Journal of Advanced Pharmacy Research

Section B: Pharmaceutical Analytical & Organic Chemistry, Medicinal & Biochemistry

Highly Sensitive High-Performance Thin-Layer Chromatographic Method for the Simultaneous Determination of Remdesivir, Nirmatrelvir and Ritonavir in their Pure Forms and Pharmaceutical Formulations

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Submitted on: 02-11-2024; Revised on: 19-02-2025; Accepted on: 20-02-2025

To cite this article: Nassar, M. W.; Serag, A.; Hasan, M. A. Kamel, M. Highly Sensitive High-Performance Thin-Layer Chromatographic Method for the Simultaneous Determination of Remdesivir, Nirmatrelvir and Ritonavir in their Pure Forms and Pharmaceutical Formulations. *J. Adv. Pharm. Res.* **2025**, *9* (2), 83-90. DOI: 10.21608/aprh.2025.333052.1297

ABSTRACT

Background: In the wake of the COVID-19 pandemic, variations of SARS-CoV-2 have emerged as a significant public health issue due to heightened transmissibility and evasion of natural immunity, vaccine efficacy, and monoclonal antibody treatments. **Objectives:** Remdesivir, Nirmatrelvir, and Ritonavir are presented as promising recently developed antiviral medicines for COVID-19, supported by clinical evidence. Therefore, optimizing an analytical method for their simultaneous determination is of significant value in quality control laboratories and subsequent confirmatory studies. A straightforward, sensitive, and selective HPTLC approach has been described and confirmed for the simultaneous quantification of these medicines in bulk and pharmaceutical formulations. **Methods:** The HPTLC method was conducted with a silica gel aluminum plate 60F254 (10*10) as the stationary phase and a mobile phase composed of dichloromethane, methanol, and triethylamine in a volume ratio of (90:10:0.1 by volume). The fabricated plates were scanned densitometrically utilizing a UV detector. Detection occurred at 254 nm across a concentration range of 10-80 µg/spot. The Rf values for Nirmatrelvir, Remdesivir, and Ritonavir were determined to be 0.13, 0.38, and 0.53, respectively. **Results:** The method has been validated for many parameters, including linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and robustness. The results fall within the acceptance criteria established by the International Conference on Harmonisation (ICH). **Conclusion:** The proposed method has been successfully applied for simultaneous determination of the studied drugs in both bulk and commercial dosage form.

Keywords: *Remdesivir; Nirmatrelvir; Ritonavir; HPTLC method development; Simultaneous determination; ICH guidelines.*

INTRODUCTION

Since the conclusion of 2019, the COVID-19 pandemic has pervaded the globe. As of March 2023, the World Health Organization (WHO) reported that the global number of individuals infected with the virus exceeded 750 million, with total mortality surpassing 6.8 million.[1] The extensive proliferation of COVID-19 in early 2020 prompted an urgent quest to discover a treatment for the causing virus to improve patient recovery and decrease mortality rates.

The medical sector was taken aback by the rapidly proliferating pandemic, prompting the exploration of multiple treatment alternatives, including immune-modulatory agents, monoclonal antibodies, and other antiviral medications. Given that the discovery, development, and evaluation of any new medicine is laborious and necessitates some time for safety and clinical assessment, previously clinically profiled antivirals were rigorously tested against the novel causal virus. Among these compounds, remdesivir (RMD) has emerged as a possible active compound against the virus.[2]

RMD, 2-ethylbutyl (2S)-2-[[[(2R,3S,4R,5R)-5-(4-aminopyrrolo[2,1- f][1,2,4]triazin-7-yl)-5-cyano-3,4-dihydroxyoxolan-2-yl]methoxy-phenoxy phosphoryl]amino]propanoate, is shown in **Figure 1 (a)**.

Remdesivir is a broad-spectrum adenosine analog prodrug. It is activated intracellular into the pharmacologically active metabolite (GS-443902). RMD is an RNA-dependent RNA polymerase inhibitor (RdRpI); binding to the viral RNA leads to the permanent inhibition of viral genome replication.[3]

In December 2021, the U.S. Food and Drug Administration (FDA) granted emergency authorization for Paxlovid® as an antiviral treatment for COVID-19[4]. Paxlovid® is composed of two tablets of nirmatrelvir and one tablet of ritonavir.

