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Simultaneous UV Spectrophotometric Method for Estimation of Nirmatrelvir and Ritonavir in Bulk and Tablet Dosage Form

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Abstract: *A simple, specific, precise, and accurate UV spectrophotometric method has been created for the simultaneous measurement of Nirmatrelvir and Ritonavir in pharmaceutical dosage forms. The absorption maxima of the drugs were found to be at 240 nm and 258 nm for Nirmatrelvir and Ritonavir respectively. Nirmatrelvir and Ritonavir obeyed Beer's law in the concentration range of 12-18 µg/ml and 8-12 µg/ml respectively. Different analytical parameters such as linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ) were determined as per ICH guidelines. Limit of detection and quantification values for Nirmatrelvir 1.25 and 3.85 µg/ml and for Ritonavir 0.34 and 1.12 µg/ml respectively. The accuracy of the methods was assessed by recovery studies and recovery values between prescribed limit of 99-101 % shows that method is free from interference of excipients present in formulation. The developed method was free from interferences due to excipients present in formulation and it can be used for routine quality control analysis. The results were validated statistically as per ICH Q2 R1 guideline and were found to be satisfactory. The proposed methods were successfully applied for the determination of for Nirmatrelvir and Ritonavir in commercial pharmaceutical dosage form.*

Keywords: Ritonavir, Nirmatrelvir, Simultaneous estimation, Absorbance maxima method, AreaICH.

I. INTRODUCTION

Nucleoside reverse transcriptase inhibitors (NRTIs) were the first class of drugs, which were introduced as antiretroviral agents for the treatment of infection with human immune deficiency virus (HIV). Additional drug classes were developed. They are protease inhibitors (PIs), non- nucleoside reverse transcriptase inhibitors (NNRTIs) and fusion inhibitors. [1,2] Nirmatrelvir is an orally bioavailable, peptidomimetic inhibitor of the severe acute respiratory syndrome coronavirus-2 (SARS-CoV2) main protease (Mpro; 3C-like protease; 3CL protease; 3CLpro; nsp5 protease), with potential antiviral activity against SARS-CoV-2 and other coronaviruses.

Upon oral administration, nirmatrelvir selectively targets, binds to, and inhibits the activity of SARS-CoV-2 Mpro. This inhibits the proteolytic cleavage of viral polyproteins, thereby inhibiting the formation of viral proteins including helicase, single-strandedRNA-binding protein, RNA-dependent RNA polymerase, 20-O-ribose methyltransferase, endoribonuclease andexoribonuclease. This prevents viral transcription and replication. [3] Ritonavir (RITO) is (5S, 8S, 10S, 11S)-10-hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4-thiazolyl]-3, 6-dioxo- 8, 11-bis (phenyl methyl)-2, 4, 7, 12-etraazatridecan-13-oic acid 5-thiazolyl methyl ester. It is official in Indian Pharmacopoeia [4] and United States Pharmacopoeia [5] . Ritonavir is an antiretroviral drug from the protease inhibitor class used to treat HIV infection and AIDS. Ritonavir is frequently prescribed with Highly Active Anti-Retroviral Therapy, not for its antiretroviral action, but as it inhibits the same host enzyme that metabolizes other protease inhibitors. These agents are metabolized by cytochrome P-450 (CYP) 3A in the liver. From the literature survey, it was found that Ritonavir estimated by analytical methods such as spectrophotometric methods [6-7], reversed-phase high-performance liquid chromatographic (RP- HPLC) method [8] and HPTLC method [9].

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II. MATERIALS AND METHODS

Materials:

Nirmatrelvir and Ritonavir were generous gift samples from Aadhaar Life Sciences Pvt. Ltd., Solapur, India. All other chemicals used were of analytical grade.

Instrumentation:

A Labman double beam UV–visible spectrophotometer, with a fixed bandwidth (2nm) and 1-cm quartz cell was used for Spectral and absorbance measurements. In addition, electronic balance, micropipette and Sonicator were used in this study.

