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Research Article

Simultaneous Quantification of Antiviral Drugs Nirmatrelvir and Ritonavir used for the Treatment of COVID-19 by HPLC Method in Bulk and Dosage Forms

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ABSTRACT

In order to avoid possibly harmful side effects and provide clients with substandard goods, quality control is crucial. Therefore, it becomes vital to establish analytical procedures enabling the quality monitoring of commercial pharmaceutical products. In this present work, we had established, optimized, and evaluated a stability-indicating approach for a co-packaged tablet containing nirmatrelvir (NMTR) and ritonavir (RTVR) using HPLC technology in compliance with ICH criteria. Using a C18 symmetric, 5 µm (Silica column) stationary phase and 0.01M dibasic phosphate buffer (pH 3.0)/methanol (60:40) (v/v) as mobile phase, chromatographic separation of NMTR and RTVR was carried out at a solvent system flow pace of 1.0-mL/min, injection size of 10 µL, and column at an ambient temperature. With R2> 0.999, the NMTR and RTVR curves of calibration were a straight line from 37.50 to 225 µg/mL and 25.00 to 150 µg/mL, respectively. The detection and quantification limits for NMTR were 0.45 and 1.363 µg/mL, while for RVTR, it was 0.301 and 0.912 µg/mL, respectively. Applying the standard addition approach, the recovery ranged from 98.819 to 99.877%; the precision for NMTR and RTVR was between 0.3256 and 0.5153 %RSD. Stress conditions, including hydrolysis (acid, alkali and water), reduction, oxidation, photodegradation, as well as thermal stress, were applied. Since the degradation products weren't hindering the NMTR and RTVR assay or detection, the approach may be characterized as stability indicating. The capacity of the suggested approach to separate NMTR and RTVR from their degradation products and excipients makes it suitable for utilization in stability studies as well as quality control assays of NMTR and RTVR in both their bulk and also dose forms (Paxlovid).

INTRODUCTION

In the UK, nirmatrelvir (NMTR), along with Ritonavir (RTVR) was initially conditionally authorized in the last month of 2021 to treat cases of COVID-19 in people who do not need extra oxygen and who are more likely to develop severe COVID-19.[1,2] The EU authorized the use of Nirmatrelvir (NMTR) with ritonavir (RTVR) in the first month of 2022. In the USA, nirmatrelvir, along with ritonavir is approved for use in emergencies. Paxlovid™ is a co-packaged tablet consisting of NMTR and RTVR that is designed for co-administration and is intended to treat and prevent COVID-19 after exposure.[3] Both NMTR and RTVR are protease inhibitors. The primary protease (Mpro) of SARS-CoV-2, an enzyme essential to viral replication, is inhibited by NMTR.[4,5] RTVR acts as a pharmacokinetic enhancing agent by permanently inhibiting the cytochrome CYP3A4 enzyme, which is responsible for NMTR's fast metabolism. This prolongs NMTR's half-life and therefore increases its bioavailability.[4,5] The structural features for NMTR and RTVR are given in Fig. 1.

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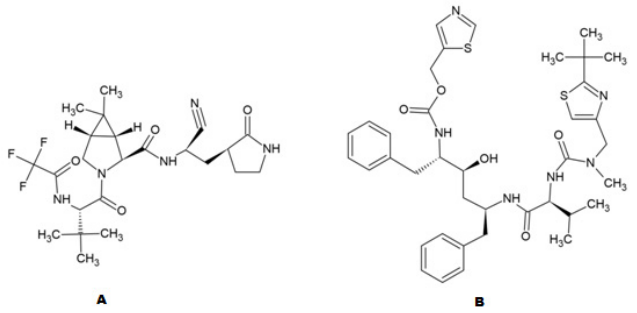

Fig. 1: Chemical structures of A) Nirmatrelvir and B) Ritonavir

Quality control is essential to preventing potentially dangerous side effects and the delivery of substandard products to customers.[6-8] Thus, the development of analytical techniques for commercial medicinal product quality control is crucial. Commercial pharmaceutical products preserved under varied settings might have considerable variations in quality even within the same batch. Consequently, it is crucial to use efficient analytical techniques to guarantee the quality of commercial pharmaceutical goods. Nowadays, a number of well-known methods, primarily liquid and gas chromatography, are used for quality control analyses. Presently, the emphasis lies on creating innovative methods to save analysis time and expense, enhance efficiency, and lessen environmental impact.[9]

