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Original Article

Simultaneous Determination of Pharmaceuticals Using Two UV-Based Methods with Different Diluents: Comparative Analysis via ANOVA and Greenness Assessment

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ABSTRACT

In this work, a UV spectrophotometric approach that is accurate, precise, and environmentally friendly Is proposed for concurrent quantification of Ritonavir and Nirmatrelvir in combination pharmaceutical form. While treating COVID-19, these antiviral medications are essential. The Q-absorbance method was created and validated in accordance with ICH criteria, and the simultaneous estimation method is constructed utilizing two distinct solvents. After being evaluated for linearity, accuracy, precision, robustness, and sensitivity, the suggested techniques showed great reproducibility and high correlation coefficients (r^2 = 0.999). One-way ANOVA statistical comparison verified that the devised methodologies were reliable and consistent for pharmaceutical analysis, with no significant differences found. Furthermore, the Green Analytical Procedure Index (GAPI), AGREE, and BAGI criteria were used to assess the Ecological sustainability of these methods. The results demonstrated that the suggested approaches provide a greener substitute for traditional analytical procedures by drastically lowering solvent usage and the production of hazardous waste. The proposed UV spectrophotometric approaches offer a straightforward, economical, and dependable solution for the routine quality evaluation of Ritonavir and Nirmatrelvir in dosage forms.

KEYWORDS: UV-spectrophotometric approach, Antiviral medications, One-way ANOVA, Environmental sustainability

1. Introduction

Nucleoside reverse transcriptase inhibitors (NRTIs) marked the initial class of antiretroviral drugs developed for combating HIV infection. Other classes of drugs were created. Protease inhibitors (PIs), fusion inhibitors, and non-nucleoside reverse transcriptase inhibitors (NNRTIs) are the three types [1]. The formula for Nirmatrelvir is $C_{23}H_{32}F_3N_5O_4$499.54 is its atomic mass. [2]. Nirmatrelvir is an orally bioavailable peptidomimetic compound exhibiting potential antiviral activity against SARS-CoV-2 and related coronaviruses. It functions by inhibiting the main protease of SARS-CoV-2 (Mpro), also known as the 3C-like protease (3CLpro or nsp5). When administered orally, nirmatrelvir selectively binds to and inhibits the SARS-CoV-2 main protease (Mpro), blocking the proteolytic cleavage of viral polyproteins. This disruption halts the synthesis of essential viral proteins—including helicase, ssRNA-binding protein, RNA-dependent RNA polymerase, 2′-O-methyltransferase, endoribonuclease, and exoribonuclease—thereby inhibiting viral transcription and

replication [3].

$C_{37}H_{48}N_6O_5S_2$ is chemical formula of ritonavir, and its atomic weight is 720.94. Ritonavir, a powerful inhibitor of the human immune virus protease and one of the few antivirals used to treat COVID-19 and HIV, is frequently used in conjunction with other antivirals to achieve a synergistic effect [4]. Ritonavir is an antiretroviral medication used to treat AIDS and HIV infection. It belongs to the protease inhibitor class. Ritonavir is commonly administered in conjunction with Highly Active Anti-Retroviral Therapy Since it targets the same host enzyme involved in breaking down other protease inhibitors, rather than because it has antiretroviral properties. In the liver, cytochrome P-450 (CYP) 3A breaks down these substances [5].

Paxlovid™ is an antiviral therapy developed by Pfizer for the treatment and post-exposure prophylaxis of infections caused by SARS-CoV-2, the virus responsible for the global COVID-19 pandemic. The recommended regimen includes 300 mg of nirmatrelvir (administered as two 150 mg tablets) alongside 100 mg of ritonavir (administered as a single 100 mg tablet). Combined, ritonavir and nirmatrelvir had an average half-life of seven hours. [6,7]

Nirmatrelvir and Ritonavir can be estimated using a variety of analytical techniques, including HPLC [8], HPTLC [9], UPLC [10]and LC-MS [11], As a monotherapy or part of a combination treatment. However, there is currently no UV spectrophotometric method available for the concurrent measurement of Ritonavir and Nirmatrelvir in a combination dosage form. By following a few easy manipulation steps, the suggested spectrophotometric techniques might be employed to determine the binary mixture of nirmatrelvir and ritonavir simultaneously with accuracy and precision. The suggested techniques could be applied to the evaluation of their PaxlovidTM tablet market formula. To evaluate the effectiveness of the suggested procedures in determining nirmatrelvir and ritonavir, a comparison of the obtained results was conducted. Therefore, the goal of this work is to create and verify two new spectrophotometric techniques for the simultaneous measurement of Ritonavir and Nirmatrelvir in a combination dosage form.

