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Development, validation and forced degradation studies of green liquid chromatographic method for determination of remdesivir in bulk drug and pharmaceutical formulation

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Abstract: Remdesivir was recently approved by Food and Drug Administration (FDA) to treat severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) disease. In this study, we report development of the green and reversed-phase liquid chromatographic method for quantitative determination of remdesivir in pharmaceutical formulations in forced degradation studies using mobile phase consisting of 0.4% trifluoroacetic acid: Acetonitrile with flow rate of 1.0mL min^{-1}. High performance liquid chromatographic stability-indicating procedure was evaluated and impurities were profiled by subjecting the remdesivir under stress conditions i.e. acidic and alkaline hydrolysis, oxidative and thermal degradation. The developed method effectively separated the parent drug response from that of degradation products and it was validated following ICH guidelines within linearity range of 5-50µg mL^{-1} exhibiting correlation coefficient greater than 0.997. At all levels, the %RSD values were less than 2.0 indicating satisfactory precision. Green profile of developed method was evaluated by NEMI and AGREE tools. Comparison of proposed method with already existing chromatographic method was established using analytical eco-scale. The proposed method is eco-friendly and resulted in reliable quantification of remdesivir in dosage formulation and impurity profiling.

Keywords: COVID-19, remdesivir, forced-degradation studies, NEMI, analytical eco-scale, AGREE

INTRODUCTION

Respiratory syndrome virus has been causing severe respiratory illness since last 100 years. Symptoms of such respiratory illness may range from mild to severe. In 1918, Spanish flue resulted in respiratory complications and caused death of 50 million global population (Jordan and Sharp, 1921; Jester *et al*., 2019). In the year 1966, isolation of novel virus from human respiratory tract of patients in Chicago having symptoms of common cold had been reported (Hamre and Procknow, 1966). According to WHO, around 8096 cases with death rate of 9.56% due to Severe Acute Respiratory Syndrome (SARS) was recorded from November 2002 to July 2003 (WHO MERS Global Summary 2003) (WHO Summary SARS cases 2003). In the year 2012, Middle East respiratory syndrome coronavirus (MERS-CoV) with symptoms of acute pneumonia and following renal failure was observed in Saudi Arabia (Zaki *et al*., 2012). WHO reported 2578 cases of MERS-CoV to-date with 34.44% mortalities worldwide ((WHO), 2021). In recent era, Coronavirus 2019 (COVID-19) has imposed significant threat to overall well-being due to fast progression of respiratory disease and deaths as consequence.

The major cause of mortality in COVID-19 was respiratory failure. Recently, another SARS-CoV-2 variant Omicron (B.1.1.529) has been announced by WHO posing high risk globally (WHO Omicron 2021). Several dru gs have emerged as options for treatment against COVID-19 and numerous products have been launched into the market with strong claims of their effectiveness which may shorten the course of the disease.

Remdesivir (fig. 1) was first approved by Gilead Sciences Biopharmaceutical in Japan in May 2020 for treatment of severe COVID-19 patients (Gilead Sciences, 2020). This drug is perceived as an effective drug for *in-vitro* and *in-vivo* antiviral activity (Grein *et al*., 2020). In the first trial, for the treatment of COVID-19 with remdesivir, in the United States, no adverse measures were observed in association with its infusion. However, reduction in lung damage in rhesus monkeys suffering from SARS-CoV-2 was observed by Ferner RE and Aronso JK (Ferner and Aronson, 2020). Furthermore, Hillaker *et al* reported an effectiveness of late initiation of remdesivir in treating the SARS-CoV-2 (Hillaker *et al*., 2020).

Based on the literature published in last two years, determination and quantification of remdesivir in bulk, pharmaceutical formulations and body fluids have been reported by LC-UV (Bulduk and Akbel, 2021; Kishore *et al*., 2021), LC-DAD (Hamdy, Abdel Moneim and Kamal, 2021; Ibrahim *et al*., 2021; Moneim, Kamal and Hamdy, 2021; Surabhi, 2021), LC-FD (Hamdy, Abdel Moneim and Kamal, 2021; Moneim, Kamal and Hamdy, 2021), LC-MS/MS (Du *et al*., 2021; Reckers *et al*., 2021), UPLC-DAD (Pasupuleti *et al*., 2021) and UPLC-MS/MS (Alvarez *et al*., 2020; Avataneo *et al*., 2020; Habler *et al*., 2021; Hu *et al*., 2021; Nguyen *et al*., 2021; Pasupuleti *et al*., 2021; Xiao *et al*., 2021) techniques. Most of these

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techniques have merits of using advanced and sophisticated detector and lesser consumption of reagents resulting in efficient separation and high sensitivity. However, due to economic constraints in laboratories of developing countries like Pakistan, India, Bangladesh etc., classical separation technique with confined resources are used for pharmaceutical testing. Liquid chromatograph equipped with conventional UV/vis detector is the usual technique for analysis in quality control laboratories to ensure the standard of finished product, in-process control, semi finishing and stability indicating throughout product's shelf life. Over the time, several greenness evaluation metrics have been developed, each having its objectives, merits and limitations. Present study is based on green liquid chromatographic determination of remdesivir in bulk and pharmaceutical formulations followed by forced degradation studies. Established analytical method has also been evaluated using NEMI, AES and AGREE metrics and compared with greenness profile of reported LC methods.

