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Stability Indicating RP-HPLC Method for Determination of Remdesivir in Sublingual Tablet Dosage Form

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Abstract— *A simple, suitable, rapid, precise and accurate RP-HPLC method was developed and validated for estimation of Remdesivir in sublingual tablets. The chromatographic separation was achieved on Inertsustain C18 (4.6 mm X 100 mm, 3 µm) column and Ammonium acetate buffer (pH 4.0) and Acetonitrile in the ratio of 60:40 % v/v was used as mobile phase at a flow rate of 1 ml/min in isocratic mode. The diluent used was Methanol and Water in the ratio of 50:50 % v/v. The detection was carried out at 245 nm by injecting 10 µl solution. Retention time of Remdesivir was found to be around 7.3 min. The method validation was carried out in accordance with ICH guidelines. The method was found to be linear in the concentration range of 5-100 µg/ml and the Correlation coefficient was found to be 0.9996. Mean percent recovery of Remdesivir sample solutions was found to be 100.19 %. LOD and LOQ values for Remdesivir were 1.08 and 3.26 µg/ml respectively. Forced degradation studies were also conducted by subjecting the drug to stress conditions such as acidic, alkaline, hydrolytic, oxidative and photolytic degradation to establish specificity of the method.*

Keywords— *Method development and Validation; Remdesivir; RP-HPLC; Stability indicating; Sublingual tablets.*

I. INTRODUCTION

Covid-19 pandemic occurred as a serious outbreak from Wuhan city of China in December 2019; the consequences of which are still faced by the world today. The causative agent Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has a unique structure containing spike protein along with other nucleoproteins, polyprotein and membrane protein called RNA polymerase [1]. Remdesivir (CAS 1809249-37-3) is an antiviral nucleotide analogue used for therapy of severe novel coronavirus disease 2019 (Covid-19) caused by SARS-CoV-2 infection [2].

It has a broad spectrum activity against members of families such as filoviruses (e.g. Ebola) and coronaviruses (e.g. SARS-CoV-2 and Middle East Respiratory Syndrome Coronaviruses [MERS-CoV]) and is found to have prophylactic and therapeutic efficacy in non-clinical models of these coronaviruses [3-6].

The IUPAC name of Remdesivir is 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-2yl]methoxyphenoxyphosphoryl]amino]propanoate (Fig. 1.) and has the molecular formula C27H35N6O8P [2]. Remdesivir is a prodrug of an adenosine triphosphate (ATP) analog, which upon administration is metabolized to its active form GS-441524. As an ATP analog, GS-441524 competes with ATP for incorporation into RNA which inhibits the action of viral RNA-dependent RNA polymerase resulting in termination of RNA transcription and decrease in viral RNA production [2].

Remdesivir received first conditional approval for use in patients severely affected by Covid-19 in Taiwan in late May 2020 [7]. Remdesivir received restricted approval for emergency use in India along with several generic formulations of Remdesivir being approved, having manufacturers such as Cipla Ltd., Hetero Drugs, Mylan and Jubilant Pharma [8-10]. A New Drug Application for Remdesivir for Covid-19 treatment was submitted on 10 August 2020 to US FDA [11].

No chromatographic method was found during the literature survey for analysis of Remdesivir in tablet dosage form. Hence attempts were made to develop suitable, simple, rapid, precise and accurate stability indicating RP-HPLC method for estimation of Remdesivir in sublingual tablets.

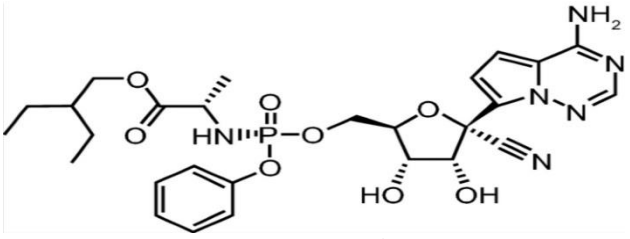

Fig. 1. Chemical structure of Remdesivir

II. MATERIALS AND METHODS

Chemicals and Reagents:

Remdesivir reference standard having defined potency of 101.36 % (on anhydrous basis) and Remdesivir sublingual tablets (20 mg) were obtained from the Central Drugs Testing Laboratory, Mumbai. Ammonium acetate (AR grade) obtained from Molychem, Methanol and Acetonitrile of HPLC grade obtained from Merck life science Pvt. Ltd. were used. Ultra-

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purified HPLC grade distilled water from Milli-Q® system (Millipore, Milford, MA, USA) water purification unit was used. High flow nylon membrane filter (0.45 µm) was purchased from Axiva Sichem Pvt. Ltd.

