RESEARCH PAPER

Experimental Design-Assisted HPTLC Method for Nirmatrelvir and Ritonavir Analysis in Pharmaceutical Dosage Forms

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Received: 14th Sep, 2025; Revised: 20th Oct 2025; Accepted: 6th Nov, 2025; Available Online: 1st December, 2025

ABSTRACT

Background: This study aimed to develop and validate a High-Performance Thin Layer Chromatography (HPTLC) method utilizing a Design of Experiments (DOE) approach for simultaneous quantification of Nirmatrelvir (NIR) and Ritonavir (RITO) in pharmaceutical dosage forms. Method: Pre-coated HPTLC aluminium plates with silica gel 60 F254 were employed as the stationary phase to optimize the mobile phase composition. Fractional factorial design (FFD) was applied to assess method robustness. The analytical procedure was validated following ICH Q2 (R2) guidelines. Results: Detection was carried out at 260 nm for both NIR and RITO. Optimal separation was achieved using a mobile phase of ethyl acetate, n-hexane, and methanol in a ratio of 8:1.2:0.8 v/v/v. The Rf values observed were 0.46 for NIR and 0.78 for RITO. The method was validated for multiple parameters, with %RSD values falling within acceptable limits. FFD results indicated that all four factors significantly influenced the retention of NIR, whereas RITO was not significantly affected. Conclusion: The developed HPTLC method is simple, rapid, selective, accurate, and precise. Statistical evaluation demonstrates reproducibility with no significant differences, indicating suitability for routine analysis of marketed formulations without prior separation.

Keywords: Nirmatrelvir, Ritonavir, HPTLC, DoE approach, Validation

How to cite this article: Musale R, Mehta H, Jamsa A, Gohil D, Uttekar P; Experimental Design-Assisted HPTLC Method for Nirmatrelvir and Ritonavir Analysis in Pharmaceutical Dosage Forms. Int J Drug Deliv Technol. 2026;16(1): 459-465. DOI: 10.25258/ijddt.16.1.48

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

Research highlights.

DoE-based HPTLC method developed for Nirmatrelvir and Ritonavir.

Optimized mobile phase: Ethyl acetate: n-hexane: methanol (8:1.2:0.8 v/v/v).

Clear separation with R_f values of 0.46 (NIR) and 0.78 (RITO).

Validated as per ICH Q2(R2) with %RSD within limits.

Rapid, precise, and suitable for routine quality control.

INTRODUCTION

A combination of Nirmatrelvir and Ritonavir tablets was developed to treat and prevent coronavirus illness. Ritonavir is an HIV-1 protease inhibitor and CYP3A inhibitor, while Nirmatrelvir inhibits SARS-COV-2[1].

Nirmatrelvir targets the coronavirus that causes severe acute respiratory syndrome. When taken orally, it binds specifically to the Mpro targets of SARS-COV-2. The SARS-COV-2Mpro can bind to its active site and is responsible for the replication of the virus. This has strong antiviral effects against several human coronaviruses, including SARS-COV and Middle East respiratory syndrome coronavirus [2].

Ritonavir is used as a protease inhibitor for HIV-1 and to treat AIDS. It is a potent in vitro inhibitor of HIV, the virus that causes acquired immunodeficiency syndrome. Ritonavir increases the half-life of Nirmatrelvir, allowing for their combined use in treating viral infections [3].

This combination is recommended for treating mild to moderate cases of Covid-19. It is available commercially as oral tablets containing 100 mg of Ritonavir and 150 mg of Nirmatrelvir [1].

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MATERIALS AND METHODS

Instrumentation

Before chromatographic separation, the TLC plates were conditioned by washing with methanol. Linear ascending development was performed using twin glass chambers of sizes 20 × 10 cm and 10 × 10 cm. Chromatographic analysis was executed on a CAMAG HPTLC system consisting of a Linomat V sample applicator, a 100 µL Hamilton microsyringe, and a CAMAG TLC Scanner-4 operated through Vision CATS software (version 3). Statistical analysis for the experimental design was carried out using Design-Expert® software (trial version 13; Stat-Ease Inc., Minneapolis, USA), while all remaining computations were completed using Microsoft Excel 2021.