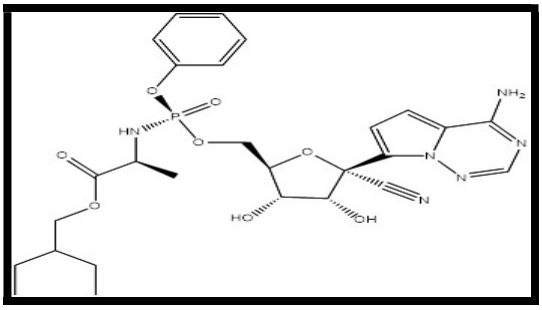

Fig. 1: Structure of remdesivir

MATERIALS AND METHODS

Materials and Reagents

Remdesivir (99.26% purity) and placebo were kindly provided by SAMI Pharmaceuticals (Pvt.) Ltd, Pakistan. HPLC grade acetonitrile, methanol, trifluoroacetic acid and HCl, NaOH and H_2O_2 were of AnalaR grade (Merck: Darmstadt, Germany). HPLC grade water was used during analysis. Pharmaceutical dosage form 100 mg Mdestiv^{TM} (lyophilized powder) intravenous infusion (B. No. 24 2F) manufactured by SAMI Pharmaceuticals (Pvt.) Ltd was purchased from local pharmacy of Karachi.

Instrumentation

Chromatographic method was developed on Agilent series 13 system (Agilent Corporation, Germany) equipped with LPG-3400RS quaternary gradient solvent delivery pump, an auto sampler WPS-3000RS and UV-visible detector VWD-3100 SD. Maximum absorption wavelength was determined by scanning the remdesivir (30µg mL^{-1}) on Shimadzu-1800 double beam UV-visible spectrophotometer in the range of 200-800 nm.

Chromatographic conditions

Chromatographic separations were achieved on Phenomenex 250 × 4.6 mm i.d., 5µm C_{18} column under isothermal condition at 40°C. Remdesivir and its degradation products were eluted using mobile phase comprised of 0.4% trifluoroacetic acid: acetonitrile following gradient steps mentioned in table 1 with the flow rate of 1.0 mL min^{-1}. Detector wavelength was adjusted at 254 nm. At the end of each day of analysis, column was washed with water then MeOH followed by purging with MeOH: water (1:1) for 15 min to maintain the column efficiency.

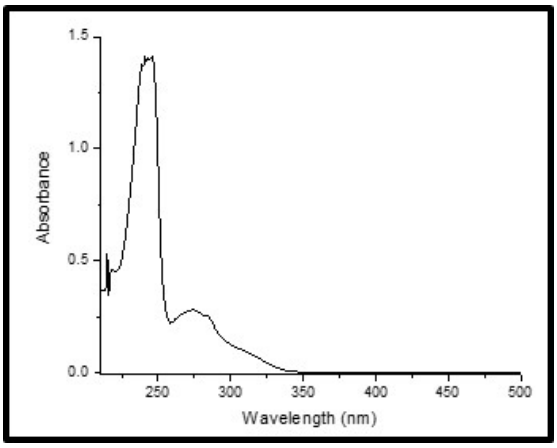

Fig. 2: UV spectra of remdesivir (20 µg ml^{-1})

Solution preparation

1000 µg mL^{-1} remdesivir stock solution was diluted with diluent to obtain 100 µg mL^{-1} remdesivir working standard solution. The stock solution was stored at 4°C for further study. Three quality control standards were prepared by spiking 20, 30 and 40µg mL^{-1} standard remdesivir in placebo to ensure the quality of analytical measurement.

Method validation

Series of calibration standards with 5-50µg mL^{-1} concentration range were prepared by diluting working standard solutions. Linearity was assessed by using linear regression analysis. Peak areas of remdesivir were plotted versus its concentrations to obtain calibration parameters such as linearity, limit of detection (LOD), limit of quantitation (LOQ) and working range. For specificity study, Mdestiv^{TM} injection excipients were mixed in appropriate proportion, followed by dilution with diluent and filtered using Whatman filter paper no 41. All solutions including placebo, 20µg mL^{-1} standard solutions of remdesivir and placebo spiked with remdesivir were analyzed. Standard addition method was used to calculate recoveries of remdesivir. It was evaluated at 80, 100 and 120% of standard remdesivir solution to three laboratory prepared sample solutions and analyzed them via developed method. Results were expressed in term of percentage of remdesivir recovered in the sample. Repeatability was assessed using dosage form solutions at 20µg mL^{-1} within the same laboratory, by same analyst, using same equipment, on the same day of analysis.

STATISTICAL ANALYSIS

Statistical analysis was performed on Minitab 17 software.

Table 1: Gradient elution of mobile phase for the chromatographic separation of remdesivir and their forced degradation products

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0.00-12.0	95	5
12.1-20.0	50	50
20.0-24.0	5	95
24.1-32.0	95	5

Table 2: System suitability parameters at optimum chromatographic condition

Drug	t_R [a]	N [b]	T_f [c]
Remdesivir	15.94	204523	1.489

[a] Retention time, [b] Theoretical plates, [c] Tailing factor

Table 3: Regression analysis for the determination of remdesivir by the proposed HPLC method according to ICH guidelines

Parameters							
Linearity (µg mL^{-1})	Slope	Y-Intercept	Retention time	R^2	SE	LOD (µg mL^{-1})	LOQ (µg mL^{-1})
5-50	8.6359	593.58	15.95	0.9989	7.399	0.91	2.76

Table 4: Evaluation of the accuracy for the determination of remdesivir by the proposed HPLC method according to ICH guidelines

Level of drug taken %	Mean % recovery[a] ± SD	Grand Mean[b]	Pooled[c]	RSD%[d]
80%	99.10 ± 0.0312	99.01%	1.65	0.281
100%	98.97 ± 0.5261			
120%	98.96 ± 0.2783			

[a]Mean of %recovery of the three different solutions at each concentration level for remdesivir.
[b]Mean of recovery of the three different concentration levels.
[c]Pooled standard deviation of all recoveries for the three different concentration levels.
[d]Percentage relative standard deviation of all determinations.