Instrumentation:

PerkinElmer UV/VIS Spectrophotometer having PerkinElmer UV WinLab ES software/version 6.0.4.0738 was used for all spectrophotometric measurements. The chromatographic separation was achieved by using Shimadzu HPLC system consisting of Quaternary Gradient pump LC-20AD, a total-volume injection type autosmpler SIL-20A HT, a dual-wavelength UV/VIS absorbance detector SPD-20A and a column oven CTO-20AC operated using a software LC LabSolutions/version 1.23 SP1. All weighings were carried out using Sartorius Analytical Balance. Forced degradation was carried out on Waters HPLC system equipped with Photo Diode Array Detector-2996.

Selection of wavelength:

50 mg of Remdesivir standard was weighed accurately and transferred to 50.0 ml volumetric flask and volume was made up to the mark with methanol (1000 µg/ml). The aliquot portion of the standard stock solution of Remdesivir was diluted appropriately with Methanol so as to obtain a solution of 10 µg/ml concentration. The above solution was scanned in the range of 400.0 nm to 200.0 nm using UV/Vis spectrophotometer using Methanol as a blank. Remdesivir showed maximum absorbance at 245.0 nm as shown in Fig. 2. Hence, the same wavelength was selected for the analysis of the Remdesivir.

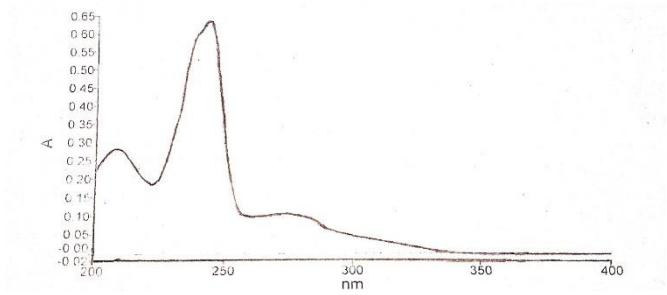

Fig. 2. UV Spectra of Remdesivir

Preparation of Buffer for Mobile Phase:

25 mM Ammonium acetate buffer (pH 4.0) was prepared by weighing 1.927 gm of Ammonium acetate, transferred to 1000 ml mobile phase bottle, added 1000 ml water and sonicated for few minutes using an ultra sonicator. Further, pH was adjusted to 4.0 and vacuum filtered through 0.45 µm high flow nylon membrane filter and was ready for use.

Selection of Diluent:

Considering the chemical nature of Remdesivir, Methanol and Water in the ratio of 50:50 % v/v was selected for all standard and sample preparations.

Preparation of Mobile Phase:

Ammonium acetate buffer pH 4.0 and Acetonitrile in the ratio of 60:40 % v/v was used as a mobile phase for the present study. The mobile phase was sonicated and degassed using an ultra sonicator.

Preparation of standard solution:

A standard solution of concentration 40 µg/ml of Remdesivir was prepared using a diluent.

Analysis of Marketed Formulation:

Twenty tablets of Remdesivir (20 mg) were weighed and their average weight was determined. The tablets were then crushed to fine powder using mortar and pestle and powder equivalent to 20 mg of Remdesivir was weighed and transferred to 100.0 ml volumetric flask and dissolved in sufficient quantity of diluent. The contents were sonicated for 10 minutes and the final volume was made up to the mark with diluent. Further dilutions were made to get 40 µg/ml of sample solution.

