

Analytical Method Development and Validation with a Stability Indicating Assay Method of a Potential Antiviral Drug through UPLC Method

Ashish M.Rathi¹, Dr. Sarita Karole²

¹ Research Scholar, Faculty of Pharmacy, Oriental University, Indore

² Faculty of Pharmacy, Oriental University, Indore

Corresponding Author: Ashish M. Rathi, Research Scholar, Department of Pharmacy, Oriental University, Indore

Email Id: ashishrathi17@gmail.com

Abstract:

Background:

The present work aimed to develop and verify an analytical quality by design (AQbD) driven RP-UPLC approach for simultaneous estimate, continuous quality evaluation and forced degradation profiling of antiviral combination of Nirmatrelvir and Ritonavir.

Methods: Chromatographic resolution was obtained using a Shimadzu Phenyl column (150 × 4.6 mm, 5 μm) coupled to a Photodiode Array (PDA) detector. Two independent factors were evaluated, mobile phase organic composition (55%, 60% and 65% v/v Acetonitrile with 0.1% formic acid) and flow rate. Nine experimental design runs were performed and analysed for response metrics including retention time (RT) and theoretical plate counts (PC) for both selected therapeutic agents.

Results: Various optimisation trials revealed that the optimal chromatographic condition was 60.0% Acetonitrile and 1.0 mL/min flow rate with a Desirability of 1.00089. The optimised approach provided quick separation with retention periods of 2.88 min for Nirmatrelvir and 4.64 min for Ritonavir, with theoretical plate counts of 8920 and 14300, and tailing factors of 1.08 and 1.02, respectively. The method validated and showed strong linear response ($R^2 = 0.998$ for Nirmatrelvir; $R^2 = 0.9993$ for Ritonavir). The precision of the method gave the %RSD values for repeatability of 0.39% (Nirmatrelvir) and 0.19% (Ritonavir) and intraday (%RSD 0.66%–0.98% and 0.96%–1.04%) and inter-day precision (%RSD 0.39%–0.83% and 0.80%–1.20%) were within the ICH acceptable limits. The mean recovery accuracy throughout 80–120% spiking levels was 100.02–101.65% for Nirmatrelvir and 100.59–100.93% for Ritonavir. The commercial formulation was tested and exhibited mean drug content tests of 101.02% (%RSD 0.21%) for Nirmatrelvir and 100.03% (%RSD 0.75%) for Ritonavir. The method was validated for its stability-indicating capacity by forced degradation stress testing, which showed the primary susceptibility under alkali hydrolysis (99.76% degradation for Nirmatrelvir; 74.68% for Ritonavir) and acid hydrolysis (66.47% and 52.78%), moderate degradation under oxidative (47.63% and 18.64%) and thermal stress (30.54% and 0.70%), and high stability under photo degradation (0.49% and 2.37%).

Conclusion: The developed AQbD-RP-UPLC technique is quick, economical, precise, specific and stability indicating and may be used as a powerful tool for regular quality control, stability studies and regulatory compliance of Nirmatrelvir and Ritonavir bulk and formulation products.

Keywords: AQbD, RP-UPLC, Nirmatrelvir, Ritonavir, Method Validation, Stability-Indicating.

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Introduction:

UPLC plays an important role in the development and validation of analytical methods, delivering a step function improvement in resolution, speed and sensitivity over traditional HPLC. This is achieved by using stationary phases packed with 2 μm particles and operating at high linear velocities of the mobile phase, as predicted by the Van Deemter equation, providing ultrahigh chromatographic efficiency and peak capacity without any loss in performance. This technique for advanced columns enables the use of shorter columns to reduce run times by 10 to 20 times while preserving or enhancing resolution, allowing

quick, high-throughput, and continuous sample analysis (Buziau et al., 2020; Cheng et al., 2010). In addition, UPLC produces sharp peaks of high concentration with low band dispersion which considerably increases the sensitivity of the detector especially for mass spectrometry (MS) coupling so that it can obtain very low limits of detection (LOD) and quantification (LOQ) of impurities, metabolites and degradation products. The fast runtimes of UPLC are well suited to AQbD and Design of Experiments (DoE) workflows, enabling developers to rapidly screen multiple chromatographic variables, build a

robust Method Operable Design Region (MODR), and simplify downstream method validation and transfer with significantly lowered regulatory risk. In general, this approach is a very green and inexpensive alternative, using just a fraction of the organic solvent needed for conventional HPLC and reducing the total operating expenses (Afonso Ulrich et al., 2021; Dongre et al., 2008).

