Available online on 15.12.2024 at http://ajprd.com

Asian Journal of Pharmaceutical Research and Development

Open Access to Pharmaceutical and Medical Research

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Research Article

Analytical Method Development and Validation for Simultaneous Estimation of Antiviral Drug in Bulk and Pharmaceutical Dosage Form by RP-HPLC

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A B S T R A C T

A new simple, specific, accurate, and precise RP-HPLC method has been developed and validated for the simultaneous estimation of Nirmatrelvir and Ritonavir in bulk and pharmaceutical dosage forms. The chromatographic separation was achieved using a Thermo fisher Scientific C18(250mm x 4.6mm, 5µm) with a mobile phase comprising a mixture of acetonitrile and phosphate buffer (70:30,), pumped at a flow rate of 1.0 mL/min and runtime of 6 minutes. The detection was performed at a wavelength of 221 nm. Under optimized chromatographic conditions, Nirmatrelvir and Ritonavir were eluted with retention times of 2.8 min and 3.9 min, respectively. Validation parameters were in compliance with ICH guidelines. These methods can be utilized for the routine determination of Nirmatrelvir and Ritonavir in bulk drug and formulation.

Keyword: Nirmatrelvir, Ritonavir, RP-HPLC, Method development, accuracy and linearity.

A R T I C L E I N F O: Received 14 July 2024; Review Complete 24 Sept. 2024; Accepted 10 Nov. 2024. ; Available online 15 Dec . 2024

Cite this article as:

Adhao VS, Kotgale NB, Rathi AM, Ambhore JP, Analytical Method Development and Validation for Simultaneous Estimation of Antiviral Drug in Bulk and Pharmaceutical Dosage Form by RP-HPLC, Asian Journal of Pharmaceutical Research and Development. 2024; 12(6):08-17. DOI: http://dx.doi.org/10.22270/ajprd.v12i6.1423

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INTRODUCTION

The coronavirus disease (COVID-19), caused by the SARS-CoV-2 virus, emerged in 2019 and quickly escalated into a global pandemic primarily spread through respiratory transmission[1]. The spectrum of clinical manifestations ranges from mild or asymptomatic cases to severe respiratory distress, multiorgan dysfunction, and fatalities[2]. By December 2021, over 272 million cases of COVID-19 had been reported globally, resulting in approximately 5.5 million deaths. The emergence of the Omicron variant in late 2021 posed a new global threat, following previous variants of concern such as alpha, beta, gamma, and delta[3]. By the end of 2021, Omicron had spread rapidly to over 108 countries, with thousands of confirmed cases and fatalities[4],[5].

In response to the pandemic, vaccination has been identified as the most effective measure to combat SARS-CoV-2[6]. Furthermore, a number of novel drugs, such as Remdesivir, Molnupiravir, and the Nirmatrelvir/Ritonavir combo, have been investigated or approved for the treatment of COVID-19[7]. Of these, the oral combination of Ritonavir and Nirmatrelvir has demonstrated encouraging therapeutic results, with reports of a large 89% decrease in hospitalization or death rates[8].Nirmatrelvir, chemically known as (1R,2S,5S)-N-((S)-1-cyano-2-((S)-2-oxopyrrolidin-3-yl) ethyl)-3-((S)-3,3-dimethyl-2(2,2,2-trifluoroacetamido) butanoyl)-6,6-dimethyl-3-azobicyclo [3.1.0] hexane-2-carboxamide. Its chemical formula is C23H32F3N5O4. Its molecular weight is 499.5 g/mol[9]. It appears as a white to pale colored powder with practical solubility in methanol, acetonitrile, methyl isobutyl ketone, 1-butanol, and isopropyl acetate, while being sparingly soluble in anisole, and insoluble in heptane[10].

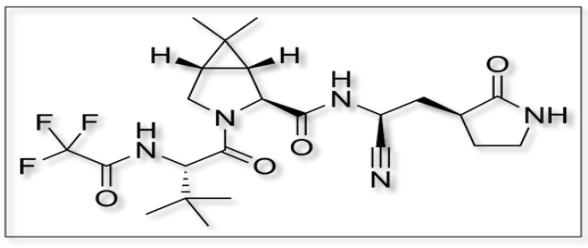

Figure 1: Molecular Structure of Nirmatrelvir

Ritonavir, known chemically as 1,3-thiazol-5-ylmethylN-[(2S,3S,5S)-3-hydroxy-5-[(2S)-3-methyl-2-{[methyl({[2-(propan-2-yl)-1,3-thiazol-4-yl] methyl}) carbamoyl] amino} butanamido]-1,6-diphenylhexan-2-yl] carbamate and marketed as Norvir. Its chemical formula is C37H48N6O5S2. Its molecular weight is 720.948 g/mol[11]. It is an antiretroviral medication used in combination with other drugs to treat HIV/AIDS. Ritonavir appears as a white to light tan powder and is practically insoluble in water, freely soluble in methanol and ethanol, and soluble in isopropanol[12].

