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Validated isocratic stability-indicating RP-HPLC method for concurrent remdesivir estimation in drug products used in bioavailability studies

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Abstract: This study presents a stability-indicating, isocratic RP-HPLC method for the quantitative analysis of remdesivir (RMD) in pharmaceutical formulations and bioavailability studies. The method was developed to ensure robustness, precision and accuracy across diverse matrices, including freeze-dried powders, microspheres, novasomes and biological samples like blood. Chromatographic separation was achieved using a Zorbax Eclipse Plus C-18 column (4.6 × 250 mm, 5 µm) with a mobile phase of 1% trifluoroacetic acid (TFA) and acetonitrile (55:45) at a 1 mL/min flow rate. Detection was carried out at 254 nm using a variable wavelength detector (VWD). Validation followed current good manufacturing practices (cGMP), covering parameters such as system suitability, linearity, specificity, precision, accuracy, robustness and forced degradation. The method showed a retention time consistent with RMD standards, a limit of detection (LOD) of 0.73 µg/mL and a limit of quantification (LOQ) of 2.22 µg/mL. Excellent linearity (R^2 = 0.999) was observed in the 12-120 µg/mL range. Long-term and accelerated stability studies confirmed the method's capability to detect degradation products without interference. The validated method supports reliable routine quality control testing and pharmacokinetic studies, making it suitable for *in-vivo* bioavailability assessments of RMD formulations.

Keywords: Remdesivir; RP-HPLC; Stability indicating method

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INTRODUCTION

The COVID-19 pandemic underscored the urgent need for effective antivirals. RMD, a key prodrug against SARS-CoV-2, requires reliable analytical methods to assess its quality, stability and pharmacokinetics, which are critical for optimizing clinical outcomes (Anitha, Rajamohan, Murugan, & Seo, 2024; Tayeb, Felimban, Almaghrabi, & Hasaballah, 2021). Reverse-phase HPLC (RP-HPLC) offers high precision and sensitivity for quantifying APIs, impurities and degradation products in complex matrices. A stability-indicating, isocratic RP-HPLC method enables accurate evaluation of RMD in formulations and biological samples (Abo-Gharam & El-Kafrawy, 2022; Hoseininezhad-Namin, Rahimpour, & Jouyban, 2024). It supports degradation analysis under stress (heat, light, oxidation and hydrolysis) and ensures product safety. Validated methods are essential for pharmacokinetic and bioavailability studies (Raasi, 2021).

RMD (Fig. 1), an adenosine analog, is recognized as a key antiviral option for COVID-19. Available in injectable and freeze-dried forms, it works by inhibiting viral RNA polymerase (Abdul Samad *et al*., 2023). Early clinical reports from critically ill patients showed promising recovery outcomes (Mahase & Kmietowicz, 2020), though more randomized trials are needed to confirm its efficacy. By late 2019, the rapid global spread of COVID-19, driven by severe respiratory symptoms, had overwhelmed healthcare systems (Cucinotta & Vanelli, 2020). Symptoms ranged from mild or absent to life-threatening complications like pneumonia, organ failure and death (Rodriguez-Morales *et al*., 2020; Wu *et al*., 2020) especially in individuals with cardiac or respiratory issues (P. Weiss & Murdoch, 2020). With no specific cure, treatment remains largely supportive focused on oxygen therapy and antibiotics (Poston, Patel, & Davis, 2020; S. R. Weiss & Leibowitz, 2011). RMD, poorly water-soluble but highly soluble in methanol and ethanol, displays broad-spectrum antiviral activity including against Ebola and MERS in preclinical studies (Nyarko, Boateng, Kahwa, & Boateng, 2020; Wang *et al*., 2020). Clinical data from around 500 individuals support its favorable safety profile (Gilead, 2020).

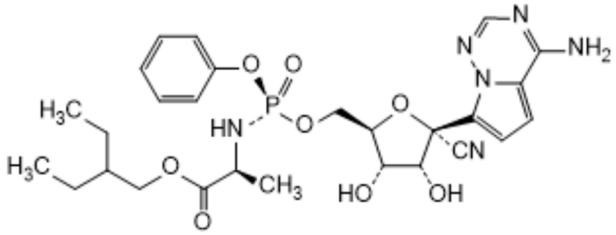

Fig. 1: RMD chemical structure.

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High-performance liquid chromatography (HPLC), owing to its speed, accuracy, precision, affordability and worldwide acceptability, is the most useful method for pharmaceutical analysis (Bhujbal, Rupenthal, & Agarwal, 2024). In addition to being a useful tool for determining the ultimate pharmaceutical product's quality, a trustworthy test method also offers information on the stability studies and kinetics of drug release (Gupta, Pancholi, & Das, 2024). Previous literature has reported the use of gradient elution systems, phosphate buffer-based methods and advanced UHPLC-MS/MS techniques for RMD analysis (Illendula *et al*., 2023a). However, these approaches often involve long injection times (e.g., 25 min in the International Pharmacopoeia, 2022), complex instrumentation and high solvent usage, making them less practical for routine analysis. RMD, a broad-spectrum antiviral drug, has significant clinical relevance in COVID-19 and other viral infections. Quantifying RMD across diverse matrices including injections, microspheres, novasomes and biological fluids is critical for quality control and pharmacokinetic evaluations. Existing methods often lack universal applicability or are matrix-specific (Avataneo *et al*., 2020; Yuan *et al*., 2022). This study aims to develop and validate a cost-effective, isocratic RP-HPLC method with a short 10-minute run time, suitable for both pharmaceutical and biological sample analysis. The method was designed following ICH guidelines to ensure specificity, accuracy, robustness and reproducibility for routine use.

