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Research Article

A Validated Stability-Indicating RP-HPLC Method for Simultaneous Determination of Nirmatrelvir and Ritonavir in Bulk Drug and Marketed Pharmaceutical Dosage Forms

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ABSTRACT

A rapid and precise reverse phase high performance liquid chromatographic method has been developed for the validated of Nirmatrelvir and Ritonavir, in its pure form as well as in marketed pharmaceutical dosage forms. This Chromatographic Separation was carried out on Develosil C18 (4.6mm×250mm) 5µm particle size Column using a mixture of Acetonitrile and Acetate buffer (pH-4.3) was taken in the ratio of 35:65% v/v as the mobile phase at a flow rate of 1.0 mL/min, the wavelength detection was carried out at 238nm. The retention time of the Nirmatrelvir and Ritonavir was found to be 2.179, 3.610 ± 0.02 min respectively. The method produce linear responses in the concentration range of 20-60µg/ml of Nirmatrelvir and 10-30µg/ml of Ritonavir respectively. The developed method precision for the determination of assay was below 2.0%RSD. The developed method is useful in the quality control of bulk and pharmaceutical formulations.

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INTRODUCTION

Nirmatrelvir (PF-07321332) is an orally bioavailable 3C-like protease (3CLPRO) inhibitor that is the subject of clinical trial NCT04756531. 3CLPRO is responsible for cleaving polyproteins 1a and 1ab of SARS-CoV-2. Without the activity of the SARS-CoV-2 3CLPRO, nonstructural proteins (including proteases) cannot be released to perform their functions, inhibiting viral replication [1]. In the US, Europe, and Canada, Nirmatrelvir, in combination with ritonavir, is indicated for the treatment of mild-to-moderate coronavirus disease 2019 (COVID-19) in adults who are at high risk for progression to severe COVID-19, including hospitalization or death. In Europe, this therapeutic indication is approved under conditional marketing authorization [2]. Nirmatrelvir is a SARS-CoV-2 main protease inhibitor. It works by preventing the growth of the virus that causes COVID-19. Ritonavir increases ("boosts") the levels of Nirmatrelvir. This helps Nirmatrelvir work better [3]. The IUPAC name of Nirmatrelvir is 5 (1R, 2S, 5S)-N-[(1S)-1-cyano-2-[(3S)-2-oxo pyrrolidin-3-yl] ethyl]-3-[(2S)-3, 3-dimethyl-2-[(2, 2, 2-trifluoro acetyl) amino] butanoyl]-6, 6-dimethyl-3-azabi cyclo [3.1.0] hexane-2-carboxamide [3]. The Chemical Structure of Nirmatrelvir is shown in follows

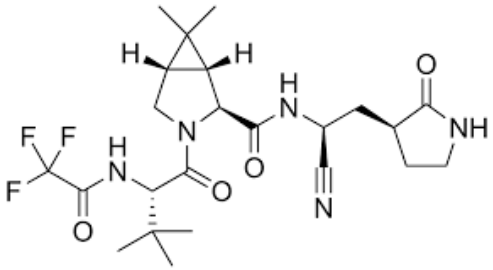


Figure None: 1: Chemical Structure of Nirmatrelvir

Ritonavir is an antiretroviral protease inhibitor that is widely used in combination with other protease inhibitors in the therapy and prevention of human immunodeficiency virus (HIV) infection and the acquired immunodeficiency syndrome (AIDS) [4]. Ritonavir can cause transient and usually asymptomatic elevations in serum aminotransferase levels and, rarely, can lead to clinically apparent acute liver injury. In HBV or HCV coinfected patients, highly active antiretroviral therapy with ritonavir may result of an exacerbation of the underlying chronic hepatitis B or C. Ritonavir is indicated in combination with other antiretroviral agents for the treatment of HIV-1 infection [5]. Ritonavir is used along with other medications to treat human immunodeficiency virus (HIV) infection. Ritonavir is in a class of medications called protease inhibitors. It works by decreasing the amount of HIV in the blood. The IUPAC name of Ritonavir is 1, 3-thiazol-5-ylmethyl N-[(2S, 3S, 5S)-3-hydroxy-5-[[(2S)-3-methyl-2-[[methyl-[(2-propan-2-yl-1, 3-thiazol-4-yl) methyl] carbamoyl] amino] butanoyl] amino]-1, 6-diphenyl hexan-2-yl] carbamate [6]. The Chemical Structure of Ritonavir is shown in following Figure-2.