10 µl volumes of standard and sample solutions of Remdesivir in triplicates were injected into the HPLC system for performing assay on the above tablets. Mean, SD, and % RSD of sample peak areas and % assay were calculated and reported. The results are depicted in Table 9 and the chromatogram of sample (40 µg/ml) solution of Remdesivir is shown in Fig. 5.

Method Optimization:

Molecular structure and solubility data showed that Remdesivir is a basic (pKa 10.23: strongest acidic, pKa 0.65: strongest basic), non-polar drug. Considering the chemical nature of the molecule, base deactivated (BDS) column was the first choice for the retention of the drug. Different mobile phase systems containing water and organic solvents such as Acetonitrile, Methanol in different ratios were tried using different makes of HPLC columns.

Initial trials on HPLC were carried out on Kromasil C18 (4.6 mm X 125 mm, 5 µm) column, with mobile phase 25 mM Potassium phosphate buffer (pH 3.5):Acetonitrile in a ratio of 70:30 % v/v at a flow rate of 1 ml/min. However, poor peak shapes with long retention times were observed with above trials.

Further trials were conducted on Inertsustain C18 (4.6 mm X 100 mm, 3 µm) column using different buffer systems with different pH. Finally, good peak shape and acceptable system suitability parameters were found with mobile phase comprising of Ammonium acetate buffer (pH 4.0) with Acetonitrile in the ratio of 60:40 % v/v on Inertsustain C18 (4.6 mm X 100 mm, 3 µm) column.

Method Validation:

Validation of developed RP-HPLC method was done for parameters such as specificity, linearity, precision, accuracy and recovery, LOD, LOQ, and robustness as per ICH guidelines [12].

Specificity:

Specificity of the method was carried out by forced degradation studies at different conditions like acidic, alkaline, hydrolytic, oxidative and photolytic conditions.

Linearity:

Linearity studies on Remdesivir standard solutions were performed in the concentration range of 5-100 µg/ml. Linearity

of Remdesivir was found to be linear with Correlation coefficient (r^2) value as 0.9996 and regression equation was found to be y=37465x-11690, having the slope 37465 and y-intercept 11690. The linearity data is shown in Table 2. The graph of peak areas obtained verses respective concentrations was plotted in terms of slope, intercept, and correlation coefficient value as shown in Fig. 6.

Precision:

System Precision:

Six replicate injections of a standard solution of Remdesivir (40 µg/ml) were injected into HPLC while performing system precision studies. The mean, SD, and % RSD of peak areas of six replicate injections were calculated and reported and the results are shown in Table 3.

Method Precision (Assay Repeatability):

Six replicate injections of standard solution of Remdesivir (40 µg/ml) and six sample solutions of Remdesivir (40 µg/ml) in triplicates were injected into the HPLC system. Mean, SD, and % RSD of % Assay were calculated and reported. The mean assay percentage results of Remdesivir sample solutions are shown in Table 4.

Intermediate Precision:

This was performed on two different days. Six replicates of standard solution of Remdesivir (40 µg/ml) and six sample solutions of Remdesivir (40 µg/ml) in triplicates were injected into the HPLC system. Mean, SD, and % RSD of % Assay were calculated and reported for the same. The results are summarized in Table 5.

Accuracy and Recovery (Standard addition method):

Accuracy is the closeness of test results obtained by a particular method to the true value. Recovery studies were done by standard addition method by adding known amount of standard solution to the preanalyzed formulation at three different levels (110 %, 120 %, 130%). At each level three determinations were performed and mean % recovery was calculated and reported. Results for accuracy studies at various concentration levels are shown in Table 6.

TABLE 1. System suitability studies of Remdesivir

Standard	Area	Retention time
1	1601780	7.358
2	1603272	7.399
3	1597225	7.351
4	1573517	7.330
5	1607511	7.322
6	1601910	7.318
Mean	1597536	7.346
SD	12219.2865	0.0303
% RSD	0.765	0.413
Limit	NMT 2.0 %	NMT 1.0 %

SD= Standard Deviation.
%RSD= Percentage relative standard deviation.
NMT= Not more than.