Nirmatrelvir (NTV), (1R,2S,5S)-*N*-[(1*S*)-1-cyano-2-[(3*S*)-2-oxo-pyrrolidin-3-yl]ethyl]-3-[(2*S*)-3,3-dimethyl-2-[(2,2,2 trifluoroacetyl) amino]butanoyl]-6,6-dimethyl-3-azabicyclo [3.1.0]hexane-2-carboxamide, is shown in **Figure 1 (b)**.

Nirmatrelvir is an inhibitor of the viral protease (3CLpro) that obstructs the cleavage of precursor proteins essential for the creation of new infectious particles, hence, ceases infection.

In this binary combination, NTV is regarded as the active component against Covid-19. Conversely, RTN functions as a CYP3A inhibitor to extend the half-life of NTV and sustain its plasma concentrations.[5]

Ritonavir (RTN), 1, 3- thiazol-5-ylmethyl N-[(2S,3S,5S)-3-hydroxy-5-[[(2S)-3-methyl-2- [[methyl-[(2-propan-2-yl-1,3-thiazol-4-yl) methyl] carbamoyl] amino] butanoyl] amino-1,6-diphenylhexan-2-yl] carbamate, is shown in **Figure 1 (c)**.

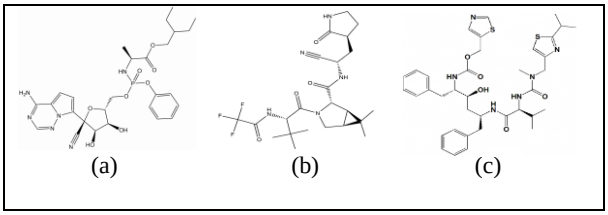

Figure 1. Structure formula of (a) Remdesivir, (b) Nirmatrelvir and (c) Ritonavir.

Ritonavir, an HIV-1 protease inhibitor, lacks direct antiviral efficacy against the new coronavirus SARS-CoV-2. It elevates the blood levels of nirmatrelvir by suppressing CYP3A enzyme activity, thereby decelerating nirmatrelvir metabolism in the body.[6]

The review of literature revealed that various analytical methods have been reported for the determination of remdesivir (RMD), mainly includes high-performance liquid chromatography[7], liquid chromatography-tandem mass spectrometry (LC-MS/MS) [8,9], densitometry[10], ultraviolet spectrophotometry[11] and spectrofluorimetry[12,13], the voltametric determination of remdesivir (RMD) including square-wave voltametry[14] and differential pulse (DP) voltammetry[15]. Few studies have focused on nirmatrelvir (NTV) in single dosage form including high-performance liquid chromatography[16]. Several analytical methods have been reported for the analytical determination of Ritonavir (RTN) as separate entities including high-performance liquid chromatography[17,18], densitometry[19] and ultraviolet spectrophotometry[20,21]. There are several analytical methods have been reported for the simultaneous determination of nirmatrelvir (NTV) and Ritonavir (RTN), these mainly includes high-performance liquid chromatography[22, 23], liquid chromatography-tandem mass spectrometry (LC-MS/MS)[24,25], densitometry[26] and spectrofluorimetry[27].

While these investigations have yielded valuable insights into the detection and quantification of these molecules, there are no publications on the concurrent determination of these antiviral drugs. These technologies can enhance quality control and monitoring of essential pharmaceuticals while decreasing the time and cost related to their individual analysis.

To our knowledge, the literature research lacked any analytical method for the simultaneous determination of the three aforementioned antiviral drugs. The current initiative is to implement a sensitive, economical, and selective HPTLC method for the simultaneous analysis of remdesivir, nirmatrelvir, and ritonavir in bulk and their pharmaceutical formulations. The established method was validated per ICH guidelines and effectively utilized for the assay of remdesivir, nirmatrelvir, and ritonavir in their combined dosage form[22].