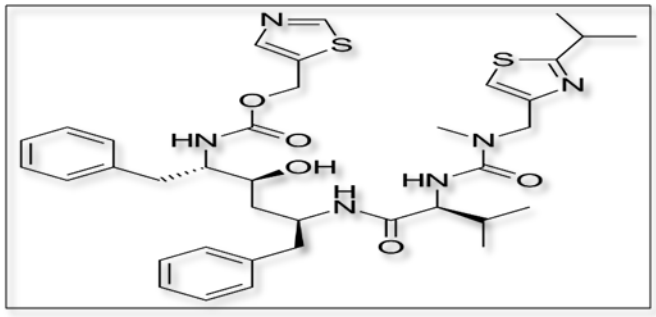

Figure 2: Molecular Structure of Ritonavir

Nirmatrelvir is an oral medication that inhibits a viral protease called MPRO, crucial for the replication of coronaviruses like SARS-CoV-2[13]. This drug has shown effectiveness against various coronaviruses known to infect humans. To enhance its effectiveness, nirmatrelvir is combined with ritonavir in a product called Paxlovid[14]. Ritonavir, acting as a cytochrome P450 (CYP) 3A4 inhibitor and pharmacokinetic booster, helps increase nirmatrelvir' s levels in the body to reach the desired therapeutic range. The US FDA approved the use of Ritonavir-boosted Nirmatrelvir in an emergency setting for the treatment of SARS-CoV-2 in December 2021[15].

MATERIALS AND METHODS

Chemical and Reagents

Acetonitrile (ACN), methanol, Ortho-phosphoric acid (OPA 0.1 %), water and other regents were supplied by Merk (India), Mumbai, India (HPLC grade). The active pharmaceutical ingredients for nirmatrelvir and ritonavir that served as reference standards (99.9 % w/w) were provided by Zydus Cadila, Ahmedabad, India as gift samples. Marketed formulation, Paxlovid (Pfizer Limited) (labeled claim, nirmatrelvir-150 mg, ritonavir- 100 mg) was purchased from licensed pharmacy.

Instrumentation

High Performance Liquid Chromatography resolution was executed with the help of Shimadzu, Kyoto, Japan's LC- 20 AD (binary pump), SPD-M20A photodiode-array detector, and Rheodyne injector with 20- loop made up the chromatographic system. These components were connected to a multi-instrument data collecting and data processing system (LC solution, software by shimadzu). A shimadzu model 1800 double beam UV/Vis spectrophotometer with a pair of 10mm match quarts cell, a Sartorious analytical balance, an ultrasonicator, and a Thermo Scientific C18 column with a dimension of 250mm x 4.6mm, 5µm, were also employed.

Selection of wavelength

An attempt was made to improve the sensitivity of the method by scanning 30 µg/mL concentrated solutions prepared from dilution of selected drugs using several detection wavelengths ranging from 200 to 400 nm in PDA detector. For all the components tested, the wavelength of 221 nm proved to be the most sensitive.

Selection of Chromatographic Conditions

Chromatographic separation was accomplished at ambient temperature using a reversed-phase isocratic high-performance liquid chromatography (HPLC) system. The mobile phase consisted of Acetonitrile and phosphate buffer in a ratio of 70:30. A flow rate of 1.0 mL/min was maintained throughout the analysis. Detection of the analytes was performed at a wavelength of 221 nm, and the total run time was set to 6 minutes.

To optimize chromatographic conditions, variables like mobile phase pH and flow rate were studied. Chromatograms were recorded under varied conditions, and responses were measured. This systematic approach helped identify the best conditions for optimal separation and detection of analytes.

Preparation of Standard Solution

1. **Preparation of Stock Solution 1 of Nirmatrelvir and Ritonavir (1000µg/ml):**
   Accurately weighed about 10 mg of Nirmatrelvir and 10 mg of Ritonavir of API working standard and transferred to a 100 ml volumetric flask. Add 50 ml of diluent and dissolved properly. Then it was shaken and sonicate for 10 minute and volume was made up to the mark with solvent.
2. **Preparation of Stock Solution 2 of Nirmatrelvir and Ritonavir (100µg/ml):**
   1ml of solution was pipetted out from (Stock solution 1) and transferred to 10 ml volumetric flask and the volume was made up to 10 ml with diluent and sonicated it for 15 min. The concentration of prepared stock solution was 100 µg/ml.

Preparation of Analytical Solution

a) **Preparation of 0.1% OPA Solution:**
   1ml of Ortho-phosphoric acid (HPLC Grade) was pipetted out and dissolve in 1000 ml of HPLC grade water. And adjust the pH (3.0), finally the solution was filtered by using 0.45 Micron membrane filter, and sonicate it for 10 min.

b) **Preparation of Mobile Phase:**
   Accurately measured 300 ml (30%) of above buffer and 700 ml (70%) of Acetonitrile for HPLC were mixed and degassed in an ultrasonic water bath for 10 min and then filter through 0.45µ filter under vacuum filtration, then this solution is used for HPLC purposes.