Chemicals and reagents

The standard Nirmatrelvir sample was kindly provided by Gonane Pharma Pvt., Ahmedabad. The Ritonavir sample was given by Bharat Parenteral Ltd., Vadodara. Primovir (150 mg Nirmatrelvir tablets co-packaged with 100 mg Ritonavir tablets) was supplied by Astrica Healthcare Pvt. Ltd. Methanol and ethanol (HPLC grade) were purchased from Rankem lab. Ethyl acetate, n-hexane, and acetonitrile (AR grade) were sourced from Suvidhinath lab, Vadodara. Pre-coated silica gel 60 F254 (20 x 20 cm, 100 µm) aluminium sheets were obtained from E. Merck Ltd, Mumbai.

Preparation of Standard stock solution

Accurately weigh 10 mg of Nirmatrelvir (NIR) and Ritonavir (RITO) separately, then transfer them into 10 ml volumetric flasks and dissolve with methanol. Fill with methanol to the mark to a 1000 µg/mL solution of NIR and RITO.

Preparation of Working solution

From the standard stock solution, pipette out 3 ml of NIR solution and 1 ml of RITO solution. Transfer this mixture to a 10 ml volumetric flask and fill with methanol. The final concentrations become 300 µg/mL of NIR and 100 µg/mL of RITO, respectively.

Chromatographic development

Band wise sample application (5 mm width) was performed using a 100 µL Hamilton microsyringe (Linomat syringe 659.0014, Hamilton-Bonaduz Schweiz, CAMAG, Switzerland) on silica gel aluminum HPTLC plates 60 F254 (20 × 10 cm, 100 µm layer thickness; E. Merck, Darmstadt, Germany) with the aid of a CAMAG Linomat V applicator. Densitometric scanning was carried out at 260 nm in absorbance-reflectance mode using a deuterium lamp. The slit dimension was fixed at 6.00 mm × 0.45 mm, scanning velocity at 100 mm/s, and data resolution at 100 µm per step. Chromatographic conditions were optimized to obtain well-resolved spots, with migration observed at Rf values ranging from 0.465 ± 0.010 to 0.782 ± 0.030, indicating reliable and reproducible separation.

Method Validation

The analytical performance of the HPTLC method was assessed in compliance with ICH Q2(R2) guidelines, including evaluation of accuracy, precision, specificity, robustness, detection limit, and quantification limit. [6].

Linearity and Range

Linearity was evaluated using six replicate determinations obtained from the working solution at 260 nm across concentration ranges of 2400–8400 ng/band for NIR and 800–2800 ng/band for RITO. Regression parameters were calculated. Various volumes of the solution were applied to examine the proportional relationship between chromatographic response and drug concentration. Calibration curves were established by plotting peak area as a function of analyte concentration.

Limit of detection (LOD) and limit of quantification (LOQ) [6]

According to ICH guidelines, the limit of detection and quantification for the developed method were calculated from the standard deviation of the response and the slope of the calibration curve using the following formulas:

Limit of detection = 3.3 * σ/S [6]

Limit of quantitation = 10 * σ/S [6]

Where "σ" represents the standard deviation of the response and "S" is the slope of the calibration curve.

Precision

The precision of the method was assessed in terms of repeatability and intermediate precision.

Repeatability

Repeatability was evaluated by scanning and measuring the responses of the NIR solution (2400 ng/band) and the RITO solution (800 ng/band) without altering the parameters of the proposed methods. This procedure was repeated six times, and the percentage relative standard deviation (% RSD) was calculated.

Intermediate precision

Intraday and interday precision of the analytical procedure were established by performing triplicate measurements on the same day and on three separate days within a one-week interval at three concentration levels of NIR (2400, 3600, and 4800 ng/band) and RITO (800, 1200, and 1600 ng/band).