Martens *et al*.[10]Zhao *et al*. [11]Zhu *et al*. [12]Komarov *et al*. [13] Liu *et al*.[14] quantitated NMTR and RTVR in human plasma applying LC-MS technique. HPLC/DAD[15], UPLC-MS[16], and TLC[17] techniques were also recommended by Abdallah *et al*; Sun *et al*. and Imam *et al*., respectively to quantitate NMTR and RTVR in plasma samples. The RP-HPLC technique was adopted by Gandhi and Mandal,[18] Venkat and Sharma,[19] Rani and Deepti[20] to quantitate NMTR and RTVR in bulk samples and formulation samples. For the quantitative evaluation of NMTR and RTVR in both their bulk along with dose forms, Pallavi and Sowjanya created one RP-UPLC[21] while Elbordiny *et al*. designed two chromatographic techniques (Micellar Electrokinetic and RP-HPLC).[22]

The sensitive LC-MS,[10-14] UPLC-MS,[16] and TLC[17] methods enable the measurement of NMTR and RTVR at nanogram quantities. However, not all analytical laboratories have access to these advanced methods. Moreover, these methods are limited to plasma samples. The HPLC/DAD[15]procedure was also limited to plasma samples. Micellar Electrokinetic technique's limitations include laborious processes and restricted instrumentation access to quality monitoring analytical labs.[22] In order to evaluate NMTR and RTVR in both its bulk and pill forms, the RP-HPLC[18-20,22,23] and RP-UPLC[21] procedures were utilized; however, the mobile phase constituent was acetonitrile. The inhalation of acetonitrile fumes or acetonitrile contact with the skin and eyes can cause acetonitrile poisoning. *In-vivo* metabolism of acetonitrile generates cyanide, which triggers cytotoxic anoxia.[22] The market pricing of acetonitrile is another problem.[24] When compared to acetonitrile plus buffer combinations, the methanol plus buffer mixes are more ecologically friendly.[25] Because of its minimal boiling point, good solubility with a wide range of compounds, low cost, eco-friendly, readily available and relatively low toxicity, we were motivated to use methanol to be a mobile phase constituent in our investigation.

In the quality assurance unit, the drug needs to be evaluated for stability over the course of its shelf lifespan.[26,27] The efficacy, including the safety of goods, are impacted by drug stability mainly since degradation products might result in potency loss and possibly dangerous deleterious effects. Consequently, in order to guarantee the effectiveness and safety of active ingredients, physical as well as chemical stability is essential.[28]

In light of the aforementioned information, environmentally friendly and cost-effective approaches must be developed for pharmaceutical quality control analyses of NMTR and RTVR. These methods should guarantee stability, minimize analysis costs, and minimize analysis times. Utilizing high-performance liquid chromatography, a novel stability indicating approach was developed in this investigation for the simultaneous quantification of NMTR and RTVR in both their bulk and also dose forms.

Materials and Methods

Dosage Form and Bulk Drugs

The investigation of method development along with validation for NMTR and RTVR and NMTR and RTVR analysis used Paxlovid tablets from Pfizer labs and bulk drugs (NMTR and RTVR) from Shree Icon Pharmaceutical Laboratories, Ricemil Road, Christurajupuram, Vijayawada, Andhra Pradesh, 520010. NMTR (150 mg per tablet) and RTVR (100 mg per tablet) are the two ingredients of Paxlovid tablets.

Chemicals

Acetonitrile and dihydrogen phosphate (H_2PO_4) of HPLC grade from Merck India limited, Dipotassium phosphate (K_2HPO_4), sodium hydroxide (NaOH), sodium hydrogen sulfate ($NaHSO_4$), hydrochloric acid (HCl) and hydrogen peroxide (H_2O_2) of analytical grade from Finar Chemicals Limited and HPLC grade Water from Milli Q were employed.

HPLC Analysis Conditions

The Water Alliance chromatographic system, which includes a diode-array detector, column oven, quaternary solvent delivery pump, auto-sampler, and controller, was utilized to analyze the NMTR and RTVR samples. The subsequent chromatographic settings were applied when performing the NMTR and RTVR analysis employing Water Alliance HPLC in the isocratic elution method. The C18

symmetry, 5 µm (Silica column) stationary phase, 4.6 mm × 250 mm column dimension, detection wavelength with 236 nm, flow rate of 1.0 mL/min, and injection volume with 10 µL were the parameters. The mobile phase, which was 0.01M dibasic phosphate buffer, pH 3.0/methanol (60:40) (*v/v*) was employed. The same mix of solvents was used as well to serve as diluent in the generation of sample NMTR and RTVR solutions.

Solutions

About 150 mg of NMTR and 100 mg of RTVR were weighed and dissolved in 100 mL of diluent. After that, it underwent a 5-min sonication to produce a standard stock solution containing a blend of NMTR and RTVR with 1.5 and 1.0 mg/mL concentration, respectively. A working NMTR and RTVR solution with 150 µg/mL (NMTR) and 100 µg/mL (RTVR) was created by diluting one mL of stock NMTR (1.5 mg/mL) and RTVR (1.0 mg/mL) solution to 10 mL using same diluent.