METHODS

Materials and Chemicals:

A pure reference standard of remdesivir (99.29%) was graciously provided by EVA- PHARMA, Giza, Egypt. Remdesivir® intravenous infusion, (100 mg/20 ml vial) was kindly provided by EVA- PHARMA, Giza, Egypt.

A pure reference standard of nirmatrelvir (99.36%) and ritonavir (99.62%) was kindly supplied by PFIZER, Inc., Egypt. Paxlovid®, consists of two pink oval nirmatrelvir film-coated tablets (150 mg/tablet) co-packaged with white ritonavir film coated tablet (100 mg/tablet), was generously provided by Pfizer, Inc., Egypt. The recommended dose is two tablets of nirmatrelvir in conjunction with one tablet of ritonavir.

Methanol, HPLC grade (Sigma Aldrich, Germany), dichloromethane and tri ethylamine (El-Nasr Company, Egypt) were utilized, with water being newly distilled for the entire procedure. Whatman filter paper Nº41.

Instruments:

The chromatography was conducted using a CAMAG HPTLC System equipped with a Linomat V Automatic Sample Applicator, and a 100 µl syringe (Hamilton, Bonaduz, Switzerland) was utilized for sample application. Densitometry scanning was conducted using a Camag HPTLC scanner III in reflectance absorbance mode at 254 nm, controlled by CATS software (Version 3.15, Camag). The radiation source employed was a deuterium lamp that emits a continuous ultraviolet spectrum ranging from 200 to 400 nm. Pre-coated silica gel aluminum plates 60F254 (10×10 cm; E. Merck) were utilized for the separation of combination components.

Preparation of standard stock solution:

A standard stock solution of remdesivir, nirmatrelvir, and ritonavir was made separately by dissolving 1000 mg of each drug powder in 50 ml of methanol using a 100 ml volumetric flask, and then the volume was adjusted with methanol to achieve a final concentration of 10 mg/ml.

Preparation of pharmaceutical sample solution:

Ten tablets of PAXLOVID® nirmatrelvir (150 mg/tablet) co-packaged with ritonavir (100 mg/tablet), were meticulously ground and weighted. A precise weight measurement was employed to ascertain the requisite quantity of powder equivalent to 1000 mg of each drug. The powder was subsequently transferred to a 100-ml volumetric flask, and the volume was increased to approximately 70 ml using methanol. Following 30 minutes of vigorous shaking and filtration, the volume was filled with methanol until a volumetric concentration of (10mg/ml) was attained. REMDESIVIR-EVA PHARMA® vial (100mg/20ml), a concentrate solution to be considered as a stock sample solution, the accurate volume of 2 ml equivalent to 10 mg of remdesivir.

Procedure:

TLC- Densitometric Conditions:

A pre-coated silica gel TLC plates were rinsed with methanol and dried at 60°C for 5 minutes in order to be activated. Samples were applied on these plates in the form of spots (6 mm length, with 10 mm spacing, and positioned 10 mm from the bottom edge of the plate). The plates were put in a chromatographic tank that had been saturated for 30 minutes at room temperature with a developing system comprising dichloromethane: methanol: tri-ethylamine in a volume ratio of (90:10:0.1 by volume). The progressive development of this technology was preceded by air drying the plates, which were subsequently scanned at 254 nm.

Construction of Calibration Graph:

Precisely measured volumes of remdesivir, nirmatrelvir and ritonavir standard solutions (10 mg/ml) were transferred into three distinct series of 10 ml volumetric flasks and diluted to volume with the methanol to achieve final concentrations of (1000 – 8000 µg/ml) for the three drugs, Triplicate applications of 10 µl from each solution were conducted on the TLC plates to obtain the concentration range of 10–80 µg/spot of remdesivir, nirmatrelvir and ritonavir, respectively. The procedure was subsequently conducted under TLC-densitometric conditions. The peak area values were calculated and plotted against the corresponding concentrations of remdesivir, nirmatrelvir and ritonavir in (µg/spot) to get the calibration graphs. The calibration graph was constructed relating the peak areas of the cited drugs to its relevant concentrations. The linear regression equations were computed and the regression data were presented in **Table 1**.