Accuracy

Accuracy of the analytical procedure was verified through recovery experiments performed at three fortification levels, namely 80%, 100%, and 120%. Predetermined quantities of NIR standard solutions (1920, 2400, and 2880 ng/band) and RITO standards (640, 800, and 960 ng/band) were incorporated into a previously analyzed marketed sample containing 2400 ng/band of NIR and 800 ng/band of RITO. Each recovery level was evaluated in triplicate.

Specificity

Specificity was established by analysing reference standards alongside sample solutions. The band obtained

from the marketed dosage form was confirmed by correlating its Rf value and spectral profile with those of the respective standards. Densitometric evaluation of peak purity for NIR and RITO was carried out at 260 nm by comparing spectra collected at three points of the peak, namely the start (S), maximum (M), and end (E).

Robustness Study Using Fractional Factorial Design: (2^{4-1})

In this study, four factors were chosen based on their criticality observed during trial runs, along with insights from previous studies. Changing factors such as those shown in Figure 5 (a) Wavelength (260 nm), (b) Volume of Ethyl Acetate (8 ml), (c) Solvent front (80 mm), and (d) Chamber saturation time (20 min) affected the retention factor from their original values. All experiments were performed in a random order to reduce the influence of uncontrolled factors. Eight experimental runs with code values for factor levels using FFD are presented. Results are shown in Table 1. [7]

Table 1 Experimental factors and levels used in FFD (2^{4-1})

Sr. No.	Factors	High level	Low level
1.	Wavelength (nm)	261	259
2.	Volume of Ethyl acetate (ml)	9	7
3.	Solvent front (mm)	81	79
4.	Chamber saturation time (min)	19	21

Analysis of Marketed Formulation

Twenty tablets labelled to contain NIR (300 mg) and RITO (100 mg) were collectively weighed and pulverized to a uniform powder. A quantity of the powdered material equivalent to 300 mg of NIR was accurately measured and transferred into a 100 mL volumetric flask. Methanol was added as the extraction solvent, and the mixture was sonicated for 25–30 minutes to facilitate complete drug dissolution. The solution volume was then adjusted to the calibration mark with methanol and filtered through a 0.45 µm Whatman membrane to obtain a stock solution containing 3000 µg/mL of NIR and 1000 µg/mL of RITO. An aliquot of 1 mL from this stock solution was further diluted to 10 mL with methanol to prepare the working sample solution for assay determination. The prepared sample was applied onto the HPTLC plate at concentrations of 300 ng/band for NIR and 100 ng/band for RITO, followed by chromatographic development and densitometric evaluation at 260 nm. The obtained results are summarized in Table 6.

Table 6 Analysis of Marketed Formulation NIR and RITO

Sample	Nirmatrelvir			Ritonavir		
	Final conc. (ng/band)	Observed peak area	Observed conc. (ng/band)	Final conc. (ng/band)	Observed peak area	Observed conc. (ng/band)
1	300	0.00945	303.11	100	0.0126	99.33
2	300	0.00937	299.05	100	0.01268	101.96
3	300	0.00939	300.38	100	0.01264	100.66
% Assay	100.24			100.65		
%RSD	0.70			1.30		

RESULTS

Preliminary method development studies

To find the right wavelength, spectra of the NIR and RITO were recorded using the Camag TLC Scanner IV. It was discovered that NIR and RITO produced high intensity together at 260 nm, as shown in Figure 1.