The QbD principles in analytical chemistry provide a systematic way to embed quality, robustness, and dependability into the method design from the beginning, rather than relying on conventional end-product testing (Prasad et al., 2020). Instead of laborious trial and error or one factor at a time assessments, QbD employs statistical DoE to uncover and quantify complicated simultaneous interactions occurring between crucial technique parameters such as mobile phase composition, flow rate and pH. This overall understanding defines a multidimensional Design Space which defines a safe operating boundary where routine adjustments do not affect method performance, thus providing regulatory flexibility and allowing operational changes within the Analytical Target Profile (ATP) without the need for formal re-approval and complete revalidation (Garg et al., 2015). In the end, this proactive framework minimises out of specification (OOS) errors, thereby greatly minimises the use of solvents and total development interruptions, and provides a significantly greater success rate for downstream method validation and multi-site transfer (Mishra et al., 2018).

Nirmatrelvir (Fig. 1A) is an orally bioavailable, peptidomimetic antiviral agent that functions as a potent, competitive, and reversible inhibitor of the SARS-CoV-2 main protease (Lamb, 2022). It is a moderately lipophilic, neutral Biopharmaceutics Classification System (BCS) Class IV compound characterized by a terminal electrophilic nitrile group, a trifluoroacetyl moiety, a pyrrolidinone side chain, and a bicyclic dimethyl-3-azabicyclo[3.1.0]hexane core (Gerhart et al., 2024). Ritonavir (Fig. 1B) is an oral antiretroviral peptidomimetic agent originally developed and approved as a human immunodeficiency virus type 1 (HIV-1) protease inhibitor. Chemically, it features a tripeptide structure incorporating two thiazole rings, carbamate functional groups, and aromatic side chains (Hashemian et al., 2023). Common short-term adverse effects include dysgeusia (altered taste), diarrhea, nausea, and vomiting (Hammond et al., 2022).

Recent literature highlights several validated chromatographic methods for ritonavir and nirmatrelvir focusing on simultaneous estimation in bulk, formulations, and stability studies. (Veerareddy

& Gandla, 2024) The methods were developed by using RP-HPLC, (Amrutha, 2025; Ashokbabu et al., 2026; Sumalatha et al., n.d.; Veerareddy & Gandla, 2024; Yassin et al., 2025) RP-HPLC with QbD, (Pandian et al., 2026) UV, (Gawand et al., 2025) and TLC (Imam et al., 2023)

This study advances AQbD by optimizing UPLC parameters for Ritonavir and nirmatrelvir as an Antiviral agent, that ensures specificity, precise and accurate amid evolving ICH Q14 standards. By addressing gaps in current literature on complex impurity profiles, the research aims to deliver a validated stability indicating method with superior resolution and minimal variability.

METHODS:

Instrumentation and Chemicals:

UPLC relies on specialized instrumentation, primarily Waters ACQUITY UPLC, Shimadzu phenyl column 150*4.6, 5micron, PDA Detector. Separations are achieved using Shimadzu Phenyl column (150 × 4.6 mm, 5 μm) was used for chromatographic separation, ultrasonic bath sonicators for extraction and degassing, vacuum filtration assemblies, The chemical mobile phases incorporate HPLC-grade organic solvents like acetonitrile paired with 0.1% formic acid. Forced degradation testing utilizes stress reagents such as 0.1N hydrochloric acid, 0.1N sodium hydroxide and 3% hydrogen peroxide.

Preparation of the standard solution:

Standard stock solution of Nirmatrelvir and Ritonavir was prepared by weighing accurately 10 mg of drug in to 10 mL volumetric flask, adding 7 mL of diluent to it, sonicating the contents and making up to the mark with diluent. Working standard solutions were prepared by transferring the required amount of standard stock solution into 10 mL volumetric flask, diluting to the mark, sonicating for 15 minutes and filtering through 0.45 μm filter.

Chromatographic Conditions:

The development of standard chromatographic method for UPLC is based on the selection of suitable stationary phase (Shimadzu phenyl column 150*4.6, 5micron). Mobile phases are mixtures of organic solvents (acetonitrile) with aqueous additions (0.1% formic acid), run in isocratic elution mode. The flow rates are usually between 0.8 and 1.2 ml/min. The column temperature is maintained at 25°C to manage separation efficiency and viscosity. Analyte monitoring is performed on compound specific absorption maxima using photodiode array (PDA) detector. Moreover, AQbD approaches with DoE (Full Factorial design), are often used in method development to systematically investigate the interaction effects of the ratio of organic solvent and

flow rate on retention time, theoretical plates and tailing factor.

Method optimization by Experimental Design:

The RP-UPLC technique for analysis of Ritonavir and Nirmatrelvir was optimised by utilising 3^2 full factorial design, where two independent variables the concentration of Acetonitrile in the mobile phase and the flow rate were considered to increase the efficiency of the method. The concentration of Acetonitrile was optimised at 55%, 60% and 65% (v/v with 0.1% formic acid) and the flow rate was studied at 0.8, 1.0 and 1.2 mL/min (Table 1). The design resulted in nine experimental runs which enabled a detailed assessment of the effect of changes in Acetonitrile concentration and flow rate on important chromatographic responses such as retention duration and number of theoretical plates. The factorial design technique allowed the calculation of the best chromatographic parameters and gave a better understanding of the interaction between the two variables, ensuring a method that is resilient and dependable according to QbD principles. Surface plots and ANOVA were used in the analysis of the experimental data to visualise the interaction effects of acetonitrile concentration and flow rate and to evaluate the statistical significance of each factor on the chromatographic results.