Test sample solution

The ten Paxlovid pills were weighed and crushed afterward. The average weight was assessed and the stock Paxlovid solution was prepared with it. The Paxlovid powder equal to 150 mg of NMTR and 100 mg of RTVR was weighed correctly in a volumetric flask (50 mL) and diluted properly with diluting solvent through 20 minutes sonication employing Model USB 5OH sonicator ultra bath and then filtered via filter membrane(0.45 microns). Concentration of stock Paxlovid solution-1.5 mg/mLNMTR and 1.0 mg/mL RTVR.

For analysis, test of Paxlovid solution was done by diluting one mL of stock Paxlovid solution (1.5 mg/mL NMTR and 1.0 mg/mL RTVR) in 10 mL volumetric flask to 10 mL using diluting solvent through 5 minutes sonication employing Model USB 5OH sonicator ultra bath. Concentration of test Paxlovid solution-150 µg/mL NMTR and 100 µg/mL RTVR.

Calibration curves and regression equations

Various aliquots, which were equal to 37.50, 75.00, 112.50, 150.00, 187.50, and 225.00 µg/mL of NMTR and 25.00, 50.00, 75.00, 100.00, 125.00, and 150.00 µg/mL of RTVR, were taken from the stock NMTR (1.5 mg/mL) and RTVR (1.0 mg/mL) solution and put into a series of volumetric flasks (10 mL), with diluent added to complete the volume. The solutions were then analysed after being injected into the HPLC column. Following that, the regression equations for NMTR and RTVR were obtained by plotting calibration curves against the ultimate NMTR and RTVR concentrations.

Quality control check of Paxlovid for NMTR and RTVR content

The NMTR and RTVR content percent of the tablets was determined by injecting the prepared test Paxlovid solution into the HPLC column and analyzing the results. The NMTR and RTVR contents in Paxlovid pills were calculated from the acquired peak area values by adopting NMTR and RTVR calibration charts or NMTR and RTVR regression equations.

Degradation studies

Paxlovid samples (1.5 mg/mL NMTR and 1.0 mg/mL RTVR) were subjected to multiple kinds of stress conditions that included hydrolysis (acid, alkali and water), reduction, oxidation, photodegradation, as well as thermal stress, as part of a forced degradation study.[29,30] Table 1 illustrates the time and degradation conditions. Stressed samples were analyzed, corresponding peak areas were examined for percent of stability/degradation, corresponding peaks were investigated for retention times, checked for any peaks interfered with, and the corresponding peak's purity was assessed. Chromatograms of Paxlovid samples after degradation were taken in order to examine the method's specificity. The tools of the empower software program were used to verify the peak purity. The NMTR and RTVR peaks were evaluated for purity using the peak purity findings.

Results and Discussion

Detection Wavelength

The spectra of the NMTR and RTVR working solutions, which had been generated with concentrations of 150 and 100 µg/mL, respectively, was measured at the wavelengths varying between 200 and 500 nm against the diluent. Plotting the absorption spectra of RTVR and NMTR was done (Fig. 2). Absorption peaks at 210.9 and 281.6 nm were observed for NMTR. Absorption maxima at 213.3, 241, and 292.2 nm were observed for RTVR. The spectra of NMTR and RTVR overlapped at 236.3 nm (isobestic point). Commonly, 236 nm was chosen as the wavelength of choice for NMTR and RTVR evaluations.

HPLC Analysis Conditions Optimization

The analytical technique was designed for choosing HPLC-PDA (reversed phase) chromatography conditions, including detection wavelength, mobile phase, and also stationary phase, based on the physical as well as chemical

Table 1: conditions applied on Paxlovid samples for degradation studies

Stress condition	*Reagent used*	*Condition*
Hydrolysis	1 N HCl 1 N NaOH Water	Heating; 30 min; 60°C
Oxidation	10% H_20_2	
Reduction	10% sodium bi-sulphate	
Thermal	Dry heat (hot air oven)	105°C; 3 hours
Photolytic	UV light (photostability chamber)	3 hours