TABLE 2. Linearity data of Remdesivir

Linearity level	Concentration (µg/ml)	Peak Area
1	5	186346.5
2	10	379277
3	20	763132
4	40	1449606.5
5	50	1830410.5
6	60	2217079.5
7	80	2987087.5
8	100	3768218

TABLE 3. System Precision (Standard)

Injection no.	Area at 245 nm	Limit
1	1601780	NMT 2.0 %
2	1603272	
3	1597225	
4	1573517	
5	1607511	
6	1601910	
Mean	1597536	
SD	12219.2865	
% RSD	0.765	

SD= Standard Deviation.
%RSD= Percentage relative standard deviation.
NMT= Not more than.

TABLE 4. Method Precision (Sample)

Sample no.	% Assay	Limit
1	99.13	NMT 2.0 %
2	100.44	
3	100.67	
4	99.44	
5	99.46	
6	99.37	
Mean	99.75	
SD	0.6367	
% RSD	0.638	

SD= Standard Deviation.
%RSD= Percentage relative standard deviation.
NMT= Not more than.

TABLE 5. Intermediate Precision/ Interday Precision

Sample no.	Day 1	Day 2	Limit
1	99.13	101.77	NMT 2.0 %
2	100.44	103.73	
3	100.67	103.71	
4	99.44	103.76	
5	99.46	101.52	
6	99.37	102.05	
Mean	99.75	102.75	
SD	0.6367	1.0838	
% RSD	0.638	1.055	

SD= Standard Deviation.
%RSD= Percentage relative standard deviation.
NMT= Not more than.

TABLE 6. Accuracy studies of Remdesivir

% level	STD spiked (µg/ml)	Amount recovered (mg)	% amount recovered	% recovery	Mean % recovery
100	0	19.9886	99.94	99.94	100.19
110	4	22.1020	110.51	100.46	
120	8	23.9684	119.84	99.87	
130	12	26.1224	130.61	100.47	

LOD and LOQ:

Following formulae were used to estimate Limit of detection (LOD) and limit of quantification (LOQ) of Remdesivir from calibration curve method:

$LOD = 3.3 \times \propto/s$, $LOQ = 10 \times \propto/s$

Where $\propto$ denotes the standard deviation of regression line response and s denotes the slope obtained from the calibration curve. Solutions of desired concentrations for LOD and LOQ were prepared and injected.

The sensitivity of measurement of Remdesivir by the current method was estimated in terms of Limit of Detection and Limit of Quantitation. The results are summarized in Table 7.

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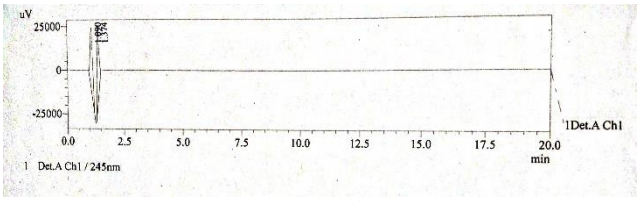

Fig. 3. Chromatogram of Blank solution

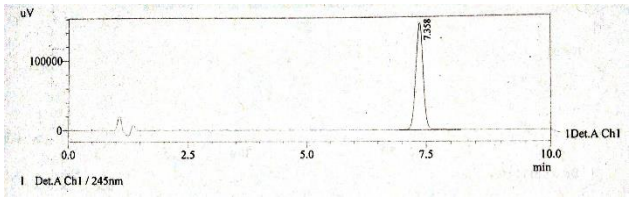

Fig. 4. Chromatogram of Standard solution of Remdesivir

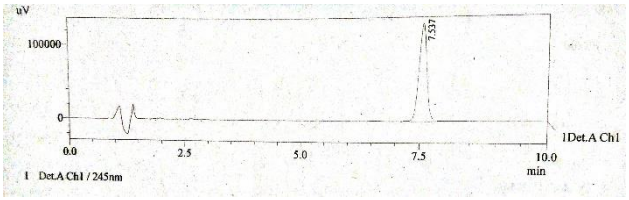

Fig. 5. Chromatogram of Sample solution of Remdesivir

Robustness:

The Robustness of the method was performed by changing flow rate (± 2 %), Mobile Phase composition (± 2 %) and wavelength (± 2 nm). Under different chromatographic conditions, three sample solutions of Remdesivir were prepared and injected in triplicates along with six replicate injections of a standard solution of Remdesivir. Mean, SD, and % RSD of % estimation were calculated and reported for the same. The results are shown in Table 8 and no significant deviation was found in the results.