Preparation of standard stock solution:

Nirmatrelvir Standard Stock Solution-I (NSSS-I):

Initially Prepare a Standard Stock Solution (MSSS-I) of by adding 15 mg of Nirmatrelvir in 10 ml volumetric flask & add 5 ml diluent, mix for 2 minutes and make the volume to 10 ml with diluent. (Conc. of Nirmatrelvir = 1500 µg/ml).

Ritonavir Standard Stock Solution-II (RSSS-II):

Then prepare a Standard Stock Solution (ASSS-II) of Ritonavir by adding 10 mg in 10 ml volumetric flask & add 5 ml diluent, mix for 2 minutes and make the volume to 10 ml with diluent. (Conc. of Ritonavir = 1000 µg/ml).

Then add 0.1 ml of MSSS-I & 0.1 ml ASSS-I in 10 ml volumetric flask and add 5 ml diluent and vortex and make up the volume with diluent. (Conc. of Nirmatrelvir= 15 µg/ml &Ritonavir = 10µg/ml).

Selection of analytical wavelength:

15µg/ml of Nirmatrelvir Working Standard and 10µg/ml of Ritonavir working Standard werescanned in the UV range of 190-400 nm. The overlay of both the spectrum was recorded. From the overlain spectra wavelengths 240 nm λmax of Ritonavir and 258 nm λmax of Nirmatrelvir were selected for analysis of both drugs using simultaneous method. (λ1-224 nm and λ2-260 nm).

The absorbance at λ1 and λ2 was measured and the concentration was calculated using following formula;

$$Cx = \frac{A2ay1 - A1ay2}{ax2ay1 - ax1ay2}$$

$$Cy = \frac{A1ax2 - A2ax1}{ax2ay1 - ax1ay2}$$

Where,

Cx and Cy are the concentrations of Nirmatrelvir and Ritonavir, respectively,

A1 and A2 are the absorbances of sample at λ1 and λ2, respectively, ax1 and ax2 are the absorptivity of Nirmatrelvir at λ1 and λ2, respectively, ay1 and ay2 are the absorptivity of Ritonavir at λ1 and λ2, respectively.

Validation:-

Linearity:

5 samples of varying concentrations ranging from 80% to 120% weremade.

The concentrations are given below The sample preparations are givenas below;

X ml of NSSS-I and Y ml of RSSS-II was diluted to 10 ml.

Table 1: Concentration

X ml of NSSS-I	Y ml of RSSS-II	Diluted to	Conc. of Nirmatrelvir (µg/ml)	Conc. of Ritonavir (µg/ml)
0.08	0.08	10 ml	12	8
0.09	0.09	10 ml	13.5	9
0.10	0.10	10 ml	15	10

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0.11	0.11	10 ml	16.5	11
0.12	0.12	10 ml	18	12

LOD/ LOQ:
Can be calculated by using AVONA Technique

$$LOD = \frac{3.3 \times Std\ Error\ of\ Intercept}{Coefficient\ of\ X\ variable\ 1}$$

$$LOD = \frac{10 \times Std\ Error\ of\ Intercept}{Coefficient\ of\ X\ variable\ 1}$$

Repeatability :
A single sample was prepared as described and 6 injections were made from same sample; checked for RSD.

Accuracy:
Samples were made of 80%, 100% and 120% concentration as per Table 1.
Samples were injected in triplicate to calculate % RSD
% recovery was also calculated.

% Conc.	Nirmatrelvir Conc. (µg/ml)	Ritonavir Conc. (µg/ml)
80	12	8
100	15	10
120	18	12

Intra- & Inter-day Precision:
The working standard and drug product samples were freshly prepared andanalysed in morning and evening for Intra-day precision.
The same working standard and drug product were used for analysis on 2ndday for inter-day precision.
% RSD for Assay was calculated for the confirmation of precision

III. RESULTS AND DISCUSSION

3.1 Selection of Wavelength

The Standard and Sample solution was scanned from 190 to 400 nm by using UV-VIS spectrophotometer against Diluent (Water: Acetonitrile (10:90)) as blank and the maximum absorption of standard and sample solution were recorded.