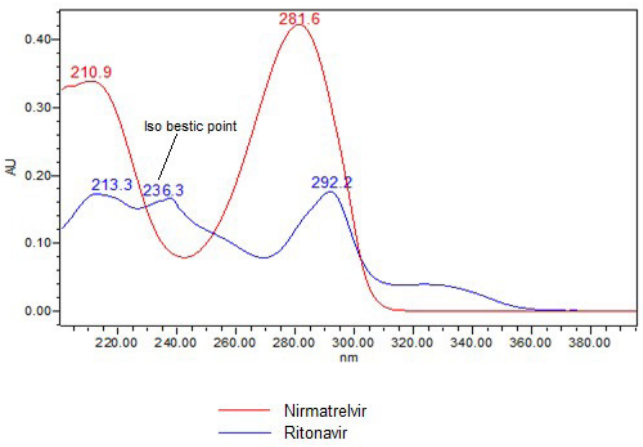

Fig. 2: Spectra of NMTR and RTVR

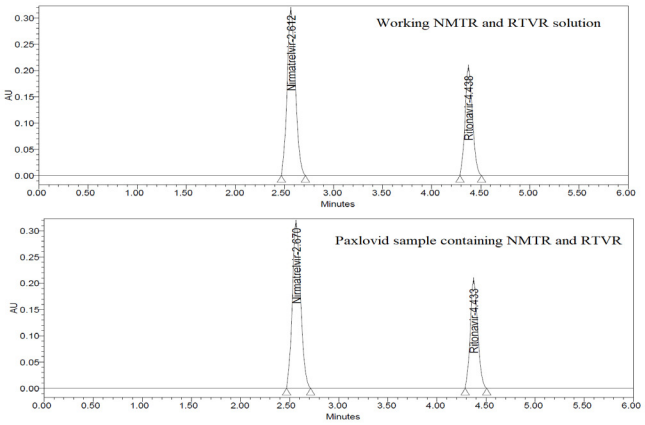

Fig. 3: Chromatogram of the Paxlovid sample and working sample utilizing the developed technique for NMTR and RTVR analysis

aspects of the NMTR and RTVR and the information gathered from the literature. In order to do that, a number of experiments were carried out, including variations in column lengths (150 and 250 mm), buffering agents (0.1% trifluoroacetic acid, pH 3.0; 0.01M dibasic phosphate, pH 3.0), and mobile phase compositions (Methanol: 0.1% Trifluoroacetic acid buffer and methanol: 0.01M Dibasic phosphate buffer) and stationary phase (Waters X-Bridge Phenyl and Symmetry C18)

The Waters X-Bridge phenyl, 5 µm stationary phase, 4.6 × 250 mm column dimensions, and methanol: 0.1% Trifluoroacetic acid buffer combination was initially chosen and utilized to separate NMTR and RTVR from one other. However, the plate count (<2000) is likewise outside of acceptable bounds (≥2000), and the NMTR and RTVR peaks were merged. When Waters X-Bridge phenyl, 5 µm stationary phase, 4.6 × 150 mm column dimensions, and methanol: 0.01M dibasic phosphate, pH 3 combination was chosen, unknown peaks in addition to NMTR and RTVR peaks were seen. Now column was changed to Waters X-Bridge phenyl, 5 µm stationary phase, 4.6 mm × 250 mm column dimensions. Methanol: 0.01M dibasic phosphate (pH 3) was tried in different ratios (25:75, 30:70, and 40:60 *v/v*) for elution of NMTR and RTVR. Good NMTR and RTVR peak symmetry along with better column efficiency were acquired with C18 symmetry, 5 µm (Silica column) stationary phase, 4.6 × 250 mm column dimension, flow rate with 1.0 mL/min, injection volume with 10 µL and mobile phase, which was 0.01M Dibasic phosphate buffer, pH 3.0/Methanol (60:40) (*v/v*). Consequently, a satisfactory analytical technique was optimized, and Fig. 3 displays the typical chromatogram of NMTR and RTVR that was produced using it.

Validation

The optimized approach for RTVR and NMTR analysis was appropriately verified in line with ICH Q2(R1) validation guidelines.[31,32]

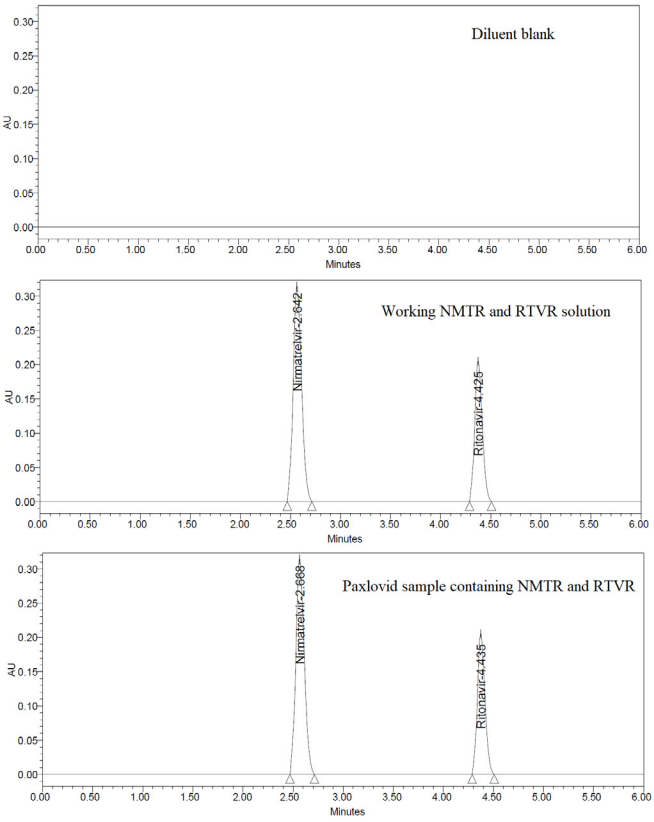

Fig. 4: Chromatograms of nimatrelvir (NMTR) and ritonavir (RVTR) in selectivity assessment