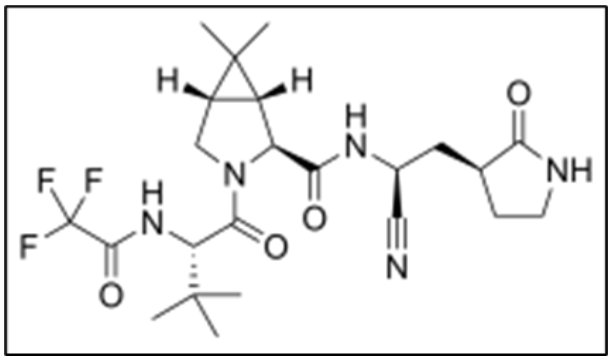


Fig. 1 Structure of Nirmatrelvir

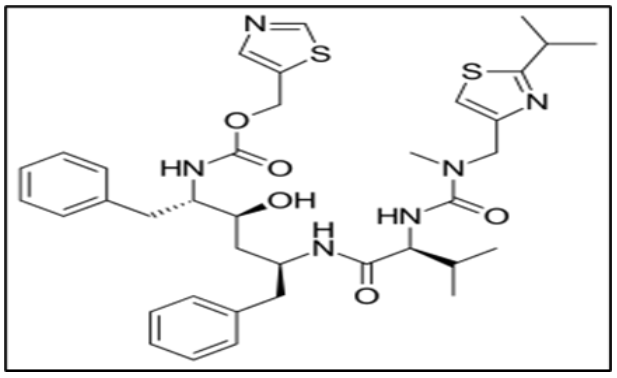


Fig.2 Structure of Ritonavir

ANOVA, or Analysis of Variance, is a statistical approach designed to evaluate differences among the means of multiple groups. Serving as a broader application of the two-sample t-test, it allows for comparison across more than two datasets. The method focuses on detecting significant variations between group means by analyzing and contrasting within-group and between-group variances. This is achieved by comparing two independent estimates of the population variance to validate or refute the hypothesis [12,13].

These days, in pharmaceutical analysis, commonly used terms include "green chemistry," "benign chemistry," and so on. These divisions focus on energy usage, waste production, substrate utilization, waste reduction or elimination, and the use of toxic or hazardous solvents. These procedures primarily aim to eliminate toxic and hazardous substances, replacing them with safer, eco-friendly alternatives that protect both the environment and analyst health. Green assessment focuses on minimizing solvent toxicity and volume during analytical operations and sample preparation, in alignment with the principles of green analytical chemistry [14].

The pictogram-based Green Analytical Procedures Index (GAPI) is a user-friendly instrument that facilitates the reading of results. Waste creation, instruments, solvent and reagent consumption, sample preparation, and sampling are all considered in this evaluation of the analytical process. GAPI offers a practical means to compare methods and identify the most environmentally sustainable option. The results of the GAPI tool and AGREE evaluation technique all concur, demonstrating the environmentally friendly nature of the new UV spectrophotometric technique. The feasibility and environmental friendliness of analytical techniques are evaluated using a new GAC metric called BAGI. BAGI evaluates the environmental impact of analytical methods using a combination of visual cues, a color gradient and a scoring system, allowing for quick and intuitive assessment of assay greenness. Although its scope can still be enhanced, BAGI remains a practical and user-friendly tool that complements established GAC metrics like AGREE and GAPI [15,16].

2. Materials and Methods

2.1 Devices and operating system:

The Shimadzu UV-1800 spectrophotometer was used for the analysis. UV-Win is the software utilized. Using 1.0 cm quartz cuvettes, the absorption spectra of the reference and test solutions were captured across the 200–400 nm UV range.

2.2 Materials

Nirmatrelvir and ritonavir were given as gift samples by Cipla Private Limited, Patalganga unit II, Rasayani, Raigad. Analytical grade methanol was used. Aceto Pharma Private Ltd., located in Ahmedabad, Gujarat, was the provider of all the chemicals. All the solvents such as methanol,acetonitrile have 99.99% purity . The commercial formulation PRIMOVIR, which comprises Nirmatrelvir and Ritonavir, was used for assay and accuracy.

2.3 Selection of solvents:

Solvents including distilled water, methanol, ethanol, and acetonitrile were used to test the solubility of both medications. Both medications were discovered to be soluble in methanol. To achieve solubility, several methanol to water ratios were also employed, a 60:40 methanol to water ratio was chosen as the solvent.

2.4 Selection of Hydrotropic Agent:

A mixture of 2M sodium acetate and 8M urea (50:50) solution was determined to be the most suitable since both medications are soluble in it and show good spectral features in it. Nirmatrelvir and Ritonavir Spectral scanning was performed in the presence of a hydrotropic agent over the UV range of 200–400 nm.

2.5 Standard stalk solution:

Standard stalk solutions of ritonavir and nirmatrelvir Were produced by dissolving precisely weighed 100 mg of each medication in certain solvents in two distinct 100 mL volumetric flasks. Two 100 mL volumetric flasks were filled with 10 mL of each drug's standard stalk solution. The volume was then adjusted to the desired level using the same solvent to create a 100 µg/mL solution.

