testing to ensure the reliability and repeatability of findings [18]. Validation characteristics like as specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), robustness, and solution stability are essential to confirm the method's suitability for its intended analytical purpose. A well validated approach ensures that findings are dependable and scientifically acceptable, which is essential for regulatory clearance, product authorization, and post-marketing monitoring [19]. The choice of analytical technique in the pharmaceutical sector depends on cost, time, sensitivity, accuracy, and the characteristics of the sample matrix. HPTLC effectively resolves these issues by separating complex mixtures rapidly without the need for sophisticated equipment. The visual documentation and densitometric analysis of the procedure render it very dependable for both qualitative and quantitative assessment [20]. Advancements in automation and software integration have enabled contemporary HPTLC systems to provide repeatable results comparable to that of HPLC, establishing it as a robust secondary analytical method [21].

This work aims to build and verify a straightforward, accurate, precise, and cost-effective HPTLC technique for the regular estimate of Relugolix, given its therapeutic importance and the need for a systematic approach. The method was established to quantify Relugolix in both bulk and pharmaceutical dose forms, according to ICH Q2(R1) criteria. The suggested approach utilizes a mobile phase of Acetonitrile, Methanol, and Orthophosphoric acid in a ratio of 6:3:1 (v/v/v) and exhibits a distinct, well-resolved peak with an Rf value about 0.45 at 254 nm. The ideal chromatographic conditions were established via several attempts to provide maximum resolution, minimal tailing, and consistent peak area.

The validation trials confirmed that the proposed technique exhibits good specificity without excipient interference, has strong linearity over the concentration range of 100–500 ng/spot, and achieves recovery rates between 99.28% and 99.38%. The intra-day and inter-day precision data fell within acceptable limits, and robustness testing demonstrated the method's resilience to minor fluctuations in chromatographic conditions. The acquired low LOD and LOQ values demonstrated great sensitivity, while stability experiments of the solution confirmed that Relugolix is stable for 24 hours under laboratory conditions. The HPTLC approach provides a straightforward, efficient, and economical analytical tool for the regular quantification of Relugolix in quality control laboratories. It may be used for dissolving testing, stability assessments, and batch-to-batch uniformity evaluations in pharmaceutical firms. The repeatability and simplicity of this technology make it particularly suitable for resource-limited developing countries, where the resource-intensive procedures of HPLC and LC–MS are less accessible. This validated HPTLC approach offers a viable alternative for ensuring the quality, safety, and effectiveness of formulations containing Relugolix.

2. Methodology

2.1. Chemicals and Reagents

Pure standards Relugolix were purchase from Tripurrte Pharmaceuticals, Pvt. Ltd., Hyderabad,

Telangana. We procured analytical-grade orthophosphoric acid, methanol, and acetonitrile from reliable vendors. Water that has been HPLC Grade was used throughout the experiment. All reagents and solvents were HPLC grade in order to preserve purity and repeatability of results.

2.2. Instrumentation and Chromatographic System

Silica gel 60 F_{254} aluminum plates (20 × 10 cm, Merck) used as the stationary phase for the HPTLC analysis. A 100 µL Hamilton syringe was used with an Automatic Sample Applicator (ATS 4) for sample application. The plates were prepared in a 20 × 10 cm twin-trough glass chamber, which had been saturated with mobile phase vapor for 20 minutes. A TLC Scanner 4, integrated with LABSERVER software, was used for densitometric scanning.

2.3. Chromatographic Conditions

Chromatographic settings were refined by several attempts to get crisp, symmetrical, and well-resolved peaks for Relugolix. The optimum mobile phase consisted of Acetonitrile, Methanol, and Orthophosphoric acid in a ratio of 6:3:1 (v/v/v). The chamber saturation duration was maintained at 20 minutes, with the solvent front distance set at 70 mm. 10 µL of sample application volume was deposited in 8 mm broad bands, positioned 8 mm from the bottom border of the plate. Densitometric detection at a wavelength of 254 nm was conducted with a slit size of 6.0 × 0.45 mm and a scanning velocity of 20 mm/s, employing a deuterium lamp as the light source. The plates were air-dried at room temperature and then scanned densitometrically at 254 nm to get the chromatograms and peak areas.

