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Research Article

Simultaneous Estimation of Analytical Method Development and Validation of Nirmatrelvir and Ritonavir by RP HPLC Method

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Abstract

The current investigation was pointed at developing and progressively validating novel, simple, responsive and stable RP-HPLC method for the measurement of active pharmaceutical ingredients of Nirmatrelvir and Ritonavir and their related substances. RP-HPLC methodology was used for the quantitative determination of Nirmatrelvir and Ritonavir. Sunfire C18 Column, (5 µm, 4.6 mm X 250 mm) using mobile phase of 0.01N KH2PO4: Acetonitrile: Methanol (80:20:10 v/v) with flow rate of 1 ml/min (Detection wavelength 238 nm) was used for the present study. Using the impurity-spiked solution, the chromatographic approach was streamlined. The proposed method to be fast, simple, feasible and affordable in RS condition. During stability tests, it can be used for routine analysis of production samples and to verify the quality of drug samples during stability studies.

Keywords: Nirmatrelvir; Ritonavir; Impurities; RP-HPLC; Validation

Introduction

The development of effective antiviral medications remains crucial in combating infectious diseases, particularly those caused by rapidly evolving viruses such as HIV and coronaviruses [1]. Nirmatrelvir (NRM) emerges as a promising antiviral agent, characterized by its role as an orally active 3C-like protease inhibitor [2]. When combined with Ritonavir (RTN), it forms a potent treatment regimen against HIV infection and Coronavirus disease. Ritonavir, by binding to the protease active site, not only inhibits the enzyme's activity but also enhances the levels of Nirmatrelvir, augmenting its therapeutic efficacy [3]. As evidenced by literature, various analytical methods, including RP-HPLC and UV-Spectrophotometric techniques, have been explored for the determination of Nirmatrelvir and Ritonavir both individually and in combination, particularly in pharmaceutical formulations [4,5]. This paper aims to contribute to this body of knowledge by detailing a reverse-phase high-performance liquid chromatographic (RP-HPLC) method for accurately estimating the Nirmatrelvir and Ritonavir combination in tablet dosage forms, furthering the understanding and optimization of this vital antiviral treatment [6,7].

Experimental

Materials and instrumentation

Waters, Separation module2695, PDA detector Instrument with Kromosil C_{18} column 4.5×150 mm x 5µmandHPLC-auto sampler–UV detector using Empower-software version-2andLab India U.V double beam spectrometer of UV 3000+ model and U.V win software used. HPLC, Water, Methanol, Acetonitrile, Ortho phosphoric acid, KH_2PO_4 was procured from Merck Enterprises, India.

Chemicals and reagents

- **0.01N KH_2PO_4 buffer**: Accurately weighed 1.36gm of Potassium dihyrogen Ortho phosphate in a 1000-mL of Volumetric flask add about 900 mL of milli-Q water added and degas to sonicate and finally make up the volume with water then PH adjusted to 3.5 with diluted Orthophosphoric acid solution.

- **0.1% OPA Buffer**: 1 mL of Orthophosphoric acid was diluted to 1000 mL with HPLC grade water.

Composition of standard solution (Concentration = 1.0 ppm)

To prepare a stock solution containing Nirmatrelvir and Ritonavir at specified concentrations, by transferring 1.5mg of Nirmatrelvir and 1.0mg of Ritonavir into a clean 100-mL volumetric flask. Ensure complete dissolution of the substances and then dilute the volume with a suitable diluent. This process yields a stock solution with concentrations of 10ppm for Ritonavir and 15ppm for Nirmatrelvir. For further dilution, take 1 mL of the prepared stock solution and transfer it into a 10-mL volumetric flask. Dilute the contents to the mark with diluent. This step results in a solution with concentrations of 1ppm for Ritonavir and 1.5ppm for Nimatrelvir. The precise concentrations of Nirmatrelvir and Ritonavir can be achieved, ensuring accuracy in subsequent analyses.

Preparation of sample solution (Concentration = 100ppm)

To prepare a solution containing Nirmatrelvir and Ritonavir from tablet dosage forms, finely crushing 10 tablets of the medication to create a powder. Then, carefully weigh out 150mg of Nirmatrelvir and 100mg of Ritonavir from the powdered tablets. Transfer these measured amounts into a clean 100-mL volumetric flask. Next, add approximately 50 mL of a suitable diluent to the flask and initiate sonication for 20 minutes with intermittent shaking. Allow the solution to return to room temperature after sonication. Once at room temperature, dilute the solution up to the mark on the volumetric flask using additional diluent, ensuring a total volume of 100 mL. Finally, thoroughly mix the contents of the flask to achieve a homogeneous solution. The accurate preparation of a solution containing the specified concentrations of Nirmatrelvir and Ritonavir is used for further analysis.