2.6 METHOD A: Simultaneous equation method (SEM)

a) To determine the proposed analytes, the UV spectrum of each of the standard analytes under investigation Was detected in the UV wavelength range between 200 and 400 nm. Solvent used here is Methanol: Water (60:40). The overlain zero order spectra of Nirmatrelvir and Ritonavir showed maximum wavelengths at 215 nm and 240 nm, respectively.

b) In this method, a 50:50 mixture of 2 M sodium acetate and 8 M urea was used as the solvent system. Nirmatrelvir exhibited maximum absorbance at 214 nm, while Ritonavir showed its absorbance peak at 222 nm. The analytical approach relies on the absorption of each drug at the wavelength maximum of the other. The formula used is,

$c_x = A_2a_{y1} - A_1a_{y2} / a_{x2}a_{y1} - a_{x1}ay_2$ (eq.1) and $c_y = A_1a_{y2} - A_2a_{y1} / a_{x2}a_{y1} - a_{x1}a_{y2}$(eq.2)

where,

A_1 and A_2 are absorbances at 215 nm and 240 nm;

a_{x1} and a_{y1} are absorptivity's of Nirmatrelvir and Ritonavir respectively at 215 nm;

a_{x2} and a_{y2} are absorptivity's of Nirmatrelvir and Ritonavir respectively at 240 nm.

2.7 METHOD B: Q-absorption ratio method

Ratio between two selected wavelengths one being the isosbestic wavelength and the other being the absorption maxima of one of the two components is used in this technique. The proposed method is applicable to drugs that obey Beer- Lambert's Law across all wavelengths and exhibit a consistent absorbance ratio between any two wavelengths, independent of path length or concentration. To get overlain spectra, the solutions of 15 µg/mL and 10 µg/mL for Nirmatrelvir and Ritonavir were scanned in the 400–200 nm Spectral range. Two wavelengths were chosen for the creation of the Q-absorbance ratio equation: 215 nm (the Nirmatrelvir absorption peak) and 225 nm (the iso-absorptive point).
The following formula was used to determine each component's concentration.
Cx=(Qm-Qy/Qx-Qy)×A1.ax(eq.3)

Cy=(Qm-Qy)/Qy-Qx)×A1/ax1(eq.4)

Where,

Qm=A2/A1, A1 is absorbance of sample at iso-absorptive point, A2 is absorbance of sample at absorbance maxima of one drug.

Qx=ax2/ax1, Qy=ay2/ay1,

Ax1 and ay2 represent absorptivity's of Nirmatrelvir at 225 nm and 215 nm respectively,

Cx and Cy be the concentration of Nirmatrelvir and Ritonavir respectively.

2.8 ASSESSMENT AND VALIDATION OF SPECTROPHOTOMETRIC TECHNIQUES:

The developed methods for the simultaneous estimation of Nirmatrelvir and Ritonavir were validated in accordance with ICH guidelines, assessing key parameters including linearity, accuracy, precision, robustness, as well as detection and quantification limits [17].

2.8.1 Linearity :

Linearity of proposed UV methods was established using six different concentration ranges of Nirmatrelvir and Ritonavir. In Method A linearity range for nirmatrelvir was 10-50 µg/mL and for ritonavir it was 10-60 µg/mL. In Method B linearity range was 10-50 µg/mL and 4-24 µg/mL for Nirmatrelvir and ritonavir simultaneously, at 215 nm and 225 nm wavelength. In Method C linearity range was 5-25 µg/mL and 5-25 µg/mL for both the drugs at 214 nm and 222 nm.

2.8.2 Accuracy:

The interference from excipients was evaluated using the standard addition method by calculating the % recovery of the drugs. In this approach, known amounts of standard nirmatrelvir and ritonavir solutions were added to the sample solution, and the recovered drug content was expressed as mean recovery with corresponding upper and lower limits, along with %RSD values.

2.8.3 Precision:

Method precision was evaluated through intra-day and inter-day studies. Repeatability was assessed by six replicate analyses of the sample solutions, with %RSD calculated from the drug responses at their respective method-specific wavelengths. By comparing this concentration on the same day and following day, the intraday and interday precisions were ascertained. Less than two was the acceptable limit for %RSD.

2.8.4 Robustness:

To evaluate the robustness of the analytical methods, the wavelengths were varied by ±3 nm from the predetermined maximum absorbance wavelengths of both nirmatrelvir and ritonavir. The results were assessed based on %RSD values.

2.8.5 Limit of detection and quantification:

The limits of detection (LOD) and quantification (LOQ) were determined using the standard deviation of the response (σ) and the slope of the calibration curve (SD). The LOD was calculated as 3.3 × σ/SD, while the LOQ was calculated as 10 × σ/SD.

2.8.6 ASSESSMENT OF TABLET FORMULATION:

Twenty marketed tablets containing nirmatrelvir and ritonavir were weighed and finely powdered. An appropriate amount of the powder was transferred to a volumetric flask and diluted with suitable solvents as per the respective methods. After complete dissolution, the solution was filtered using Whatman filter paper. The filtrate was collected and further diluted to bring the concentrations of both drugs within the working range. The absorbances of the final dilutions were measured at selected wavelengths, and the drug concentrations were determined using all proposed methods.

2.8.7 Statistical comparison by one way ANOVA:

Using one-way analysis of variance, statistical analysis was done to evaluate the impact of two approaches in the simultaneous estimation of ritonavir and nirmatrelvir.

