RESEARCH ARTICLE

Development of a Simple Accurate Method, Validation and It's Degradation Studies of Nirmatrelvir, Ritonavir in Bulk and Marketed Formulation by RP-HPLC

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ABSTRACT

An easy, accurate, and precise method used for simultaneous quantification of nirmatrelvir, ritonavir in bulk and marketed formulation by reverse phase high performance liquid chromatography (RP-HPLC) using a standard column Inertsil ODS (150 x 4.6 mm, 5 µm) at a rate of flow 1-mL/min, acetonitrile and buffer containing hexane sulphonic acid, 50:50 v/v as a mobile phase was introduced through the HPLC column, detected at a wavelength 258 nm. Nirmatrelvir and ritonavir retention times were 2.481 and, 3.873 minutes, respectively. %Recovery rates for nirmatrelvir and ritonavir were 100 and 100.3%, respectively. LoD and LoQ values for nirmatrelvir and ritonavir were 1.5, 1 and 4.5, 3 g/mL, respectively. Nirmatrelvir regression equation is y = 32885.25x + 4223.04 while ritonavir is y = 39086.65x + 1680.21. Stability indicating studies done by acid, alkali, peroxide, Reduction, thermal, neutral and ultra violet light. The newly created reverse phase HPLC method, for bulk and marketed formulation, was rapid, stability indicating and accurate.

Keywords: Accurate, Nirmatrelvir, Ritonavir, RP-HPLC, Stability indicating.

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INTRODUCTION

Nirmatrelvir (Figure 1) and ritonavir, is a novel combination drug that has been licensed in the UK, Europe and US for the cure of COVID-19 in people who don't need extra oxygen but are at high risk of developing severe symptoms.[1-3] Nirmatrelvir chemically designated as "(1R,2S,5S)-N - ((1S)- 1 - Cyano – 2 - ((3S) - 2- oxopyrrolidin – 3 - yl) ethyl) – 3 - ((2S) - 3, 3 – dimethyl -2-(2,2,2-trifluoroacetamido) butanoyl)-6,6-dimethyl-3-azabicyclo (3.1.0) hexane-2 carboxamide". It's atomic mass is 499.54. Its chemical formula is $C_{23}H_{32}F_3N_5O_4$. It is available in powder form, and has colorless to pale color, practically soluble in 1-butanol, isopropyl acetate and methyl isobutyl ketone and low solubility in 1- 1-acetoxy butane, anisole 1- acetoxy propane and insoluble in dipropyl methane.[4,5]

Ritonavir [5] is one of the few antivirals used to treat HIV and COVID-19, is also a powerful human immunovirus protease inhibitor[6] and is frequently used along with other antivirals to create a synergistic effect.[7,8]

Ritonavir is "5-Thiazolylmethyl [(αS)-α- [(1S,3S)-1 -hydroxy-3- [(2S)-2- [3- [(2- isopropyl-4-thiazolyl) methyl] - 3 - methylureido] – 3 - methylbutyramido] – 4 - phenylbutyl] phenethyl] carbamate". It's chemical formula - $C_{37}H_{48}N_6O_5S_2$; Atomic weight - 720.94. Ritonavir is almost colorless powder, practically soluble in methylene dichloride, methanol and poor solubility in acetonitrile.[9,10]

The literature review reveals that few efficient methods were available for spectrophotometric,[11] HPTLC,[12] UPLC,[13] and RP-HPLC[14-16] analysis with different combinations of drugs. No published data on simultaneous estimation of nirmatrelvir and ritonavir in bulk and marketed formulation exists. Hence, an attempt has been made to develop a cost-effective, simple and precise RP-HPLC method to estimate both drugs in bulk and marketed formulation.