MATERIALS AND METHODS

RMD, with a potency of 99.30% ± 0.45% on an as-is basis, was obtained from Ameer Adnan Pharmaceutical, Lahore, Pakistan. Laboratory-scale batches of RMD microspheres and novasomes were prepared as research formulations in the lab. Trifluoroacetic acid (Fisher Scientific, U.K.) and acetonitrile (Chemico Group Limited, U.K.), along with all other analytical-grade chemicals and reagents used in the study, were supplied by Saffron Pharmaceutical (Pvt.) Ltd., Faisalabad, Pakistan.

Apparatus

In this study, a Shimadzu HPLC (20A series) system fitted out with a quaternary pump (LC-20AT), prominence UV/visible detector (SPD-M20A), auto-sampler (SIL-20ACHT), column oven (CTO-20AC), degasser (DGU-20A5R) and "Lab-Solution" software was employed. A Zorbax Eclipse Plus C18 (4.6 × 250 mm, 5 µm particle size, Agilent, USA) was used as a column. Filtration was performed using a Sartorius filtration assembly (Goettingen, Germany), with a "Rocker 300" vacuum pump (Taiwan) to generate vacuum and a 0.45 µm polyamide membrane filter (250xx). A bath sonicator (Elmasonic Elma E 30H, Sr. No. 100925124, Germany) and an analytical balance (AL204, Mettler Toledo, USA) were also used. For stability studies, a "Temperature and Humidity Chamber" (Model PL-3FP, Osaka, Tabai Espec Corp, Japan) and for accelerated studies, a "Constant Climate Chamber" (Model HPP750, Memmert, Germany) were utilized.

Chromatographic conditions

Chromatographic separation of RMD from formulation excipients and biological matrices was achieved using an isocratic reverse-phase HPLC system equipped with a Zorbax Eclipse Plus C18 column (4.6 × 250 mm, 5 µm particle size, Agilent, USA). The mobile phase consisted of a mixture of 1% trifluoroacetic acid (TFA) and acetonitrile in a 55:45 (v/v) ratio. The mobile phase was vacuum-filtered through a 0.45 µm polyamide membrane filter (250xx) and degassed by sonication before use. The injection volume was 20 µL, the flow rate was maintained at 1.0 mL/min and detection was carried out at 254 nm using a UV detector (Patel, Tiwari, & Patel, 2021). The analysis was conducted at ambient temperature. Under these optimized conditions, the internal standard and RMD eluted at approximately 4.7 and 7.7 min, respectively, with a total run time of 10 min.

Analytical method development

Several mobile phase combinations were tested, including phosphate buffers and methanol. Ultimately, a mixture of 1% TFA and acetonitrile was selected for its robustness and accuracy in estimating RMD in formulations and blood, as shown in table 1.Palermiti *et al*., 2025 noted that advanced methods were unsuitable for routine HPLC due to complexity and solvent use. A 55:45 v/v TFA:ACN ratio was chosen based on optimal peak shape, resolution, retention time and method reproducibility.TFA was chosen over phosphate buffers because it improved RMD's solubility and polarity, enabled faster elution, reduced carry-over and ensured a cleaner baseline while preventing column fouling or salt buildup. The isocratic method using a 55:45 v/v mixture of 1% TFA and ACN proved to be cost-effective, reproducible and ideal for quality control, enabling accurate RMD quantification within 10 minutes. To prepare the internal standard, 16 mg of methylparaben was dissolved in 50 mL of water, sonicated and volume-adjusted to obtain stock solution B1. Separately, 12 mg of RMD was dissolved in 50 mL of 1% TFA: ACN (55:45 v/v) to prepare stock solution A (0.24 mg/mL). 2 mL of stock solution A and 5 mL of stock solution B1 were mixed in a 10 mL flask, sonicated and diluted with diluent to obtain 0.048 mg/mL (48 µg/mL) RMD.

Analytical method validation

To evaluate the system's suitability, six replicate injections of the same standard were performed prior to beginning the validation tests. System suitability parameters, including theoretical plates (N), tailing factor (T), resolution and % RSD of the area under the curve, were determined by injecting six replicates of a reference solution containing 48 µg/mL RMD and 160 µg/mL internal standard (IS).

Preparation of calibration curve

By creating a series of dilutions of the pure medication with known analyte concentrations, the technique must be calibrated in order to establish linearity and range in instrumental procedures. The calibration curve was assessed using two popular statistical tests: Pearson's correlation coefficient (r), which should be near +1 for high linear correlation and eye examination to verify linearity. Where,

$$r = \frac{n\Sigma x_1 y_1 - \Sigma x_1 \Sigma y_1}{\sqrt{[n\Sigma x_1^2 - (\Sigma x_1)^2][n\Sigma y_1^2 - (\Sigma y_1)^2]}} \quad (1)$$

Finding the best-fit straight line for a calibration curve using linear regression more especially, the method of least squares is the most reliable and accurate approach. This technique ensures an accurate estimation by lowering the sum of squared variances between the observed and anticipated values. The equation of a straight line is

$$y = ax + b \quad (2)$$

When x, the independent variable (analyte concentration), is shown against y, the dependent variable (AUC). With the use of linear regression, these parameters a (slope) and b (intercept) are extracted from the data, enabling accurate y value (AUC) prediction from known x values (analyte concentrations).

$$a = \frac{n\Sigma x_1 y_1 - \Sigma x_1 \Sigma y_1}{n\Sigma x_1^2 - (\Sigma x_1)^2} \quad (3)$$

A 0.24 mg/mL stock solution of pure RMD in the mobile phase was used to create a calibration curve for RMD utilizing concentrations of 12, 24, 48, 72, 96 and 120 µg/mL. Plotting the peak area against the matching RMD concentrations produced the curve. Regression findings (determined by the least squares approach), average peak area values and statistical information are computed for this process (Arayne, Sultana, & Hussain, 2011).