Result:
The maximum absorption for Nirmatrelvir was found to be 258 nm.
The maximum absorption for Ritonavir was found to be 240 nm.The UV scans for both the drugs is given below:

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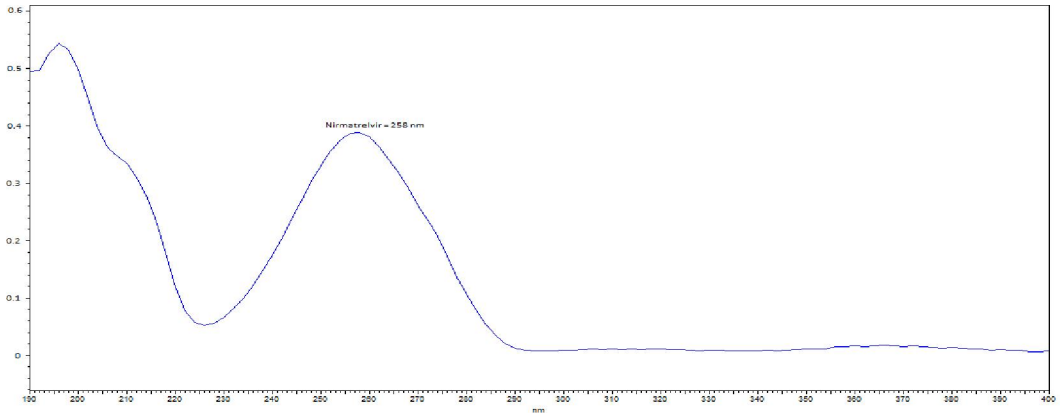

Figure 1: UV Scan of Nirmatrelvir

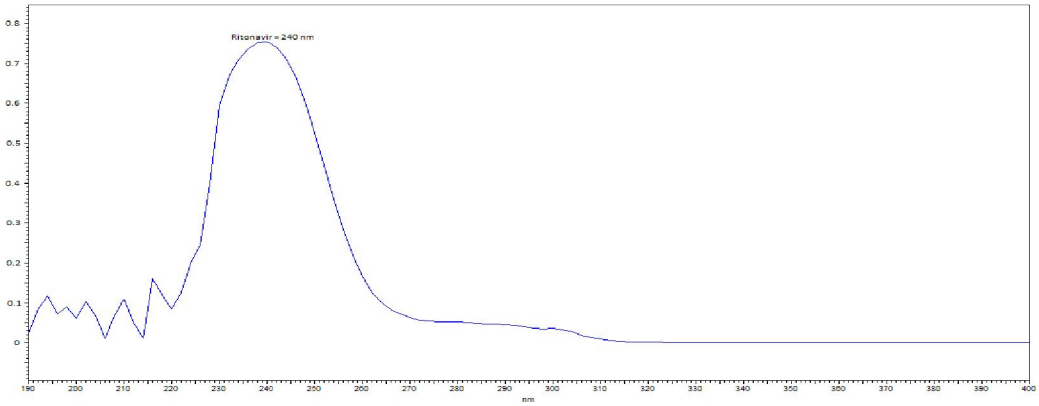

Figure 2: UV Scan of Ritonavir

Analysis of Tablet Formulation

Assay was performed using drug product, the data is given below:

Table 11: Assay of Nirmatrelvir & Ritonavir

Sample	**Nirmatrelvir**		**Ritonavir**	
	Conc. (µg/ml)	**% Assay**	**Conc. (µg/ml)**	**% Assay**
DP-1	15.12	100.80	10.11	101.10
DP-2	15.22	101.47	10.05	100.50
DP-3	15.01	100.07	10.15	101.50
DP-4	14.95	99.67	9.85	98.50
DP-5	14.94	99.60	9.97	99.70
AVG		100.32	**AVG**	100.26
STDEV		0.80	**STDEV**	1.19
%RSD		0.80	**RSD**	1.19

The Average % assay for Nirmatrelvir was found to be 100.32% and % RSD was found to be 0.80%. The Average % assay for Ritonavir was found to be 100.26% and % RSD was found to be 1.19%.