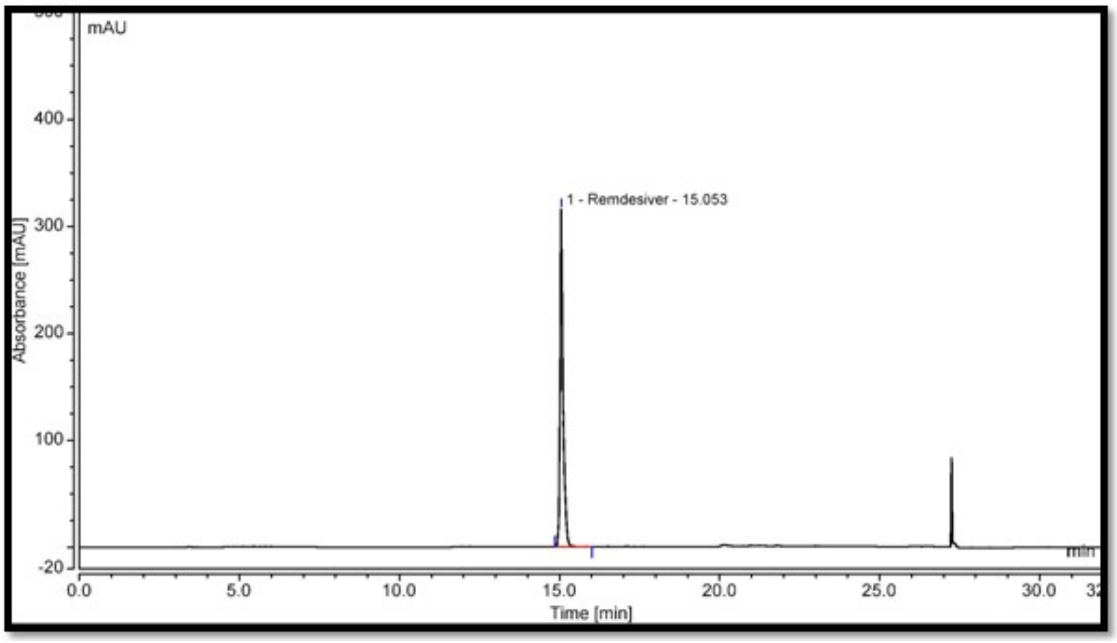

Fig. 3: Representative chromatogram of standard solution of remdesivir 20µg mL^{-1} obtained by the proposed HPLC method

Intermediate precision was evaluated on two consecutive days of analysis. Robustness studies were performed by making small deliberate changes in chromatographic conditions. Minor alterations like flow rate 1.0±0.2 mL min^{-1} and column temperature 40◦C±5°C were intentionally made in optimum parameters and instrumental responses were observed. Reliability of analytical measurement was confirmed by assessing instrument and method performance. Responses were evaluated on each day of method validation by injecting calibration standards and quality control standards into chromatograph before assessing each validation parameter.

Table 5: Evaluation of the intra-day precision for the determination of remdesivir by the proposed HPLC method according to ICH guideline

Assay number	Mean recovery %[a]
1	98.50
2	99.51
3	98.28
4	99.92
5	98.79
6	100.16
Mean [b]	99.19
SD [c]	0.78
%RSD [d]	0.79

[a]Recovery of each individual assay, for a concentration of 20 µg mL^{-1} remdesivir.
[b]The mean of all recoveries of all determinations (n = 6).
[c]Standard deviation of the recoveries of all determinations.
[d]Percentage relative standard deviation of all determinations.

Force degradation study

Acidic hydrolysis

In 50 mL volumetric flask, 25 mL of stock remdesivir solution and 5 mL of 1N HCl were transferred followed by heating at 80°C for 4 hrs. The content was then cooled and neutralized with 1N NaOH. Lastly, the volume was made up to 50 mL with diluent to obtain 0.5 mg mL^{-1} final concentration of remdesivir.

Alkaline hydrolysis

The alkaline degradation studies was performed by taking 5 mL of 0.01 N NaOH into a 50 mL volumetric flask and 25 mL of stock remdesivir solution, followed by heating at 80°C for 4 hrs. The content was then cooled, then it was neutralized with 5 mL of 0.01N HCl, and finally, the volume was made up to 50 mL with diluent to obtain 0.5 mg mL^{-1} final concentration of remdesivir.

Oxidative degradation

Into a 50mL volumetric flask, 25mL of stock remdesivir solution and 5mL of 3% H_2O_2 solution were transferred followed by heating at 80°C for 4 hrs. After cooling, content was then incorporated in 50mL volumetric flask and volume was brought up to the mark using diluent to obtain 0.5 mg mL^{-1} final concentration of remdesivir.

Thermal degradation

For thermal degradation study, 25 mL of the stock remdesivir solution was transferred into a 50mL conical flask followed by heating at 80°C for 4 hrs. After cooling, content was moved in a 50 mL volumetric flask and volume was brought up to the mark with diluent to obtain 0.5 mg mL^{-1} final concentration of remdesivir.

All degradation solutions were compared with standard remdesivir (0.5 mg mL^{-1}) to identify decrease in the area of standard remdesivir for degradation.

Placebo preparation

19 mL of placebo was introduced into 100 mL volumetric flask. Diluent was added gradually with vigorous shaking to dissolve the placebo and then finally volume was made up to 100mL using diluent. 25 mL of this solution was further diluted to 50mL. This solution was used as control.

RESULTS

Development and optimization of liquid chromatographic method

A series of trials including different compositions and mode of mobile phase (isocratic and gradient) and column lengths, were performed to optimize resolution, run time and shape of the peaks. Initially, HPLC grade organic solvents and water combinations were tried as mobile phase in isocratic and gradient elution systems at the maximum wavelength of remdesivir i.e. 254 nm (fig. 2). Considering the factor of green chemistry, methanol: water in various ratios were also tried. Increasing the methanol ratio, although decreased the retention time, but resulted in distortion of peak shape and symmetry. To obtain an enhanced peak shape and response, mobile phase composition were altered. Different ratios of acetonitrile: water and also buffer: acetonitrile were evaluated. At isocratic mode, the peak shape was improved in buffer: acetonitrile, however retention time was high. The combination of 0.4% trifluoroacetic acid and acetonitrile at constant flow of 1.0 mL min^{-1} with the gradient mode resulted in better peak shape and optimum separation between the impurities. fig. 3 shows representative chromatogram of standard remdesivir solution (20 µg mL^{-1}) acquired by the developed method.