Application to Pharmaceutical Preparation:

Various aliquots from pharmaceutical sample solutions, corresponding to (10-80 mg) of nirmatrelvir and ritonavir, along with (10-40 mg) of remdesivir, were put into a series of 10 ml volumetric flasks, and the volume was adjusted to the mark with methanol. 10 µl of each solution were applied to a TLC plate following the above mentioned specific chromatographic conditions and scanned at 254 nm. The analysis was conducted in triplicate. The quantity of each drug in the formulation was determined by substituting the corresponding response into the regression line equation for remdesivir, nirmatrelvir, and ritonavir. The percentage recovery of the drugs was computed, and the results are given in **Table 2**.

Table 1. Regression and validation data for determination of Remdesivir, Nirmatrelvir and Ritonavir by the proposed TLC densitometric method.

Parameter		Remdesivir	Nirmatrelvir	Ritonavir
Slope		161.681	85.917	173.021
Intercept		2659.6	940.1	2672.1
Coefficient of determination(r^2)		0.9995	0.9994	0.9993
Range (µg/spot)		10–80	10–80	10–80
Accuracy (mean %R) *		99.853	99.908	100.491
Repeatability (%RSD) **		1.484	1.608	1.515
Intermediate precision (%RSD) **		1.529	1.659	1.543
LOD (µg/spot)		1.891	1.911	2.024
LOQ (µg/spot)		5.732	5.790	6.135
Robustness (%R ± %RSD)***	Mobile phase contents ratio ± 2%	100.332 ±0.935	100.851±1.023	99.054±0.973
	Saturation time ± 2 min	99.254±0.689	100.171±1.521	100.063±0.861

** Average of nine determinations (three concentrations repeated three times).*
*** %RSD of nine determinations (three concentrations repeated three times).*
**** Values for three determinations.*

Table 2. Recovery study of Remdesivir, Nirmatrelvir and Ritonavir by applying standard addition technique using the proposed TLC densitometric method.

Drug	Pharmaceutical taken(µg/spot)	Pharmaceutical found* (µg/spot)	Pure added (µg/spot)	Pure found** (µg/spot)	%Recovery
Remdesivir	30	30.267	10	10.033	100.336
			30	29.564	98.553
			50	49.868	99.737
		Mean±%RSD			99.541 ± 0.911
Nirmatrelvir	30	30.279	10	10.144	101.447
			30	30.045	100.162
			50	49.607	99.215
		Mean±%RSD			100.274 ± 1.116
Ritonavir	30	30.304	10	10.119	101.199
			30	29.971	99.901
			50	49.529	99.058
		Mean±%RSD			100.053 ± 1.078

**Average of five determinations.*
***Average of three determinations.*

Table 3. System suitability testing parameters for the determination of Remdesivir, Nirmatrelvir and Ritonavir by the proposed TLC–densitometric method.

Parameters	Remdesivir*	Nirmatrelvir*	Ritonavir**	Reference value
Retardation factor (Rf)	0.387	0.134	0.534	——
Capacity factor (K´)	4.033	1.064	7.411	1-10
Tailing factor (T)	0.976	0.673	1.162	< 2
Resolution factor (R)	4.166		2.502	> 2

** Resolution factor between peaks of nirmatrelvir and remdesivir.*
*** Resolution factor between peaks of remdesivir and ritonavir.*

Table 4: Determination of Remdesivir in REMDESIVIR® vail and Nirmatrelvir, Ritonavir in PAXLOVID® tablets by the proposed TLC densitometric and the reported methods.