2.4. Preparation of Standard Stock Solution

A 10 mL volumetric flask was used to dissolve a precisely weighed quantity of Relugolix pure standard in a 6:3:1 combination of acetonitrile, methanol, and orthophosphoric acid, creating a stock solution with a concentration of 1000 µg/mL. The correct disintegration was aided by sonication for 5 minutes.

2.5. Preparation of Working Standard Solutions

To make working standard solutions of 100, 200, 300, 400, and 500 ng/spot, portions of the stock solution were diluted in the same solvent combination in the same proportions. The concentrations mentioned were used to create the Relugolix calibration curve. Using an automated applicator, the dilutions were placed to the plate in distinct bands.

2.6. Preparation of Sample Solution

An exact measuring volume of a Relugolix-like formulation was transferred to a volumetric flask and diluted with 6 parts Acetonitrile, 3 parts Methanol, and 1 part Orthophosphoric acid. To put the concentration within the linear range, the solution was diluted appropriately after being sonicated for 10 minutes and filtered through a 0.45 µm membrane filter.

2.7. Plate Development

On the activated TLC plate, the spots for the standard and sample were arranged in 8 mm bands. Twenty minutes were spent developing the plate in a twin-trough chamber that had been pre-saturated with the optimal mobile phase. Up to 70 mm of migration of the solvent front was allowed from the starting position. Following development, the plate was air-dried and densitometrically scanned at 254 nm.

2.8. Calibration Curve for Relugolix

A calibration plot was prepared by plotting peak area against concentration (ng/spot) for the five concentrations (100–500 ng/spot). Three replicates of each concentration were analyzed. Calibration equation was determined by linear regression analysis. Regression equation obtained was: $y = 3.778x + 0.9285$ ($R^2 = 0.998$) demonstrating good linear relationship between peak area and concentration.

2.9. Method Validation

The developed procedure was validated in line with ICH Q2 (R1) guidelines for the following parameters:

Specificity: Specificity was established by comparing standard and sample solutions. Rf values and spectral overlay evidenced the non-interference from excipients. Peak purity data proved the specificity of the method was 97.78% for Relugolix.

Linearity: Linearity was tested at five concentration levels (100–500 ng/spot). The correlation coefficient (R^2) value of 0.998 proved excellent linearity.

Accuracy (Recovery Studies): Accuracy was established by standard addition technique at three concentration levels (80%, 100%, and 120%). The mean percentage recovery was 99.28% to 99.34%, which demonstrates good accuracy of the method.

Precision: Precision was evaluated as **intra-day** and **inter-day** variation using three different concentration levels.

- Intra-day precision (%RSD): 0.59–0.62%
- Inter-day precision (%RSD): 0.69–0.71%These values were within the acceptable limit of ≤2%.

Limit of Detection (LOD) and Limit of Quantification (LOQ): LOD and LOQ were calculated based on the standard deviation of the response and slope of the calibration curve. The obtained values were:

- **LOD:** 18 µL

- **LOQ:** 84 µL

Robustness: Robustness was tested by introducing minor variations in chromatographic parameters such as mobile phase ratio, saturation time, and detection wavelength. The results were consistent, indicating the method's robustness.

System Suitability: System suitability was confirmed by repeated application of standard solution (n=6). The %RSD of Rf and peak area was found to be less than 2%, ensuring reliable system performance.

2.10. Solution Stability

The stability of standard and sample solutions was examined by storing them at room temperature and reanalyzing at regular intervals. The solutions remained stable for 24 hours without any significant change in peak area or Rf value.