Preparation of Sample of Nirmatrelvir and Ritonavir in Marketed Formulation

Weight and takes 20 Paxlovid Table No. The Table No t was crushed to fine powder and amount of powder equivalent to 150 mg of Nirmatrelvir and 100 mg of Ritonavir were weighed and transferred to a 100 ml volumetric flask containing 50ml of diluent. The flask was shaken and sonicate for 10 minute and volume was made up to the mark with diluent. From this solution appropriate dilutions of Nirmatrelvir and Ritonavir were made to get the final

concentrations and 20 µl sample was injected into the system to measure peak height, area, retention time. The chromatogram obtained and the area obtained in each chromatogram of five replicate was correlated with regression equation and the amount found is calculate which was within the limit of label claim. The obtained assay values were within the acceptable limit (90-102 %) against the amount claimed in the Table No.

METHOD VALIDATION

The technique validation characteristics, such as specificity, linearity, accuracy, precision, limit of detection, limit of quantitation and robustness, were examined in accordance with ICH guideline Q2(R1)[16],[17].

System suitability

System suitability is a test to determine the suitability and effectiveness of chromatographic system prior to use. This was done to make sure the chromatographic system was appropriate for the planned purpose. Six replicate injections of standard preparation (30µg/ml) were injected into the liquid chromatographic system and chromatograms were recorded[18].

Linearity (Calibration Curve)

The linearity of an analytical method is its capability to elicit check consequence which might be at once, or with the aid of well describe mathematical adjustment, proportional to the concentration of analytes in within a given range

Linearity is determined by injecting a series of standards of stock solution/diluent using the solvent/mobile phase, at a minimum of five different concentration in the range of 10-50µg/ml. The absorbance was measured at wavelength 221nm[19].

Range

The range of an analytical technique is the range of concentrations (amounts) of analyte in the sample, including these concentrations, for which the analytical procedure has been shown to have an appropriate degree of linearity, accuracy, and precision[20].

Accuracy

To evaluate the closeness of the measured value to the true value, the accuracy of an analytical method is established. A method's accuracy is generally assessed through the drug candidate's percentage recovery, which is spiked into a placebo matrix. Percent recovery of the commenced investigation has been performed at 80, 100, and 120 % levels. It was done by the addition to the pre-studied sample a known amount of standard drug and further it was re-examined through the same investigation[21].

Precision

Precision of the commenced investigation was accomplished through the intra-day and inter-day precision and repeatability (system precision) and was assessed as RSD%. According to ICH Q2R1 procedure, RSD % value must be less than 2%. Three distinct concentration 20, 30, and 40 µg/mL were selected for intra-day and inter-day precision and repeatability of present investigation was performed using 30 µg/mL[22].

Limit of Detection (LOD)[23]

Limit of detection is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. LOD was calculated by the standard deviation of the response and the slope.

$$LOD = \frac{3.3\ x\ \sigma}{s}$$

Where, σ = the,standard deviation of the response,

S = the slope of the calibration curve

Limit of Quantitation (LOQ)[24]

It is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. LOQ was calculated by the standard deviation of the response and the slope. The data was obtained from linearity curve and the LOQ was calculated.

$$LOQ = \frac{10\ x\ \sigma}{s}$$

Where, σ = the standard deviation of the response,

S = the slope of the calibration curve

Robustness

Robustness is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameter.

For HPLC robustness was carried out by changing wavelength, Mobile phase and flow rate. Robustness of a method was done by change in wavelength, Mobile phase or change in flow rate. Injection of 30 µg/mL was prepared from the stock solution and the recorded[25].

RESULTS AND DISCUSSIONS

Optimized Method Development

The assay method was developed and validated using a Shimadzu Liquid Chromatographic System, comprising an LC-20 AD pump, an SPD-20A UV-Visible detector, and a universal loop injector with a 20µl injection capacity. Separation was conducted on a Thermo fisher Scientific C18 (250mm x 4.6mm, 5µm) with a flow rate of 1.0 ml/min. A 30 µl sample volume was injected, and detection was performed at a wavelength of 221 nm. The mobile phase, consisting of Acetonitrile and Phosphate Buffer in an (70:30 %(v/v) ratio, was employed. The run time was set at 6. Before analyte injection, the column was equilibrated for 15 minutes with the mobile phase.