2.8.8 Greenness assessment

The GAPI pictogram's five pentagrams assess and display the greenness of each stage in the analytical process, serving as a reliable tool for Green Analytical Chemistry (GAC) evaluation. From the sample collection until the final analysis, it may offer a thorough assessment of the entire analytical process, both qualitatively and numerically. Using a three-level color scale, the GAPI pictogram illustrates how green specific stages of the entire analytical process are. The colors green, yellow, and red, respectively, indicate low, medium, and high impacts of the analytical process on the environment and human health.

Consequently, various factors are considered, such as energy consumption and the environmental and health hazards posed by chemicals. Moreover, GAPI offers insights into the entire analytical procedure. Importantly, the compact design of the GAPI pictogram allows for easy side-by-side comparison of multiple methods, aiding in the selection of the most eco-friendly option for a given study.

AGREE is an intuitive and versatile assessment tool that assesses the environmental friendliness of analytical tests and provides understandable, unambiguous results. The GREEN metric is computed using the 12 GAC principles. which are transformed into a 0–1 scale. The AGREE symbol's central area shows the final greenness score along with its corresponding color. The twelve GAC Principles are conveyed by the twelve segments that surround the pictogram's core area. The AGREE pictograph has segments that represent the breadth of each principle's weight. Furthermore, the AGREE symbol's dark green color can be changed to red for each part. Hence, the AGREE pictogram serves as a user-friendly tool to evaluate the greenness of the analytical assay, guided by the 12 GAC principles. The BAGI measure uses ten characteristics to assess the environmental friendliness and feasibility of different testing. This GAC measure's attributes, scores, and related colors. The scoring range for this GAC metric is 25–100. A higher score on the BAGI meter indicates better assay greenness [18].

3 Results

It is believed that the projected spectrophotometric techniques are most suited for application in quality control areas where evaluation speed and cost are crucial. Because of their simplicity, speed, affordability, and repeatability, UV-spectrophotometric techniques are widely used for routine examination of pharmaceutical preparation. These spectrophotometric techniques are excellent and have several benefits when compared to other analytical techniques. It can be challenging to analyze all analytes without prior separation when dealing with multi-component compositions that Exhibit overlapping ultraviolet spectra The suggested approach develops a few easy and affordable methods for the simultaneous analysis of ritonavir and nirmatrelvir. This section should provide a concise and precise description of the experimental results, their interpretation, as well as the experimental which can be drawn from the individual parts of research. English format of numbers must be applied.

3.3.1 Determination of wavelength of maximum absorbance (λ max)

Quantitative analysis requires that the wavelength with the highest absorption be identified. Nirmatrelvir and ritonavir solutions' complete scans revealed that their respective λ max were 215 nm and 240 nm. Spectra for Nirmatrelvir shown in Fig 3 and spectra for Ritonavir is shown in fig,4. The iso-absorptive point, or 225 nm, is represented by the overlapped spectra of both medications. Both the iso-absorptive and nirmatrelvir wavelengths were employed in the Q-absorbance ratio approach. Nirmatrelvir and ritonavir have wavelengths of 214 and 222 nm, respectively, in the mixed hydrotropy method. Figure 3 and 4 shows UV spectra of Nirmatrelvir and Ritonavir simultaneously, and figure no.5 shows the overlay spectra of Nirmatrelvir and Ritonavir indicating isosbestic point of both drugs. As shown in Fig.5, To make the key points of the data more visible, we adjusted the graph's absorbance scale (y-axis). This could entail removing extremely high absorbance values that are unnecessary for the comparison or focusing on lower absorbance values.

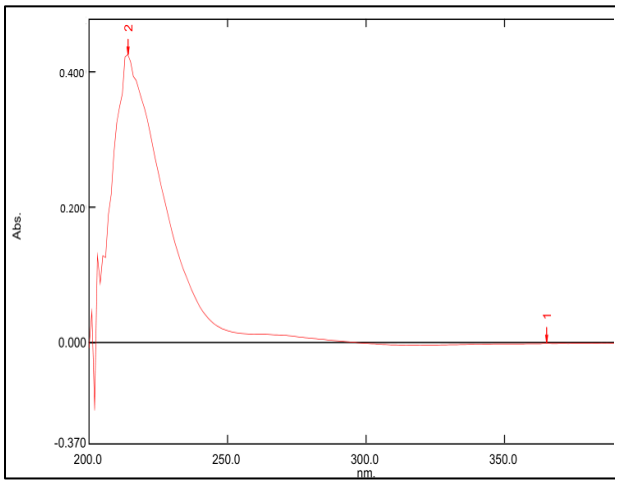


Fig.3 Zero order UV spectra of Nirmatrelvir (15 µg/mL) using methanol: water (60:40) as blank

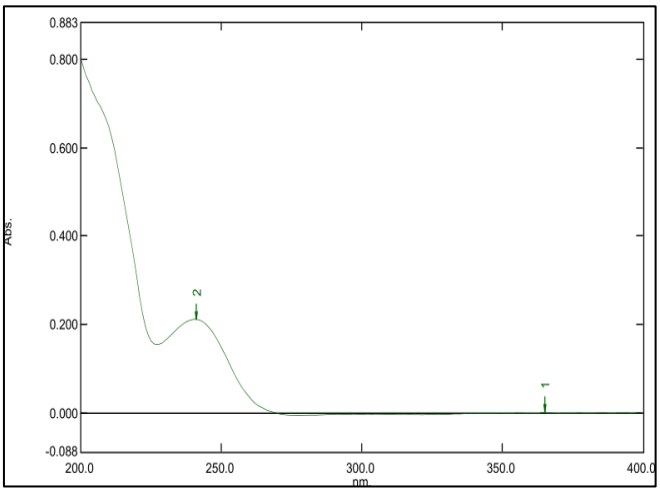


Fig.4 Zero order UV spectra of Ritonavir (10 µg/mL) using methanol: water (60:40) as blank