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Validation of UV Method

Validation is the systematic process of assessing and providing objective evidence that specific requirements for a particular intended use are met. It involves evaluating a method's performanceand demonstrating its capability to meet specific criteria. [10-12]

Specificity

It was confirmed with blank and working standard run that there was zero absorbance ofblank at set lambda in UV Spectrophotometer.

Linearity

The peak response is directly proportional to the concentration of drug and was found to belinear in the range of 8-12µg/ml.

The linearity data for Nirmatrelvir and Ritonavir is give below

Table 3: Linearity data for Nirmatrelvir

% Level	Concentration (µg/ml)	Absorbance
80	12	0.608
90	13.5	0.688
100	15	0.755
110	16.5	0.825
120	18	0.911

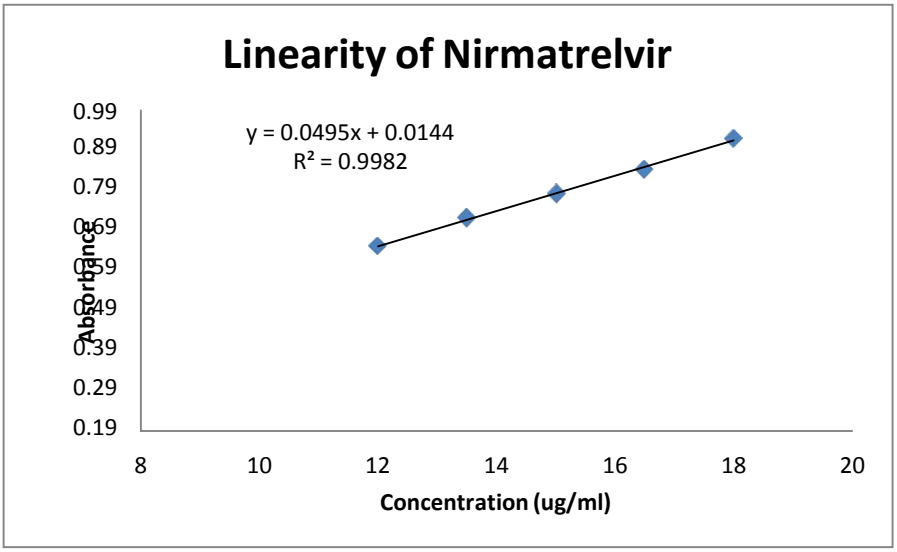

Figure 3: Linearity graph of NirmatrelvirTable 4: Linearity data for Ritonavir

% Level	Concentration (µg/ml)	Absorbance
80	8	0.317
90	9	0.355
100	10	0.394
110	11	0.435
120	12	0.476

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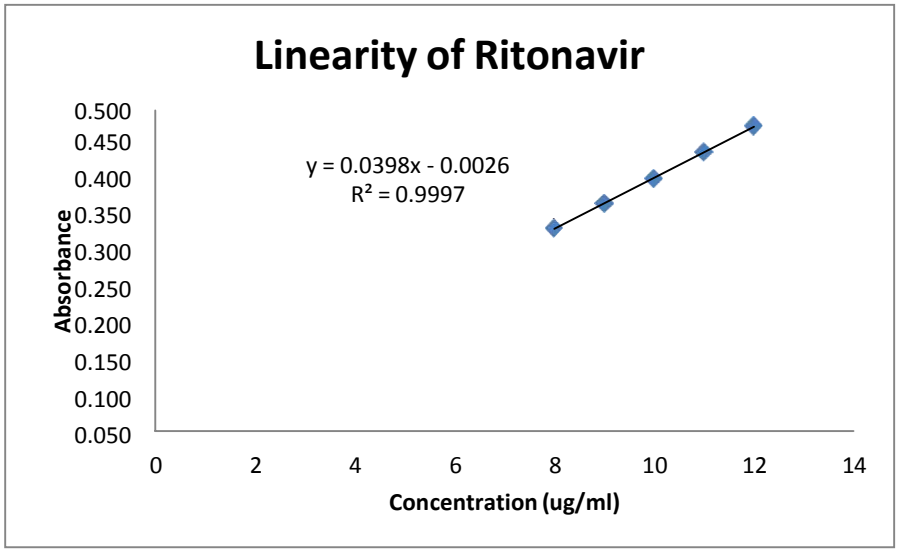