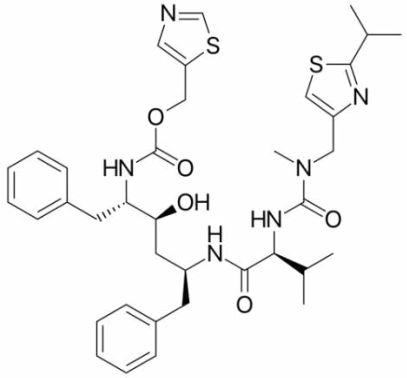


Figure None: 2: Chemical Structure of Ritonavir

MATERIALS AND METHODS

Instruments Used:

Table None: 1: Instruments Used

S.No.	Instruments and Glasswares	Model
1	HPLC	WATERS Alliance 2695 separation module, Software: Empower 2, 996 PDA detector.
2	pH meter	Lab India
3	Weighing machine	Sartorius
4	Volumetric flasks	Borosil

S.No.	Instruments and Glasswares	Model
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil
7	Digital Ultra Sonicator	Labman

Chemicals Used:

Table None: 2: Chemicals Used

S.No.	Chemical	Brand Names
1	Nirmatrelvir (Pure)	Synpharma Research Lab, Hyderabad
2	Ritonavir (Pure)	Synpharma Research Lab, Hyderabad
3	Water and Methanol for HPLC	LICHROSOLV (MERCK)
4	Acetonitrile for HPLC	Merck
5	Acetic Acid	Merck

HPLC Method Development:

Preparation of Standard Solution:

Accurately weigh and transfer 10 mg of Nirmatrelvir and Ritonavir 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 0.4ml of Nirmatrelvir and 0.2ml of Ritonavir from the above 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 guidelines [27].

Mobile Phase Optimization:

Initially the mobile phase tried was Methanol: Water and ACN: Water with varying proportions. Finally, the mobile phase was optimized to Acetonitrile and Acetate buffer (pH-4.3) in proportion 35:65% v/v respectively.

Optimization of Column:

The method was performed with various C18 columns like Symmetry, X terra and ODS column. Develosil C18 (4.6mm×250mm) 5µm particle size Column was found to be ideal as it gave good peak shape and resolution at 1ml/min flow [7].

Preparation of Mobile Phase:

Accurately measured 350ml of Acetonitrile (35%) of and 650ml of Acetate buffer (65%) were mixed and de-gassed in a digital ultra sonicater for 20 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:

The Mobile phase was used as the diluent.

Method Validation Parameters

System Suitability

Accurately weigh and transfer 10 mg of Nirmatrelvir and Ritonavir 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 [8]. (Stock solution)**.** Further pipette out 0.4ml of Nirmatrelvir and 0.2ml of Ritonavir from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

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 limits.

Specificity Study of Drug:

Preparation of Standard Solution:

Accurately weigh and transfer 10 mg of Nirmatrelvir and Ritonavir 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 out 0.4ml of Nirmatrelvir and 0.2ml of Ritonavir from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent [9].

Preparation of Sample Solution:

Take average weight of Tablet and crush in a mortar by using pestle and weight 10 mg equivalent weight of Nirmatrelvir and Ritonavir 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. Filter the sample solution by using injection filter which contains 0.45µ pore size. Further pipette out 0.4ml of Nirmatrelvir and 0.2ml of Ritonavir Sample solution from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent [10].

Procedure:

Inject the three replicate injections of standard and sample solutions and calculate the assay by using formula [11-13]:

%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 and Range:

Accurately weigh and transfer 10 mg of Nirmatrelvir and Ritonavir 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 [14]. (Stock solution)

Preparation of Level – I (20ppm of Nirmatrelvir and 10ppm of Ritonavir):

Pipette out 0.2ml of Nirmatrelvir and 0.1ml of Ritonavir in to a 10ml volumetric flask and make the volume upto mark by using diluent and sonicate for air entrapment.

Preparation of Level – II (30ppm of Nirmatrelvir and 15ppm of Ritonavir):

Pipette out 0.3ml of Nirmatrelvir and 0.15ml of Ritonavir in to a 10ml volumetric flask and make the volume upto mark by using diluent and sonicate for air entrapment.

Preparation of Level – III (40ppm of Nirmatrelvir and 20ppm of Ritonavir):

Pipette out 0.4ml of Nirmatrelvir and 0.2ml of Ritonavir in to a 10ml volumetric flask and make the volume upto mark by using diluent and sonicate for air entrapment [15].