Method Validation:

Selectivity/Specificity:

Standard solutions of both the drugs and blank were injected separately into the UPLC machine for assessment. The process entails the analysis of the resultant chromatograms to ensure that there is no co eluting peaks which interfere with the principal analyte peaks.

Linearity:

It was assessed by preparing serial dilutions of standard stock solutions of ritonavir and Nirmatrelvir at numerous concentration levels ranging from 5-30 ug/ml and 7.5-45 ug/ml respectively. Each concentration level is injected into the chromatographic system. Calibration graphs was created as a function of peak area against analyte concentration. Linear regression analysis was used to compute the correlation coefficient (R^2) and the slope and intercept.

Precision:

Repeatability:

The performance was tested by creating and analysing six independent test preparations and injecting the duplicate standard solutions within one day under the same operating conditions. The peak areas were collected for all injections and used to calculate the %RSD.

Intraday Precision:

It was evaluated by injecting freshly prepared standard solutions across multiple concentration levels in triplicate within a single day (Morning, Afternoon and Evening). The peak area responses were recorded across all runs to determine intraday variation and calculated the %RSD.

Interday Precision:

It was performed by analyzing freshly prepared standard solutions for multiple consecutive days (Day 1 to Day 3). Results from the different days are compiled and evaluated to confirm method reproducibility, expressed as the overall %RSD for peak areas.

Accuracy:

Accuracy is evaluated using standard addition method, where known quantities of pure reference standards are spiked into pre-analyzed sample solutions at multiple concentration levels (80%, 100% and 120%) in triplicate. The spiked samples are extracted and analyzed using the UPLC method to measure the recovered amounts and calculate the mean percentage recovery and %RSD.

Stability Studies:

Stability studies encompass forced degradation testing. Forced degradation involves intentionally stressing drug samples under acid hydrolysis, alkali hydrolysis, oxidative stress, thermal stress and photolytic exposure and results were recorded.

RESULTS AND DISCUSSION

Factorial design:

Table 2 represents a two factor, multi response experimental design dataset evaluating the effects of mobile phase organic concentration and flow rate on the chromatographic retention time (RT) and plate count (PC) for Nirmatrelvir (Nir) and Ritonavir (Rit) in a RP-UPLC system. Factor 1 measures the percentage of acetonitrile (ACN) in the mobile phase, ranging from 55% to 65%, while Factor 2 represents the mobile phase flow rate across three levels (0.8, 1.0, and 1.2 mL/min). The experimental responses record the resulting elution characteristics, showing clear inverse relationships between both independent factors and the measured chromatographic outputs.

The data analysis revealed that raising the acetonitrile percentage or flow rate led to a substantial reduction in retention time and plate count for both analytes. Higher organic strength will reduce hydrophobic interactions with the stationary phase, making elution easier, and higher flow rates will enhance linear velocity, reducing run durations. For example, Run 8, with the lowest ACN content (55%) and lowest flow rate (0.8 mL/min), shows the longest retention durations (3.58 to 7.73 min) and the greatest plate counts (13250 for Nir and 22850 for Rit). In contrast, Run 4 with the greatest organic content (65%) and maximum flow rate (1.2 mL/min) gives the quickest

elution (2.07 and 3.02 min) but lower plate counts (6540 and 9450). As expected due to its increased lipophilicity under these reverse phase circumstances, Ritonavir generally showed longer retention and higher peak responses than Nirmatrelvir in all experimental runs.

Optimized Condition Obtained:

The numerical optimisation (table 3) of the 3^2 full factorial design resulted in an excellent chromatographic condition: mobile phase with 60.00% ACN and flow rate of 1.000 mL/min, with a perfect overall Desirability score of 1.000 (Figure 2). The model with these optimised parameters predicts rapid, well-resolved retention periods of 2.88 min for Nirmatrelvir and 4.6 min for Ritonavir with robust detector response values of 8920 and 14300, respectively. Overlay of these surfaces identifies the prediction flags exactly at the center point (60% ACN, 1.0 mL/min) indicating that this location gives the best combination of quick separation, sharp peak symmetry and highest analytical sensitivity.