Figure 4: Linearity graph of Ritonavir

From the above data it was found that the correlation coefficient of Nirmatrelvir and Ritonavir were 0.998 and 0.999 respectively, which was found to be within the acceptance criteria of 0.998.

LOD and LOQ

Based on the linearity data, LOD and LOQ was calculated and reported as below:

Table 5: LOD & LOQ of Nirmatrelvir

Regression Statistics					
Multiple R	0.99967472				
R Square	0.999349546				
Adjusted R Square	0.999132728				
Standard Error	0.003521363				
Observations	5				
ANOVA					
	df	SS	MS	F	Significance F
Regression	1	0.0571536	0.0571536	4609.16129	7.04204E-06
Residual	3	3.72E-05	1.24E-05		
Total	4	0.0571908			
	Coefficients	Standard Error	t Stat	P-value	
Intercept	-0.0198	0.011246333	-1.76057391	0.176533624	
X Variable 1	0.03024	0.000445421	67.89080417	7.04204E-06	

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LOD	1.25	ug/ml
LOQ	3.85	ug/ml

Table 6: LOD & LOQ of Ritonavir

Regression Statistics					
Multiple R	0.999798052				
R Square	0.999596144				
Adjusted R Square	0.999461525				
Standard Error	0.00101653				
Observations	5				
ANOVA					
	df	SS	MS	F	Significance F
Regression	1	0.0076729	0.0076729	7425.387097	3.44494E-06
Residual	3	3.1E-06	1.03333E-06		
Total	4	0.007676			
	Coefficients	Standard Error	t Stat	P-value	
Intercept	-0.001	0.003246537	- 0.308020552	0.778214513	
X Variable 1	0.034625	0.000401819	86.17068583	3.44494E-06	

LOD	0.34	ug/ml
LOQ	1.12	ug/ml

From the above data it was found that:
The LOD & LOQ for Nirmatrelvir were found to be 1.25µg/ml and 3.85 µg/ml.
The LOD & LOQ for Ritonavir were found to be 0.34 µg/ml and 1.12µg/ml.

Repeatability
Repeatability was performed for both the APIs, the recorded absorbance is shown below:
Table 7: Repeatability of Nirmatrelvir and Ritonavir

Sample ID	**Nirmatrelvir ABS**	**Ritonavir ABS**
100% Rep 1	0.754	0.394
100% Rep 2	0.751	0.393
100% Rep 3	0.753	0.395
100% Rep 4	0.754	0.394

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100% Rep 5	0.755	0.392
100% Rep 6	0.755	0.391
AVG	0.754	0.393
STDEV	0.002	0.00
% RSD	0.20	0.37

From the above data, it can be seen that the % RSD for 6 replicate injections of Nirmatrelvir and Ritonavir are 0.20% and 0.37% respectively. The percentage RSD (<2) values obtained showed that the method developed was precise at repeatability precision level.

Accuracy

Accuracy of an analysis was determined by systemic error involved. Accuracy may often be expressed as % Recovery by the assay of known, added amount of analyte. It is measure of the exactness of the analytical method. Recovery studies carried out for both the methods by spiking standard drug in the powdered formulations 80%, 100%, 120% amount of each dosage content asper ICH guidelines.[13-14]