Preparation of Level – IV (50ppm of Nirmatrelvir and 25ppm of Ritonavir):

Pipette out 0.5ml of Nirmatrelvir and 0.25ml of Ritonavir in to a 10ml volumetric flask and make the volume upto mark by using diluent and sonicate for air entrapment.

Preparation of Level – V (60ppm of Nirmatrelvir and 30ppm of Ritonavir):

Pipette out 0.6ml of Nirmatrelvir and 0.3ml of Ritonavir in to a 10ml volumetric flask and make the volume up to mark by using diluent and sonicate for air entrapment.

Procedure:

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

Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient [16-18].

Precision

Repeatability

Preparation of Nirmatrelvir and Ritonavir Product Solution for Precision:

Accurately weigh and transfer 10 mg of Nirmatrelvir and Ritonavir 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 out 0.4 ml of Nirmatrelvir and 0.2ml of Ritonavir from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

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 limits [19].

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:

Day 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 [20].

Day 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 limits.

Accuracy:

For preparation of 50% Standard stock solution:

Accurately weigh and transfer 10mg of Nirmatrelvir and Ritonavir 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 out 0.2ml of Nirmatrelvir and 0.1ml of Ritonavir from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent [21].

For preparation of 100% Standard stock solution:

Accurately weigh and transfer 10 mg of Nirmatrelvir and Ritonavir 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 out 0.4ml of Nirmatrelvir and 0.2ml of Ritonavir from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

For preparation of 150% Standard stock solution:

Accurately weigh and transfer 10 mg of Nirmatrelvir and Ritonavir 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 out 0.6ml of Nirmatrelvir and 0.3ml of Ritonavir from the above stock solutions into a 10ml volumetric flask and dilute up to the 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 Nirmatrelvir and Ritonavir and calculate the individual recovery and mean recovery values [22].

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 Nirmatrelvir and Ritonavir 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 out 0.4ml of Nirmatrelvir and 0.2ml of

Ritonavir from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Effect of Variation of Flow Conditions:

The sample was analyzed at 0.9 ml/min and 1.1 ml/min instead of 1ml/min, remaining conditions are same. 20µl of the above sample was injected and chromatograms were recorded [23].

Effect of Variation of Mobile Phase Organic Composition:

The sample was analyzed by variation of mobile phase i.e. Acetonitrile: Acetate buffer was taken in the ratio 40:60 and 30:70 instead of 35:65 remaining conditions are same. 20µl of the above sample was injected and chromatograms were recorded.

RESULTS AND DISCUSSION

Optimization of an Analytical Method:

Optimized Chromatographic Conditions:

Mobile phase ratio : Acetonitrile and Acetate buffer (pH-4.3) (35:65% v/v)

Column : Develosil C18 (4.6mm×250mm) 5µm particle size Column

Column temperature : Ambient

Wavelength : 238nm

Flow rate : 1ml/min

Injection volume : 20µl

Run time : 6minutes

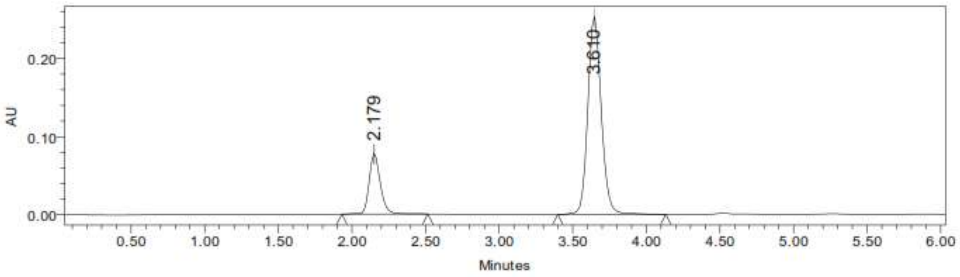


Figure None: 3: Optimized Chromatogram of Nirmatrelvir and Ritonavir

Method Validation

The validation parameters, such as linearity, specificity, precision, and accuracy, limit of detection (LOD), limit of quantification (LOQ), robustness and ruggedness repeatability were assessed in accordance with the ICH Q2 (R1) guidelines [27].

System Suitability:

Five distinct injections of Nirmatrelvir and Ritonavir solutions were used to evaluate the technique's applicability. The retention time, peak area, column efficiency, peak symmetry, and theoretical plates were calculated for standard solutions [24].