Forced Degradation Studies/ Specificity of the Method

Forced degradation studies also known as stress testing happen to be an intrinsic part of pharmaceutical product development. Stability of Active Pharmaceutical Ingredient or formulation product can be predicted by carrying out stress testing. Impurities developed during the storage of drug products in various environmental conditions can be studied with the help of forced degradation studies. Forced degradation studies help in formulation and packaging development by studying the chemical behaviour of the molecule. Forced degradation of Remdesivir was carried out under acidic, alkaline, hydrolytic, oxidative and photolytic conditions.

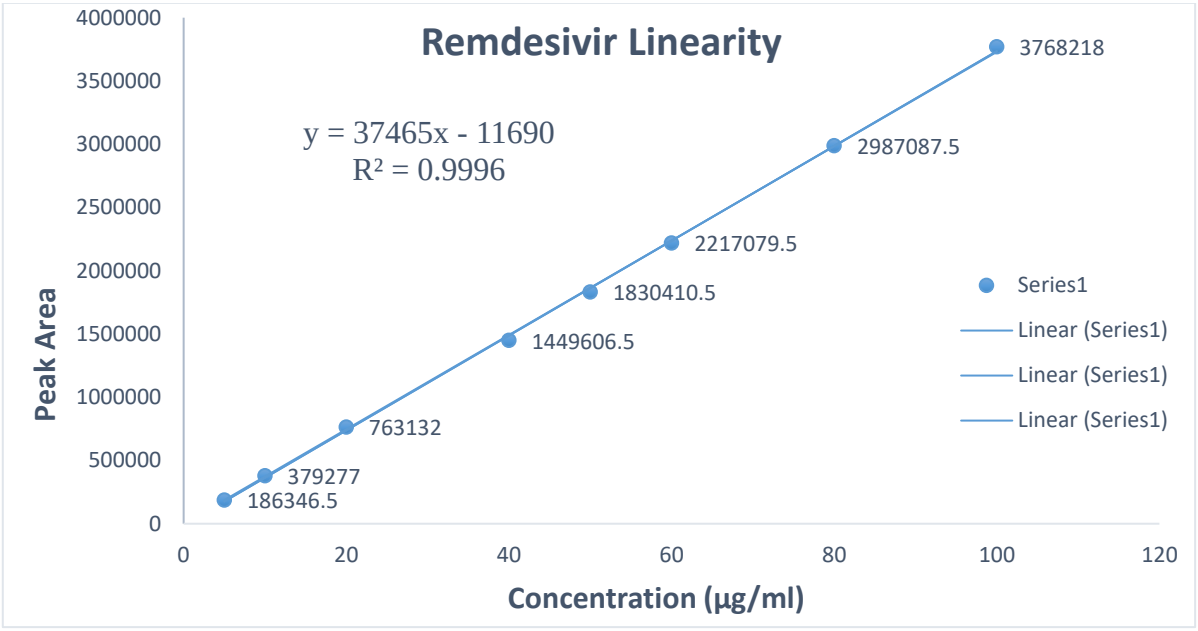

Fig. 6. Linearity graph of Remdesivir

PDA detector was used to evaluate the peak purity of Remdesivir in each condition and the detection was carried out at different wavelengths to confirm the stability indicating nature of the proposed method.

Preparation of stock solution:

Accurately weighed and transferred 25 mg of Remdesivir standard into 100.0 ml of volumetric flask, added diluent and sonicated to dissolve it completely and made volume up to the mark with the same diluent (250 µg/ml).