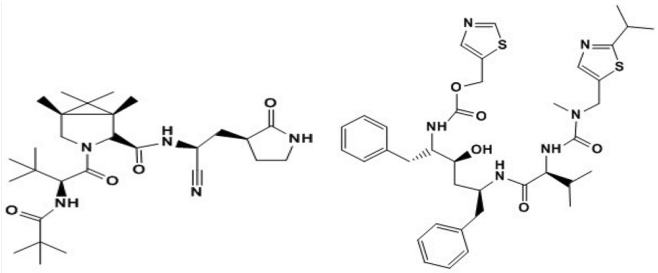

Figure 1: Chemical structure of Nirmatrelvir and Ritonavir

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MATERIAL AND METHODS

Acetonitrile (ACN) - HPLC grade
Hexane sulphonic acid - HPLC grade
Water HPLC grade (Milli Q or equivalent)

Equipments and Techniques

"Waters Alliance" HPLC pump with e2695, photo-diode array (PDA) detector were used in the development of method, and validation process. Software called Windows Empower-2 was employed to collect and process the data. A magnetic stirrer, a Eutech 700 pH metre, a Mettler Toledo ME 204 weighing balance, a distillation still, and Hot air oven (Kemi) used in the method development and validation process.

Conditions for Chromatographic Optimization

The data was collected using Empower® version 2 and a "Waters Alliance" HPLC system with a 2695 separation unit and a 2998 type PDA (photo diode array) detector for HPLC analysis. An Intersil ODS (150x4.6 mm, 5 µm) column was used for the chromatography. Acetonitrile and buffer containing Hexane sulphonic acid, 50:50 v/v as a mobile phase. A rate of flow 1-mL/min, 10 µL injection volume, a 6 minutes run time, and a constant temperature were used to test the samples. The substances were detected and their purity was assessed using a photodiode array detector with a 258 nm wavelength.

Buffer of Ammonium Formate Preparation

Accurately weigh 2.5 gms of hexane sulphonic acid in 1lt of water, and then use orthophosphoric acid to adjust the pH 3.0.

Preparation of the Mobile Phase

Buffer and acetonitrile should be combined 50:50 ratio. Afterward, 0.45 µ membrane filter paper was used to filter the produced mobile phase.

METHOD DEVELOPMENT

Standard Solution preparation

Ritonavir (100 mg), and nirmatrelvir (150 mg) working standards should be prepared by accurately weighed quantity transferred into a 100 mL graduated flask diluted with diluent up to volume. Using diluent (Nirmatrelvir -1500 µg/mL and Ritonavir -1000 µg/mL), 5 mL were further diluted to 50 mL.

Preparation of 100% Complete Standard working Solutions

In 10 mL volumetric flask, 1mL of each stock solution was taken, then make up to the mark with diluent. (100 µg/mL of Ritonavir, 150 µg/mL of Nirmatrelvir).

Sample Solution Preparation

Weigh 320 mg sample precisely, then transfer it to a 100 mL graduated flask. Add 70 mL of diluent and sonicate to dissolve the material, then add other ingredients. The solution was further diluted to 5 mL in a 50 mL graduated flask diluted with diluent to the required volume. (Nirmatrelvir -1500 µg and Ritonavir -1000 µg)

Preparation of 100% Complete Sample working Solutions

In 10 mL volumetric flask, 1mL of each stock solution was taken, then make up to the mark with diluent. (100 µg/mL of Ritonavir, 150 µg/mL of Nirmatrelvir)

Procedure

After injecting the samples under different chromatographic conditions, record the chromatograms, noting the ideal peak elution conditions to carry out validation variables in accordance with International Council for Harmonisation (ICH) criteria.

METHOD VALIDATION

Parameters of System Suitability

Nirmatrelvir (150 ppm) and ritonavir (100 ppm) standard solutions were prepared, then above solutions were introduced to determine the characteristics such USP plate count, resolution, and peak tailing.

Specificity

The involvement with the ideal approach is examined. In this method, no interference peaks in the placebo or blank were seen during the retention times of these medicines. It was, therefore asserted that this approach was unique.