Table None: 3: Results of System Suitability for Nirmatrelvir

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Nirmatrelvir	2.152	526358	86598	5695	1.56
2	Nirmatrelvir	2.157	526548	86254	5652	1.57
3	Nirmatrelvir	2.141	526854	86598	5627	1.56
4	Nirmatrelvir	2.133	526598	86245	5692	1.57
5	Nirmatrelvir	2.166	524874	86521	5641	1.56
Mean			**526246.4**			
Std. Dev			**787.353**			
% RSD			**0.149617**			

Table None: 4: Results of System Suitability for Ritonavir

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing	Resolution
1	Ritonavir	3.674	1682821	1686958	8659	1.56	9.8
2	Ritonavir	3.631	1682726	1685745	8675	1.57	9.9
3	Ritonavir	3.625	1687361	1685421	8692	1.56	9.8
4	Ritonavir	3.692	1682811	1685242	8642	1.57	9.8
5	Ritonavir	3.629	1683816	1685364	8635	1.58	9.8
Mean			**1683907**				
Std. Dev			**1982.03**				
% RSD			**0.117704**				

Specificity (Assay)

In accordance with ICH guidelines, the assay method must be capable of quantifying the active pharmaceutical ingredient in the presence of excipients, impurities, and degradation products without interference. The assay was performed on tablet formulations. The percentage of assay for the drug content was determined. The results demonstrate that the method is both accurate and specific, enabling precise measurement of the active ingredient in the presence of excipients in the tablet matrix [25].

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

CONCLUSION:

The % purity of Nirmatrelvir and Ritonavir in pharmaceutical dosage form was found to be 99.89%

Linearity

A precisely weighed amount of 10 mg of the drug was transferred into a 10 mL volumetric flask and diluted with solvent to the calibration mark, yielding a concentration of 1000 µg/mL. To assess the drug's linear response, concentrations ranging from 20 to 60µg/mL and 10 to 30µg/mL for Nirmatrelvir and Ritonavir respectively were prepared from this stock solution. A calibration curves was constructed by plotting the peak area against the corresponding concentrations. The linearity of the response was evaluated, and the correlation coefficients (r2) and y-intercepts were determined from the resulting curves [26].

Table None: 5: Chromatographic Data for Linearity Study of Nirmatrelvir

Concentration µg/ml	Average Peak Area
20	272897
30	402986
40	526389
50	649785
60	769287

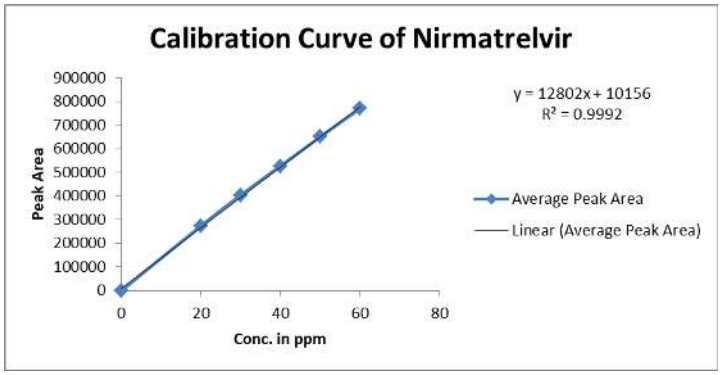


Figure None: 4: Calibration Curve of Nirmatrelvir

Table None: 6: Chromatographic Data for Linearity Study of Ritonavir

Concentration µg/ml	Average Peak Area
10	1000237
15	1448768
20	1887285
25	2365897
30	2826845

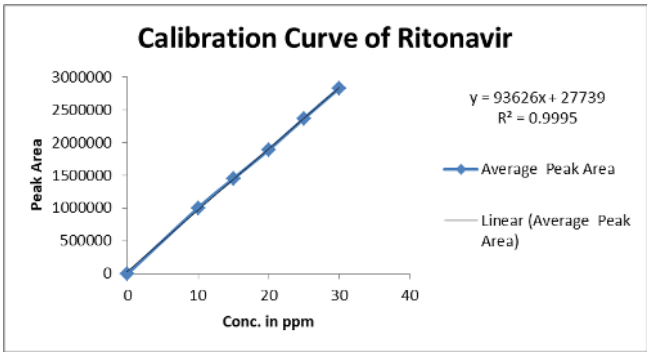


Figure None: 5: Calibration Curve of Ritonavir

Precision

A standard solution of 40µg/ml and 20µg/ml was prepared and injected 5 times into the system. A chromatogram was recorded and the %RSD of the Peak Area was calculated [28].