System suitability

Testing for system appropriateness was done to confirm column and system reliability. The %RSD (system precision/ instrument precision), theoretical plates, resolutions and tailing factors were determined for both NMTR and RTVR following five duplicate injections of the exact same working NMTR (150 µg/mL) and RTVR (100 µg/mL) solution. Table 2 displays the NMTR and RTVR findings

Table 2: Summary of the system suitability assessment

Drug →	*NMTR*			*RTVR*			
Sample ↓	*Area*	*TP*	*Tailing*	*Area*	*TP*	*Res*	*Tailing*
Inj-1	2952365	10158	1.15	1958749	7628	8.14	0.97
Inj-2	2953230	10121	1.11	1945766	7615	8.17	0.96
Inj-3	2956958	10123	1.11	1931354	7617	8.19	0.95
Inj-4	2952962	10125	1.1	1949235	7619	8.21	0.94
Inj-5	2953124	10127	1.09	1935891	7621	8.23	0.93
Inj-6	2956888	10129	1.12	1955648	7623	8.27	0.92
Mean →	2954254.5	10130.5	1.113333	1946107.1	7620.5	8.201	0.945
SD→	2088.666	13.766	0.021	10796.010	4.637	0.046	0.019
%RSD→	0.0707	0.1359	1.8553	0.5547	0.0608	0.5583	1.9797
Criteria →	RSD - <2.0	≥2000	0.9 - 1.2	RSD - <2.0	≥2000	>2.0	0.9-1.2

Inj – injection; TP – theoretical plates (column efficiency); Res – resolution

along with the permitting criteria. The chromatographic equipment was determined to be suitable for the NMTR and RTVR evaluation.

Selectivity

The test was executed through the comparison of the chromatograms (Fig. 4)of the working NMTR (100 µg/mL) and RTVR (150 µg/mL) solution with the diluent (dibasic phosphate buffer 0.01 N, pH 3.0:MeOH, 60:40, *v/v*) and Paxlovid sample (NMTR-150 µg/mL; RTVR-100 µg/mL). There were no discernible peaks in the diluent chromatogram at the same retention times for RTVR and NMTR. The Paxlovid sample and the working NMTR and RTVR solution did not significantly differ in terms of retention times and areas for NMTR and RTVR. These results proved the specificity of the procedure by showing that the excipients had no influence on the NMTR and RTVR peaks and their assay (Table 3).

Linearity

The responses of RVTR and NMTR to concentrations between 25.0 and 150.0 µg/mL and 37.50 to 225 µg/mL, respectively, were shown to be linearly correlated. After computing the concentrations of the NMTR and RVTR against their corresponding responses, linear regression curves for NMTR and RVTR were generated, as seen in Fig. 5. Strong linearity was demonstrated through a regression coefficient (R^2) value better than 0.999 (Fig. 5).

LoD and LoQ

According to the ICH, LoD, also referred as the detection limit (DL), may be computed as "LoD = 3.3 × σ/S", whereas the quantitation limit (QL), also referred as the limit of quantification, is "LoQ = 10 × σ/S". In this instance, 'S' represents the calibration curve's slope while 'σ' denotes the response's standard deviation. The DL and QL for NMTR were 0.45 and 1.363 µg/mL, while for RVTR, it was 0.301 and 0.912 µg/mL, respectively. Strong sensitivity for the recommended NMTR and RVTR analysis procedure was demonstrated through low DL and QL values.

Table 3: Summary of selectivity assessment

Drug→	*NMTR*		*RVTR*	
Sample ↓	*Retention time*	*Area*	*Retention time*	*Area*
Diluent blank	-	-	-	-
Working solution	2.642	2953230	4.425	1945766
Paxlovid sample	2.688	2954423	4.435	1947890
Mean →	2.665	2953826.5	4.43	1946828
SD→	0.0325	843.5784	0.0071	1501.8948
%RSD→	1.2205	0.0286	0.1596	0.0771
Criteria →	%RSD - ≤2.0			