Linearity

Preparation of standard stock solution

Ritonavir (100 mg) and Nirmatrelvir (150 mg) working standards solution should be prepared by accurately measured quantity and transferred to a 100 mL graduated flask diluted with diluent to volume. 5mL were further diluted to 50 mL with diluent (Nirmatrelvir -1500 µg/mL and Ritonavir -1000 µg/mL)

Standard working Solution Preparation

From each stock solution, pipette out 1-mL and taken into a 10 mL volumetric flask before adding the diluent. (100 µg/mL of Ritonavir and 150 µg/mL of Nirmatrelvir)

25% Standard solution

From each of the two stock solutions, pipette out 0.25 mL and taken into a 10 mL volumetric flask, added diluent in order to get a final volume. Nirmatrelvir (37.5 µg/mL) with ritonavir (25 µg/mL).

50% Standard solution

Pipette out 0.5 mL from each of the two stock solutions in order to get a final volume of 10 mL. Nirmatrelvir (75 µg/mL) with ritonavir (50 µg/mL).

75% Standard solution

Pipette 0.75 mL from each of the two stock solutions in order to get a final volume of 10 mL. nirmatrelvir (112.5 µg/mL) with ritonavir (75 µg/mL).

100% Standard solution

Pipette 1-mL from each of the two stock solutions in order to get a final volume of 10 mL nirmatrelvir (150 µg/mL) with ritonavir (100 µg/mL).

125% Standard solution

To make a total of 10 mL, pipette 1.25 mL from each of the two standard stock solutions nirmatrelvir (187.5 µg/mL) with ritonavir (125 µg/mL).

150% Standard solution

To make a total of 10 mL, pipette 1.50 mL from each of the two standard stock solutions: Nirmatrelvir (225 µg/mL) with ritonavir (150 µg/mL).

Procedure

Measure the peak area after each quantity has been injected into the chromatographic system. Plot a linear peak response vs. drug concentration graph to get the R^2 (correlation coefficient).

Precision

Standard stock solution preparation

Ritonavir (100 mg), and nirmatrelvir (150 mg) working standards should be prepared by weighed accurate quantity and taken into a 100 mL graduated flask diluted with diluent to volume. With diluent (1500 µg/mL of Nirmatrelvir and 1000 µg/mL of ritonavir), 5 mL were further diluted to 50 mL.

Preparation of 50% concentration solution

From each of the two stock solutions, pipette out 0.5 mL and taken into a 10 mL volumetric flask, added diluent in order to get a final volume.

Preparation of 100% concentration solution

From each of the two stock solutions, pipette out 1-mL and taken into a 10 mL volumetric flask, added diluent in order to get a final volume.

Preparation of 150% concentration solution

A final volume of 10 mL can be obtained by pipetting 1.5 mL from each of the two standard stock solutions.

Acceptance Criteria

The percentage recovery of individual level should be lies in between 9 to 101%

Robustness

Minor intentional modifications were applied to the technique, such as organic phase ratio, flow rate, but the results remained within the ICH guideline range and could not be distinguished. The robustness criteria were upheld, and duplicate samples were injected. These settings comprised organic minus (40:60), organic plus (60:50), flow rate minus (0.9 mL/min), and flow rate plus (1.1 mL/min). Since they had no effect, all of the system suitability requirements were satisfied. The %RSD was within allowable limits.

Limit of Detection

The detection limit of a technique is the smallest amount of analyte in a sample that can be detected but not always quantitated as an exact amount.

Sample Preparation

After obtaining 0.25 mL from each standard stock solution, the two separate 10 mL volumetric flasks were filled with diluents. From the aforementioned solutions, 0.1 mL of nirmatrelvir and 10 mL of ritonavir solutions were transferred and diluted with the same diluents.

"Limit of detection" is equal to "$3.3 \times \sigma / s$."

Where σ indicates "standard deviation of response."

S indicates "slope of calibration curve"

Limit of Quantitation

The smallest amount of analyte in a sample that may be quantitatively identified is known as the quantitation limit of a certain analytical method.