3. Results

3.1. Optimization of HPTLC Conditions

A crisp, well-resolved, and compact peak for Relugolix was achieved by testing several solvent solutions. At 254 nm, a clear and repeatable spot with an Rf value of 0.45 ± 0.02 was obtained by the optimized mobile phase Acetonitrile : Methanol : Orthophosphoric acid (6 : 3 : 1, v/v/v). The method's appropriateness was confirmed by the densitometric chromatogram, which showed symmetrical peak shape with minimum tailing [22].

3.2. Linearity and Calibration Curve

Linearity of Relugolix was established in the range of **100–500 ng/spot**. The mean peak area was plotted against the corresponding concentration to obtain the calibration curve. The regression analysis data are presented in **Table 1**.

Table 1. Calibration Data for Relugolix (100–500 ng/spot)

Concentration (ng/spot)	Mean Peak Area ± SD	%RSD
100	380 ± 2.1	0.55
200	750 ± 3.8	0.50
300	1128 ± 5.4	0.48
400	1502 ± 7.8	0.52
500	1888 ± 8.6	0.46

Regression Equation: y = 3.778x + 0.9285

Correlation Coefficient (R^2): 0.998

Slope: 3.778 **Intercept:** 0.9285

These results confirm excellent linearity of response between peak area and concentration in the specified range [23].

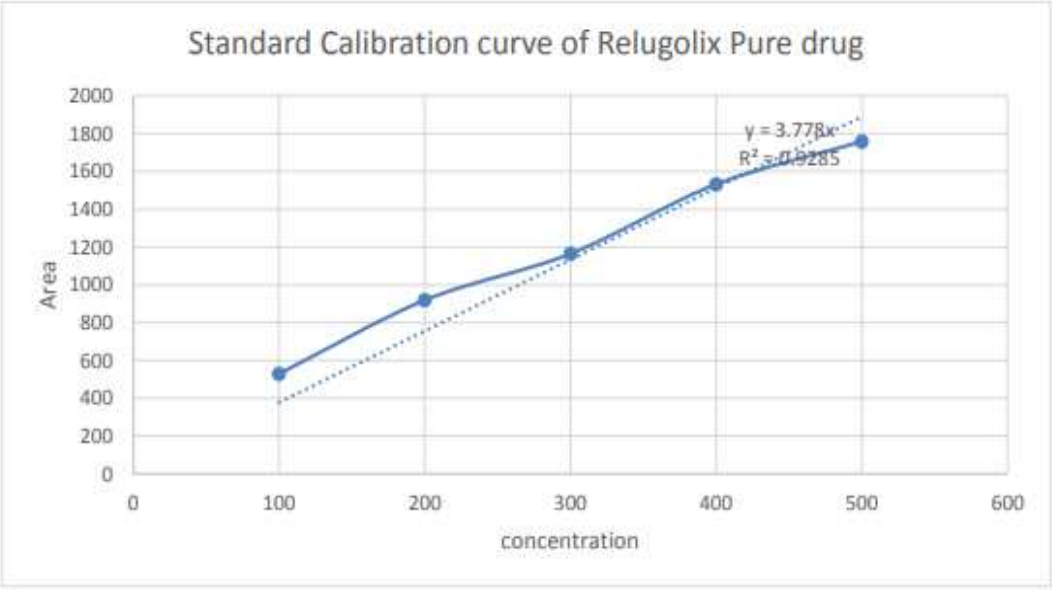

Fig. no. 1: Standard Calibration curve of Relugolix

3.3. Precision

The method's precision was evaluated by analyzing three replicates of Relugolix at three concentration levels (100, 300, and 500 ng/spot) within the same day (intra-day) and on different days (inter-day). The results are summarized in Table 2 [24].

Table 2. Precision Studies for Relugolix

Parameter	Concentration (ng/spot)	Mean Area ± SD	%RSD
Intra-day Precision	100	382 ± 2.3	0.60
	300	1126 ± 7.0	0.62
	500	1884 ± 10.3	0.59
Inter-day Precision	100	384 ± 2.7	0.69
	300	1128 ± 7.9	0.71
	500	1890 ± 10.2	0.70

All %RSD values were below 2%, indicating high precision of the developed method.