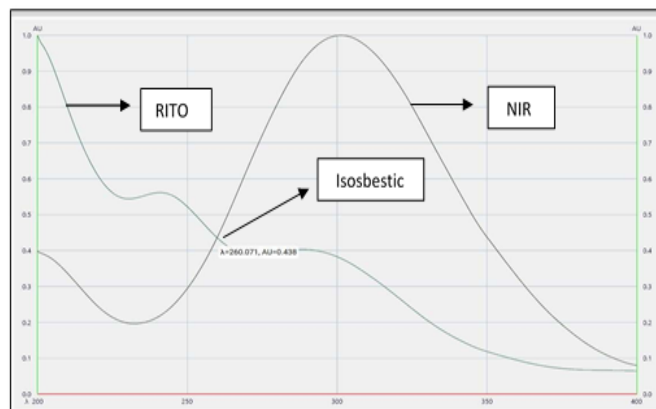


Figure 1: HPTLC Spectrum of NIR and RITO

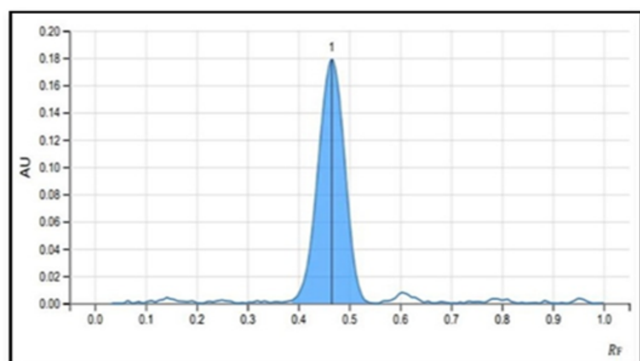
Various combinations of mobile phases were tested to optimize the peaks of NIR and RITO using a trial-and-error method. Literature review showed that the HPTLC method had been reported for NIR and RITO, either alone or with other drug combinations. The selected mobile phase consisted of methanol, chloroform, toluene, ethyl acetate, glacial acetic acid, and ammonia. These solvents were used in different combinations and amounts during the preliminary study. The optimized mobile phase of Ethyl acetate: n-Hexane: Methanol (8:1.2:0.8 v/v/v) was chosen for the experiment. Results are summarized in Table 2 and shown in Figure 2.

Table 2 Optimized chromatographic conditions

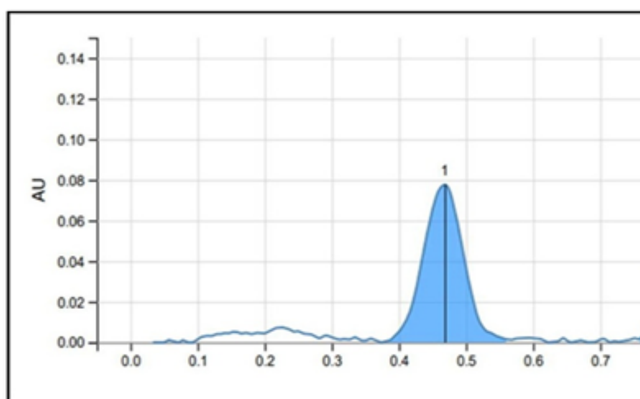
Parameters	Conditions
Mobile Phase	Ethyl acetate: n-hexane: methanol (8:1.2:0.8 v/v/v)
Saturation time	20 min
Bandwidth	5 mm
Detection Wavelength	260 nm
Micro-Syringe	100 µl
Solvent front	80 mm
Pre-coated silica gel thickness	100 µm
Chamber	Twin through glass chamber (10 x 20 cm)

Table 3 R_f value of drugs

Sr. No.	Name of Drug	R_f value in solution	R_f value in formulation
1.	Nirmatrelvir	0.465 ± 0.010	0.468
2.	Ritonavir	0.782 ± 0.030	0.808



(a) Std. Drug of NIR



(c)Formulation (NIR+RITO)

Figure 2 HPTLC Densitogram of Standard Drug and Formulation

Method validation

The analytical parameters of the proposed HPTLC method are listed in Table 4.

Table 4 Analytical parameters of proposed HPTLC method

Parameter	Nirmatrelvir	Ritonavir
Analytical Wavelength(nm)	260	260
R_f Value	0.46	0.78
Linear regression parameters		
Calibration Range (ng/band)	2400-8400	800-2800
Regression line equation	y = 0.00002x+0.0031	y = 0.00003x+0.0096
Regression coefficient (R^2)	0.9944	0.9945
Slope	0.00002095	0.0000302
Intercept	0.00312	0.00960
Sensitivity		
Limit of Detection (ng/band)	245.56	107.75
Limit of Quantitation (ng/band)	744.14	326.54
Precision		
Repeatability (%RSD)	0.83	1.57
Intraday precision (%RSD)	0.33-0.88	0.75-0.80
Interday precision (%RSD)	0.87-1.16	0.97-1.52
Accuracy		
80 % (Mean% recovery ± %RSD)	0.47	1.38
100 % (Mean% recovery ± %RSD)	0.43	1.31
120 % (Mean% recovery ± %RSD)	0.36	1.65
Specificity		
r (s, m)	0.999994	0.999839
r (m, e)	0.999685	0.998688