Selection of the column type

The role of column length for better separations was assessed by using Phenomenex 250 × 4.6 mm i.d., 5µm C_{18} column and Phenomenex 150 × 4.6 mm i.d., 5µm C_{18} column under isothermal conditions at 40°C. Analysis was performed using mobile phase 0.4% trifluoroacetic acid: acetonitrile with gradient elution at a flow rate of

1.0 mL min^{-1} and detector wavelength 254 nm. It was observed that on reducing the column length, the retention time of remdesivir was reduced to 2.87 min from 15.05 min, however the peak shape and symmetry was distorted and chromatogram showed tailing with high pump pressure (fig. 4). Therefore, the suitable column for remdesivir analysis was found to be Phenomenex 250 × 4.6 mm i.d., 5µm C_{18} column.

Method validation

ICH guidelines and requirements were followed for the validation of the developed HPLC method with following parameters.

System suitability test

After selection of suitable column for remdesivir analysis, system suitability of developed method was evaluated by injecting 20 µg mL^{-1} standard remdesivir solution into the system six times on each day of method validation. The obtained results with 204523 theoretical plates (N) and 1.489 tailing factor (T) confirmed good system suitability (table 2).

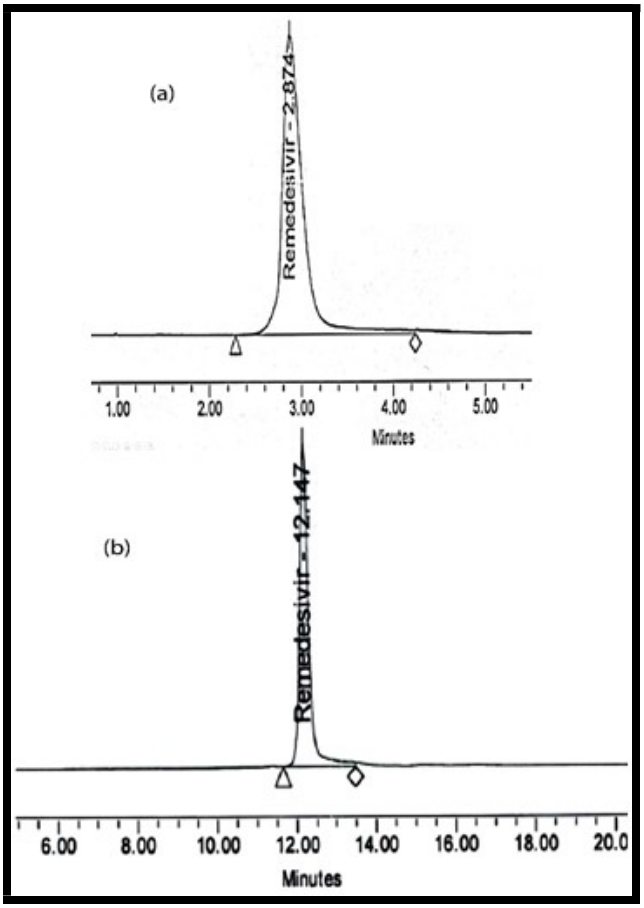

Fig. 4: HPLC chromatogram of standard solution of remdesivir 20µg mL^{-1} obtained using (a) Phenomenex 150 × 4.6 mm i.d., 5µm C_{18} column and (b) Phenomenex 250 × 4.6 mm i.d., 5µm C_{18} column

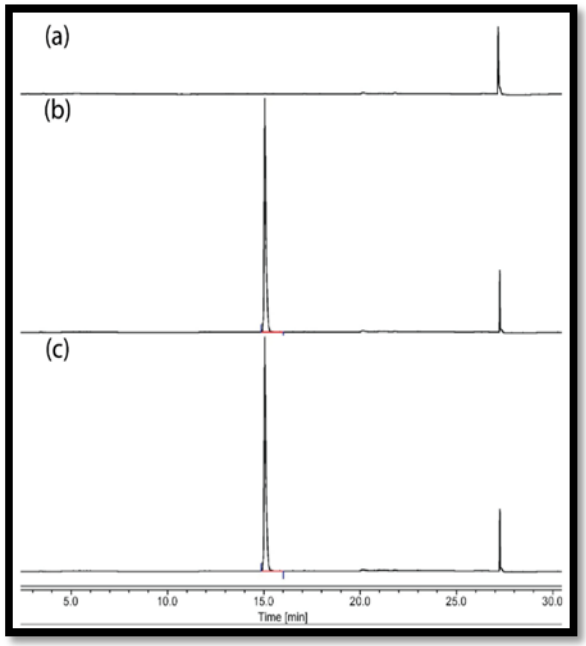

Fig. 5: Chromatogram obtained for (a) placebo (b) MdestivTM diluted to 20 µg mL^{-1} remdesivir and (c) placebo spiked solution with 20 µg mL^{-1} remdesivir

Linearity

A series of five different concentrations were prepared to assess linearity of the developed method. Each level was repeated six times and an average peak areas values was used for calculation of calibration parameters. Peak area was plotted against remdesivir concentration to construct the calibration curve. An excellent linearity within range of 5-50 µg mL^{-1} was obtained with regression equations y = 8.6359x + 593.58 for remdesivir. Regression results (R^2>0.997) suggested that the proposed method has good linearity. Parameters of the linearity data in terms of correlation coefficient, slope, and intercept are presented in table 3.

Detection and quantitation limits

The sensitivity of measurement for remdesivir by using developed RP-HPLC method was assessed as LOD and LOQ. LOD and LOQ were taken as 3.3 and 10 times the ratio of standard deviation of peak area of drug to the slope of regression curve respectively. These were found to be 0.91 and 2.76 µg mL^{-1} respectively (table 3).