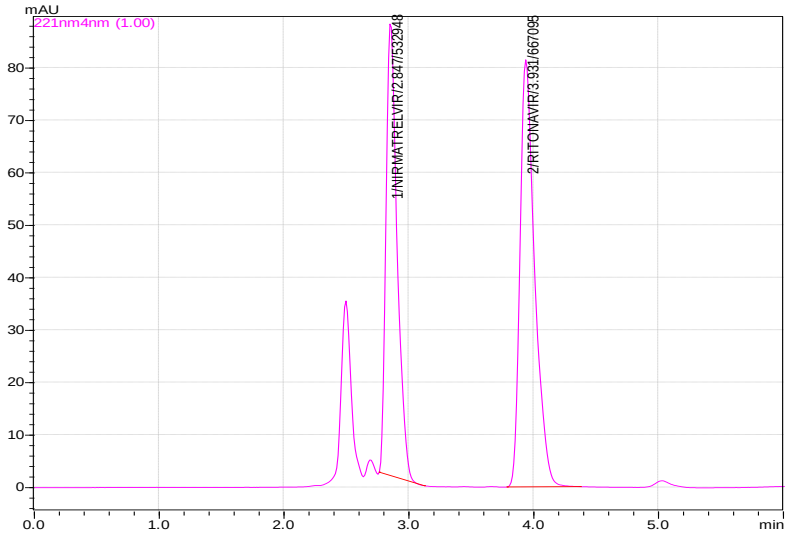

Figure 3: Optimized Chromatogram of Standard Drug at Ratio 70:30 of ACN: Phosphate Buffer containing 30 µg/mL Nirmatrelvir and Ritonavir at Wavelength 221 nm.

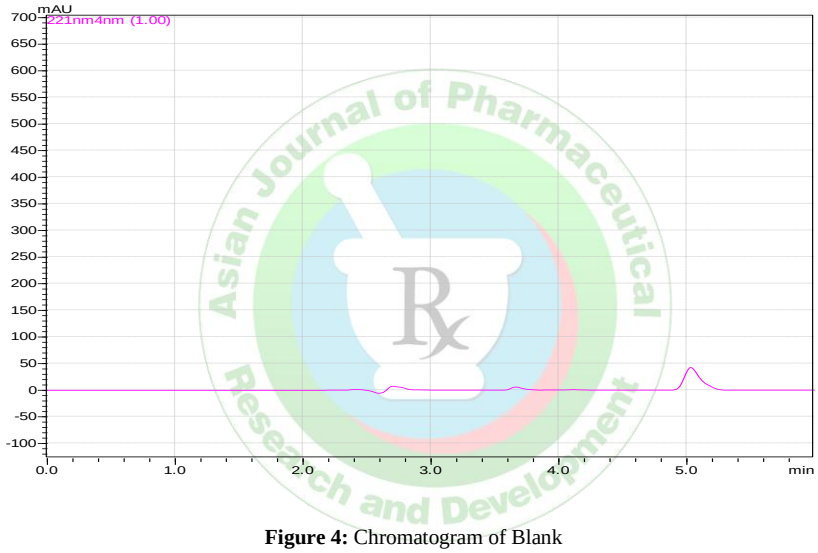

Figure 4: Chromatogram of Blank

Validation of the Developed Method

SYSTEM SUITABILITY

Table 1: System Suitability of Nirmatrelvir and Ritonavir

Test/ parameters	Retention time (Rt)		Theoretical plates		Tailing factor	
Analytes	**NRR**	**RTR**	**NRR**	**RTR**	**NRR**	**RTR**
Average (n=6)	2.901	3.951	3981.826	4764.614	1.567	1.542

The % RSD of system-suitability test parameters was found satisfactory. The results are listed in Table No 1.n= number of determinations

LINEARITY

A. Linearity Curve for the Nirmatrelvir

From the (Standard stock solution 2) of Nirmatrelvir and Ritonavir ((100µg/ml), 1ml, 2ml, 3ml, 4ml, 5ml were pipetted out and transferred to separate 10 ml of volumetric flask and the volume was made up to 10 ml with the help of diluent. The linearity of the relationship between peak area and concentration was determined by analyzing five working standards over the concentration range of 10, 20, 30, 40, 50 µg/ml for Nirmatrelvir. The injection was given at time interval of 10 minutes with run time of 6 minutes. The linearity was obtained in selected conc. ranges. Linearity of Nirmatrelvir is shown in Table No 2 and calibration plot in Figure No 5.