Table 8: Accuracy for Nirmatrelvir

% Level	Reps	SpikedConc. (µg/ml)	Abs	Amount Recovered (µg/ml)	% Recovery	%AVG	STDEV	% RSD
80	Rep 1	12.00	0.61	12.12	100.99	100.99	0.33	0.33
	Rep 2	12.00	0.608	12.08	100.66			
	Rep 3	12.00	0.612	12.16	101.32			
100	Rep 1	15.00	0.754	14.98	99.87	99.78	0.28	0.28
	Rep 2	15.00	0.755	15.00	100.00			
	Rep 3	15.00	0.751	14.92	99.47			
120	Rep 1	18.00	0.91	18.08	100.44	100.77	0.48	0.48
	Rep 2	18.00	0.911	18.10	100.55			
	Rep 3	18.00	0.918	18.24	101.32			

The %RSD of three replicates of Nirmatrelvir for accuracy level 80%, 100% and 120% was found to be 0.33%, 0.28% and 0.48% respectively.

The % recoveries for accuracy level 80%, 100% and 120% was found to be 100.99%, 99.78% and 100.77% respectively.

Table 9: Accuracy for Ritonavir

% Level	Reps	SpikedConc. (µg/ml)	Abs	Amount Recovered (µg/ml)	% Recovery	%AVG	STDEV	% RSD
80	Rep 1	8.00	0.321	8.15	101.84	101.10	0.66	0.65
	Rep 2	8.00	0.318	8.07	100.89			
	Rep 3	8.00	0.317	8.05	100.57			
100	Rep 1	10.00	0.394	10.00	100.00	100.00	0.25	0.25
	Rep 2	10.00	0.393	9.97	99.75			
	Rep 3	10.00	0.395	10.03	100.25			
120	Rep 1	12.00	0.478	12.13	101.10	100.89	0.21	0.21

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	Rep 2	12.00	0.476	12.08	100.68		
	Rep 3	12.00	0.477	12.11	100.89		

The %RSD of three replicates of Ritonavir for accuracy level 80%, 100% and 120% was found to be 0.65%, 0.21% and 0.21% respectively.
The % recoveries for accuracy level 80%, 100% and 120% was found to be 101.10%, 100.00% and 100.89% respectively.

Intra & Inter day Precision
The Standard solution of Nirmatrelvir and Ritonavir were examined for Intra and Inter day Precision, the data is shown below:

Table 10: Intra & Inter day Precision of Nirmatrelvir and Ritonavir

Condition	Sample ID	Interval	Nirmatrelvir		Ritonavir	
			Conc. (µg/ml)	% Assay	Conc. (µg/ml)	% Assay
Intraday	**WS**	**Mrng**	10.00	-	10.00	-
	DP	**Mrng**	10.05	100.50	10.12	101.20
	WS	**Evng.**	10.00	-	10.00	-
	DP	**Evng.**	10.03	100.30	10.01	100.10
Interday	**WS**	**Day 2**	10.00	-	10.00	-
	DP	**Day 2**	9.85	98.50	9.81	98.10
			% RSD	**1.10**	**% RSD**	**1.57**

The % Assay for Nirmatrelvir for Morning, Evening and Day 2 were found to be 100.50%, 100.30% and 98.50%, respectively.
The % Assay for Ritonavir for Morning, Evening and Day 2 were found to be 101.20%, 100.10% and 98.10%, respectively.
The %RSD for intra and Inter day Precision of Nirmatrelvir and Ritonavir were found to be 1.10% and 1.57%, respectively.
Hence, the working standard of Nirmatrelvir and Ritonavir is stable for 2 days as nosignificant variation was observed.

IV. CONCLUSION

This research was aimed to develop and validate UV method for the estimation of Nirmatrelvir and Ritonavir in bulk and tablet Formulation. The proposed methods were found to be appropriate due to its simplicity, reliability, sensitivity, rapidness and selectivity for detection at very low concentrations. The short chromatographic time makes this method suitable for processing of multiple samples in short time. The method showed no interference of the Excipients present in Nirmatrelvir and Ritonavir. The statistical parameters and recovery data reveals the good accuracy and precision of the proposed methods. The UV method developed forthe estimation of Nirmatrelvir and Ritonavir was validated as per the ICH guidelines
Validation data demonstrates that, these methods are accurate, precise, simple and economic and can be used in the routine analysis of Nirmatrelvir and Ritonavir in various formulations.

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