Methodology

20µL of the blank, standard and sample were injected into the chromatographic system and areas for the Nirmatrelvir and Ritonavir peaks was used for calculation.

Process validation

The proposed High Performance liquid chromatographic process was validated as per the accordance of ICH guidelines with aspect to linearity, accuracy, precision, limit of quantification (LOQ), limit of detection, specificity and robustness [8,9].

Preparation of stock solutions

- **Stock-A**: Weigh and transfer 1.5mg of Nirmatrelvir acid and 1.5 mg of Bicycloamide and 1 mg of Ritonavir impurity into 10-mL volumetric flask, dissolved in diluent and dilute to volume with diluent (1ppm and 1.5 ppm). Further 1 mL of above stock transfer into a 10ml volumetric flask, dilute to volume with diluent.
- **Stock-B**: Transfer 1.5mg of Nirmatrelvir and 1.0mg of Ritonavir into 100-mL volumetric flask, dissolve, and dilute volume with diluents. (Concentration=10ppm of Ritonavir and 15ppm of Nirmatrelvir). Further 1 mL of above stock transfer into a 10-mL volumetric flask, dilute to volume with diluent. (Concentration=1ppm of Ritonavir and 1.5ppm of Nirmatrelvir).

Linearity [10]

Spiking solution for Linearity

Transfer 1.5mg of Nirmatrelvir and 1.0mg of Ritonavir into 100-mL volumetric flask, dissolve, and dilute volume with diluents. Weigh and transfer 1.5mg of Nirmatrelvir acid, 1.5 mg of Bicycloamide and 1 mg of Ritonavir impurity into 10-mL volumetric flask, dissolved in diluent and dilute to volume with diluent.

From above solution, prepare below dilutions.

Precision [11]

Spiking solution for precision

Pipette 1.0 mL of Stock-A and 1.0 mL of Stock-B into a 100-mL of volumetric flask, dilute to volume with diluent, mix well.

100% spike sample

Calculate the average weight of 10 tablets and crush to fine powder. Weigh accurately powder equivalent to 100mg of Nirmatrelvir and Ritonavir into a 50-mL volumetric flask. Add 5 mL of Spiking solution for Precision and about 35 mL of diluent, sonicate for 10 minutes with intermittent shaking. Attain to room temperature. Dilute up to the volume with diluent and mix well.

Accuracy [12]

50%, 100% and 150% spike sample

Calculate the average weight of 10 tablets and crush to fine powder. Weigh accurately powder equivalent to 150mg of Nirmatrelvir and 100mg Ritonavir into a 50-mL volumetric flask. Add 1, 2 and 3 mL of Spiking solution for Precision and about 35 mL of diluent, sonicate for 10 minutes with intermittent shaking. Attain to room temperature. Dilute up to the volume with diluent and mix well.

Robustness

As part of the robustness, deliberate change in the flow rate, mobile phase composition was made to evaluate the impact on the method. The flow rate was varied at 1.0ml/min ± 0.1ml/min. Standard solution of Nirmatrelvir and Ritonavir was prepared and analysed using the varied flow rates along with method flow rate with column temperature 30°C ± 5°C. The organic composition in the mobile phase ± 10% standard solution, Nirmatrelvir and Ritonavir was prepared and analysed using the varied mobile phase composition along with the actual mobile phase composition in the method.

Results and Discussion

The simultaneous estimation of Nirmatrelvir and Ritonavir and their impurities using HPLC technique from the optimized chromatogram where retention time is 5.876, 3.770, 4.638, 7.764 and 2.289 minutes respectively. The results were summarized in Table 1 and the optimized chromatogram was shown in Figure 2.

Table 1: System suitability.