Linearity and range

Linearity was evaluated by plotting the AUC against a series of RMD solutions (0.012-0.12 mg/mL in the mobile phase). The correlation coefficient (*r*), coefficient of determination (r^2), intercept (b) and slope (a) of the regression line were calculated to assess the linear relationship (Ryu, Lee, & Whang, 2021). The method's range includes the lowest and highest concentrations where accurate, reproducible results are achievable; the reportable and working ranges may be identical. Additionally, a one-way ANOVA test was conducted upon the values of AUC obtained from each pure RMD concentration, with three imitate measurements of standard solution.

Synthesis of RMD containing microspheres

Microspheres containing RMD were synthesized using a solvent evaporation method. First, a 500 mL solution of 1% polyvinyl alcohol (PVA) was freshly prepared and stirred continuously at 300 rpm using a magnetic stirrer. Separately, 20 mL of dichloromethane was mixed with Eudragit E100 (E100), RMD and polycaprolactone (PCL) and stirred at 300 rpm for uniform dispersion. To this mixture, 10 mL of ethanol was added and the solution was heated to 50°C until the volume reduced to 10 mL after the addition of K15. The organic phase was then gradually introduced into the PVA solution using a 5 mL syringe under continuous stirring at 900 rpm for 30 minutes. The resulting emulsion was filtered through a 42 µm Whatman filter and dried in a desiccator at 40°C for 6 hours. The dried microspheres were then placed in Eppendorf containers and kept at room temperature in a vacuum desiccator set to 2.5 kPa until they were needed again (Qiu *et al*., 2024).

RMD sample preparation

A 50 mL volumetric flask was filled with around 100 mg of microspheres that contained roughly 12 mg of RMD. The flask was sonicated for 15 min in 20 mL of diluent to shatter the microspheres and the volume was adjusted using the first dilution of the diluent. After that, the mixture was centrifuged at 3500 rpm for 10 min. Next, 2 mL of the supernatant was transferred to a 10 mL volumetric flask and 5 mL of stock solution B was added. To get the target concentration of 48 µg/mL, diluent was added to the mixture. The dilution was filtered through a 0.45 µm nylon filter before to being fed into the auto-sampler. After preparation, six samples were added to the HPLC apparatus (Jubele, 2018).

Solution state stability

To evaluate the stability of the solution over time, the 1st dilution was stored for 24 hours at room temperature (max. 25°C) and then the procedure was repeated to achieve the final concentration of 48 µg/mL. The samples were then filtered and injected to calculate solution state stability (Paul, Astier, & Vieillard, 2018).

Specificity and selectivity

To evaluate interference from formulation excipients, by-products, degradants, blood serum contaminants and solvents during forced degradation, stability testing and analysis, the distinction between the analyte (RMD) and other sample components was examined. A simple and efficient protein precipitation method using acetonitrile was developed for RDV extraction from whole blood. It achieved >85% recovery, minimal matrix effects (±15%) and confirmed dilution integrity, ensuring reliable and reproducible quantification in biological matrices. Specificity was assessed by analyzing and comparing the chromatograms of six repeats of the RMD reference solution (48 µg/mL) with those of placebo microspheres containing polyvinyl alcohol, polycaprolactone, Eudragit-E100 and Methocel-K15. The comparison focused on the retention time at 254 nm to confirm that no significant overlapping peaks or interferences were present, indicating the method's specificity in detecting RMD in the presence of potential contaminants and excipients (Illendula, Sindhuja, Suresh, & Rao, 2023b).

Robustness

By deliberating changing some parameters such flow rate, detection wavelength and mobile phase composition, the analytical method's pliability was evaluated. In order to evaluate any influence on the assay content, the RMD concentration was calculated after these modifications. The impact on the assay output and system suitability parameters were remained within allowable limits, validated the method's consistency and dependability.

Limit of detection and limit of quantification

RMD's limit of quantification (LOQ) and limit of detection (LOD) were calculated using the following formulas:

$$LOD = \frac{3.3\sigma}{S} \qquad (4) LOQ = \frac{10\sigma}{S} \qquad (5)$$

Here, "S" stands for the slope of the calibration curve and "σ" for the response's standard deviation. A particular calibration curve was created utilizing six values ranging from 12 to 120 µg/mL in order to ascertain "σ." To calculate the standard deviation "σ," the regression line's residual standard deviation also known as the root mean square error (RMSE) or root mean square deviation was calculated. This method ensured dependable sensitivity in identifying and measuring RMD by providing precise LOD and LOQ values.