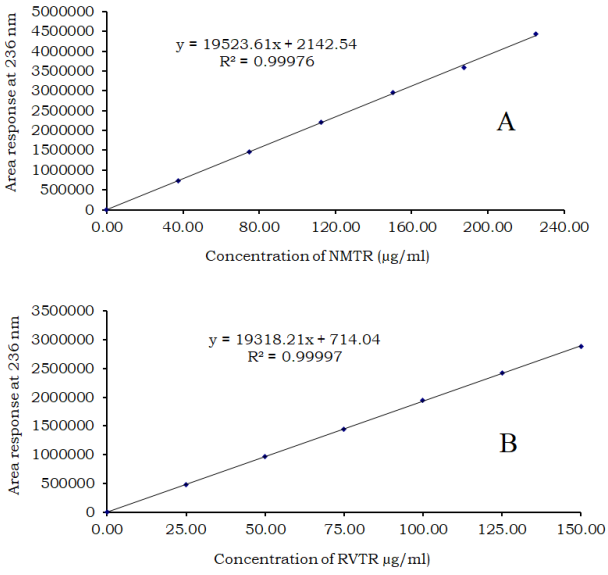

Fig. 5: Linear regression curves for NMTR [A] and RVTR [B]

Table 4: Summary of the method precision assessment

Drug →	*NMTR*			*RTVR*		
Sample ↓	*Quantity (µg/mL)*	*Area*	*Assay (%)*	*Quantity (µg/mL)*	*Area*	*Assay (%)*
Inj-1	150	2951859	99.818	100	1953461	100.419
Inj-2	150	2954859	100.139	100	1948933	99.980
Inj-3	150	2952365	99.908	100	1960465	100.696
Inj-4	150	2953648	100.062	100	1951689	100.039
Inj-5	150	2951255	99.834	100	1945421	99.800
Inj-6	150	2915496	98.733	100	1958748	100.112
Mean →	-	-	99.749	-	-	100.174
SD→	-	-	0.514	-	-	0.3262
%RSD→	-	-	0.5153	-	-	0.3256
Criteria →	-	-	RSD-≤2.0	-	-	RSD-≤2.0

Table 5: Summary of the ruggedness assessment

Drug →	*NMTR*			*RTVR*		
Sample ↓	*Quantity (µg/mL)*	*Assay[1*] (%)*	*Assay[2*] (%)*	*Quantity (µg/mL)*	*Assay[1*] (%)*	*Assay[2*] (%)*
Inj-1	150	99.818	100.035	100	100.419	100.442
Inj-2	150	100.139	99.929	100	99.980	100.050
Inj-3	150	99.908	99.964	100	100.696	100.711
Inj-4	150	100.062	99.916	100	100.039	99.758
Inj-5	150	99.834	100.024	100	99.800	99.865
Inj-6	150	98.733	99.890	100	100.112	99.722
Mean →	-	99.854		-	100.133	
SD→	-	0.3656		-	0.3516	
%RSD→	-	0.3661		-	0.3512	
Criteria →	-	RSD-≤2.0		-	RSD-≤2.0	

1* = analysis with analyst 1; laboratory 1; day 1,
2* = analysis with analyst 2; laboratory 2; day 2.

Method precision

The results (mean assay percent, SD, and %RSD) were collected for NMTR and RVTR from the assessment of the six working samples (NMTR-150 µg/mL and RVTR-100 µg/mL) preparations that was done that same day. The method was precise since the RSD readings for NMTR and RVTR in dilution seemed less than 2% (Table 4).

Ruggedness

On day 1, analyst 1 used six working samples (NMTR 150 µg/mL and RVTR-100 µg/mL) in laboratory 1 to repeatedly determine the NMTR and RVTR assay percent. The appropriate peak responses of NMTR and RVTR for each of the six samples were used to calculate the assay percent for NMTR and RVTR using their respective calibration curve. Separately, on day two in laboratory 2, analyst 2 assessed six working samples (NMTR- 150 µg/mL and RVTR-100 µg/mL) samples in a manner identical to analyst 1. The appropriate peak responses of NMTR and RVTR for every sample were utilized to figure out the assay percent for NMTR and RVTR. For analysts 1 and 2, overall mean assay percent, SD, and % RSD were computed (Table 5). The experiment's %RSD was computed to be 0.3661% for NMTR and 0.3512% for RVTR, whereas the ruggedness acceptability criterion was a value below 2.0% of RSD. These results unequivocally demonstrate that the repeatability of this approach in two distinct environments is acceptable.

Accuracy

Quantities of known NMTR (75 µg/mL -50% level accuracy; 150 µg/mL-100% level accuracy; 225 µg/mL -150% level accuracy) and RVTR (50 µg/mL -50% level accuracy; 100 µg/mL-100% level accuracy; 150 µg/mL -150% level accuracy) were added to the Paxlovid sample in order to establish accuracy data. The corresponding responses of NMTR and RVTR were used to compute their concentration values. It was computed to get the spike recoveries

Table 6: Summary of the accuracy assessment for NMTR

Spiked (µg/mL)	*Area*	*Found (µg/mL)*	*Recovered (%)*	*Mean ± SD*	*%RSD*
50% quantity level accuracy test					
75	1457127	73.984	98.645	98.819 ±0.1849	0.1871
75	1462548	74.26	99.013		
75	1459356	74.098	98.797		
100% quantity level accuracy test					
150	2935586	149.052	99.368	99.523 ±0.1683	0.1691
150	2945459	149.553	99.702		
150	2939465	149.249	99.499		
150% quantity level accuracy test					
225	4421691	224.508	99.781	99.877 ±0.0899	0.0890
225	4426548	224.755	99.891		
225	4429495	224.904	99.957		