TABLE 1: Optical parameters and linearity measurements of NTV(Nirmatrelvir) and RTV (Ritonavir)

Method	Method A				Method B			
Solvent	a)Methanol: Water(60:40)		b)8M Urea:2M sodium acetate (50:50)		Methanol: Water (60:40)			
Drug	NTV	RTV	NTV	RTV	NTV	RTV	NTV	RTV
Wavelength	215 nm	240 nm	214 nm	222 nm	225 nm		215 nm	
Linearity Range (µg/mL)	0-50	10-60	0-25	0-25	0-50	4-24	0-50	4-24
Correlation coefficient (r^2)	0.9991	0.9991	0.9990	0.9998	0.9993	0.9990	0.9993	0.9993
Slope (m)	0.0181	0.0148	0.0382	0.0331	0.0115	0.0181	0.0184	0.0393
Intercept (c)	0.0102	0.0023	-0.0174	0.0053	0.002	0.0237	0.0031	0.051
LOD (µg/mL)	0.5586	0.7237	0.3248	4.3802	0.4723	0.2951	0.4861	0.2610
LOQ (µg/mL)	1.8623	2.4126	0.9844	13.2735	1.5744	0.9839	1.6204	0.8700
t-Test	1.2394	0.2647	-1.8920	-1.4720	0.4531	-5.4522	0.4231	-6.0852
p value	0.2882	0.8042	0.1314	0.2149	0.6739	0.0054	0.6939	0.0036

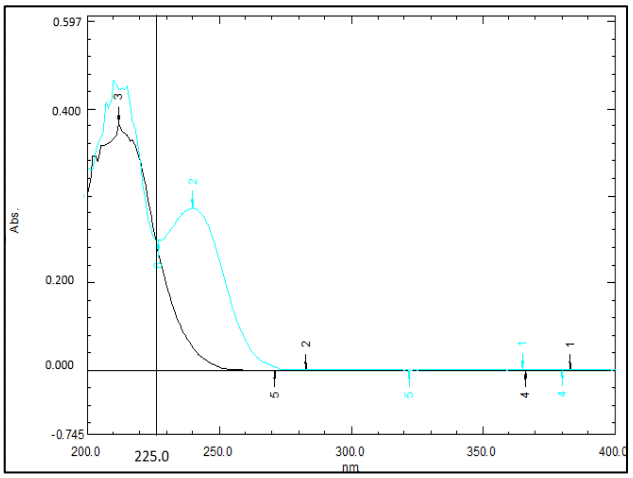


Fig .5 Zero order overlay spectra of Nirmatrelvir (15 µg/mL) and Ritonavir 10 (µg/mL) using methanol: water (60:40) as blank

3.3.2 Linearity:

The crucial analytical technique parameter that shows the upper limit of the desired approach to be used for its best performance is linearity. Table 1. lists the concentration and optical characteristic values of Ritonavir and Nirmatrelvir, respectively. It was discovered that every calibration curve in every method was linear over the concentration range that was being examined. It was discovered that the suggested UV approach was linear and adhered to Beer- Lambert's law. In addition, ANOVA was employed to statistically compare the outcomes of the established spectrophotometric procedures for both medications. The P values that were found to be within the threshold range indicate that there are no appreciable differences between the two recommended approaches. These outcomess demonstrated that every proposed approach offers higher and comparable analytical performance.

3.3.3 Accuracy:

Accuracy describes the degree of agreement between the observed value and the actual value. The extent to which measured values correspond to a conventional true value or an accepted reference value defines the accuracy of an analytical procedure. The percentage of drug recovery was used to calculate the interference of the excipients using the usual addition method. This approach involved adding a standard solution of ritonavir and nirmatrelvir to the test solution, and calculating the standard medication stated as mean recovery value with associated limits along with % RSD. Table 2. presents the findings from the recovery studies. Suggested approach was found to be accurate.

3.3.4 Precision:

The outstanding consistency of all the proposed techniques, both within a single day and across multiple days, is demonstrated by the intra-day and inter-day precision results, expressed as %RSD ensure ICH recommendation limits (<2). Table 3. indicates precision data of both drugs.