Peak name	Rt (min)	Peak area	USP Plate count	USP Resolution	USP Tailing
RTN Impurity-B	2.289	366454	8638.1	-	1.2
NRM Imp-Acid	3.770	306566	7107.5	9.2	1.3
Bicyclo amide	4638	327567	8924.5	4.6	1.4
NRM	5.876	2105179	77245	7.5	14
RTN	7.764	327801	7088.6	12.1	1.4

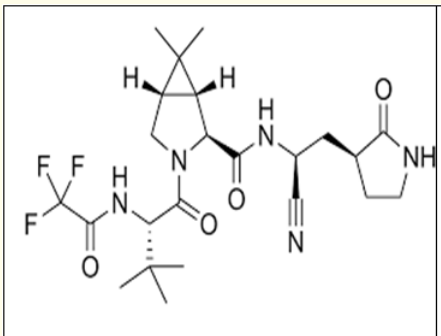

Figure 1A: Structure of Nirmatrelvir (NRM).

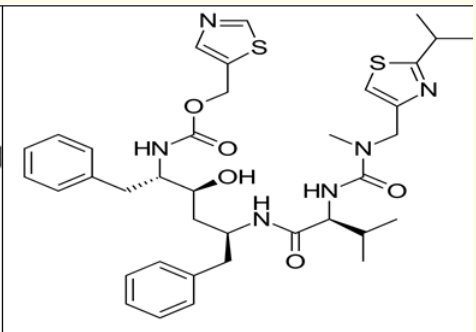

Figure 1B: Structure of Ritonavir (RTN).

Figure 1

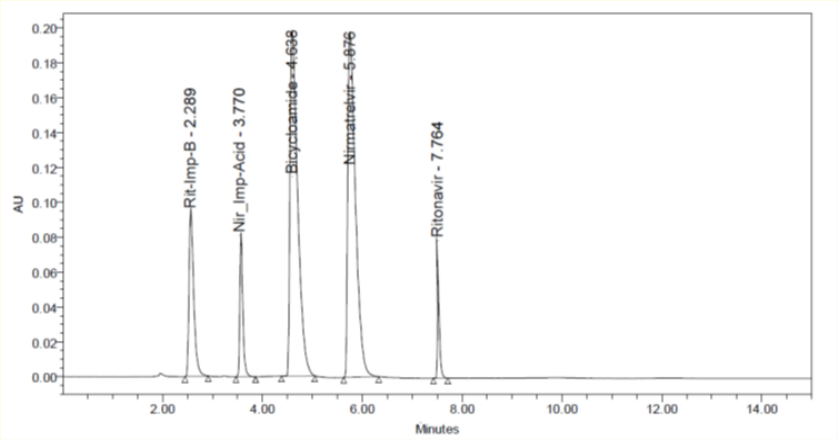

Figure 2: Optimized Chromatogram.

Optimized conditions

Chromatographic conditions:

Mobile phase: 0.01N KH_2PO_4: Acetonitrile: Methanol (80:20:10 v/v)

Flow rate: 1 ml/min

Column: SunFire C18 Column, 100Å, 5 µm, 4.6 mm X 250 mm.

Detector wave length: 238 nm

Column temperature: 30°C

Injection volume: 10µL

Run time: 15min

Diluent: Water: Acetonitrile (30:70, v/v)

Linearity

150 mg Nirmatrelvir and 100mg Ritonavir working standard was accurately weighed and were transferred into a 10-mL clean dry volumetric flask, add about 7 mL of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Each level was injected into the chromatographic system and peak area was measured Table 2. Calibration graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) was drawn and the correlation coefficient was found to be 0.999 (Figure 3A and Figure 3B). The Limit of detection for Nirmatrelvir and Ritonavir is 2.97, whereas limit of quantification for Nirmatrelvir and Ritonavir is 9.92.g/mL and 10.02 g/mL respectively.

Accuracy

The % recovery range of Nirmatrelvir and Ritonavir is shown in Table 5. Therefore no interference from the additives commonly present in the tablets and developed technique constitute to be precise as the percent relative standard deviations for repeatability and intermediate precision is less than 2 as per proposed ICH guidelines. For Nirmatrelvir and Ritonavir is 1.0% which indicates that the method has good repeatability. The findings of recovery studies were summarized in Table 3.

Robustness

The robustness of method was determined by assessing the ability to develop a method which remains intact by the small changes in the criteria's such as pH of mobile phase, percent organic content, buffer strength, temperature injection volume and flow rate. Thus, the developed LC method was robust in the findings of Nirmatrelvir and Ritonavir along with impurities in combined tablet dosage formulations and the results were summarized in Table 4.