The effect of Retention Time of Nirmatrelvir:

The statistical analysis and the 3D response surface plot (Figure 3) for the retention period of Nirmatrelvir validate a good 2FI model with strong prediction ability throughout the whole experimental design space. The analysis of variance (ANOVA) indicated that the whole model was statistically significant with F value of 140.97 ($p < 0.0001$). This implies a very low likelihood of 0.01% that such an F value is attributable to experimental noise. The two main independent factors have a significant effect on the retention of Nirmatrelvir. The most significant variable is Factor B (Flow Rate, $p < 0.0001$, $F = 268.83$) as it shows the highest sum of squares (2.29), and is closely followed by Factor A (Mobile Phase ACN Concentration, $p < 0.0001$, $F = 149.86$). The interaction term AB ($p = 0.0948$, $F = 4.23$) is non-significant at 5% level ($p > 0.05$) indicating the effect of mobile phase organic strength and linear velocity on Nirmatrelvir elution are independent of each other. The 3D response surface map shown beside presents a distinct, even slanting plain descending from a retention time of 4.26 minutes (55% ACN, 0.8 mL/min) to 2.07 minutes (65% ACN, 1.2 mL/min). This structural trend suggests that increasing concentrations of the organic modifier decrease hydrophobic interactions with the non-polar stationary phase, while increasing the flow rate increases the linear mobile phase velocity, allowing Nirmatrelvir retention to be accurately adjusted to preserve short run times without compromising peak resolution.

The effect of Theoretical plate of Nirmatrelvir:

Statistical analysis and 3D response surface plot (Figure 4) for Nirmatrelvir plate counts showed a

good 2FI model with strong prediction ability throughout the whole design space studied. The ANOVA shows that the model is extremely significant with an F value of 88.58 ($p < 0.0001$) showing only a 0.01% chance that a F value of this size can be attributed to experimental noise. The primary variables, Factor B (Flow Rate, $p < 0.0001$, $F = 173.37$) and Factor A (Mobile Phase ACN Concentration, $p = 0.0002$, $F = 89.52$), had a significant influence on the peak intensity with Factor B having a major effect, as shown in the sum of squares (2.128 times 107). The binary interaction term AB ($p = 0.1530$, $F = 2.84$) was statistically insignificant ($p > 0.05$), showing that the major variables acted separately, without a significant non-linear interaction.

The corresponding 3D surface plot shows a smooth descending plane surface indicating that the highest peak counts of 13250 is obtained at 55% ACN and 0.8 mL/min flow rate and the lowest peak counts of 6,540 is obtained at 65% ACN and 1.2 mL/min flow rate. This trend demonstrates that lower flow rates boost peak response by increasing analyte residence time in the flow cell, while decreased organic strength increases analyte focussing during column loading, thus maximising detector sensitivity for Nirmatrelvir.

The effect of Retention Time of Ritonavir:

The ANOVA analysis and the related 3D response surface plot (Figure 5) showed a high degree of statistical validity and prediction capacity of the retention time of Ritonavir using 2FI model. The whole model is highly significant with an F value of 91.55 ($p < 0.0001$) suggesting a 0.01% possibility that the observed variation is attributable to random experimental noise. Both main parameters have a statistically significant influence on analyte retention: The most significant contributor was Factor A (Mobile Phase ACN Concentration, $p < 0.0001$, $F = 169.39$), followed by Factor B (Flow Rate, $p = 0.0002$, $F = 100.62$). The interaction term AB ($p = 0.0840$, $F = 4.63$) is not statistically significant at 5% alpha ($p > 0.05$) and confirms that organic strength and linear velocity operate mainly as independent factors on retention behaviour. Visually, the 3D surface plot shows a steep, downwards sloping plane with retention times decreasing gradually from a high of 7.73 min (55% ACN, 0.8 mL/min) to a minimum of 3.02 min (65% ACN, 1.2 mL/min). Higher concentrations of organic modifier decrease the hydrophobic interaction with the stationary phase, which results in faster elution, while higher flow rates increase the linear mobile phase velocity, which provides fine control over the retention time of Ritonavir.

The effect of Theoretical plate of Ritonavir:

The plate counts for Ritonavir were evaluated using 2FI model and found to be statistically very significant and had a clear parametric dependency throughout the experimental design space. The Analysis of Variance (ANOVA) validated the statistical significance of the whole model with a F value of 106.02 ($p < 0.0001$), indicating that there is only a 0.01% likelihood of such a model being a result of experimental noise. The individual main effects of Mobile Phase ACN Concentration (A, $p < 0.0001$, $F = 200.02$) and Flow Rate (B, $p = 0.0001$, $F = 115.18$) are significantly contributing to the variability of Ritonavir peak area, with the relative contribution of the mobile phase composition being more due to its higher sum of squares (8.513 times 107). In contrast, the binary interaction term (AB) is statistically not significant ($p = 0.1526$). This means that the variables do not interfere with each other in a synergistic or antagonistic way but function independently. This linear multi-variable behaviour is visibly confirmed in the 3D surface plot (Figure 6): a flat slope gradually dropping from maximum peak counts (22850) at 55% ACN and 0.8 mL/min to minimum values (9450) at 65% ACN and 1.2 mL/min. The consistent slope demonstrates that lower flow rates improve signal intensity by extending residence time in the detector flow cell and lower organic strength sharpens peak focus, both leading to higher total chromatographic counts.