Parameters	Proposed method			Reported method[29,30]		
	Remdesivir	Nirmatrelvir	Ritonavir	Remdesivir	Nirmatrelvir	Ritonavir
n*	5	5	5	5	5	5
%R**	100.116	99.533	100.091	99.813	99.962	99.894
%RSD	1.167	1.212	1.058	0.803	0.737	0.996
t-test (2.306) ***	0.430	0.350	1.179	———	———	———
F-test (6.388) ***	2.262	2.515	1.231	———	———	———

** Number of experiments.*
*** The mean of percent recovery of pharmaceutical preparation.*
**** The values in parenthesis are tabulated values of "t" and "F" at (P = 0.05).*

RESULTS AND DISCUSSION

In the recent scientific work, the strategy was concerned with the application of HPTLC densitometric technique for simultaneous determination and quantification of remdesivir, nirmatrelvir and ritonavir in raw materials and its combined dosage forms. The first reported method is a chromatographic method (RP-HPLC) for simultaneous estimation of nirmatrelvir and ritonavir[22]. The second reported method is a chromatographic method (RP-HPLC) utilized for the estimation of remdesivir[28]. The simultaneous determination of co-formulated or co-administrated drugs is crucial in pharmacy, since it minimizes extraction effort and time. This finding encourages our pursuit to development of a simple and sensitive HPTLC method for the simultaneous quantitative determination of cited drugs. HPTLC technique provides advantages over the previously reported technique in terms of higher sensitivity, fast analysis times, smaller quantities of solvents.

Method Optimization:

The selection of an appropriate mobile phase is conducted through systematic testing and literature review. Multiple trials were conducted to identify the optimal mobile phase. Various developing systems with distinct compositions and component ratios were tested, including methanol-acetonitrile-triethylamine (20:80:0.1, by volume), methanol-hexane-acetic acid (40:60:0.1, by volume), and methanol-ethyl acetate-triethylamine (50:50:0.1, by volume), which resulted in inadequate resolutions, spot broadening, tailing, and asymmetric peaks. Utilizing a mixture of dichloromethane, methanol, and triethylamine in several ratios yielded resolution with some degree of tailing until the optimal ratio of 90:10:0.1 (by volume) was employed, resulting in the best resolution. The quantitative determination of remdesivir, nirmatrelvir and ritonavir was conducted by scanning the spots at 254 nm. The R_f values for Nirmatrelvir, Remdesivir, and Ritonavir were 0.13, 0.38, and 0.53, respectively **Figure 2, 3.**

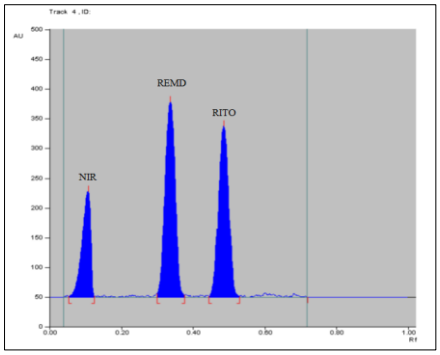

Figure 2. Two-dimensional TLC-densitogram of Nirmatrelvir (40 µg/spot), Remdesivir (40 µg/spot) and Ritonavir (40 µg/spot) using dichloromethane: methanol: tri-ethylamine (90:10:0.1 by volume) as a mobile phase with UV detection at 254 nm.

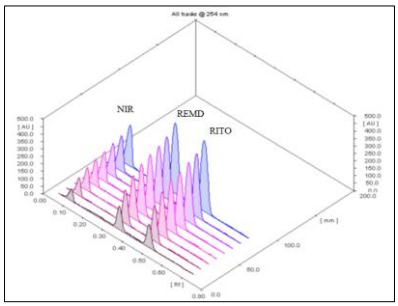

Figure 3. Three-dimensional TLC-densitogram of Nirmatrelvir (40 µg/spot), Remdesivir (40 µg/spot) and Ritonavir (40 µg/spot) using dichloromethane: methanol: tri-ethylamine (90:10:0.1 by volume) as a mobile phase with UV detection at 254 nm.