Assay

The proposed technique was applied for the assay of commercial formulation consisting 20µL of the blank, standard and sample were injected into the chromatographic system and areas for the Nirmatrelvir, Ritonavir and impurities where the peak was used

Table 2: Linearity Study.

%Level	Conc. (µg/mL)		Peak area			Peak area	
	NRM and impurities	RTN and impurities	NRM	NRM acid	Bicyclo amide	RTN	RTN Imp
25%	0.375	0.25	524019	75642	78819	81579	91261
50%	0.75	0.5	1039332	152085	157781	165152	181278
75%	1.125	0.75	1571135	226471	234455	246102	276629
100%	1.5	1	2045263	305139	314284	326212	367498
125%	1.875	1.25	2502558	376262	387038	405676	455393
150%	2.25	1.5	2994590	454487	469305	485253	543216

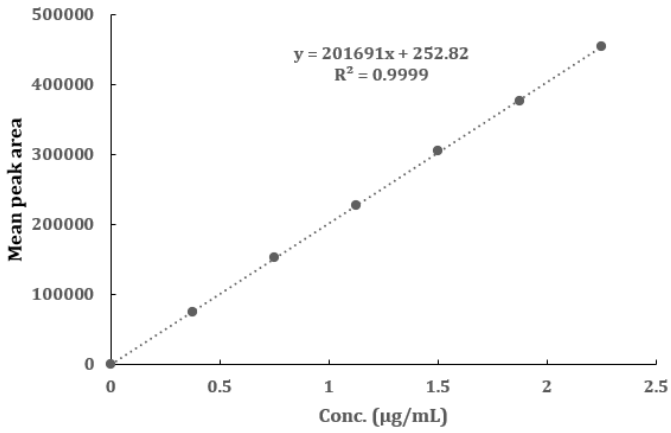

Figure 3A: Linearity graph of Nirmatrelvir Acid

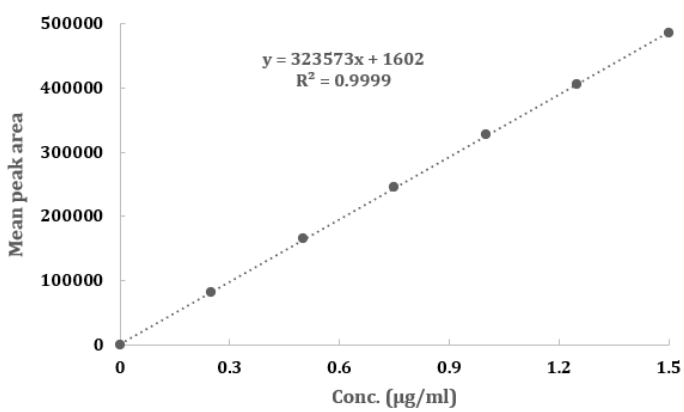

Figure 3B: Linearity graph of Ritonavir

Table 3: Accuracy study.

Spiked Levels		Peak area		Drug added		% Recovered		%RSD	
NRM	RTN	NRM	RTN	NRM	RTN	NRM	RTN	NRM	RTN
50	50	43822760	34987553	50	50	99.46	99.9	0.1	0.4
100	100	43829729	32336522	100	100	100.25	99.8	0.4	0.5
150	150	4397130	34762862	150	150	99.05	100	0.3	0.5

Table 4: Robustness study.

Flow minus					
S. No	**RTN**	**RTN Impurity-B**	**NRM**	**NRM Imp-Acid**	**Bicyclo amide**
1	326455	367532	2089575	298545	328754
2	324837	367843	2095774	301776	327666
3	324844	368375	2085545	302755	327556
4	323254	364788	2066656	304645	326444
5	323446	362457	2086766	305734	325644
Avg.	324567	366199	2084863	302691	327213
SD	1293.7	2510.4	10918.3	2788.8	1199.0
%RSD	0.4	0.7	0.5	0.9	0.4
Flow plus					
1	338745	359876	1986545	317565	332556
2	335466	355866	2019656	315465	335545
3	337756	353487	2038455	317655	331456
4	337667	352345	2018676	315345	335656
5	332567	358766	2025465	317686	332656
Avg.	336440	356068	2017759	316743	333574
SD	2474.8	3253.6	19147.2	1223.1	1909.5
%RSD	0.7	0.9	0.9	0.4	0.6
Temperature Minus					
1	348576	368955	2189566	308223	324686
2	346732	368933	2206765	305454	326456
3	347565	362876	2165567	308656	328665
4	342755	366354	2164534	302457	326457
5	348665	367656	2196543	307567	321568
Avg.	346859	366955	2184595	306471	325566
SD	2427.3	2520.1	18864.5	2558.9	2643.3
%RSD	0.7	0.7	0.9	0.8	0.8
Temperature plus					
1	338765	354356	2254667	309756	327856
2	332546	352556	2275656	308677	325677
3	332767	357578	2264555	301665	327897
4	332656	356567	2275465	304667	328666
5	337655	352324	2254556	307677	326545
Avg.	334878	354676	2264980	306488	327328
SD	3068.1	2351.9	10477.5	3296.3	1197.0
%RSD	0.9	0.7	0.5	1.1	0.4