Accuracy

Accuracy of the developed method was assessed by calculating recoveries of remdesivir by method of standard addition. It was evaluated by spiking 80%, 100% and 120% remdesivir solutions to three laboratory prepared sample solution and analyzing them via developed method. Table 4 represents satisfactory values of recovery (>98%.) with small value of pooled standard deviation obtained from triplicate analysis (fig. 5).

Table 6: Evaluation of the inter-day precision for the determination of remdesivir by the proposed HPLC method according to ICH guidelines

Day	Mean % recovery[a]	Grand Mean (%)[b]	Pooled SD[c]	RSD%[d]
1	98.76	99.19	1.061	0.735
2	99.62			

[a]Mean recovery for a concentration of 20 µg mL^{-1} remdesivir for each day.
[b]The grand mean of recoveries of all determinations in two different days.
[c]Pooled standard deviation of all recoveries for two different days.
[d]Percentage relative standard deviation of all determinations.

Table 7: Evaluation of the robustness for the determination of remdesivir by the proposed HPLC method according to ICH guidelines

Parameters		Area[a]	% Recovery[b]	Mean recovery[c]	% RSD[d]
Flow rate (mL min^{-1})	0.8	1069.21	100.12	99.93	0.474
		1069.79	100.28		
		1062.79	99.39		
	1.0	835.76	98.10	98.36	0.663
		843.49	99.11		
		834.75	97.89		
	1.2	715.79	100.03	99.65	0.376
		712.39	99.66		
		711.12	99.28		
Column temperature (˚C)	45	731.76	101.96	101.60	1.666
		739.12	103.09		
		716.69	99.76		
	40	835.76	98.10	98.36	0.663
		843.49	99.11		
		834.75	97.89		
	35	715.95	99.76	100.39	1.540
		732.44	102.16		
		713.00	99.25		

[a]The area of remdesivir samples at each flow rate value and temperature for a concentration of 20 µg mL^{-1}.
[b]% recovery of remdesivir.
[c]Mean of %recoveries of remdesivir at different flow rate values and temperatures.
[d]Mean of % RSD of remdesivir at different flow rate values and temperatures.

Table 8: Results of force degradation studies

Degradation type	Degradation condition	% Assay	Average
Control	-	100.89	100.33
		99.77	
Acidic hydrolysis	5N HCl at 80˚C for 4 hrs	1.63	1.55
		1.46	
Basic hydrolysis	0.01N NaOH at 80˚C for 4 hrs	68.65	68.31
		67.97	
Oxidative agent	3% H_2O_2 at 80˚C for 4 hrs	98.25	97.79
		97.34	
Thermal degradation	80˚C for 4 hrs	98.75	98.65
		98.55	

Table 9: Analysis of remdesivir in different dosage forms by the external standard method

Dosage form	% Recovery [a]	Mean %recovery ± RSD%[b]
Mdestiv™	98.5	99.069%
	99.9	
	98.8	

[a] The %recovery of triplicate determinations of 20 µg mL^{-1} remdesivir
[b] Mean %recovery ± %RSD of duplicate determinations

Table 10: Comparison of proposed analytical method on the basis of figure of merit and greenness assessment of the developed and reported methods for the determination of remdesivir by analytical eco-scale approach

Applied Instrument and chromatographic condition	Figure of Merits (µg mL^{-1})	Eco scale assessment		Ref
		Penalty points	AES Score	
Chromatography technique: HPLC-DAD HPLC-FD Mobile phase: Water acidified with *o*-phosphoric acid (pH 4): ACN gradient elution 0-4 min (70:30), 4-6 min, linearly changed till (45:55), 6-10 min (45:55) Diluent: Methanol Flow rate: 1 mL min^{-1} Time of analysis: 10 min	Linearity: 0.02-15 LOD: 0.02 LOQ: 0.06 Linearity: 0.01-15 LOD: 0.01 LOQ: 0.03	Reagents		(Moneim, Kamal and Hamdy, 2021)
		H_3PO_4	2	
		ACN	4	
		Methanol	6	
		Instrument	1	
		Occupational Safety	0	
		Waste	3	
			16	
			84	
Chromatography technique: HPLC-DAD HPLC-FD Mobile phase: ACN:Water (acidified with *o*-phosphoric acid (pH 4)) (55:45) Diluent: Methanol Flow rate: 1 mL min^{-1} Time of analysis: 10 min	Linearity: 0.1–15 LOD: 0.030 LOQ: 0.015 Linearity: 0.05-15 LOD: 0.015 LOQ: 0.05	Reagents		(Hamdy, Abdel Moneim and Kamal, 2021)
		H_3PO_4	2	
		ACN	4	
		Methanol	6	
		Instrument	1	
		Occupational Safety	0	
		Waste	3	
			16	
			84	
Chromatography technique: HPLC-UV Mobile phase: Mixture of 0.025 M Brij-35, 0.1 M sodium lauryl sulfate (SLS), and 0.02 M disodium hydrogen phosphate (pH 6.0) Flow rate: 1 mL min^{-1} Time of analysis: 10 min Diluent: MeOH and mixture of 0.025 M Brij-35, 0.1 M sodium lauryl sulfate (SLS) and 0.02 M disodium hydrogen phosphate	Linearity: 5–100 LOD: 0.5 LOQ: 2.0	Reagents		(Ibrahim *et al.*, 2021)
		H_3PO_4	2	
		SLS	12	
		Na_2HPO_4	0	
		Methanol	6	
		Instrument	1	
		Occupational Safety	0	
		Waste	3	
			24	
			76	
Chromatography technique: HPLC-MS/MS Mobile phase: Water: ACN, gradient elution: ACN (%) 0-0.3 min 5%, 0.3-0.35 min 30%, 0.35-1 min 70%, 1-1.3 min 90%, 1.3-3.3 min 90%, 3.3-3.4 min 100%, 3.4-4.4 min 100%, 4.4-4.5 min 5%, 4.5-10 min 5%. Diluent: MeOH: water (1:1) Flow rate: 0.55-0.75mL min^{-1} Time of analysis: 10 min	LOQ: 135 ng ml^{-1} LOD: 0.0375 ng ml^{-1}	Reagents		(Reckers *et al.*, 2021)
		ACN	4	
		Methanol	6	
		Instrument	2	
		Occupational Safety	0	
		Waste	3	
			15	
			85	
Chromatography technique: HPLC-MS/MS Mobile phase: A=(ACN+water+ formic acid (95 + 5, 0.1%), B=Water+ACN+ formic acid (99 + 1, 0.1) Gradient elution (%B): 0-1.2 min 99%, 1.2-3.5 min 5%, 3.5-3.6 min 99%, 3.6-4.5 min 99%. Diluent: MeOH:Water (1:1) Flow rate: 0.4 mL min^{-1} Time of analysis: 4.5 min	Linearity: 20–1000 ng ml^{-1} LOD: 1 ng ml^{-1} LOQ: 2 ng ml^{-1}	Reagents		(Du *et al.*, 2021)
		ACN	4	
		Methanol	6	
		Formic acid	1	
		Instrument	2	
		Occupational Safety	0	
		Waste	3	
			16	
			84	
Chromatography technique: HPLC-UV Mobile phase: 20 mM KH_2PO4 solution (pH 7.50): ACN (50:50 v/v) Diluent: MeOH Flow rate: 1.2 mL min^{-1} Time of analysis: 10 min	Linearity: 10-60 LOD: 2.40 LOQ: 7.30	Reagents		(Bulduk and Akbel, 2021)
		Methanol	6	
		KH_2PO_4	0	
		ACN	4	
		Instrument	1	
		Occupational Safety	0	
		Waste	5	
			16	
			84	