Robustness study using DoE Software

The experimental results were calculated using Design Expert Trial Version 13. A multivariate approach was used, where method factors changed simultaneously through a design matrix. The robustness study used FFD (24-1), as summarized in Table 5. [7]

Table 5 Statistical Parameters from ANOVA

Sr. No.	Parameters	R_f of NIR	R_f of RITO
1	Std. Dev.	0.0010	0.0087
2	Mean	0.468	0.769
3	% C. V	0.21	1.13
4	PRESS	0.0015	0.0024
5	R-Squared	0.9808	0.9769
6	Adj R-Squared	0.9727	0.9367
7	Predicted R squared	0.6923	0.4734
8	Adequate precision	13.85	5.223
9	Polynomial equation	0.4680-0.0008A-0.0003B+0.0022C-0.0018D+0.0020AD	0.7695+0.0057A+0.0085B+
10	Model (p-value)	Significant (0.0474)	Non – Significant (0.2146)

Factor screening studies

In this Pareto chart, effects above the Bonferroni limit are considered significant factors. Effects above the t-value limit suggest the possibility of a significant factor, while those below indicates a chance of a non-significant factor.

Figure 3 shows that the solvent front has a significant effect on robustness compared to other factors, making it important to control carefully. Additionally, Figure 4 reveals that the retention factor of RITO was not affected by variations in all factors, ensuring the robustness of the analytical method. Refer to Figure 3 and Figure 4.