Table 2. Accuracy data of NTV(Nirmatrelvir) and RTV(Ritonavir) by the proposed methods

Mehod	Solvent	Drug and wavelength	Level	Conc. Present (µg/mL)	Spiked conc. (µg/mL)	Total conc. Taken (µg/mL)	Mean of total conc. found(µg/mL)	%Recovery	%RSD
Method A	a)Methanol:Water (60:40)	NTV(215 nm)	80%	20	16	36	35.53	98.72%	0.95
			100%		20	40	40.29	100.73%	1.75
			120%		24	44	43.93	99.85%	1.98
		RTV(240 nm)	80%	20	16	36	36.31	100.87%	0.48
			100%		20	40	40.46	101.17%	0.92
			120%		24	44	44.45	101.03%	0.76
	B) 8M Urea: 2M Sodium acetate (50:50)	NTV(214 nm)	80%	10	8	18	18.34	101.94%	0.87
			100%		10	20	19.72	98.63%	1.02
			120%		12	22	21.99	99.96%	0.40
		RTV(222 nm)	80%	10	8	18	18.27	101.56%	0.45
			100%		10	20	20.04	100.20%	0.53
			120%		12	22	22.01	100.05%	0.72
Method B	Methanol:Water (60:40)	NTV(225 nm)	80%	20	16	36	36.39	101.10%	1.86
			100%		20	40	40.45	101.14%	0.26
			120%		24	44	44.10	100.24%	0.68
		RTV(225 nm)	80%	8	6.4	14.4	14.37	99.79%	0.21
			100%		8	16	16.18	101.17%	1.68
			120%		9.6	17.6	17.68	100.48%	0.63

NTV(215 nm)	80%	20	16	36	36.18	100.51%	0.88
	100%		20	40	40.17	100.43%	0.45
	120%		24	44	44.36	100.82%	0.44
RTV(215 nm)	80%	8	6.4	14.4	14.39	100.00%	0.04
	100%		8	16	16.17	101.11%	0.04
	120%		9.6	17.6	17.25	98.05%	0.04

3.3.5 Robustness:

The method's ability to remain effective under minor modifications, intentional changes are made to its parameters is known as its robustness. This is a vital component of the analytical methodology, since little, Inadvertent variation in method parameters like wavelength may emerge in the process of normal use and may impede the efficiency of the procedure. It is anticipated that this modification won't affect how well the analytical procedure performs. The %RSD figures, which are less than 2%, show that the approach is resilient. The robustness of the suggested UV technique was demonstrated by %RSD values being less than 2.

3.3.6 Limit of quantification and limit of detection

LOD stands for the lowest concentration that can be examined with a reasonable level of precision and accuracy. All two approaches' LOD and LOQ values turned out to be extremely low, indicating the degree of sensitivity of the anticipated procedures. Table 1. contains information of LOD and LOQ values.

3.3.7 Assessment of tablet formulation:

The suggested methods were successfully used to evaluate ritonavir and nirmatrelvir. To obtain a statistically validated data set, six replicate determinations were performed, and the results ranged from 97 to 102% for both analytes. Thus, the known techniques can be used to evaluate both medications in combination at the same time. Table no.4 shows findings of tablet analysis.

3.3.8 Statistical comparison by one-way ANOVA:

To ascertain the influence of each anticipated methodology, a statistical analysis of the accuracy data was conducted. For every test, the significance criterion was set to $p<0.05$. The results of a one-way ANOVA are shown in Table 5, and it was discovered that the developed techniques did not differ significantly from one another.

Table 3. Precision data of NTV (nirmatrelvir) and RTV (ritonavir)

Method	Method A				Method B			
Solvent	a)Methanol:Water(60:40)		b)8M Urea:2M sodium acetate(50:50)		Methanol:Water(60:40)			
Drug	NTV	RTV	NTV	RTV	NTV	RTV	NTV	RTV
Wavelength	215 nm	240 nm	214 nm	222 nm	225 nm	225 nm	215 nm	215 nm
Conc.taken(µg/mL)	20	20	10	10	20	8	20	8
				INTRADAY PRECISION				
Mean conc. found(µg/mL)	19.96	20.25	10.13	9.99	20.11	7.99	20.03	7.94
Standard deviation	0.08	0.15	0.03	0.004	0.01	0.09	0.01	0.01
%RSD	0.40	0.75	0.36	0.04	0.08	1.20	0.07	0.16
				INTERDAY PRECISION				
Mean conc. found(µg/mL)	19.96	20.23	10.19	9.96	20.06	7.90	19.99	8.01
Standard deviation	0.04	0.16	0.01	0.002	0.01	0.12	0.01	0.009
%RSD	0.20	0.82	0.11	0.02	0.08	1.52	0.07	0.11

Table 4. Results for tablet analysis

Method	Drug	Label claim	Amount found	%Recovery
Method A	Nirmatrelvir	150	147.8	98.53
	Ritonavir	100	98.8	98.8
	Nirmatrelvir	150	149.9	99.9
	Ritonavir	100	99.2	99.2
Method B	Nirmatrelvir	150	148.5	99.0
	Ritonavir	100	99.5	99.5