Acid degradation:

Pipetted 10 ml of stock solution into 25.0 ml of volumetric flask and added 1 ml of 0.1 N HCl. Then the volumetric flask was kept at room temperature for 15 min and then neutralized with 0.1 N NaOH and made up to 25.0 ml with diluent (100 µg/ml). Filled the solution in vial and injected into HPLC system. The chromatogram was recorded as shown in Fig. 7.

Alkali degradation:

Pipetted 10 ml of stock solution into 25.0 ml of volumetric flask and added 1 ml of 0.1 N NaOH. Then the volumetric flask was kept at room temperature for 15 min and then neutralized with 0.1 N HCl and made up to 25.0 ml with diluent (100µg/ml). Filled the solution in vial and injected into HPLC system. The chromatogram was recorded as shown in Fig. 8.

Water hydrolysis:

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Pipetted 10 ml of stock solution into 25.0 ml of volumetric flask and added 3 ml of water. Then the solution was heated on water bath at 100 °C for 2 hours. Further the solution was cooled and made up to 25.0 ml with diluent (100 µg/ml). Filled the solution in vial and injected into HPLC system. The chromatogram was recorded as shown in Fig. 9.

Oxidative degradation:

Pipetted 10 ml of stock solution into 25.0 ml of volumetric flask and added 1 ml of 3 % Hydrogen Peroxide solution. Then the solution was heated on water bath at 80 °C for 15 min. Further the solution was cooled and made up to 25.0 ml with diluent (100 µg/ml). Filled the solution in vial and injected into HPLC system. The chromatogram was recorded as shown in Fig. 10.

TABLE 7. LOD and LOQ data of Remdesivir

Injection no.	Area at 245 nm
1	1601780
2	1603272
3	1597225
4	1573517
5	1607511
6	1601910
Mean	1597536
SD	12219.2865
% RSD	0.765
Regression equation	y=37465x-11690
Slope (S)	37465
LOD=3.3σ/S (µg/ml)	1.08
LOQ=10σ/S (µg/ml)	3.26

SD= Standard Deviation.
%RSD= Percentage relative standard deviation.
LOD= Limit of Detection.
LOQ= Limit of Quantitation.

TABLE 8. Robustness studies of Remdesivir

Parameter	Change in parameter (±)	% Estimation	Mean	SD	% RSD	Limit
Wavelength (± 2 nm)	243	99.81	100.87	1.082043463	1.0727409	NMT 2.0 %
	245	101.97				
	247	100.82				
Mobile Phase composition (± 2 %)	58:42	101.16	100.98	0.840152705	0.8320217	
	60:40	100.06				
	62:38	101.71				
Flow rate (± 2 %)	0.98	100.03	100.64	0.527375974	0.5240273	
	1.00	100.93				
	1.02	100.95				

SD= Standard Deviation.
%RSD= Percentage relative standard deviation.
NMT= Not more than.

TABLE 9. Assay results of Remdesivir

Sample no.	Weight of standard (mg)	Sample Weight (equivalent to 20 mg)	Mean Area of standard at 245 nm	Area of sample at 245 nm	% Assay
1	10.00	308.36	1440810	1405288	99.13
2		309.38		1428567	100.44
3		310.20		1435590	100.67
4		311.29		1423090.5	99.44
5		312.35		1428219	99.46
6		310.10		1416590.5	99.37
			Mean	1422890.83	99.75
Average weight	309.202 mg		SD	10690.8453	0.6367
Limit	NMT 2.0 %		% RSD	0.751	0.638

SD= Standard Deviation.
%RSD= Percentage relative standard deviation.
NMT= Not more than.

Photolytic degradation:

Pipetted 10 ml of stock solution into 25.0 ml of volumetric flask and exposed to UV radiation at 254 nm for 24 hours at room temperature. Further the solution was made up to 25.0 ml with diluent (100 µg/ml). Filled the solution in vial and injected into HPLC system. The chromatogram was recorded as shown in Fig. 11.

III. Results and Discussion

Forced Degradation Studies

Chromatograms of acidic and oxidative degradation showed extra peaks indicating mild degradation i.e. 1.32 % degradation in acidic condition and 1.79 % degradation due to oxidative degradation. Most significant degradation was observed under alkaline condition i.e. 38.37 %. Remdesivir was found to be stable to water hydrolytic stress conditions and hence no degradation was observed. Moderate degradation of the drug was observed when subjected to UV degradation i.e. 5.88 %.