Accuracy

Accuracy was determined by the standard addition method at three levels: 80%, 100%, and 120% of the nominal concentration. Results are shown in Table 3 [25,26].

Table 3. Recovery Studies of Relugolix

Level of Addition	Amount Added (ng/spot)	Amount Recovered (ng/spot)	% Recovery ± SD	%RSD
80%	80	79.43 ± 0.38	99.28 ± 0.48	0.48
100%	100	99.34 ± 0.52	99.34 ± 0.52	0.52
120%	120	119.25 ± 0.66	99.38 ± 0.55	0.55

The recovery results (99.28–99.38%) confirm the accuracy and reliability of the proposed HPTLC method.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ were determined using the signal-to-noise ratio method. The results are presented in **Table 4** [27,28].

Table 4. LOD and LOQ for Relugolix

Parameter	Value	Detection Method
Limit of Detection (LOD)	18 µL	Signal-to-noise (S/N = 3:1)
Limit of Quantification (LOQ)	84 µL	Signal-to-noise (S/N = 10:1)

Both parameters indicate the high sensitivity of the developed HPTLC method.

Specificity

The densitogram of Relugolix standard and sample showed identical Rf values (~0.45), with no interference from excipients or solvents. Peak purity index confirmed specificity at 97.78%, indicating that the method can selectively quantify Relugolix in the presence of formulation excipients.

Robustness

Small, deliberate changes in chromatographic conditions such as mobile phase composition (±0.1 mL), chamber saturation time (±5 min), and detection wavelength (±2 nm) did not significantly affect the Rf or peak area values. The %RSD remained below 2%, confirming the robustness of the method (Table 5).