Analytical Method Validation

System suitability/Specificity:

System suitability parameters (table 4 and figure 7-10) confirm column efficiency and chromatographic separation under optimized UPLC conditions. Nirmatrelvir demonstrated a retention time of 2.88 minutes, 8920 theoretical plates, and a tailing factor of 1.08. Ritonavir exhibited a retention time of 4.64 minutes, 14300 theoretical plates, and a tailing factor of 1.02. There is no interference of placebo/blank at the RT of both the drugs.

Linearity:

Linearity was evaluated across multiple concentration levels to verify a direct proportional response between analyte concentration and peak area. The linear regression equation for Ritonavir was $y = 3316x + 526.91$ with a correlation coefficient of 0.9993. For Nirmatrelvir, the regression equation was $y = 1951.4x + 1088$ with a correlation coefficient of 0.998 (table 5 and figure 11-12).

Accuracy:

Accuracy was assessed using standard addition recovery studies across 80%, 100%, and 120% spiked levels in triplicate. For Ritonavir, the mean percentage recoveries were 100.931% (%RSD 0.219%) at the 80% level, 100.59% (%RSD 1.553%) at the 100% level, and 100.77% (%RSD 0.811%) at

the 120% level. For Nirmatrelvir, mean percentage recoveries were 100.350% (%RSD 1.093%) at the 80% level, 101.65% (%RSD 0.718%) at the 100% level, and 100.02% (%RSD 0.707%) at the 120% level (Table 6).

Precision:

Precision was established across repeatability, intraday precision, and inter-day precision evaluations. In repeatability testing across six independent preparations, Ritonavir achieved a mean assay of 100.2805% with a %RSD of 0.194185%, while Nirmatrelvir achieved a mean assay of 100.3243% with a %RSD of 0.391813%. Intraday precision showed %RSD values ranging between 0.963% and 1.044% for Ritonavir and 0.65575% and 0.98489% for Nirmatrelvir. Inter-day precision over consecutive days yielded %RSD values between 0.79999% and 1.20000% for Ritonavir and 0.39362% and 0.83175% for Nirmatrelvir (Table 7-9).

Sensitivity:

The LOD and LOQ for both the drugs were mentioned in table 10.

Analysis of Marketed Formulation:

Marketed formulation analysis confirmed the practical applicability of the method for quantifying drug content in commercial samples. Ritonavir yielded a mean peak area of 84,505.29, resulting in a mean assay of 100.03% and a %RSD of 0.753%. Nirmatrelvir showed a mean peak area of 75,006.33, resulting in a mean assay of 101.0179% and a %RSD of 0.20789%.

Stability Study:

Forced degradation studies verified the stability-indicating capacity of the method under varied environmental stress conditions (Table 12). Acid hydrolysis caused 52.78336 % degradation in Ritonavir and 66.47286 % in Nirmatrelvir. Alkali hydrolysis resulted in 74.676% degradation for Ritonavir and 99.757% for Nirmatrelvir. Oxidative stress induced 18.643% degradation in Ritonavir and 47.63227 % in Nirmatrelvir. Photo degradation led to 2.374615 % for Ritonavir and 0.493828% for Nirmatrelvir. Thermal stress resulted in 0.697622% degradation in Ritonavir and 30.54113 % in Nirmatrelvir.

Conclusion:

The developed AQbD-driven RP-UPLC method provides a rapid, precise, cost-effective, and stability-indicating technique for the simultaneous estimation of Nirmatrelvir and Ritonavir. Through numerical optimization, ideal chromatographic separation was achieved using a mobile phase of 60.0% Acetonitrile (with 0.1% formic acid) at a 1.0 mL/min flow rate, resulting in fast, well-resolved retention times of 2.88 minutes for Nirmatrelvir and 4.64 minutes for Ritonavir. Validation confirmed

strong linearity, high precision, and mean recovery accuracies of 100.02%–101.65% for Nirmatrelvir and 100.59%–100.93% for Ritonavir, alongside successful assay performance on commercial formulations. Additionally, forced degradation stress testing verified its stability indicating capacity, identifying primary drug susceptibility under alkali and acid hydrolysis. Overall, this robust method offers an efficient tool for routine quality control, stability evaluation, and regulatory compliance of bulk and formulation antiviral products.