Table 2: Linearity of Nirmatrelvir at wavelength 221 nm

	ncentration (µg/mL)	ea (µV. sec)
		5294
		4916
		2948
		2582
		9613

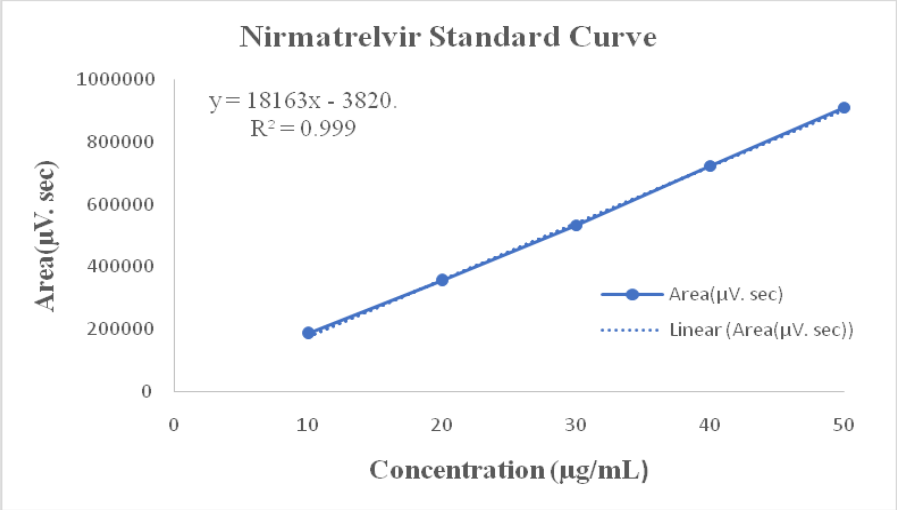

Figure 5: Calibration Curve of Nirmatrelvir at wavelength 221 nm

B. Linearity Curve for the Ritonavir

From the (Standard stock solution 2) of Nirmatrelvir and Ritonavir ((100µg/ml), 1ml, 2ml, 3ml, 4ml, 5ml were pipetted out and transferred to separate 10 ml of volumetric flask and the volume was made up to 10 ml with the help of diluent. The linearity of the relationship between peak area and concentration was determined by analyzing five working standards over the concentration range of 10, 20, 30, 40, 50 µg/ml for Ritonavir. The injection was given at time interval of 10 minutes with run time of 6 minutes. The linearity was obtained in selected conc. ranges. Linearity of Ritonavir is shown in Table No 3 and calibration plot in Figure No 6.

Table 3: Linearity of Ritonavir at wavelength 221 nm

Sr. No	Conc. (µg/mL)	Area (µV. sec)
1	**10**	227809
2	**20**	447477
3	**30**	677095
4	**40**	892945
5	**50**	1135645