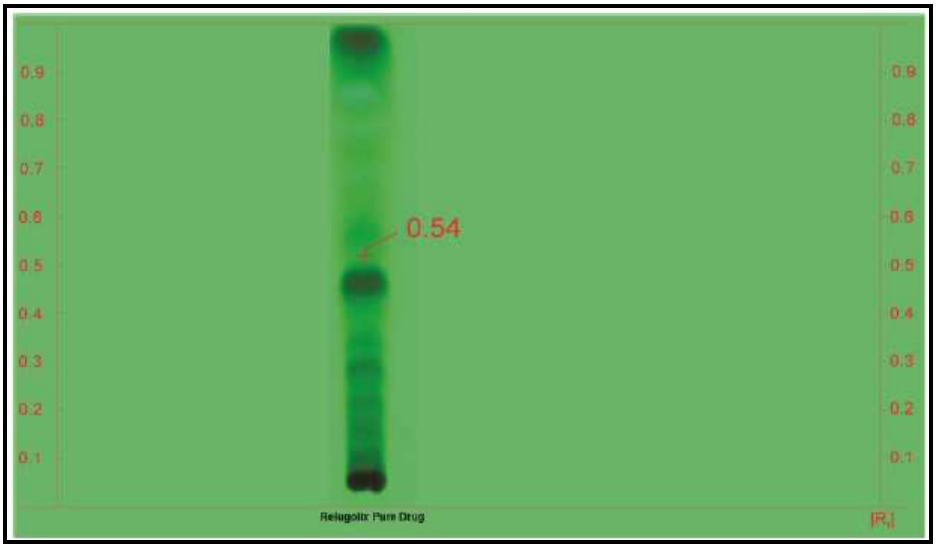

Fig. no.1; HPTLC Plate Visualization

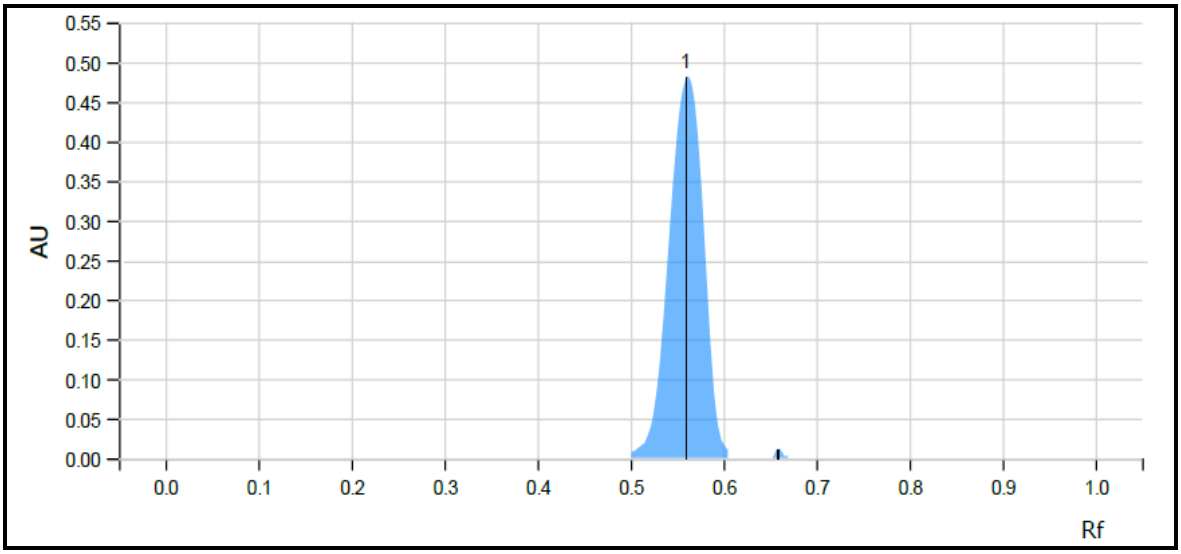

Fig. no. 2; Densitogram of Relugolix standard area demonstrating %RSD within acceptable limits.

Table 5. Robustness Study for Relugolix

Parameter Varied	Condition	Rf ± SD	%RSD
Mobile phase ratio	6:3:1	0.45 ± 0.01	0.62
Saturation time	20min	0.44 ± 0.02	0.74
Detection wavelength	254 nm	0.45 ± 0.01	0.70

System Suitability

System suitability was confirmed by analyzing standard Relugolix (n = 6). The %RSD of peak area and Rf values was found to be less than 2%, indicating the system's suitability for routine use.

Table 6. System Suitability Parameters for Relugolix

Parameter	Mean ± SD	%RSD	Acceptance Criteria
Rf value	0.45 ± 0.01	0.60	≤ 2.0
Peak area	1128 ± 7.3	0.65	≤ 2.0

Solution Stability

The standard and sample solutions of Relugolix" were found to be stable for 24 hours at room temperature, with less than 1% change in area and no shift in Rf value.

Conclusion

A novel, simple, accurate, precise, and strong High-Performance Thin-Layer Chromatography (HPTLC) technique was successfully established and authenticated for the quantitative determination of Relugolix in bulk and dosage forms. Optimized mobile phase system — Acetonitrile : Methanol : Orthophosphoric acid (6 : 3 : 1, v/v/v) — was yielding a distinct and reproducible peak with an Rf value of 0.45 ± 0.02.Validation of the method following ICH Q2(R1) guidelines was ensuring its acceptability for analytical purposes. The method demonstrated good linearity (R^2 = 0.998) within the range of 100–500 ng/spot, good accuracy (99.28–99.38%), and precision with %RSD values under 1%. The low LOD (18 µL) and LOQ (84 µL) demonstrated the sensitivity of the method, while robustness testing determined its reliability under minimal intentional variations in conditions. In general, the proposed HPTLC method is fast, cost-effective, and reproducible, well-suited for routine quality control, stability, and formulation analysis of Relugolix in drug laboratories.

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