Continue…

Chromatography technique: HPLC-UV Mobile phase: ACN: MeOH: 0.1% *o*-phosphoric acid (65:30:5) Diluent: ACN: ACN: MeOH: 0.1% *o*-phosphoric acid (65:30:5) Flow rate: 0.8 mL min^{-1} Time of analysis: 20 min	Linearity: 10-70 ng ml^{-1} LOD: NA LOQ: 10 ng ml^{-1}	Reagents		(Kishore *et al.*, 2021)
		ACN	8	
		MeOH	6	
		H_3PO_4	2	
		Instrument	1	
		Occupational Safety	0	
		Waste	5	
			22	
			78	
Chromatography technique: HPLC-UV Mobile phase: TFA: ACN Gradient elution (% ACN) 0-12 min 5%, 12-20 min 50%, 20-24 min 95%, 24-32 min 5%. Diluent: ACN: Water(50:50) Flow rate: 1.0 mL min^{-1} Time of analysis: 32 min	Linearity: 5-50 LOD: 0.91 LOQ: 2.76	Reagents		Proposed method
		TFA	8	
		ACN	4	
		Instrument	1	
		Occupational Safety	0	
		Waste	5	
			18	
			82	

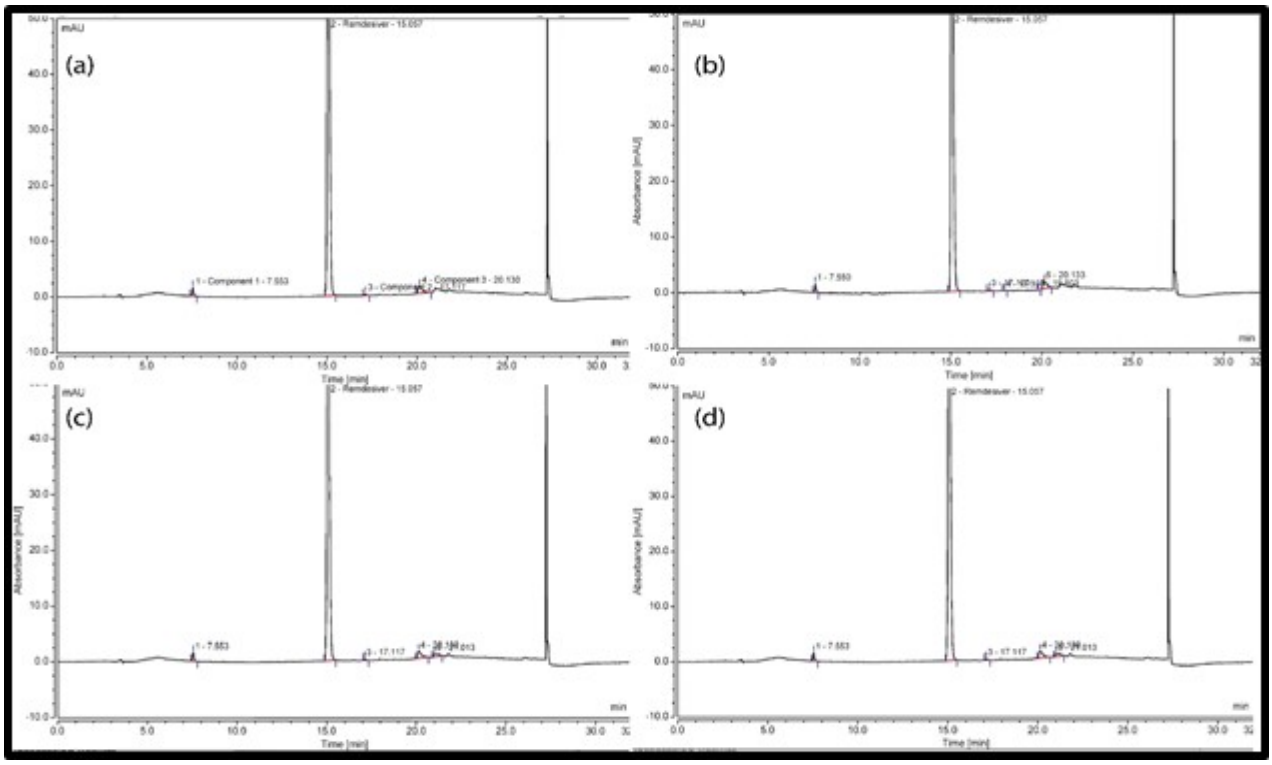

Fig. 6: HPLC chromatograms of a working standard solution of remdesivir 500µg ml^{-1} after (a) acidic hydrolysis with 5N HCl, (b) basic hydrolysis with 0.01 N NaOH, (c) oxidative degradation with 3% H_2O_2 (d) thermal degradation at 80 for 4hrs