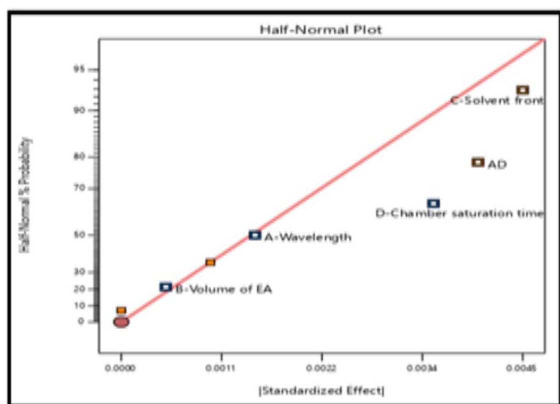


Figure 3 Pareto chart of NIR

R_f of drugs in decreasing order for NIR: C>AD>D>A>B

R_f of drugs in decreasing order for RITO: B>AB>A>C>D

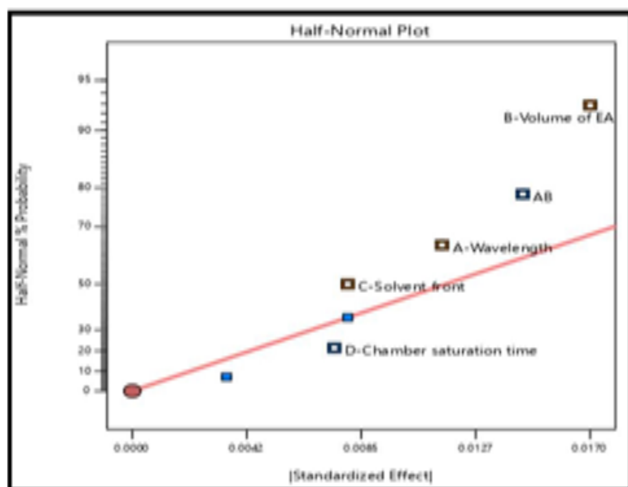


Figure 4 Pareto chart of RITO

3D Response Surface Plot

The 3D response surface plots, based on the equation, were generated to show significant variables for NIR and non-significant variables for RITO. These plots provide a visual representation of response variation. Refer to Figure 5(a) for wavelength, Figure 5(b) for the volume of ethyl acetate, Figure 5(c) for solvent front, and Figure 5(d) for chamber saturation time. This study includes a 3D response surface plot, revealing which factors most and least affect the R_f of drugs. Figure 5(a) indicates that the R_f of NIR decreased as the wavelength increased from a lower to a higher level, and it also decreased as chamber saturation time rose. Figure 5(b) shows that the R_f of NIR decreased as the wavelength increased, and it also dropped with higher volumes of ethyl acetate. Figure 5(c) illustrates that the R_f of NIR decreased with increasing wavelength and increased as the solvent front rose. Figure 6(d) shows that the R_f of RITO increased with higher wavelengths and decreased as chamber saturation time went up. Figure 6(e) indicates that the R_f of RITO increased as the wavelength and volume of ethyl acetate rose. Figure 6(f) confirms that the R_f of RITO increased with higher wavelengths and increased as the solvent front rose.

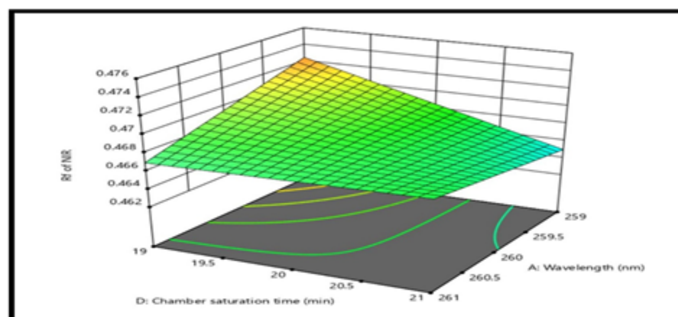


Figure 5 (a) 3D Response Surface Plot for R_f of NIR (A and D)

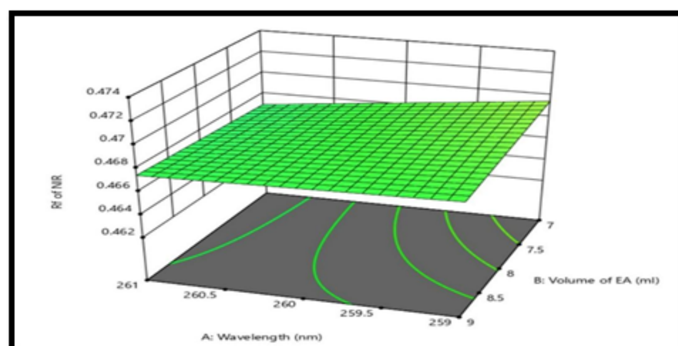


Figure 5 (b) 3D Response Surface Plot for R_f of NIR (A and B)

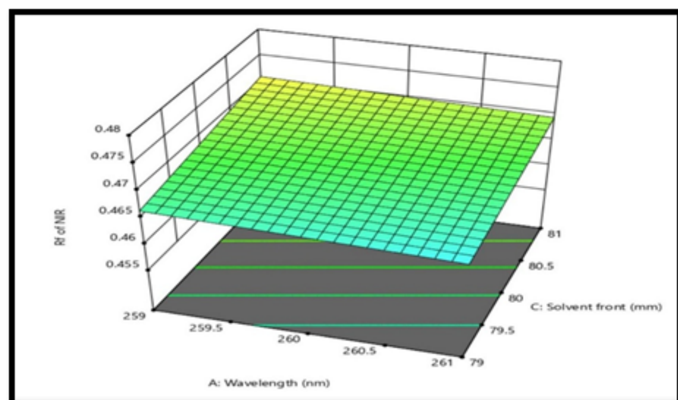


Figure 5 (c) 3D Response Surface Plot for R_f of NIR (A and C)