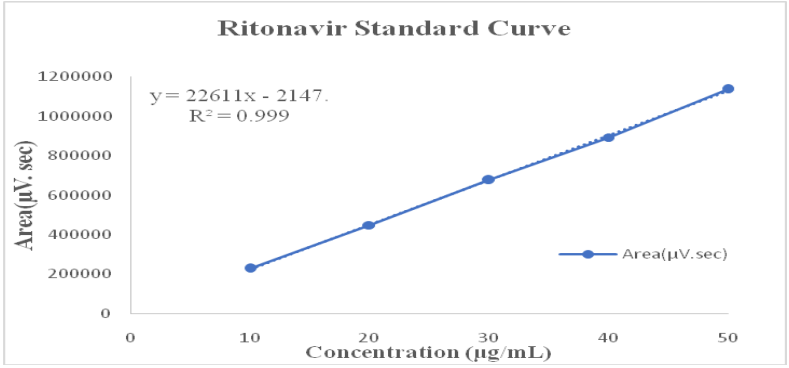

Figure 6: Calibration Curve of Ritonavir at wavelength 221 nm

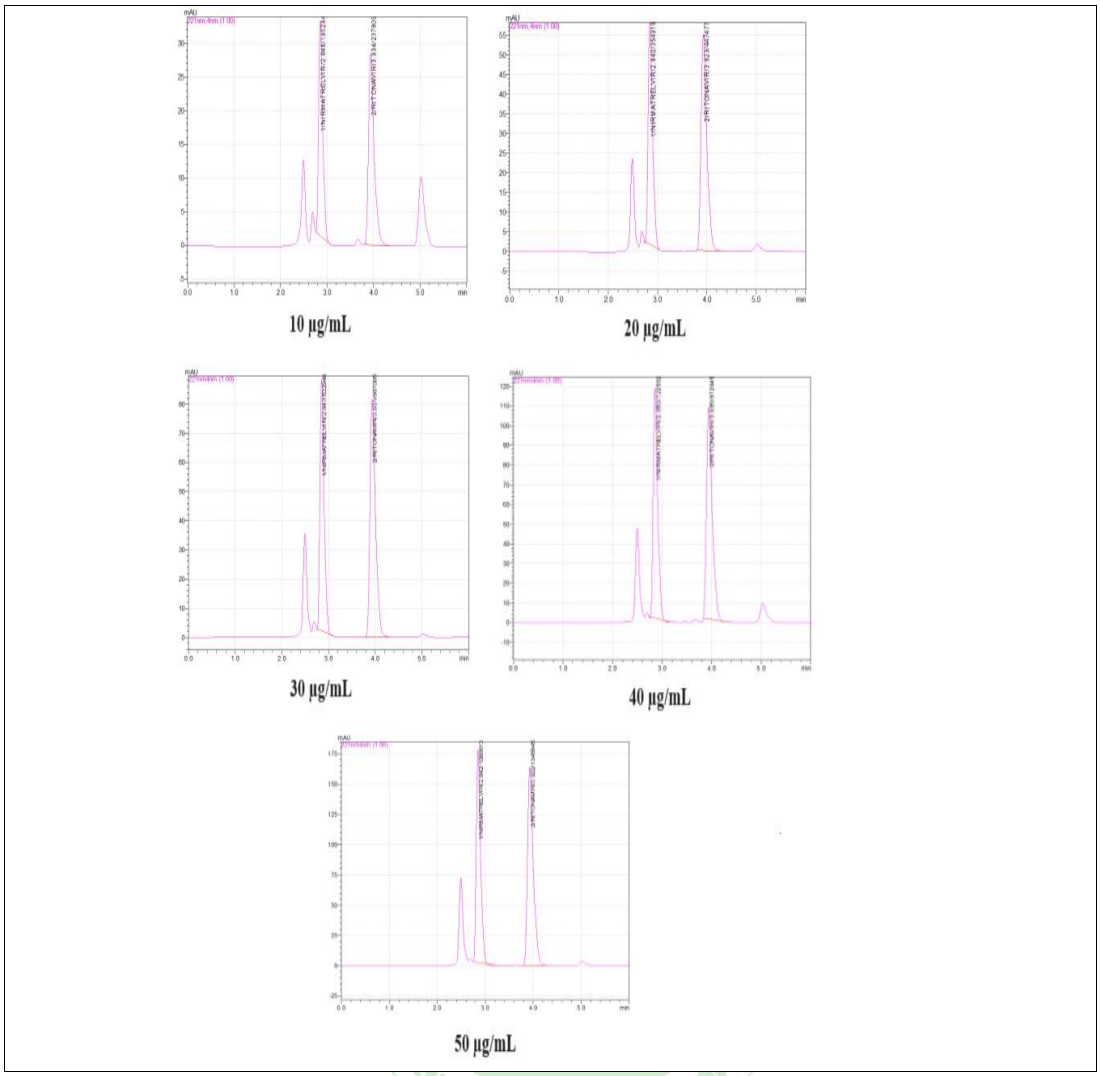

Figure 7: Various Linearity Chromatograms of Nirmatrelvir and Ritonavir

ACCURACY

Accuracy is the closeness of the test results obtained by the method to the true value. Recovery studies were carried out by addition of standard drug to the sample at 3 different levels of spiking i.e. 80%, 100% and 120% of the actual amount taking into consideration percentage purity of added bulk drug sample. Accuracy of Nirmatrelvir and Ritonavir are shown in Table No 4 & 5 respectively.

Table 4: Accuracy of Nirmatrelvir at wavelength 221 nm

Sr. No	level of Recovery %	Conc. (µg/mL)	Spiked	Total	Amount Recovery %	% Recovery
1	80%	30	24	54	53.95	99.91
2	80%	30	24	54	54.87	101.61
3	80%	30	24	54	53.85	99.72
4	100%	30	30	60	59.95	99.92
5	100%	30	30	60	59.99	99.98
6	100%	30	30	60	60.89	101.48
7	120%	30	36	66	65.94	99.91
8	120%	30	36	66	66.89	101.35
9	120%	30	36	66	65.85	99.77

Table 5: Accuracy of Ritonavir at wavelength 221 nm

Sr. No	level of	Conc.	Spiked	Total	Amount Recovery	% Recovery
1	80%	30	24	54	53.96	99.93
2	80%	30	24	54	54.91	101.69
3	80%	30	24	54	53.82	99.67
4	100%	30	30	60	59.92	99.87
5	100%	30	30	60	59.98	99.97
6	100%	30	30	60	60.82	101.37
7	120%	30	36	66	65.92	99.88
8	120%	30	36	66	65.96	99.94
9	120%	30	36	66	66.81	101.23

PRECISION

Precision is one of the key parameters assessed during method validation. The precision of an analytical method is the degree closeness of agreement between a series of measurements obtained from the multiple sampling of the same sample. Precision include repeatability, inter and intraday precision and reproducibility

a. Repeatability

For repeatability minimum of 6 determinants were prepared of 30µg/mL conc. of Nirmatrelvir and Ritonavir, respectively. The chromatogram responses were obtained by injecting one by one. The standard deviation & relative standard deviation was calculated for each type of precision. Repeatability of Nirmatrelvir and Ritonavir are shown in Table No 6& 7 respectively.

Table 6: Repeatability of Nirmatrelvir at wavelength 221 nm

Sr. No	Peak Area of Nirmatrelvir (µV. sec)
1	532948
2	533568
3	533941
4	529546
5	534146
6	531445
MEAN	532599
SD	1783.67
%RSD	0.334900

Table 7: Repeatability of Ritonavir at wavelength 221 nm

Sr. No	Peak Area of Ritonavir (µV. sec)
1	667095
2	673208
3	674511
4	677426
5	678975
6	685524
MEAN	676123.2
SD	6177.56
%RSD	0.913673

b. Intra-Day Precision

Intra-day precision, also known as within-day precision, evaluates the precision of analytical methods within a single day. It assesses the variation in results obtained from multiple analyses of the same sample within a single day. Intraday precision of conc. 20, 30, 40 µg/mL was prepared and data was obtained for Nirmatrelvir and Ritonavir. Intra-day precision of Nirmatrelvir and Ritonavir are shown in Table No 8& 9 respectively.