Precision and accuracy

Six duplicates of sample solutions containing 48 µg/mL of the analyte were generated and spiked with a placebo solution in order to evaluate precision through repeatability. Furthermore, repeatability was assessed to take into consideration variations across various analysts, devices and days. A minimum of nine measurements at three concentration levels (80%, 100% and 120% of the typical 48 µg/mL concentration of RMD) were used to evaluate accuracy. Nine samples in all, as well as one reference solution, were examined. Each sample was injected three times and the recovery % was calculated.

Force degradation study

Forced degradation tests were carried out in compliance with ICH guideline Q2 (R1) in order to assess the degradation behavior of RMD and validate the specificity of the stability-indicating analytical approach. Various stress factors, including as acid, alkali, heat, oxidative and photostability stress, were applied to RMD. A 5 mL RMD solution (1.25 ± 0.05 mg/mL) in the mobile phase was combined with 2 mL of either 1N HCl or 1N NaOH and heated under reflux at 80°C for 24 hours in order to hydrolyze in the acid and alkali. The RMD solution was heated under the same circumstances after being treated with water for thermal deterioration. To apply oxidative stress, 5 mL of RMD solution was combined with 2 mL of 3% v/v hydrogen peroxide (H_2O_2) and the reaction was let to run for 24 hours at 25 ± 2°C in the dark. Using a photostability chamber (LHH-250GSD, Keelrein Instrument Co., Ltd., China) at 25 ± 2°C, RMD in the mobile phase was subjected to UV light equal to 1.2×10^6 lux hours for photostability testing. To guarantee technique specificity, Agilent ChemStation software (Agilent, Germany) was used to evaluate peak purity after all stressed samples were subjected to HPLC analysis (Hoseininezhad-Namin *et al*., 2024).

Stability studies

Stability studies were conducted on three replicate trials of tablets formulated from the optimized microsphere formulation. The tablets were blister-packed in alu-alu blister packs and stored under both long-term and accelerated conditions in accordance with WHO stability guidelines. For the long-term study, tablets were stored in a temperature and humidity-controlled chamber (Model PL-3FP, Osaka, Tabai Espec Corp, Japan) at 30 ± 2°C and 65 ± 5% relative humidity (RH). For accelerated conditions, tablets were placed in a constant climate chamber (Model HPP750, Memmert) at 40 ± 2°C and 75 ± 5% RH. Samples were analyzed initially and subsequently at the 3rd and 6th months to evaluate any changes in the formulation's stability.

RESULTS

The study developed an HPLC technique for valid quantification of RMD in microsphere formulations and blood samples, using a mobile phase of 1% trifluoroacetic acid and acetonitrile. Various solvent ratios were tested to optimize method sensitivity, resolution, stability and efficiency. Adjustments in acid and acetonitrile ratios improved peak shape and resolution. The final optimized mobile phase had a 55:45 v/v ratio, achieving ideal retention time and separation at an ambient column temperature of 25 ± 3°C. The chromatogram showing the retention time of RMD along with the internal standard is shown in fig. 2. The robustness of the developed method was confirmed by deliberately varying flow rate (±0.1 mL/min), detection wavelength (±2 nm) and mobile phase composition (TFA: ACN from 65:35 to 45:55). Minor changes in flow rate slightly influenced retention time and theoretical plates, but all system suitability parameters, including tailing factor (<1.2) and resolution (>11), remained within acceptable limits. Similarly, wavelength shifts showed negligible impact on assay performance. Mobile phase variations slightly affected retention and resolution, with optimal performance at 55:45. Assay values remained between 98.17% and 100.23%, demonstrating the method’s reliability and stability under deliberate variations in analytical conditions.

DISCUSSION

The absorbance of RMD and its degradants was monitored at 254 nm, ensuring accurate detection under stress conditions. The acetonitrile-based protein precipitation method enabled rapid and efficient extraction of RMD from whole blood, with matrix effect variation within ±15%, confirming minimal interference

and good method selectivity. The method achieved high precision and specificity, suitable for RMD analysis. The ultraviolet absorptivity of RMD is primarily due to the pyrrolol-1,2,4-triazine and phenoxy-phosphoryl nuclei within its molecular structure.

The method was found to be linear with a strong coefficient correlation throughout the range of 12-120 µg/mL for RMD. With the correlation coefficient and coefficient of determination found to be 0.999 and 0.999, respectively, the mean linear regression equation for RMD was y = 20073x + 3870 (x = RMD concentration, y = average peak area). This suggests a strong linear relationship between the drug concentration and the area under the curve (AUC). Table 2 presents the specific data and findings from the regression analysis using the least squares approach.

This observation corresponds to the low standard error values recorded for the mean AUCs of the solutions used in generating the calibration curve, reinforcing the method's accuracy and reliability. To determine if the intercept significantly deviated from zero, a t'test was performed. The calculated 't' value was 0.0023 (with five degrees of freedom), compared to the tabulated value of 2.015 at the same degrees of freedom and 95% confidence interval. Consequently, accepting the null hypothesis confirmed that the intercept was not significantly different from zero. No interference was observed from the solvent (i.e., mobile phase) used in the method.

The limit of detection (LOD) for RMD was determined to be 0.73 µg/mL, representing the smallest detectable amount of RMD using this method. The limit of quantification (LOQ) was calculated as 2.22 µg/mL, denoting the smallest amount of RMD that can be quantified with acceptable precision and accuracy. The stability of RMD in the mobile phase was assessed over a 24-hour period. ANOVA of the mean absorbance values for solutions at various concentrations and preselected intervals revealed no significant differences, confirming that RMD remains stable in the mobile phase for at least 24 hours. By performing recovery experiments in the drug product (microspheres) with known levels of pure drug tested in triplicate at 80%, 100% and 120% of the standard dilution (48 µg/mL), the accuracy of the approach was evaluated. For every concentration level, the standard deviation (S.D.), coefficient of variation and confidence limits were computed as part of the evaluation.