Sample Preparation

The two separate 10 mL volumetric flasks were filled with diluents after receiving 0.25 mL from each standard stock solution. From the aforementioned solutions, 0.3 mL of nirmatrelvir and 10 mL of ritonavir solutions were transferred and diluted with the same diluents**.**

"Limit of quantitation" is equal to "$10\times\sigma/S$"

Where σ indicates "standard deviation of response"

S indicates "slope of calibration curve"

Stress Degradation Studies

According to the ICH rules Q2B, attempts were made to forced degradation under circumstances including basic, acidic, reduction, oxidation, hydrolysis, thermal and photolytic degradation.

RESULTS AND DISCUSSION

The optimized conditions were shown in Table 1. The typical chromatogram of nirmatrelvir and ritonavir was shown in Figure 2. The current HPLC method was validated by precision, linearity, robustness, and accuracy per the ICH guidelines. Linear graph, shows that the range of nirmatrelvir is 37.5 to 225 µg/ mL and ritonavir is 25 to 150 µg/mL. For nirmatrelvir, the equations of regression were y =32886.25x + 4223.04 (R^2 = 0.9997) and y = 39086.65x +1680.21 (R^2 = 0.9998) for ritonavir respectively. Linearity results were demonstrated in Table 2 and Figure 3.

Method and intraday precision RSD values were 0.47, 0.50 and 0.40, 0.54 for Nirmatrelvir and Ritonavir shown in Table 3. The average %recovery results were 100 and 100.3 for Nirmatrelvir and Ritonavir Tables 4 and 5. Nirmatrelvir and ritonavir had a LoD and LoQ results are tabulated in Table 6.

Intentional alterations had no impact on system appropriateness characteristics like tailing factor, or the theoretical plates, RSD, resolution of nirmatrelvir and ritonavir. The findings and the factors affecting system appropriateness were shown in Table 7.

These forced degradation studies was carried out by using 1N Hydrochloric acid, 1N Sodium hydroxide, 30% H_2O_2 (Hydrogen peroxide) and H_2O at 60°C for 15 minutes. In acid degradation, 15.5% of nirmatrelvir and ritonavir has subjected to degradation. In alkali degradation, about 14.5% degraded and with oxidation degradation 13.2% degraded for both drugs. Photodegradation of nirmatrelvir and ritonavir were found to be 14.3 and 12.6. Very low degradations i.e less than 6 were observed with thermal and hydrolysis. The findings were shown in Table 8.

Table 1: Optimized chromatographic conditions

Variables	*Appropriate conditions*
Column	Inertsil ODS (150 x 4.6 mm, 5 µm)
Mobile phase	Acetonitrile: Buffer (50:50 v/v)
Injection volume	10 mL
Rate of flow	1 mL/min
Column temperature	Ambient
Wavelength	258 nm
Time duration	6 minutes
Retention time of nirmatrelvir	2.461 minutes
Retention time of ritonavir	3.873 minutes

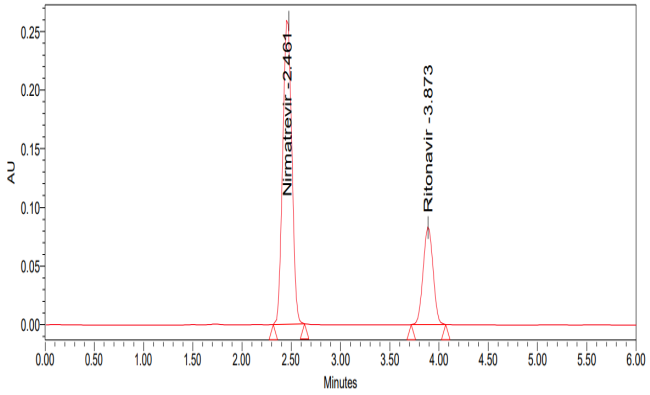

Figure 2: A Typical optimized chromatogram

Table 2: Linearity results of Nirmatrelvir and Ritonavir

S. No.	*Nirmatrelvir*		*Ritonavir*	
	Conc. (µg/mL)	*Peak Response*	*Conc. (µg/mL)*	*Peak Response*
1	37.50	411818	25.00	147356
2	75.00	838822	50.00	288229
3	112.50	1223374	75.00	455265
4	150.00	1645546	100.00	586456
5	187.50	2100828	125.00	737004
6	225.00	2441815	150.00	875525