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List of Figures:

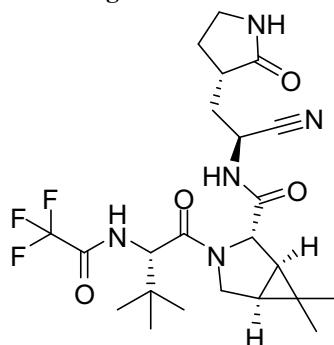


Figure 1A: Chemical structure of Nirmatrelvir

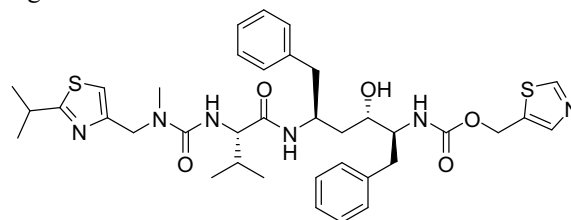


Figure 1B: Chemical structure of Ritonavir

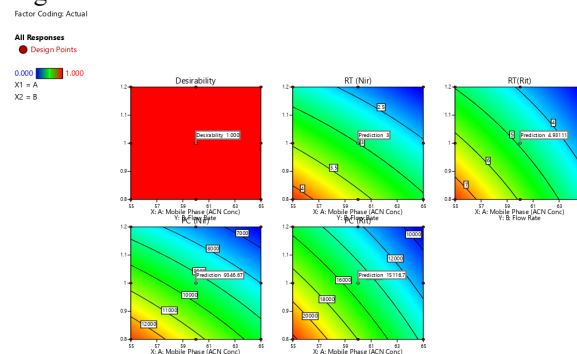


Figure 2: Contour plot of desirability for obtaining optimized trail

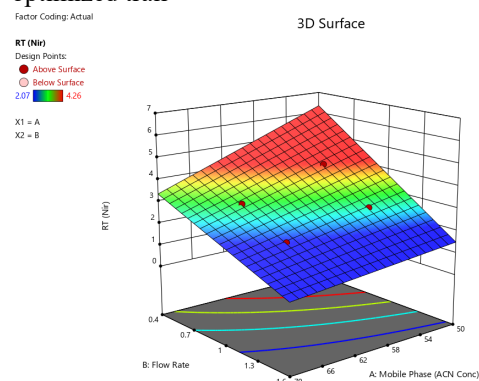


Figure 3: The effect of Retention Time of Nirmatrelvir

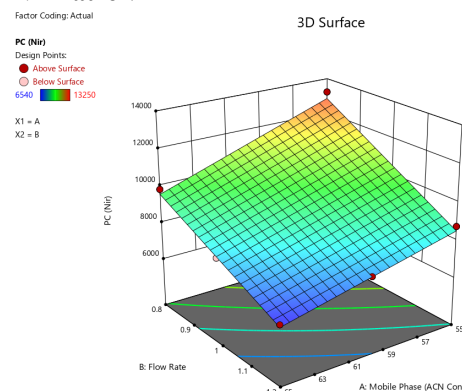


Figure 4: The effect of Theoretical plate of Nirmatrelvir

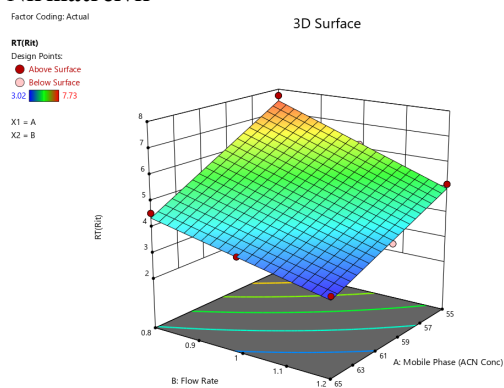


Figure 8: Chromatogram for Standard Nirmetravir.

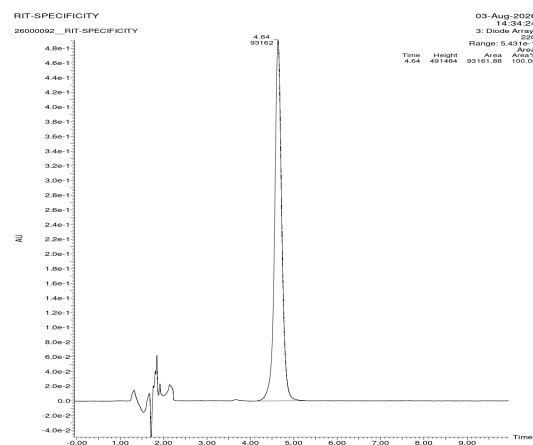


Figure 5: The effect of Retention Time of Ritonavir

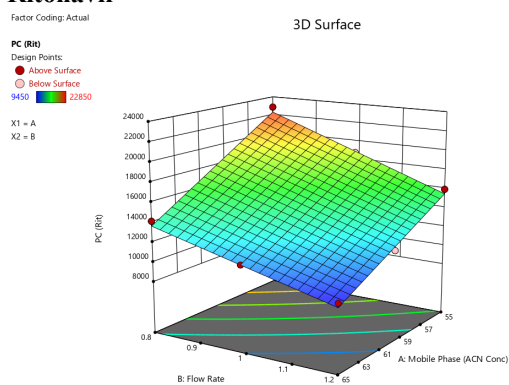


Figure 9: Chromatogram for Standard Ritonavir.