The method's low standard deviation and coefficient of variation show great accuracy, according to the results, which is summed up in table 3. The broad confidence bounds shown at various levels further corroborate this accuracy and attest to the method's dependability for precise calculations. The method's high accuracy was demonstrated by the low standard deviation and minimal coefficient of variation. Accuracy was evaluated using nine determinations, with a maximum relative mean error (RME) of 0.542, indicating excellent precision. The maximum coefficient of variation, calculated as 0.94, reflects strong consistency among individual results.

The precision of the method was assessed by estimating RMD spiked onto the blank microspheres, using six replicates with a final dilution of 48 µg/mL. Table 3 reveals no significant disparity between the added amount of RMD in the drug product and the recovered amount, indicating that excipients such as polyvinyl alcohol, polycaprolactone, Eudragit E100 and Methocil-K15, as well as the internal standard methylparaben, did not interfere with its estimation. Furthermore, the filtration medium showed no measurable absorption of the drug.

The retention times for the internal standard (IS) and RMD were observed at 4.7 min and 7.7 min, respectively. No peaks were detected in the placebo chromatogram, confirming the method's specificity for RMD. The reproducibility across all conditions (days, instruments and analysts) is statistically consistent and acceptable, as the F-values are significantly lower than the tabulated F-value of 5.05 (Table 4).

The calculated F values for different analysts (0.201), different days (0.178) and different instruments (0.327) were all lower than the tabulated F value of 5.05 at a 95% confidence level with five degrees of freedom, indicating no significant differences between the datasets. This confirms the method's precision. Additionally, the relative mean error (RME) values were low at each level, further supporting the method's precision (Mohyeldin, Daabees, Talaat, & Kamal, 2024).

Under oxidative stress (3% H_2O_2 at 25 ± 2 °C for 24 hours), remdesivir exhibited 6.62 ± 0.02% degradation, with an early-eluting peak between 2–3 minutes. This peak is attributed to GS-441524, a known nucleoside metabolite of remdesivir formed through phosphoramidate bond cleavage, as reported in previous literature (Bakheit, Darwish, Darwish, & Al-Ghusn, 2023). A similar degradation pattern was observed under thermal stress (80°C for 24 hours), resulting in 15.11 ± 0.09% degradation and two additional peaks: One again between 2-3 minutes (GS-441524) and a minor peak at 4.75 minutes, possibly corresponding to a phosphate or alanine derivative, although not confirmed by spectral identification as shown in fig. 3. No interference was observed in the chromatograms and all assay-related quality attributes remained within acceptable limits, confirming the chemical stability of the RMD-loaded microsphere tablets. Furthermore, long-term (30 ± 2 °C / 65 ± 5% RH) and accelerated (40 ± 2 °C / 75 ± 5% RH) stability studies confirmed product stability under storage conditions. Details of the forced degradation and stability studies are presented in table 5 and table 6, respectively.

Table 1: Process for optimizing the mobile phase.

(TFA:ACN) ratio	Retention time	Theoretical plates	Tailing factor	Resolution	Peak quality
8:92	2.44	447	Split Peaks	NIL	Fail
10:90	2.44	602	Split / broad Peaks	NIL	Fail
12:88	2.4	642	Split Peaks	NIL	Fail
20:80	2.6	1784	1.78 / broad peak	NIL	Fail
25:75	2.7	4008	1.29 / negative baseline	NIL	Fail
30:70	2.8	2889	1.86	NIL	Fail
55:45	7.1	45491	1.38	9.7000	PASS