Table None: 7: Results of Repeatability for Nirmatrelvir

S. No.	Peak Name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Nirmatrelvir	2.157	526358	86598	5689	1.56
2	Nirmatrelvir	2.159	524856	86542	5687	1.57
3	Nirmatrelvir	2.186	526985	86578	5684	1.56
4	Nirmatrelvir	2.160	528654	86354	5689	1.56
5	Nirmatrelvir	2.170	528457	86958	5639	1.56
Mean			**527062**			
Std Dev			**1569.114**			
%RSD			**0.297709**			

Table None: 8: Results of Repeatability for Ritonavir:

S. No.	Peak Name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Ritonavir	3.603	1687589	367859	8659	1.79
2	Ritonavir	3.608	1685987	368547	8679	1.80
3	Ritonavir	3.600	1685987	367985	8645	1.80
4	Ritonavir	3.696	1685754	365874	8695	1.79
5	Ritonavir	3.629	1685985	364589	8625	1.79
Mean			**1686260**			
Std Dev			**749.493**			
%RSD			**0.044447**			

Intermediate Precision

The intermediate precision was calculated by measuring the responses of a standard peak on another day of the same solution concentration.

Day-I:

Table None: 9: Results of Intermediate Precision for Nirmatrelvir (Day-1)

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USPPlate count	USPTailing	%Assay
1	Nirmatrelvir	2.198	546585	87589	5898	1.58	100%
2	Nirmatrelvir	2.196	548758	87985	5879	1.59	100%
3	Nirmatrelvir	2.160	549854	87452	5868	1.58	100%
4	Nirmatrelvir	2.160	548798	87421	5847	1.59	100%
5	Nirmatrelvir	2.160	542659	87963	5896	1.58	100%
6	Nirmatrelvir	2.186	548754	87254	5874	1.59	100%
Mean			547568				
Std. Dev			2631.576				
% RSD			0.480593				

Table None: 10: Results of Intermediate Precision for Ritonavir

S.No.	Peak Name	Rt	Area (µV*sec)	Height (µV)	USPPlate Count	USPTailing	Resolution
1	Ritonavir	3.623	1698587	385482	8789	1.81	9.8
2	Ritonavir	3.611	1698574	385698	8759	1.80	9.8
3	Ritonavir	3.696	1698532	385748	8754	1.81	9.9
4	Ritonavir	3.696	1698574	386958	8754	1.81	10.01
5	Ritonavir	3.696	1698532	385755	5798	1.80	9.98
6	Ritonavir	3.642	1698547	386558	8762	1.80	10.02
Avg			**1698558**				
Std. Dev			**23.77113**				
% RSD			**0.001399**				

Day-II:

Table None: 11: Results of Intermediate Precision Day 2 for Nirmatrelvir

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USPPlate Count	USPTailing
1	Nirmatrelvir	2.198	536854	8758	5789	1.58
2	Nirmatrelvir	2.196	536985	8795	5726	1.59
3	Nirmatrelvir	2.178	536587	8746	5742	1.58
4	Nirmatrelvir	2.142	532546	8754	5746	1.59
5	Nirmatrelvir	2.177	534587	8725	5798	1.58
6	Nirmatrelvir	2.177	538598	8726	5785	1.59
Mean			**536026.2**			
Std. Dev			**2131.492**			
% RSD			**0.397647**			

Table None: 12: Results of Intermediate Precision Day 2 for Ritonavir

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USPPlate Count	USPTailing	Resolution
1	Ritonavir	3.611	1678598	356875	8875	1.82	9.9
2	Ritonavir	3.623	1678985	358985	8856	1.83	10.01
3	Ritonavir	3.684	1678984	358754	8862	1.82	9.9
4	Ritonavir	3.697	1678985	352412	8849	1.83	10.01
5	Ritonavir	3.684	1678549	358987	8873	1.82	9.9
6	Ritonavir	3.684	1678984	358986	8842	1.83	10.01
Mean			**1678848**				
Std. Dev			**212.8048**				
% RSD			**0.012676**				

Accuracy

The accuracy of the method was determined by spiking with a known amount of drug to result in sample solutions with the following levels of drug 0%, 100% and 150% relative to the working concentration in triplicate according to the method of analysis and analyzed as per the method. The % recovery was calculated [29].