Table 7: Summary of the accuracy assessment for RVTR

Spiked (µg/mL)	*Area*	*Found (µg/mL)*	*Recovered (%)*	*Mean ± SD*	*%RSD*
50% quantity level accuracy test					
50	963158	49.492	98.984	99.036 ±0.0616	0.0622
50	964336	49.552	99.104		
50	963514	49.51	99.020		
100% quantity level accuracy test					
100	1934580	99.408	99.408	99.650 ±0.2904	0.2914
100	1945569	99.972	99.972		
100	1937747	99.57	99.570		
150% quantity level accuracy test					
150	2897658	148.895	99.263	99.187 ±0.1669	0.1683
150	2889851	148.494	98.996		
150	2898795	148.954	99.303		

percentage for NMTR (Table 6) and RVTR (Table 7). The percentage recovery that was obtained fell below the acceptable limit (90% to 110%), ranging from 98.819 to 99.877% for NMTR and from 99.036 to 99.650% for RVTR.

Robustness

Using working samples (NMTR-150 µg/mL and RVTR-100 µg/mL) and a ±0.1 mL/min flow rate difference, robustness data for this approach were examined. The response and assay percent were determined at 0.9, 1.0, and 1.1 mL/min flow rates for NMTR and RVTR. Flow rate variation results were expressed as a %RSD of assay percent (Table 8). Similarly, robustness data for this approach were further examined while keeping the methanol fraction of the mobile phase at a ±5.0% volume difference. The assay percent and responses for NMTR and RVTR were determined with three different mobile phases containing volumes of 35, 40, and 45% Methanol, respectively. For the fluctuation in methanol volume proportion, the percentage of RSD for assay percent of NMTR and RVTR was computed (Table 8). The method's robustness is made clear by the findings in Table 8, where the %RSD in the flow rate variation condition is 0.83% and in the methanol fraction variation is 0.73%.

Degradation studies

Paxlovid samples (1.5 mg/mL NMTR and 1.0 mg/mL RTVR) were subjected to multiple kinds of stress conditions that include hydrolysis (acid; alkali; water), reduction, oxidation, photodegradation, as well as thermal stress, as part of a forced degradation study. NMTR and RVTR have better stability in stressful water-based hydrolysis and reduction conditions, as evidenced by the absence of breakdown products (Fig. 6) and less percent of degradation upon exposure to hydrolysis and reduction (Table 9). The Paxlovid sample yields three degradation products with retention times of 1.824,

Table 8: Summary of the robustness assessment

Parameter	*Value*	*NMRT area counts*	*NMRT assay (%)*	*RVTR area counts*	*RVTR assay (%)*
Flow rate (mL/min)	0.9	2715618	99.428	1854868	100.395
	1.0	2951859	99.818	1953461	100.419
	1.1	3236528	99.918	2168925	100.306
Mean			99.721	-	100.373
SD			0.2589	-	0.0595
%RSD			0.2596	-	0.0593
Parameter	*Value*	*NMRT area counts*	*NMRT assay (%)*	*RVTR area counts*	*RVTR assay (%)*
Methanol volume proportion	35	2632789	99.669	1659292	100.216
	40	2951859	99.818	1953461	100.419
	45	3341157	100.255	2259548	99.718
Mean			99.914	-	100.117
SD			0.3046	-	0.3607
%RSD			0.3048	-	0.3603