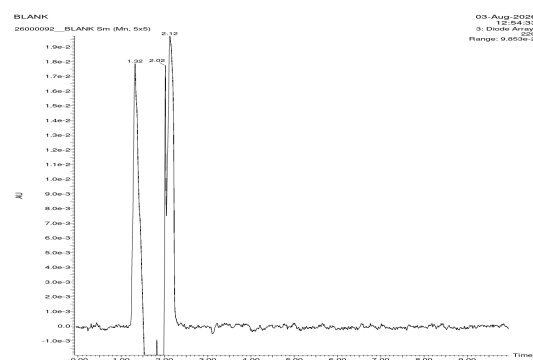


Figure 6: The effect of Theoretical plate of Ritonavir

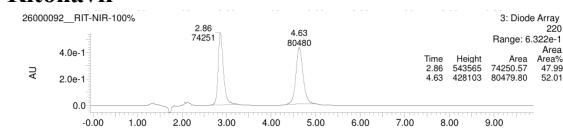


Figure 7: Suitability of the Device Standard Chromatogram

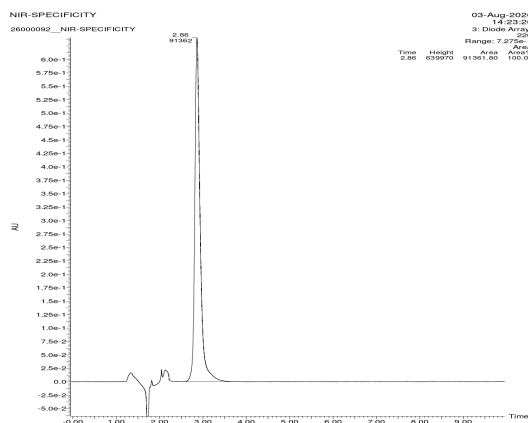


Figure 10: Chromatogram for Blank

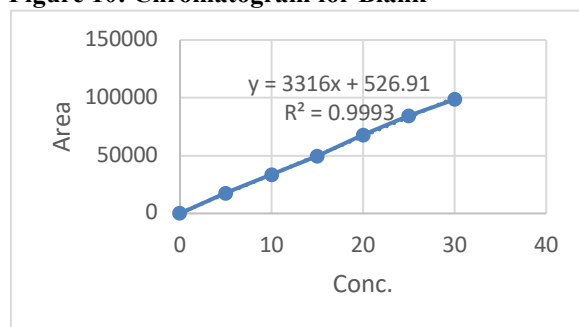


Figure 11: Linearity graph of Ritonavir

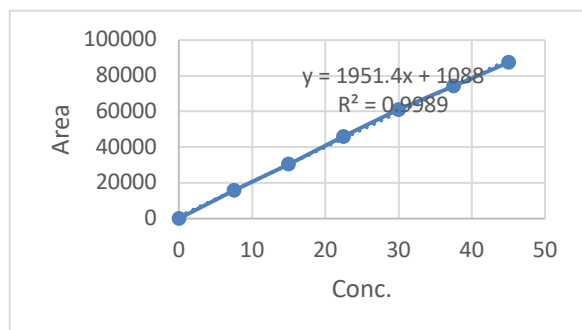


Figure 12: Linearity graph of Nirmatrelvir

List of Table:

Table 1: Factors for independent variables

Factor	Name	Level	Low Level	Medium Level	High Level
A	Mobile phase composition (% of Acetonitrile)	3	55	60	65
B	Flow Rate	3	0.8	1.0	1.2

Table 2: Optimization of parameters for analysis of Ritonavir and Nirmatrelvir by using 3² full factorial design

	Factor 1	Factor 2	Response 1	Response 2	Response 3	Response 4
R	A: Mobile Phase (ACN Conc)	B: Flow Rate	RT (Nir)	RT (Rit)	PC (Nir)	PC (Rit)
1	65	0.8	3.13	4.55	9850	14150
2	55	1	3.39	6.18	10420	18750
3	60	0.8	3.58	5.73	10980	17400
4	65	1.2	2.07	3.02	6540	9450
5	60	1.2	2.37	3.8	7480	11850
6	60	1	2.88	4.6	8920	14300
7	55	1.2	2.82	5.14	8760	15950
8	55	0.8	4.26	7.73	13250	22850

9	65	1	2.5	3.63	7920	11350
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Table 3: Obtained solution for optimized trail of Ritonavir and Nirmatrelvir by using 3² full factorial design