Table None: 13: The Accuracy Results for Nirmatrelvir

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	267011.3	20	20.063	100.315%	100.28%
100%	523752.3	40	40.118	100.295%	
150%	778457.3	60	60.133	100.221%	

Table None: 14: The Accuracy Results for Ritonavir

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	972876.3	10	10.094	100.94%	100.48%
100%	1900122	20	19.998	99.99%	
150%	2851152	30	30.156	100.52%	

Limit of Detection: 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 value.

LOD = 3.3 × σ / s

Where

σ = Standard deviation of the response and S = Slope of the calibration curve

Nirmatrelvir = 1.04µg/ml

Ritonavir = 3.12µg/ml

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

LOQ = 10×σ/S

Where

σ = Standard deviation of the response and S = Slope of the calibration curve

Nirmatrelvir =2.1µg/ml

Ritonavir = 6.3µg/ml

Robustness: Nirmatrelvir and Ritonavir were tested for resilience at flow rates ranging from 0.9 to 1.1 ml/min and mobile phase ratios varying from more to less organic phase. Only at low flow rates is the technique robust, and it is also resistant to a 10% shift in the mobile phase. The standard and samples of Nirmatrelvir and Ritonavir were injected using a variety of chromatographic settings. The resolution, tailing factor, and plate count properties remained unchanged [30].

Table None: 15: Results for Robustness of Nirmatrelvir

Parameter Used For Sample Analysis	Peak Area	Retention Time	Theoretical Plates	Tailing Factor
Actual Flow rate of 1.0 mL/min	526389	2.133	5679	1.56
Less Flow rate of 0.9 mL/min	542685	2.210	5264	1.54
More Flow rate of 1.1 mL/min	526483	2.184	5426	1.52
Less organic phase	516854	2.200	5163	1.57
More Organic phase	506898	2.172	5098	1.51

Table None: 16: Results for Robustness of Ritonavir

Parameter Used For Sample Analysis	Peak Area	Retention Time	Theoretical Plates	Tailing Factor
Actual Flow rate of 1.0 mL/min	1687285	3.692	8685	1.79
Less Flow rate of 0.9 mL/min	1725468	4.498	8265	1.68
More Flow rate of 1.1 mL/min	1652847	3.505	8415	1.59
Less organic phase	1687485	4.504	8326	1.62
More organic phase	1674524	3.512	8415	1.63

Stability Studies

The specificity of the method can be demonstrated by applying stress conditions using acid, alkaline, peroxide, thermal, UV, water degradations. The sample was exposed to these conditions the main peak of the drug was studied for peak purity that indicating the method effectively separated the degradation products from the pure active ingredient.

Table None: 17: Results of Forced Degradation Studies of Nirmatrelvir

S.No.	Stress Condition	Peak Area	% of Degraded Amount	% of Active Amount	Total % of Amount
1	Standard	526389	0	100%	100%
2	Acidic	371683.27	29.39	70.61	100%
3	Basic	411794.11	21.77	78.23	100%
4	Oxidative	480645.79	8.69	91.31	100%
5	Thermal	327045.48	37.87	62.13	100%

S.No.	Stress Condition	Peak Area	% of Degraded Amount	% of Active Amount	Total % of Amount
6	Photolytic	477118.99	9.36	90.64	100%

Table None: 18: Results of Forced Degradation Studies of Ritonavir

S.No.	Stress Condition	Peak Area	% of Degraded Amount	% of Active Amount	Total % of Amount
1	Standard	1687285	0	100%	100%
2	Acidic	1359614.25	19.42	80.58	100%
3	Basic	1445497.05	14.33	85.67	100%
4	Oxidative	1644427.96	2.54	97.46	100%
5	Thermal	1297353.43	23.11	76.89	100%
6	Photolytic	1632954.42	3.22	96.78	100%

SUMMARY AND CONCLUSION

In the present investigation, a simple, sensitive, precise and accurate RP-HPLC method was developed for the quantitative estimation of Nirmatrelvir and Ritonavir in bulk drug and pharmaceutical dosage forms. This method was simple, since diluted samples are directly used without any preliminary chemical derivatization or purification steps. Acetonitrile: Acetate Buffer (pH-4.3) (35:65 v/v) was chosen as the mobile phase. The solvent system used in this method was economical. The %RSD values were within 2 and the method was found to be precise. The results expressed in Tables for RP-HPLC method was promising. The RP-HPLC method is more sensitive, accurate and precise compared to the Spectrophotometric methods. This method can be used for the routine determination of Nirmatrelvir and Ritonavir in bulk drug and in Pharmaceutical dosage forms.

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