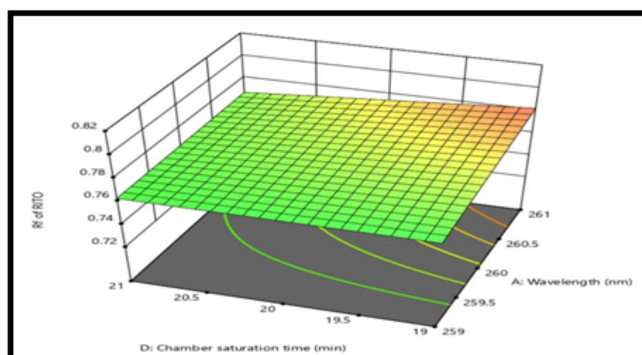


Figure 6 (d) 3D Response Surface Plot for R_f of RITO (A and D)

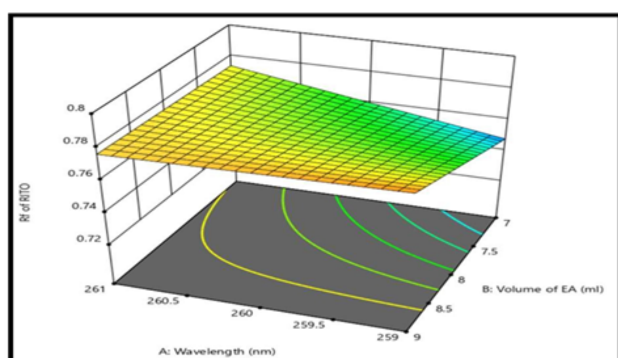


Figure 6 (e) 3D Response Surface Plot for R_f of RITO (A and B)

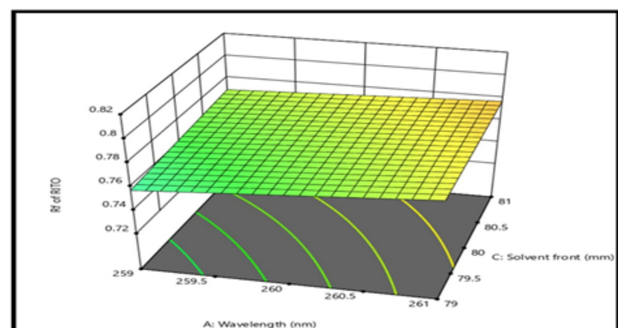


Figure 6 (f) 3D Response Surface Plot for R_f of RITO (A and C)

Figure 6 (d, e, f): 3D Response Surface Plots of RITO

Analysis of Marketed formulation

The developed and validated analytical procedure was successfully employed to evaluate the marketed tablet formulation. The assay results indicated drug contents of 99.68 ± 0.33% for Nirmatrelvir and 99.33 ± 2.63% for Ritonavir.

DISCUSSION

In the HPTLC method, 260nm wavelength was selected for NIR and RITO respectively for quantification. R_f for NIR and RITO was 0.46 and 0.78 nm using optimized mobile phase; Ethyl acetate: n-Hexane: Methanol (8:1.2:0.8 v/v/v).

The chromatographic method was validated for various parameters and % RSD which was within desirable limits. From the result of FFD, it seems that all four selected factors studied had a significant effect on the retention factor of NIR and a non-significant factor for RITO.

CONCLUSION

A practical, rapid, selective, dependable, and highly precise HPTLC method was successfully optimized for simultaneous quantification of both drugs and can be employed for quality control analysis of marketed dosage forms. The proposed method exhibited robustness. Data generated through fractional factorial experimental design confirmed the robustness of the developed analytical procedure. An advantage of the HPTLC technique lies in its ability to analyze multiple samples concurrently while using minimal mobile phase compared with HPLC, thereby lowering analysis duration, sample preparation requirements, and analytical cost for marketed formulations of both drugs without cross interference and with satisfactory recovery, precision, and consistent method performance.

ACKNOWLEDGEMENT

The authors express their sincere gratitude to the Parul Institute of Pharmacy and Research for their support.

DECLARATION OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

FUNDING SOURCES

The research was conducted without any financial support.

ABBREVIATIONS

RITO- Ritonavir

NIR- Nirmatrelvir

LOD-Limit of Detection

LOQ- Limit of Quantification

FFD- Full fractional Design

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