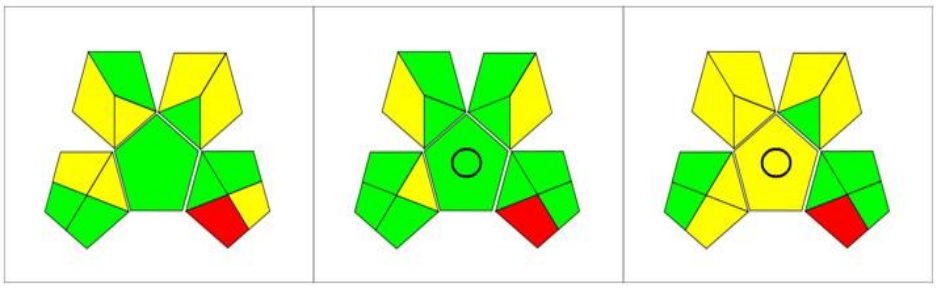
Table 5. One-way ANOVA employed for statistical comparison of accuracy data

Drug	Method	Mean	Variance	F value	F crit	p-value
Nirmatrelvir	Method A(a)	0.9976	0.00010	1.0194	4.2564	0.3989
	Method A(b)	1.0017	0.00027			
	MethodC	1.0070	0.00013			
Ritonavir	Method A(a)	1.0102	0.00000225	1.0016	4.2564	0.4048
	Method A(b)	1.0060	0.0000692			
	Method C	1.001	0.000132			

3.3.9 Greenness assessment:

A cutting-edge semi-quantitative assessment tool called GAPI was created to measure the environmental sustainability of analytical processes. GAPI is a special combination of components from the Environmental Sustainability Assessment (ESA) and National Environmental Methods Index (NEMI) provide a thorough evaluation of the general environmental friendliness of analytical techniques. This program makes it easier to select the most environmentally friendly course of action by offering an easily navigable platform for assessing and contrasting alternative analytical techniques. The following parameters are part of

3.3.9.1 GAPI: sample collection and preparation, possible health and safety hazards related to chemicals and reagents substances, methods for managing waste, equipment, and pre-analysis procedures, including workup, purification, green economics, yield, and occupational hazards are measured in GAPI [19]. The GAPI pictograms shows that the method has a low environmental impact, with most fields marked green, indicating minimal hazards in terms of reagent toxicity, waste generation and energy consumption [20]. Fig 6 displays the results of the evaluation of the green analytical performance of the mixed hydrotropy method, Q-absorbance method, and simultaneous equation approach utilizing GAPI. The GAPI assessment revealed that simultaneous estimation of drugs by using methanol: water solvent have 7 green zones, 7 yellow zones and 1 red zone. By using Q-absorbance method it shows 6 green zones, 7 yellow zones and 1 red zones and in mixed hydrotropy method it have 11 green zones, 3 yellow zones and 1 red zone, which indicates that mixed hydrotropy method is largely environmentally benign, compare to other two methods.



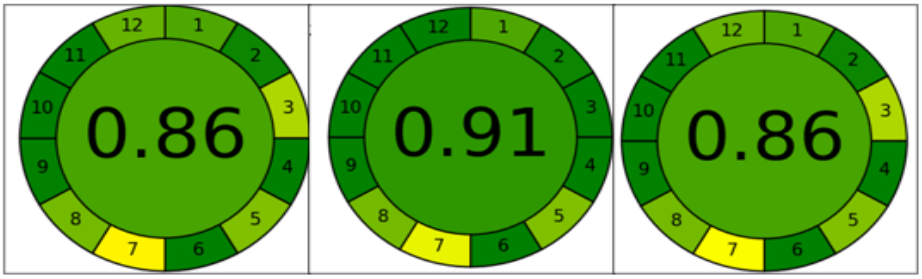
A) a.Simultaneous equation method b. Mixed hydrotropy B)Q-absorbance method

Fig 6.GAPI of all methods

3.3.9.2 AGREE

The specified parameters align with the 12 SIGNIFICANCE principles, which can be assigned different levels of

importance to enable a degree of flexibility. Each of the 12 input factors is transformed onto a standardized scale from 0 to 1. The final assessment is determined by the cumulative results across all principles. Consequently, a clock-style chart is produced at the center, displaying the overall score along with its corresponding color.[19] The greenness evaluation report from two investigative techniques is shown in Fig. 7. AGREE score foe all methods was 0.86, 0.91 and 0.86 respectively. This scores indicates that methods are highly compliant with the principles of Green analytical chemistry.

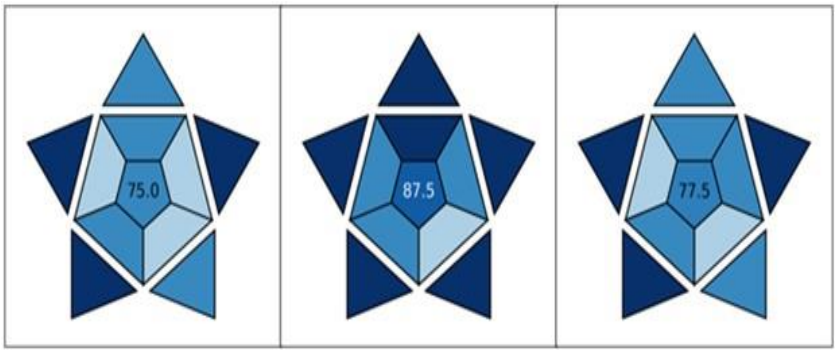


A) a.Simultaneous equation method b.Mixed Hydrotropy B)Q-absorbance method

Fig 7. AGREE plot of all the methods

3.3.9.3 BAGI

The BAGI evaluation is based on four different scores that have Uniform weights. Each score corresponds to a different shade of color and adds to the overall score. In BAGI, the colors white, light blue, blue, and dark blue are represented by score values of 2.5, 5.0, 7.5, and 10, respectively. The strategy needs to score at least 60 points in order to be considered "practical." Figure 8 illustrates the assessment of our recently developed approach for asteroid pictograms. The score demonstrates the viability and relevance of our methodology in an authentic bioanalytical context. BAGI score was 75.0, 87.5 and 77.5, respectively. From which it shows mixed hydrotropy method is highly sustainable than other two methods.