Method validation:

The validation of the outlined procedures was conducted in accordance with International Conference of Harmonization (ICH) guidelines.[27]

Linearity and range:

Calibration graphs for remdesivir, nirmatrelvir, and ritonavir were established under optimal TLC-densitometric conditions by graphing the peak area values of the separated spots against the drug concentrations in µg/spot. The regression figure exhibited linearity within the range of 10–80 µg/spot for remdesivir, nirmatrelvir, and ritonavir, respectively. The values of slopes, intercepts and coefficient of determination (r^2) are shown in **Table 1.**

Limits of detection and quantitation:

The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated according to ICH guidelines from the following equations:

$$LOD = 3.3\ \sigma / S$$

$$LOQ = 10\ \sigma / S$$

Where, σ is the standard deviation of y-intercepts of regression lines and S is the slope of the calibration curve.

The standard solutions of remdesivir, nirmatrelvir, and ritonavir were analyzed using the established procedure, and the minimum detectable and quantifiable limits were determined; the results are presented in **Table 1.**

Accuracy:

Accuracy was calculated as a mean percent recovery of three determinations for three concentration levels of standard solutions covering the linearity range (20, 40, 60 µg /spot) for remdesivir, nirmatrelvir and ritonavir, respectively. The results in **Table 1** indicated the accuracy of the proposed method. Moreover, standard addition technique was applied to assess the accuracy, with no interference from excipients, as shown in **Table 2**.

Precision:

Three replicate determinations of three different concentrations of remdesivir, nirmatrelvir and ritonavir in their pure forms within linearity range were conducted on the same day (repeatability) and on three successive days (intermediate precision) to evaluate the three selected concentrations using the proposed method. The acceptable % RSD confirms the method's precision, as shown in **Table 1**.

Specificity:

The specificity of the proposed procedure was ensured through the application of the standard addition technique. Various quantities of remdesivir, nirmatrelvir and ritonavir are analyzed using the proposed method in presence of the same concentration of pharmaceutical formulation of each drug. Moreover, the peak purity and homogeneity were evaluated by means of HPTLC scanner. The specificity and selectivity of the proposed method was confirmed through the peak purity profiling. The obtained results were revealed that the method was sufficient selective for determination of the cited drugs without interference from the excipients as shown in **Table 2**. The proposed method proved appropriate for the determination of the remdesivir, nirmatrelvir and ritonavir in raw materials and pharmaceutical formulation.

Robustness:

The method was found to be robust, as it wasn't appreciably influenced by minor deviation in experimental parameters, e.g.: changing methanol volume in the developing system ±2% and changing saturation time ±2 min. These proved by smaller values of RSD as shown in **Table 1**.

System suitability:

System suitability parameters were applied to a representative chromatogram to confirm that, the system is working correctly during the analysis operation. Parameters including Retardation factor (Rf), capacity factor (K´), tailing factor (T) and resolution factor (R) were computed to assess the correct functioning of the operating system. The acquired values fell within the permitted ranges, as illustrated in **Table 3**.

Applications for pharmaceutical applications

The described method was applied for the determination of PAXLOVID® nirmatrelvir (150 mg/tablet) co-packaged with ritonavir (100 mg/tablets) and REMDESIVIR-EVA PHARMA® remdesivir (100 mg/vial). Results were satisfactory and aligned with the label claim. The standard addition technique was employed, and the findings demonstrate the absence of matrix interference. Statistical studies utilizing the t-test and *F*-test at 95% confidence level of the results obtained by the proposed method and those obtained by the reported methods[22,28], indicate no significant differences, as shown in **Table 4**.

CONCLUSION

This paper presents a sensitive and selective TLC-densitometric method for the simultaneous quantification of remdesivir, nirmatrelvir, and ritonavir in both pure form and pharmaceutical formulations, which has been developed and validated. The developed TLC-densitometric technique in comparison to the reported methods, offers enhanced sensitivity and selectivity. This TLC-densitometric process can

substitute the stated HPLC method when HPLC resources are inaccessible. The developed method is time saving where many spots can be run at the same time. This method is also cost-effective, as it utilizes a minimal volume of mobile phase as a developing system, in contrast to HPLC methods. Finally, we can conclude that the TLC- densitometric method described, can be used in routine analysis of remdesivir, nirmatrelvir and ritonavir in their pure forms and pharmaceutical dosage form without previous separation.

Acknowledgments

No external funding was received.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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