Number	Mobile Phase (ACN Conc)	Flow Rate	RT (Nir)	RT (Rit)	PC (Nir)	PC (Rit)	Desirability
1	60.00	1.00	2.88	4.3	8920	14300	1.000

Table 4: System suitability parameters

Sr.	Parameter	Nirmatrelvir	Ritonavir
1.	Theoretical	8920	14300
2.	Retention Time	2.88	4.64
3.	Tailing factor	1.08	1.02

Table 5: Linearity results for Ritonavir and Nirmatrelvir

Level	Area	Level	Area (Nirmatrelvir)
0	0	0	0
5	17401.77	7.5	15765.24
10	33665.42	15	30410.13
15	49718.48	22.5	46090.52
20	68030.09	30	61049.55
25	84479.8	37.5	74250.57
30	98573.3	45	87393.71
Regression	y = 3316x		y = 1951.4x +
Correlation	0.9993		0.998

Table 6: Accuracy study for Ritonavir and Nirmatrelvir

Ritonavir			
Concentration % of the spiked level	Amount added (ppm)	Statistical Analysis of % Recovery	
80 %	18	Mean % Recovery	100.931
		% RSD	0.219
100 %	20	Mean % Recovery	100.59
		% RSD	1.553
120 %	22	Mean % Recovery	100.77

		% RSD	0.811
Nirmatrelvir			
80 %	27	Mean % Recovery	100.350
		% RSD	1.093
100 %	30	Mean % Recovery	101.65
		% RSD	0.718
120 %	33	Mean % Recovery	100.02
		% RSD	0.707

Table 7: Repeatability data for Ritonavir and Nirmatrelvir

Sr.	Ritonavir		Nirmatrelvir	
	Area	%Assay	Area	%Assay
1.	84522.0	100.05	74963	100.959
2.	84761.7	100.333	74270.7	100.027
3.	84880.3	100.474	74544.5	100.395
4.	84690.1	100.249	74512.2	100.352
5.	84907.4	100.506	74106.6	99.8061
6.	84539.1	100.070	74550.9	100.404
Mean	84716.7	100.280	74491.3	100.324
SD	164.507	0.19473	291.867	0.39308
%RS	0.19418	0.19418	0.39181	0.39181

Table 8: Intraday Precision data of Ritonavir

Sr. no.	Ritonavir			Nirmatrelvir		
	Conc. (µg/ml)	Mean Peak Area	% RSD	Conc. (µg/ml)	Mean Peak Area	% RSD
1	10	33665.42	1.040	15	3041013	0.81853
2	15	49744.8133	0.963	22.5	4609052	0.98489
3	20	68030.09	1.044	30	6104955	0.65575

Table 9: Inter-day Precision data of Nirmatrelvir

Sr.	Ritonavir			Nirmatrelvir		
	Conc. (µg/ml)	Mean Peak Area	% RSD	Conc. (µg/ml)	Mean Peak Area	% RSD

no.				ml)		
1	10	3366542	0.79999	15	3041013	0.39362
2	15	4971848	0.99999	22.5	4609052	0.83175
3	20	6803009	1.20000	30	6104955	0.74127

Table 10: LOD and LOQ values

Parameters	Ritonavir	Nirmatrelvir
LOD (ug/ml)	0.404 µg/ml	0.35 µg/ml
LOQ (ug/ml)	1.224 µg/ml	1.05 µg/ml

Table 11: Analysis of marketed formulations for Ritonavir and Nirmatrelvir

Injection	Ritonavir		Nirmatrelvir	
	Peak Areas	% Assay	Peak Areas	% Assay
1	83522.02	98.86	74892	100.8639
2	85318.47	100.99	75145	101.2046
3	84691.35	100.25	74786	100.7211
4	84157.82	99.61	75038	101.0605
5	84976.14	100.58	74974	100.9743
6	84365.96	99.86	75203	101.2827
Mean	84505.29	100.03	75006.333	101.0179
SD	636.325	0.753	155.9316089	0.210007
% RSD	0.753	0.753	0.207891257	0.207891

Table 12: Stability Study data for Ritonavir and Nirmatrelvir

Stress type	Stress conditions	% Degradation of Ritonavir	% Degradation of Nirmatrelvir
Acid Hydrolysis	1 N HCl, 6 h	52.78336	66.47286

Analytical Method Development and Validation with a Stability Indicating Assay Method of a Potential Antiviral Drug through UPLC Method

Alkali Hydrolysis	1 N NaOH, 6 h	74.67625	99.75721
Oxidative Hydrolysis	3% H ₂ O ₂ , 6 h	18.64312	47.63227
Photo Degradation	UV under 254 nm, 24 h	2.374615	0.493828
Thermal Hydrolysis	60 °C, 24 h	0.697622	30.54113