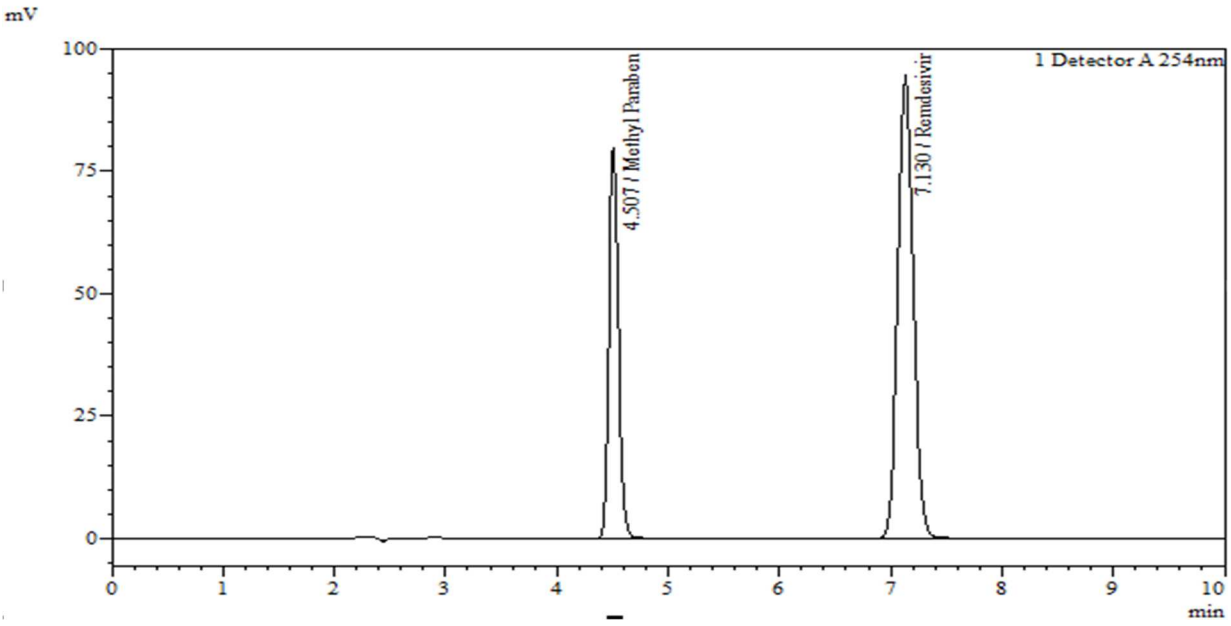

Fig. 2: Chromatogram showed peaks of RMD and IS.

Table 2: Calibration curve for the estimation of remdesivir.

Concentration (µg/mL)	Mean AUC[A]	±	(S. E)	Residual sumof least squares[B]
12	243395	±	0.071	244741
24	487089	±	0.061	485613
48	969135	±	0.057	967356
72	1449590	±	0.050	1449098
96	1924675	±	0.050	1930841
120	2416350	±	0.022	2412584

Table 3: Evaluation of RMD precision (repeatability).

Added amount (mg)	Recovered amounts (mg)	Recovery %	Standard deviation	coefficient of variation	relative mean error	confidence limits[B]
Mean	Mean	Mean	S. D[A]	CV	RME	
11.27	11.22	99.54	0.62	0.63	0.259	98.69 ± 0.51
11.32	11.16	98.62				
11.34	11.15	98.32				
11.28	11.21	99.37				
11.39	11.16	97.99				
11.33	11.14	98.32				

Table 4: Evaluation of RMD reproducibility.

Sample ID	Different days		Different instruments		Different analysts	
	Day 1	Day2	HPLC 1	HPLC 2	Analyst 1	Analyst 2
1	99.54	96.82	99.54	97.76	99.54	98.80
2	98.62	96.77	98.62	98.54	98.62	97.06
3	98.32	99.76	98.32	96.70	98.32	98.79
4	99.37	96.48	99.37	99.09	99.37	96.35
5	97.99	97.14	97.99	96.82	97.99	97.44
6	98.32	97.48	98.32	98.63	98.32	99.25
Mean	98.69	97.41	98.69	97.92	98.69	97.95
SD	0.63	1.20	0.63	1.00	0.63	1.16
F- Value		0.178		0.327		0.201
Value of tabulated F-test			5.05			
5 degree of freedom and 95% confidence interval						

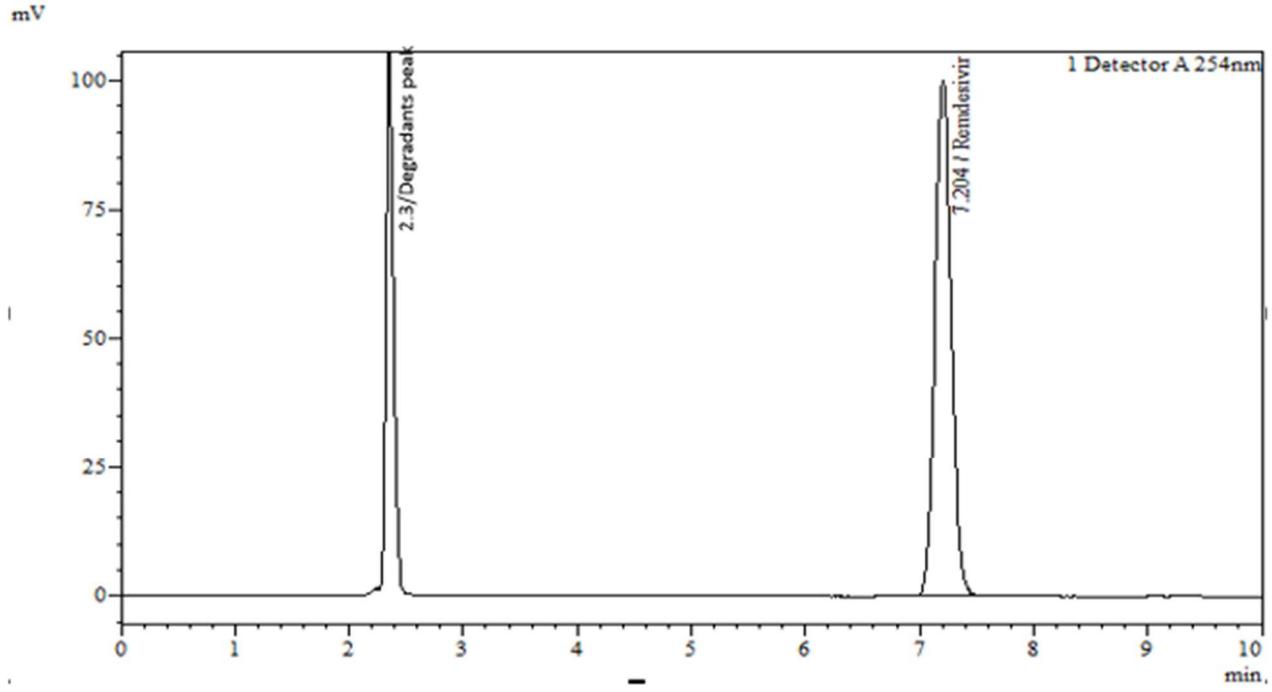

Fig. 3: Chromatogram showed resolution of RMD with the degradants appeared during forced degradation study (Oxidative stress)