Table 8: Intra-Day Precision of Nirmatrelvir at wavelength 221 nm

Conc.	20µg/mL	30µg/mL	40µg/mL
Area (µV. sec)	354916	531712	724682
	355001	531820	734758
	360810	540875	734841
MEAN	356909	534802.33	731427
SD	3378.63	5259.361	5841.489
%RSD	0.9466369	0.9834214	0.7986427

Table 9: Intra-Day Precision of Ritonavir at wavelength 221 nm

Conc.	20µg/mL	30µg/mL	40µg/mL
Area (µV. sec)	445812	677095	882945
	446924	687157	883075
	448836	687241	873155
MEAN	447190.67	683831	879725
SD	1529.535	5833.698	5690.16
%RSD	0.342032	0.8530906	0.646811

c. Inter-Day Precision

Inter-day precision, also known as between-day precision, assesses the precision of analytical methods over multiple days. It evaluates the variation in results obtained from multiple analyses of the same sample conducted on different days. Inter-Day precision of conc. 20, 30, 40 µg/mL was prepared and data was obtained for Nirmatrelvir and Ritonavir. Inter-day precision of Nirmatrelvir and Ritonavir are shown in Table No 10& 11 respectively.

Table 10: Inter-Day Precision of Nirmatrelvir at wavelength 221 nm

Conc.	20µg/mL (Day 1)	30µg/mL (Day 2)	40µg/mL (Day 3)
Area (µV. sec)	361510	548529	726724
	366411	548615	736823
	366312	544718	736924
MEAN	364744.333	547287.33	733490.333
SD	2801.452	2225.523	5860.034
%RSD	0.7680591	0.4066462	0.7989245

Table 11: Inter-Day Precision of Ritonavir at wavelength 221 nm

Conc.	20µg/mL	30µg/mL	40µg/mL
Area (µV. sec)	447482	677101	876460
	447510	687122	876575
	451620	688045	886621
MEAN	448870.667	684089.3	879885.333
SD	2381.03	6069.64	5833.542
%RSD	0.5304498	0.8872590	0.6629888

LIMIT OF DETECTION (LOD)

The lowest concentration of the analyte that can be consistently detected but not always quantified is known as the limit of detection, or LOD. This guarantees the sensitivity and reliability of the approach by ensuring that singles detected above the LOD can be distinguished from background noise. The LOD of Nirmatrelvir and Ritonavir are shown in Table No 12 respectively.

Table 12: LOD of Nirmatrelvir and Ritonavir at wavelength 221 nm

Sr. No	Drug	LOD
1	**Nirmatrelvir**	0.317529 µg/mL
2	**Ritonavir**	0.893981 µg/mL

LIMIT OF QUANTITATION (LOQ)

The lowest concentration of the analyte that can be accurately and precisely measured and quantified is known as the limit of quantification, or LOQ. The lowest concentration standard solutions, when the single-to-noise ratio hits a predetermined level, are analysed to ascertain this. The LOQ of Nirmatrelvir and Ritonavir are shown in Table No 13 respectively.

Table 13: LOQ of Nirmatrelvir and Ritonavir at wavelength 221 nm

Sr. No	Drug	LOQ
1	**Nirmatrelvir**	0.962211 µg/mL
2	**Ritonavir**	2.709034 µg/mL

ROBUSTNESS

Robustness is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameter. For HPLC robustness was carried out by changing wavelength, flow rate and mobile phase composition. The impact of changing in detection wavelength, within ±2 nm, was specifically studied. The robustness data for Nirmatrelvir and Ritonavir are shown in Table No 14, 15, & 16 respectively.

Table 14: Robustness of Nirmatrelvir and Ritonavir at Wavelength 221±2 nm

Sr. No	Wavelength (nm)	Rt. of Nirmatrelvir	Rt. of Ritonavir	Peak Area of Nirmatrelvir	Peak Area of Ritonavir
1	219	2.847	3.931	532942	677080
2	221	2.847	3.931	532948	677095
3	223	2.847	3.931	532951	677098
			MEAN	532947	677091
			SD	4.582576	9.643651
			%RSD	0.000860	0.001424

Table 15: Robustness of Nirmatrelvir and Ritonavir by changing the Flowrate

Sr. No	Flow rate (ml/min)	Rt. of Nirmatrelvir	Rt. of Ritonavir	Peak Area of Nirmatrelvir	Peak Area of Ritonavir
1	0.1	2.841	3.924	533047	677190
2	1	2.844	3.927	532951	677097
3	1.1	2.847	3.931	532882	676901
			MEAN	532960	677062.667
			SD	82.86736	147.52740
			%RSD	0.015549	0.02179