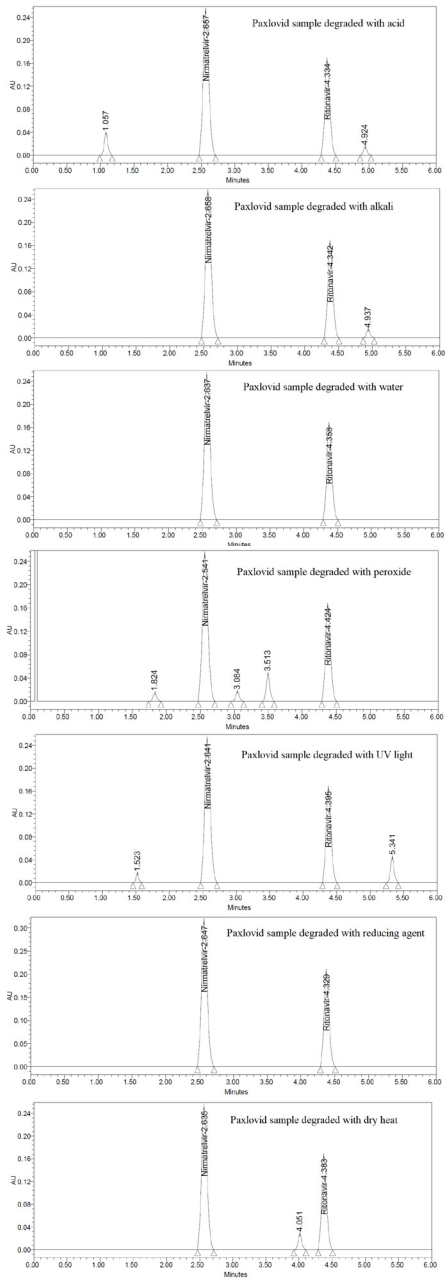

Fig. 6: Paxlovid sample chromatograms after their degradation

3.084, and 3.513 minutes when treated with hydrogen peroxide. Comparing the percentage of NMTR and RVTR deterioration in hydrogen peroxide conditions to other exposed conditions, a higher percentage was discovered. This proved that NMTR and RVTR have lesser stability in the presence of peroxide. It was discovered that under acidic, alkaline, thermal, and photo-applied conditions, NMTR degraded by more than 10%(Table 9). In alkaline and thermally applied contexts, RVTR was degraded less than 10% but more than 10% in acidic and photo-applied conditions (Table 9). Only one new degradant peak was observed under alkali (degradant RT - 4.937 minutes) and dry heat (degradant RT-4.051 minutes) conditions, while two more were visible under acid (degradants RT -1.057 and 4.924 minutes) and photodegradation (degradants RT -1.523 and 5.341 minutes) conditions (Fig. 6). The NMTR and RVTR peaks found well set apart from degradant peaks within the adapted chromatographic conditions, suggesting superior specificity.

Peak purity was assessed as a variance between the purity angle and purity threshold; a higher threshold is preferred since it shows that there truly is no discernible co-elution across the threshold angle's range, indicating the impact of noise. For all NMTR and RVTR peaks, the purity angle along with purity threshold was assessed during all degradation instances (Table 9). For both NMTR and RVTR, the purity angle was shorter compared to the purity threshold across every condition, demonstrating the lack of co-elution and the purity of their peaks.

Assay of NMTR and RVTR in formulation

To find out application of the developed method, assay studies were run on a chosen brand of marketed medication (Paxlovid tablet) and determined the concentrations of NMTR and RVTR in it. Equations for each calibration curve were applied to figure out the NMTR and RVTR quantities in the paxlovid tablet (Table 10). These assays produced findings for NMTR and RVTR that were 100.053 and 99.847%, respectively, of the claimed label. A low precision score (RSD = 0.0414% for NMRT and RSD = 0.4852% for RVTR) suggests that the approach is reliable for estimating the precise quantities of NMTR and RVTR in formulations.

Table 9: Stability and peak purity assessment outcomes

Drug →	*NMTR*				*RVTR*			
Condition applied ↓	*Percentage of*		*Purity*		*Percentage of*		*Purity*	
	Stability	*Degraded*	*Angle*	*Threshold*	*Stability*	*Degraded*	*Angle*	*Threshold*
Acid	87.822	11.85	0.581	10.336	89.574	10.695	1.865	8.469
Thermal	89.196	10.476	0.587	10.134	91.087	9.182	1.779	8.937
Alkali	88.597	11.075	0.593	10.158	90.581	9.688	1.824	8.573
Reduction	97.933	1.739	0.583	10.147	99.253	1.016	1.831	8.487
Photo	84.037	15.635	0.647	10.559	82.374	17.895	1.864	8.946
Water	95.194	4.478	0.689	10.774	96.091	4.178	1.965	11.761
Peroxide	81.743	17.929	0.547	10.367	80.594	19.675	1.875	8.264

Table 10: Quality control assay of NMTR and RVTR in formulation

Drug	*NMRT*		*RVTR*	
Label claim (mg)	150		100	
Concentration found (mg)	150.123	150.035	99.505	100.190
Assay (%)	100.082	100.023	99.504	100.189
Assay % Mean	100.053		99.847	
SD	0.0414		0.4844	
%RSD	0.0414		0.4852	

Conclusion

A novel stability-indicating approach was developed using the HPLC technique for the simultaneous quantification of NMTR and RTVR in both their bulk and also dose forms. In adherence to ICH guidelines, the technique to separate and quantify the NMTR and RTVR in co-packaged tablets (Paxlovid) was verified. Peak qualities, their resolution, and analysis time were optimized and assessed. It has been found that the NMTR and RTVR analysis approach was linear, sensitive, selective, precise, robust, rugged, and accurate. The degradation experiments validated the efficacy, stability indicative and specificity of the approach. Thus, during manufacture or regular pharmacovigilance or stability investigations, the suggested approach may be put to use for the simultaneous quantification of NMTR and RTVR in both their bulk and also dose forms (Paxlovid).

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