Table 5: Force degradation study of RMD

Stress condition	Weight taken (mg)	Storage temperature	Storage time (hours)	AUC	Assay Mean ± SD (n = 3)	Degradation (%) Mean ± SD (n = 3)
Fresh sample	12.7	25 ◦C	0	1085194	100.92 ± 1.80	NIL
1 N HCl	13.3	80 ◦C under reflex	24	1064354	94.51± 1.80	6.40 ± 0.05
1 N NaOH	12.0	80 ◦C under reflex	24	1000558	98.47 ± 2.75	2.44 ± 0.05
3 % H_2O_2	12.4	25 ◦C	24	990078	94.30± 0.69	6.62 ± 0.02
UV light (liquid)	12.9	25 ◦C	24	1035396	94.79± 1.02	6.12 ± 0.03
Heat (liquid)	13.4	25 ◦C	24	973519	85.80 ± 0.98	15.11 ± 0.09

Table 6: Evaluation of stability study

Parameter	Specification	40°C ± 2°C / 75% RH ± 5%			30°C ± 2°C / 65% RH ± 5%		
		Initial	3rd month	6th month	Initial	3rd month	6th month
Appearance	Almost white, oblong tablets	Complies	Complies	Complies	Complies	Complies	Complies
Hardness	40-70 N	58N	55N	59N	58N	61N	56N
Avg. Weight (mg)	951 mg ± 5%	950.7	950.5	951.2	950.7	951.5	950.9
Disintegration Time	NMT 15 min	1.0	1.0	1.0	1.0	1.0	2.0
Assay (Drug Content)	85-115% of labeled content	98.36	97.6	97.5	98.36	96.9	97.2
Degradation Products	NMT 1.0	<1 %	<1 %	<1 %	<1 %	<1 %	<1 %
Moisture Content	2.5 - 4.0 %	2.9	3.1	3.15	2.9	3.15	3.2

CONCLUSION

This study successfully developed and validated a robust, stability-indicating RP-HPLC method for the quantification of remdesivir (RMD) in pharmaceutical formulations and biological samples. The method demonstrated high sensitivity, specificity and reproducibility, meeting stringent validation parameters under cGMP guidelines. Its accuracy, low detection limits and robustness make it suitable for routine quality control and pharmacokinetic applications. In addition to filling the current gap in pharmacopeial standards, the method offers a more economical and efficient alternative compared to compendial and other reported analytical methods.

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Authors' contributions
Muhammad Ibrahim: Conceptualization, methodology, validation and writing the original draft of article. Muhammad Irfan: Supervision, conceptualization, project administration, software and writing the original draft of article. Syed Haroon Khalid: Methodology and review the original draft of article.

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Data availability statement
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethical approval
The use of animals for the study was approved by the ethical committee of Government College University Faisalabad, Pakistan through Notification No. GCUF/290/2023, dated, 6th June, 2023.

Conflict of interest
The authors declare that there is no conflict of interest regarding the publication of this manuscript.

REFERENCES

Abdul Samad H, Nadir Ali S, Qayoom A, Haroon U, Saad M and Mumtaz Alavi G (2023). COVID-19 pandemic: perspectives on an unfolding crisis. *Pak. J. Pharm. Sci.,* **36**(1): 159-170.

Abo-Gharam AH and El-Kafrawy DS (2022). Eco-friendly stability-indicating HPTLC micro-determination of the first FDA approved SARS-CoV-2 antiviral prodrug Remdesivir: Study of degradation kinetics and structural elucidation of the degradants using HPTLC-MS. *Sustan. Chem. Pharm.,* **29**: 100744.

Anitha A, Rajamohan R, Murugan M and Seo JH (2024). Inclusion complexation of remdesivir with cyclodextrins: A comprehensive review on combating coronavirus resistance-Current state and future perspectives. *Molecules,* **29**(19): 4782.

Arayne MS, Sultana N and Hussain F (2011). Validated RP-HPLC method for determination of permethrin in bulk and topical preparations using UV-vis detector. *J. Chromatogr. Sci.,* **49**(4): 287-291.

Avataneo V, de Nicolo A, Cusato J, Antonucci M, Manca A, Palermiti A and D'Avolio A (2020). Development and validation of a UHPLC-MS/MS method for quantification of the prodrug remdesivir and its metabolite GS-441524: A tool for clinical pharmacokinetics of SARS-CoV-2/COVID-19 and Ebola virus disease. *J. Antimicrob. Chemother.,* **75**(7): 1772-1777. doi: 10.1093/jac/dkaa152

Bakheit AH, Darwish H, Darwish IA and Al-Ghusn AI (2023). Remdesivir. *Profiles of drug substances, excipients and related methodology,* **48**: 71-108.

Bhujbal S, Rupenthal ID and Agarwal P (2024). Development and validation of stability-indicating HPLC method for assay of tonabersat in pharmaceutical formulations. *Methods*, **231**(2024): 178-185.