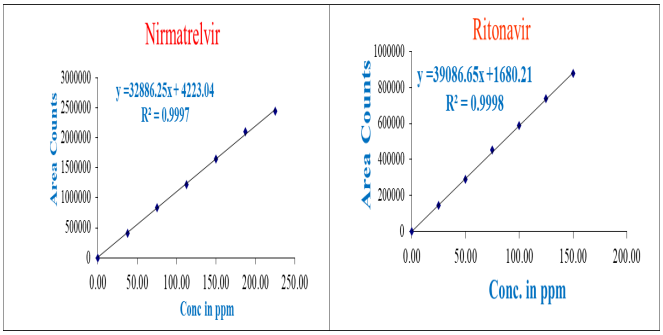

Figure 3: Linearity graph of Nirmatrelvir and Ritonavir

Table 3: Method and intermediate precision of Nirmatrelvir and Ritonavir

S. No	*Nirmatrelvir*	*Ritonavir*	*Nirmatrelvir*	*Ritonavir*
1	1665540	580286	1665540	580286
2	1652520	583277	1652520	583277
3	1653847	580624	1653847	580624
4	1666328	588056	1666328	588056
5	1663809	581947	1663809	581947
6	1672728	585119	1660409	582838
Mean	1662462	583218	1660409	582838
SD	7806.24	2965.28	6674.91	3147.56
%RSD	0.47	0.508	0.402	0.54

Table 4: Accuracy results of Nirmatrelvir

Level in%	*API Added amount (mg)*	*Recovered amount (mg)*	*Recovery%*	*Average %recovery*
	75.02	74.15	98.9	
50	75.11	75.38	100.5	
	75.06	76.15	101.5	
	150.1	149.01	99.3	
100	150.5	150.43	100.3	100.0
	150.2	150.92	100.6	
	225.5	223.82	99.5	
150	225.1	222.62	98.9	
	225.9	225.25	100.1	

Table 5: Accuracy results of Ritonavir

Level in%	*API Added Amount (mg)*	*Recovered Amount (mg)*	*Recovery%*	*Average %Recovery*
	50.07	50.25	100.5	
50	50.02	49.96	99.9	
	50.07	50.79	101.6	
	100.1	99.75	99.8	
100	100.2	100.23	100.2	100.3
	100.08	100.61	100.6	
	150.05	149.66	99.8	
150	150.1	150.88	100.6	
	150	149.21	99.5	

Table6: LOD and LoQ values of Nirmatrelvir and Ritonavir

Drug name	*LoD*	*LoQ*
Nirmatrelvir	1.5	4.5
Ritonavir	1	3

Table 7: Outcomes of robustness

Drug name	*Flow plus (1.1 mL/ min) %RSD*	*Flow minus (0.9 mL/ min) %RSD*	*Organic plus (60:40) %RSD*	*Organic ninus (40:60) %RSD*
Nirmatrelvir	0.51	0.34	0.76	0.51
Ritonavir	0.4	0.19	0.23	0.39

Table 8: Results of Forced Degradation

Degradation	*%Deg. of Nirmatrelvir*	*%Deg. of Ritonavir*
Control	0.1	0.2
Acid	15.5	14.8
Alkali	14.5	15.4
Peroxide	16.6	16.3
Reduction	13.2	13.2
Thermal	3.4	4.3
Photo	14.3	12.6
Hydrolysis	4.1	5.9

The suggested HPLC technique can be used routinely for the simultaneous determination of Nirmatrelvir and Ritonavir in API's as well as in marketed preparations because it is accurate, sensitive, and repeatable. The results of the statistical overview show that this method is highly precise and accurate. The %RSD for each parameter was identified to be not more than one, indicating the procedure validity and the acceptable agreement between the test results produced using this method. The method validation parameters' results also showed that the overall RSD value was less than two.

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