Precision

Repeatability was assessed by analyzing dosage form sample solutions of remdesivir (100mg Mdestiv^{TM}) at 100% of test concentration (20 µg mL^{-1}) using the developed method in to the same laboratory, by same analyst on the same instrument. The intra-day variation was carried out using six determinations each injected twice, by computing the %RSD values attained on same day of analysis (table 5). Intermediate precision was assessed in the same way for two consecutive days, each of three determinations. Precision, reported in terms of % RSD for remdesivir, showed good agreement among the individual test results (table 6).

Robustness

Preliminary screening of process variables i.e. mobile phase composition, pH, column temperature, wave length of detector and flow rate of mobile phase suggested that only flow rate of mobile phase and column temperature are worth exploring further to establish robustness of the method. Therefore, deliberate changes were made in the flow rate (1.0±0.2 mL min^{-1}) and column temperature (40°C±5) for elution of remdesivir dosage form (100mg MdestivTM). The %RSD value of the recovery attained by analyzing the same analyte after above mentioned deliberate variations was found to be <2% representing the robustness of method (table 7).

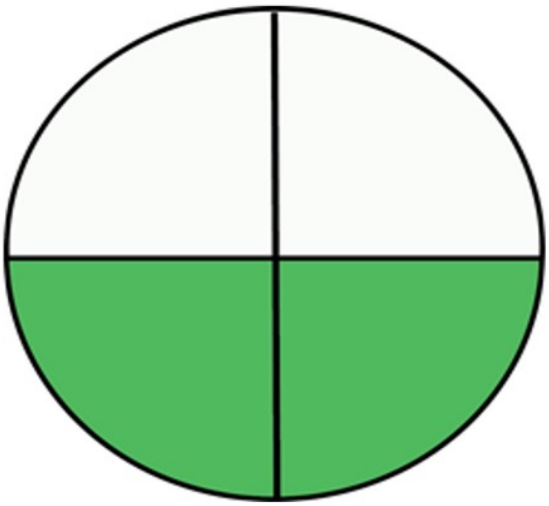

Fig. 7: NEMI pictogram of the proposed LC-UV method for remdesivir determination using mobile phase TFA: ACN with gradient elution at 1.0 mL min^{-1} flow rate

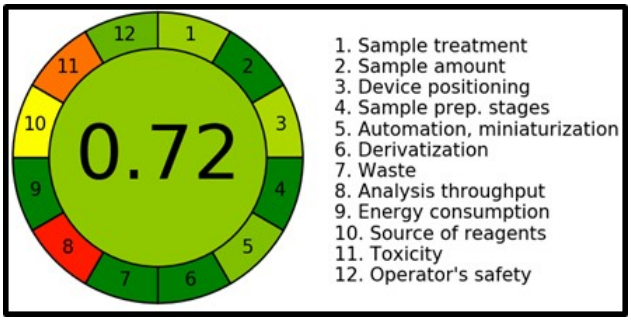

Fig. 8: Greenness evaluation of the developed LC-UV method for the determination of remdesivir using AGREE approach

Specificity

For establishing specificity, placebo solution was prepared as per test procedure for blank solution. Chromatograms of blank placebo, dosage form of drug (100 mg Mdestiv^{TM}) diluted to 20 µg mL^{-1} and placebo spiked with 20 µg mL^{-1} standard remdesivir solution were recorded in order to verify the interference of inactive components at the retention time of remdesivir. It was observed that existing additives and excipients did not interfere at the retention time of remdesivir confirming the specificity of the proposed method (fig. 5).

Forced degradation study

Stability of remdesivir drug substance and its product in solid and solution were investigated for normal and stressed conditions and results have been summarized in table 8. The studies were carried out as per ICH guidelines under acid, base hydrolysis, oxidation and thermal conditions. Remdesivir degraded at all stressed condition, however was found to be stable under oxidative and thermal conditions. For the acidic hydrolysis, the peak of remdesivir was totally decomposed with the appearance of three degradation peaks at 7.55, 17.11 and 20.13 min, far from the retention times of remdesivir peak (Fig. 6a). The percentage of remdesivir recovered after acidic hydrolysis for 4 hrs was around 1.55%. Remdesivir showed 31.67% degradation in basic hydrolysis for 4 hrs. The chromatograms represented in Fig. 6b explain the degradation behavior of remdesivir under basic hydrolysis (7.55, 17.12, 17.93, 19.80 and 20.13 min). The peak of remdesivir was not much affected by oxidative and thermal degradation (fig. 6c and d). Their peak areas did not show major change i.e. percent recovery of remdesivir under both stress conditions was 97.79% and 98.65% respectively.

Analysis in pharmaceutical formulation

20 mL Water for Injection (WFI) was added to 100 mg Mdestiv^{TM} lyophilized powder for infusion to convert into solution form. Freshly prepared content were then transferred into 100 mL volumetric flask and the volume was brought to mark with diluent (50% acetonitrile) to bring the final concentration 1000 µg mL^{-1}. This solution was further diluted with diluent to prepare sample solution for the determination of percent recovery. The percent recovery of remdesivir was found to be 99.06% shown in (table 9 fig. 5).