Cucinotta D and Vanelli M (2020). WHO declares COVID-19 a pandemic. *Acta bio medica: Atenei parmensis,* **91**(1): 157.

Gilead R (2020). Summary on Compassionate Use. *Amsterdam, European Medicines Agency*.

Gupta PK, Pancholi SS and Das P (2024). *In-vitro*drug release testing method for nepafenac ophthalmic suspension. *J.Pharm. Sci.,* **113**(4): 1061-1067.

Hoseininezhad-Namin MS, Rahimpour E and Jouyban A (2024). Favipiravir, remdesivir and lopinavir: Metabolites, degradation products and their analytical methods. *Drug Metab. Rev.,* **56**(2): 127-144.

Illendula S, Sindhuja PL, Suresh CV and Rao K (2023b). A Novel Val.dated RP-HPLC method for the estimation of remdesivir in bulk and pharmaceutical dosage form. *Int. J. Adv. Res. Med. Pharm. Sci. (IJARMPS),* **8**(1): 58-67.

Jubele A (2018). *Chromatographic determination of amines in food samples.* M. Sc. thesis University of Helsinki Department of Chemistry, Laboratory of Analytical Chemistry.,

Mahase E and Kmietowicz Z (2020). Covid-19: Doctors are told not to perform CPR on patients in cardiac arrest: BMJ 368-m1282

Mohyeldin SM, Daabees HG, Talaat W and Kamal MF (2024). Efficient integration of analytical quality-by-design and tri-hued HPLC-DAD approach for synchronized assessment of gemigliptin and rosuvastatin in their fixed-dose pills: Application to stability study. *Sustain. Chem. Pharm.,* **42**: 101777.

Nyarko RO, Boateng E, Kahwa I and Boateng PO (2020). A comparison analysis on remdesivir, favipiravir, hydroxychloroquine, chloroquine and azithromycin in the treatment of corona virus disease 2019 (COVID-19)-A review. *World J. Pharm. Pharm. Sci.,* **9**(5): 121-133.

Palermiti A, De Nicolo A, Antonucci M, Soloperto S, Billi M, Manca A and Calcagno A (2025). A novel HPLC-MS/MS method for the intracellular quantification of the active triphosphate metabolite of remdesivir: GS-443902. *J. Xenobiot.,* **15**(4): 107.

Patel V, Tiwari N and Patel K (2021). Stability indicating RP-HPLC method development and validation for the estimation of Remdesivir in API form. *World J. Pharm. Pharm. Sci.,* **10**: 1544-1551.

Paul M, Astier A and Vieillard V (2018). Extended stability of a biosimilar of trastuzumab (CT-P6) after reconstitution in vials, dilution in polyolefin bags and storage at various temperatures. *GaBI J,* **7**(3): 101-110.

Poston JT, Patel BK and Davis AM (2020). Management of critically ill adults with COVID-19. *JAMA,* **323**(18): 1839-1841.

Qiu C, Zhang Y, Fan Y, Li S, Gao J, He X and Zhao X (2024). Solid dispersions of genistein via solvent rotary evaporation for improving solubility, bioavailability and amelioration effect in HFD-induced obesity mice. *Pharmaceutics,* **16**(3): 306.

Raasi KM (2021). Analytical method development and validation of Remdesivir in bulk and pharmaceutical dosage forms using reverse-phase-high performance liquid chromatography. *Int. J. Phram. Sci. Clin. Res.,* **1**(2): 282-329.

Rodriguez-Morales AJ, Cardona-Ospina JA, Gutiérrez-Ocampo E, Villamizar-Peña R, Holguin-Rivera Y, Escalera-Antezana JP and Henao-Martinez AF (2020). Clinical, laboratory and imaging features of COVID-19: A systematic review and meta-analysis. *Travel Med. Infect. Dis.,* **34**: 101623.

Ryu HS, Lee SJ and Whang WK (2021). Isolation of anti-diabetic active compounds from benincasae exocarpium and development of simultaneous analysis by HPLC-PDA. *Molecules,* **27**(1): 9.

Tayeb HH, Felimban R, Almaghrabi S and Hasaballah N (2021). Nanoemulsions: Formulation, characterization, biological fate and potential role against COVID-19 and other viral outbreaks. *Colloid. Interface. Sci. Commun.,* **45**: 100533.

Wang M, Cao R, Zhang L, Yang X, Liu J, Xu M and Xiao G (2020). Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) *in-vitro*. *Cell Research,* **30**(3): 269-271.

Weiss P and Murdoch DR (2020). Clinical course and mortality risk of severe COVID-19. *The Lancet,* **395**(10229): 1014-1015.

Weiss SR and Leibowitz JL (2011). Coronavirus pathogenesis. *Adv.Virus Res.,* **81**: 85-164.

Wu C, Chen X, Cai Y, Zhou X, Xu S, Huang Hand Zhang Y (2020). Risk factors associated with acute respiratory distress syndrome and death in patients with coronavirus disease 2019 pneumonia in Wuhan, China. *JAMA Internal Medicine,* **180**(7): 934-943.

Yuan M, Hu W, Feng Y, Tong Y, Wang X, Tan B and Liu J (2022). Development and validation of an LC-MS/MS method for simultaneous determination of remdesivir and its hydrolyzed metabolite and nucleoside and its application in a pharmacokinetic study of normal and diabetic nephropathy mice. *Biomed. Chromatogr.,* **36**(7): e5380.