for calculating the % assay by using the formulae. The % purity of Nirmatrelvir and Ritonavir was found to be 99.87 and 99.05 % respectively.

Forced degradation studies

The purpose of this study was to work out Nirmatrelvir and Ritonavir stress degradation studies using the suggested methods, such as acidic, peroxide degradation, alkaline hydrolytic degradation, thermal degradation, UV and hydrolytic degradation and the results were illustrated in Table 5.

Table 5: Forced degradation studies.

Degradation Studies	Peak area		Percent degraded	
	NRM	RTN	NRM	RTN
Standard	43504184	38606365	-	-
Acid	39675814	29425847	6.51	3.23
Base	43932866	38553846	2.24	1.32
Peroxide	36049390	30553846	5.11	2.28
Thermal	43606723	38789967	1.25	1.32
UV	4361888	38796310	2.52	2.24
Hydrolytic	43530343	38508405	1.08	1.35

Conclusion

The suggested RP-HPLC method for concurrent assay Nirmatrelvir and Ritnonavir along with impurities was validated and It is appropriate for routine quantitative analysis of Nirmatrelvir and Ritonavir in bulk and its pharmaceutical dosage forms. The proposed technique is accurate, rapid and sensitive. It does not interfere from any hindrance due to common additives present in pharmaceutical dosage forms and can be readily accepted for quality control analysis.

Bibliography

1. Bekerman E and Einav S. "Combating emerging viral threats". *Science* 348 (2015): 282-283.

2. Lea AP and Faulds D. "Ritonavir". *Drugs* 52.4 (1996): 541-546.

3. Eatemadi A., *et al*. "Role of protease and protease inhibitors in cancer pathogenesis and treatment". *Biomed Pharmacotherapy* 86 (2017): 221-231.

4. Geneva: IFPMA. International Conference on Harmonization. Guidance on validation of analytical procedure: Text and methodology. ICH - Q2 (R1) (2005).

5. Saravolatz LD., *et al*. "Molnupiravir and Nirmatrelvir-Ritonavir: Oral COVID Antiviral Drugs". *Clinical Infectious Disease* (2022).

6. Hiremath S., *et al*. "Prescribing Nirmatrelvir/Ritonavir for COVID-19 in Advanced CKD". *CJASN* 17.8 (2022): 1247-1250.

7. Bioanalytical method validation, European Medicines Agency (2012).

8. Aboras SI and Maher HM. "Green adherent degradation kinetics study of nirmatrelvir, an oral anti-COVID-19: Characterization of degradation products using LC-MS with in silico toxicity profile". *BMC Chemistry* 17.1 (2023): 23.

9. ICH, International conference on harmonization of technical requirements for registration of pharmaceuticals for human use ICH harmonized tripartite guideline stability testing of new drug substances and products Q1A (R2) (2003).

10. International Conference on Harmonization. ICH Q2 (R1): Validation of analytical procedures: Text and methodology. ICH Secretariat, Geneva (2005).

11. Patil TS and Deshpande A. "Development of an innovative quality by design (QbD) based stability-indicating HPLC method and its validation for clofazimine from its bulk and pharmaceutical dosage forms". *Chromatographia* 82.2 (2019): 579-590.

12. Secretan P., *et al*. "Unequal behaviour between hydrolysable functions of nirmatrelvir under stress conditions: Structural and theoretical approaches in support of preformulation studies". *Pharmaceutics* 14.8 (2022): 1720.