A)a.Simultaneous equation method b. Mixed hydrotropy B) Q-absorbance method

Fig 8. BAGI plot of all the methods

4 Discussion

The current study successfully presents and validates two UV-spectrophotometric methods—Simultaneous Equation Method (SEM) and Q-absorbance Method—for the simultaneous estimation of Nirmatrelvir and Ritonavir in pharmaceutical formulations using different solvents. These methods were evaluated for key analytical parameters in accordance with ICH guidelines and statistically compared using ANOVA.

The linear response obtained for both drugs across a wide concentration range (with correlation coefficients consistently near 0.999) confirms the high reliability and suitability of the proposed UV methods for quantitative analysis. Notably, the Beer-Lambert's law was followed in all methods, further strengthening their analytical credibility. The comparison using one-way ANOVA confirmed no significant difference ($p > 0.05$) among the methods, suggesting equivalence in performance despite different solvents or wavelength strategies.

Accuracy, determined via recovery studies, yielded percent recoveries within the range of 98–102% for both drugs in all methods and across all spike levels (80%, 100%, and 120%). This demonstrates minimal interference from tablet excipients and ensures suitability for routine quality control. Precision, assessed through intra- and inter-day studies, yielded %RSD values well below 2%, signifying excellent repeatability and intermediate precision. This supports the methods' robustness for use in routine laboratory settings. Robustness evaluation, involving minor changes in analytical wavelength, did not significantly affect results. The low %RSD values confirm that the methods are stable under small deliberate variations, which is essential for method transferability and day-to-day consistency. Sensitivity, in terms of LOD and LOQ, was

found to be remarkably high, with values indicating the capability to detect and quantify very low concentrations of both drugs. This highlights the potential for use in bioanalytical or degradation studies where sensitivity is crucial.

Tablet analysis using the proposed methods further validated their practical application. Assay results closely matched label claims, reaffirming the accuracy and applicability of the methods in commercial product evaluation.

Importantly, this study went a step beyond traditional analytical validation by incorporating a comprehensive greenness assessment using three independent tools: GAPI, AGREE, and BAGI. These evaluations confirmed the environmentally friendly nature of the developed methods. The use of methanol: water mixtures and hydrotropic agents (urea and sodium acetate) reduced reliance on hazardous solvents, aligning well with green analytical chemistry principles. BAGI scoring and ComplexGAPI visuals demonstrated favorable greenness metrics, making these methods ideal candidates for sustainable pharmaceutical analysis. The hydrotropic method, in particular, showcased not only environmental advantages but also analytical efficacy, making it a strong alternative for laboratories aiming to adopt green practices.

In conclusion, the developed UV spectrophotometric methods are precise, accurate, robust, sensitive, and environmentally sustainable. These qualities make them highly valuable for routine estimation of Nirmatrelvir and Ritonavir in pharmaceutical dosage forms, offering a viable and greener alternative to more resource-intensive spectrophotometric techniques.

5 Conclusions

The present study efficiently designed and validated ultraviolet spectroscopic methods for the simultaneous quantification of Ritonavir and Nirmatrelvir in combination dose forms: The simultaneous equation method with two different solvents and Q-absorbance method. Excellent linearity, accuracy, precision, and resilience were displayed by these techniques, and their sensitivity was confirmed by their low LOD and LOQ values. There were no discernible variations amongst the suggested approaches, according to statistical validation using one-way ANOVA, guaranteeing their dependability for pharmaceutical study.

Additionally, GAPI, AGREE, and BAGI metrics were used to assess the environmental sustainability of these methods, confirming their compliance with green chemistry by reducing the amount of hazardous waste and solvent used. The findings demonstrate that these UV spectrophotometric approaches offer a straightforward, economical, and environmentally responsible substitute for traditional spectrophotometric methods, which makes them ideal for routine quality monitoring of Ritonavir and Nirmatrelvir in pharmaceutical formulations.

Author's Contributions: Reshma Jadhav conceptualized the study, designed the Quality by Design (QbD) approach, and supervised the research. Prapti gawand and Priya jagtap conducted the experimental work, performed the method development and validation, and analyzed the data. Bhavesh Mahajan and Prathamesh chaudhari contributed to data interpretation and statistical analysis. Prapti gawand drafted the manuscript, while Ashish jain and Aishwarya patil reviewed and revised it critically. All authors read and approved the final manuscript. All authors have read and agreed to the published version of the manuscript.

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10 Conflicts of Interest: The authors declare no conflict of interest.

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