Degradation products formed were well resolved under developed conditions. Under each condition, peak purity of main peak was found to be 100.00 % as the peaks of degradation products were successfully separated and resolved from the main peak of Remdesivir without any interference. This confirms the stability indicating nature of the proposed method. The detailed results are depicted in Table 10.

System Suitability Studies

Analysis and evaluation of System Suitability parameters was done to check the system performance by injecting standard solution of Remdesivir (six replicates) of a working

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concentration of 40 µg/ml and a blank preparation (single injection) into the HPLC. The chromatograms were recorded and % RSD of parameters such as Area and retention time were evaluated. Tailing factor and Theoretical plates were also evaluated. In this method, all parameters were found to be within the acceptance limits. The results of system suitability studies are summarized in Table 1 and the chromatograms of blank and standard (40 µg/ml) solution of Remdesivir are shown in Fig. 3, and 4 respectively.

Method Validation

The proposed method for determination of Remdesivir in sublingual tablets is specific which was established by Forced Degradation studies. The method was found to have good linearity over the concentration range of 5-100 µg/ml with a correlation coefficient of 0.9996.

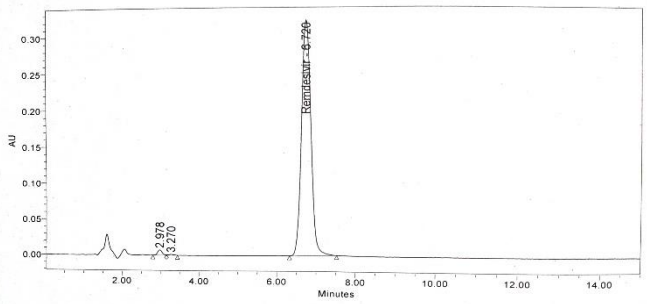

Fig. 7. Chromatogram of Acid degradation of Remdesivir

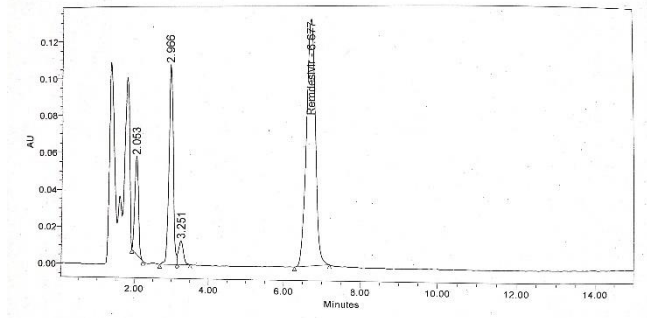

Fig. 8. Chromatogram of Alkali degradation of Remdesivir

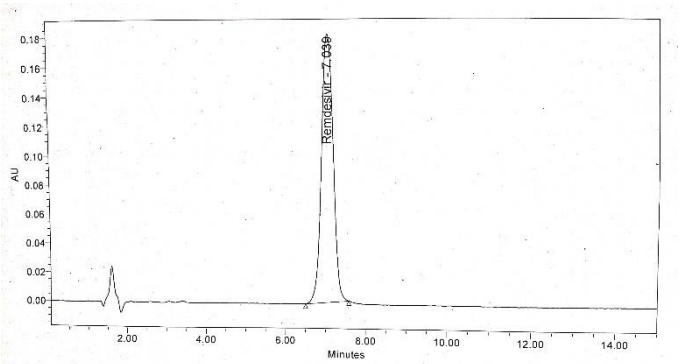

Fig. 9. Chromatogram of Water hydrolysis of Remdesivir

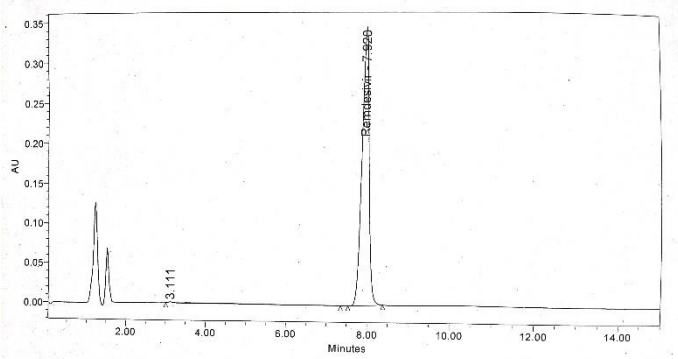

Fig. 10. Chromatogram of Oxidative degradation of Remdesivir

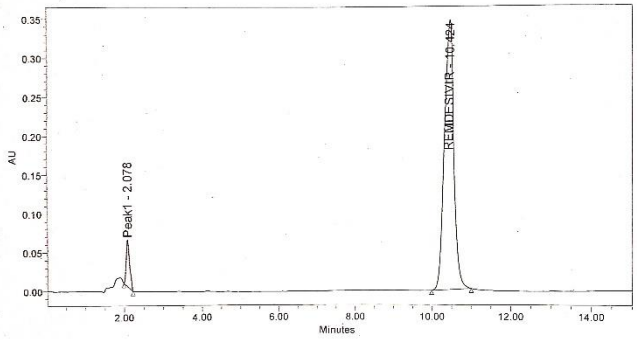

Fig. 11. Chromatogram of Photolytic degradation of Remdesivir

TABLE 10. Data for Forced Degradation studies of Remdesivir

S. No.	Degradation	Condition	Duration	Retention time of degradation products (Min)	% Residual drug	Observed peak purity (%)	Acceptance limit of peak purity
1	Acid degradation	1 ml of 0.1 N HCl at room temperature	15 min	2.978; 3.270	98.68	100.00	NLT 99 %
2	Alkali degradation	1 ml of 0.1 N NaOH at room temperature	15 min	2.053; 2.966; 3.251	61.64	100.00	
3	Water hydrolysis	3 ml of water at 100° C	2 h	-	100.00	100.00	