Table 16: Robustness of Nirmatrelvir and Ritonavir by changing Mobile phase

Sr. No	tio (V/v %)	Rt.of Nirmatrelvir	Rt. of Ritonavir	Peak Area of Nirmatrelvir	Peak Area of Ritonavir
1	65:35	2.843	3.926	532946	677094
2	70:30	2.846	3.929	533052	678096
3	75:25	2.849	3.932	533155	679098
			MEAN	533051.00	678096
			SD	104.5035885	1002
			%RSD	0.01960	0.14777

ANALYSIS PREPARATION OF TABLE NO T FORMULATION

The RP-HPLC (Reverse Phase High-Performance Liquid Chromatography) method effectively determined the content of Nirmatrelvir and Ritonavir in tablet dosage form. The content was found to be consistent with the label claim, indicating that the tabletcontains the expected amount of these medications. This suggests that the manufacturing process is reliable

and the tablet are likely to provide the intended therapeutic effect.

Table 17: Analysis of Nirmatrelvir and Ritonavir Marketed formulation (Paxlovid)

Sr. No	Amount Present In (mg)		Amount Found In (mg)		% label claim	
	Nirmatrelvir	Ritonavir	Nirmatrelvir	Ritonavir	Nirmatrelvir	Ritonavir
1	30	20	29.95	19.92	99.83	99.6
2	30	20	29.93	19.91	99.77	99.55
3	30	20	30.21	19.87	100.70	99.35
4	30	20	29.85	20.05	99.50	100.25
5	30	20	29.78	19.91	99.27	99.55

DISCUSSION

The method was developed for the simultaneous estimation of Nirmatrelvir and Ritonavir in bulk and pharmaceutical dosage forms are essential steps towards ensuring the reliability and accuracy of pharmaceutical analysis. SThe specificity of the method was demonstrated by the absence of interference from other components present in the pharmaceutical matrix, ensuring accurate determination of Nirmatrelvir and Ritonavir content. Validation studies conducted in accordance with ICH guidelines confirmed the method's accuracy, precision, linearity, and robustness, meeting the required acceptance criteria. The method development phase, the composition of the mobile phase was carefully optimized, and suitable wavelengths were chosen for simultaneous estimation. Thermo fisher Scientific C18 (250mm x 4.6mm, 5µm) column was used, and the resolution and runtime were sufficient. The best separation was made possible by the mobile phase that was selected a 70:30, v/v mixture of acetonitrile and phosphate buffer. The flow rate was maintained at 1 ml/min, retention time of Nirmatrelvir and Ritonavir was determined to be 2.8 min and 3.9 min. Wavelength selection for simultaneous estimation was based on overlaying UV spectra, resulting in the selection of 221mm.Validation of the developed RP-HPLC method encompassed assessing various parameters such as linearity, precision, accuracy, ruggedness, robustness, LOD, and LOQ. The method displayed excellent linearity, with a regression coefficient (R^2) close to 1. The limit of detection was found to be 0.317529 µg/mL and 0.893981 µg/mL respectively for Nirmatrelvir and Ritonavir. The limit of quantitation was found to 0.962211 µg/mL and 2.709034 µg/mL respectively for Nirmatrelvir and Ritonavir.Precision analysis, indicated by %RSD values of repeatability of Nirmatrelvir is 0.334900 and ritonavir is 0.913673, Intra-day precision of Nirmatrelvir is 0.9466369, 0.9834214, 0.7986477 and Ritonavir is 0.342032, 0.8530906, 0.646811 and Inter-day precision of Nirmatrelvir is 0.7680591, 0.4066462, 0.7989245 and Ritonavir is 0.5304498, 0.8872590, 0.6629888. Accuracy was assessed through recovery studies, with % recovery values of Nirmatrelvir ranging from 99.72% to 101.61% and % recovery values of Ritonavir ranging from 99.67% to 101.69%.The method's robustness and reliability make it well-suited for routine analysis in pharmaceutical laboratories because it consistently delivers accurate and consistent results, even when subjected to variations in experimental conditions or external factors.

CONCLUSION

In conclusion, the developed RP-HPLC method offers a reliable and efficient approach for the simultaneous estimation of Nirmatrelvir and Ritonavir in bulk and pharmaceutical dosage forms, facilitating quality control and ensuring the safety and efficacy of drug products containing these active ingredients. The study proposes utilizing validating its efficacy across various parameters such as calibration curve, accuracy, precision, sensitivity, robustness, ruggedness, selectivity, and specificity. This approach enables efficient estimation in both combined marketed preparations and other pharmaceutical formulations. It simplifies sample and stock preparation for Nirmatrelvir and Ritonavir, allowing for direct solution introduction into the system in a single step. With a total analysis time of less than 6 minutes, it minimizes solvent wastage, making it highly efficient. Moreover, it exhibits remarkable sensitivity in detecting and quantifying microgram quantities of the analytes. Consequently, this method holds promise for routine analysis of Nirmatrelvir and Ritonavir in diverse marketed preparations and for estimating metabolites of these drugs.

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