DISCUSSION

A good chromatographic method results in well resolved peaks with symmetrical shape, higher peak area and theoretical plates. Chromatographic parameters such as solvent composition, type of elution, column type, temperature, detector wavelength are optimized to obtain most suitable responses. For development of proposed method, chromatographic conditions were optimized to develop a reliable and repeated method with ability to separate chromatographic peak corresponding to remdesivir from those of its possible degradation products. Initially environmentally benign solvents such as methanol, water, buffers etc. were tried in isocratic and gradient elution mode but better peak shape and symmetry of remdesivir and degradation products were obtained by using 0.4% trifluoroacetic acid: acetonitrile at constant flow of 1.0 mL min^{-1} with gradient mode. The developed method was validated and system suitability was established as mentioned in the results.

Comparison and evaluation to previously reported methods

Since last four decades, HPLC is the imperative technique widely used in quality control and R&D laboratories worldwide. Liquid chromatographic methods are significantly improved with passage of time in terms of speed, sensitivity, resolution etc. LC-UV is still most commonly used technique used for pharmaceutical determinations because of the its ease of use and

relatively lower chemical and electrical background noises. Different liquid chromatographic methods including LC-UV (Bulduk and Akbel, 2021; Kishore *et al*., 2021), LC-DAD (Du *et al*., 2021; Hamdy, Abdel Moneim and Kamal, 2021; Moneim, Kamal and Hamdy, 2021; Reckers *et al*., 2021), LC-FD (Hamdy, Abdel Moneim and Kamal, 2021; Moneim, Kamal and Hamdy, 2021), LC-MS/MS (Du *et al*., 2021; Reckers *et al*., 2021), UPLC-DAD (Pasupuleti *et al*., 2021) and UPLC-MS/MS (https://www.nemi.gov/about/, no date; Alvarez *et al*., 2020; Avataneo *et al*., 2020; Habler *et al*., 2021; Hu *et al*., 2021; Nguyen *et al*., 2021) are reported in literature for detection and quantification of remdesivir (table 10). In comparison to the proposed method, though reported UPLC technique or even LC with DAD or MS/MS detector show improved efficiency and high sensitivity, however cost and maintenance limitations restrict the applicability of these methods in low-budget laboratories.

Greenness profiling of the proposed method

The greenness profile was evaluated by NEMI (www.nemi.gov; Keith, Gron and Young, 2007) AES and AGREE metrics approaches. According to NEMI, an internet-searchable database greenness of the analytical method is implicated by a pictogram containing four quadrants each representing separate parameter for greenness evaluation. Quadrant 1 is green if the chemicals used in analysis are not mentioned in EPA's TRI list i.e. none of the reagent is persistent, bioaccumulative and toxic (PBT). Second quadrant is colored green if chemicals used in analysis are not listed in EPA's TRI list of K, F, P & U. Quadrant 3 of ideal method is green if sample pH is greater than 2 or less than 12 and fourth quadrant is green if waste generation is less than 50 mL (g). fig. 7 represents NEMI pictogram for proposed method, showing two of the quadrants green corresponding to corrosiveness and waste generation. Liquid chromatographic methods represented in table 10 also show similar pattern of pictogram because of using hazardous/PBT solvents listed in EPA's TRI lists. According to AES approach, the ideal green analytical method is one which utilizes benign solvent, possess no environmental hazard, waste generation is zero and power consumption per sample is <0.1 kwh. On AES, the score of such method is 100. Penalty points are allocated on deviation from ideal conditions; which are subtracted from 100. Resultant score >75 represents remarkable green method, score >50 represents an acceptable green method, whereas a score <50 indicates a poor green method (Ibrahim *et al.*, 2021). The proposed method collected 18 penalty points, thus analytical eco-scale score is 82. This score point was compared with those of reported method (table 10) and it was observed that resultant score is very close to the chromatographic techniques equipped with advanced detectors i.e DAD, FD or MS/MS which restrict the applicability of method for routine analysis. Analytical score point of developed method is also well comparable with reported LC-UV methods. To the best of our knowledge, only two articles based on HPLC-UV method are available for determination of remdesivir (Bulduk and Akbel, 2021; Kishore *et al.*, 2021). Method reported by I. Bulduk and E. Akbel fulfills the greenness criteria but sensitivity of method (LOD = 2.74 μg mL^{-1}) is lower than that of proposed method having detection limit 0.91 μg mL^{-1}. The other method reported by D. Kishore *et. al* scored 22 penalty points, thus analytical eco-scale score is less that the proposed method (Table 10). Another green chemistry metrics i.e. AGREE calculator is represented by clock-shaped pictogram with twelve divisions, one for each GAC principle. In the middle of the AGREE Pictogram, a numeric value from 0.0-1.0 presents greenness evaluation score. AGREE score point for the proposed method is 0.72 (fig. 8). This score was calculated by taking average of twelve points for each principle based on employing direct analysis on auto sampler, without sample pre-treatment, derivatization and preservation, using 0.100g remdesivir for stock solution preparation with waste generation less than 50 mL per sample. According to agree tool criteria, score deduction is due to use of acetonitrile and TFA as mobile phase and 32 min analysis time. Therefore, we suggest that proposed HPLC-UV method is reliable, sensitive and environmental friendly for the determination and impurity profiling of remdesivir in bulk and dosage formulation.

CONCLUSION

A green stability indicating HPLC-UV method for the quantification of anti-COVID drug remdesivir have been developed and validated following the ICH guidelines. The established method has adequate results in terms of specificity, linearity, accuracy, precision, system suitability and sensitivity. Green profile of the proposed method evaluated via NEMI, analytical eco-scale and AGREE tools confirms that the developed method is environmental friendly and appropriate for QC laboratories in routine analysis of remdesivir in dosage formulation without interference of excipients.

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