4	Oxidative degradation	1 ml of 3 % H2O2 at 80° C	15 min	3.111	98.21	100.00	
5	Photolytic degradation	UV lamp (254 nm)	24 h	2.078	94.12	100.00	

NLT= Not less than.

For system precision, the % RSD for peak areas of Remdesivir standard solution was found to be 0.765 and the mean assay percentage results of Remdesivir sample solutions were found to be within limits and % RSD was found to be 0.638, hence the method was found to be precise. The % RSD values of intermediate precision and assay were found to be within the acceptance limits.

The method was found to be accurate as the mean percent recovery of Remdesivir sample solutions was found to be 100.19 % which was within limit i.e. between 98 %-102 %. The

LOD and LOQ values were found to be 1.08 µg/ml and 3.26 µg/ml respectively for Remdesivir. Lower values for LOD and LOQ demonstrate that the method developed is sensitive, accurate and precise as it can detect and quantify the analyte at very low concentration.

Reproducible results were obtained which proves the method to be robust. % RSD of % assay during changes in method parameters was less than 2.0 % and the results were not adversely affected by these changes. High percent recovery values and very low SD and % RSD values confirm that the current developed method is suitable for routine analysis of Remdesivir in its pharmaceutical dosage form i.e. sublingual tablets.

IV. CONCLUSION

The proposed RP-HPLC method was successfully validated for parameters such as specificity, linearity, precision, accuracy and recovery, LOD, LOQ, and robustness as per ICH guidelines. The method was found to be simple, rapid, economical, accurate and precise. No any method was reported for estimation of Remdesivir in sublingual tablet dosage form before; hence this method development is worthwhile. All validation parameters were within the acceptance limits. The degradation products were successfully separated from the active pharmaceutical ingredient without any interference during forced degradation studies; hence the stability indicating nature of method was confirmed. This stability indicating method uses simple reagents along with minimum preparation procedures. Hence this method can be used for routine analysis and quality control of Remdesivir in sublingual tablet dosage form in Pharmaceutical industry.

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