Development and Validation of Novel Rapid Stability Indicating Mass Compatible UPLC Method for the Simultaneous Estimation of Assay and Related Substances of Remdesivir in Bulk and Injectable Dosage Form

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Developed and validated a simple, rapid, new mass compatible reverse phase ultra-performance liquid chromatography (UPLC) method for the simultaneous estimation of assay and related substance of remdesivir in bulk and injectable dosage form. The developed method is capable of separating all the potential process and degradation impurities within 15 min. Chromatography was carried out using Waters Acquity BEH C18 column (50 mm length, 2.1 mm internal diameter and 1.7 µ particle size) with gradient elution. Ghost buster column (30 mm length, 2.1 mm internal diameter) was used as inline filter to suppress the response of peaks originating from mobile phase. Mobile phase A composed of 10 mM ammonium acetate buffer pH 3.5 and acetonitrile in the ratio of 90:10 v/v and mobile phase B composed of 10 mM ammonium acetate buffer pH 3.5 and acetonitrile in the ratio of 10:90 v/v. The flow rate of the mobile phase was set at 0.35 mL/min and the column temperature was maintained at 35 ºC. The detection was carried out using UV detector at 245 and 270 nm. Method validation was performed according to ICH guidelines. The validated method is simple, rapid, mass compatible, economical, specific, accurate, linear, precise, rugged, sensitive, robust and stability indicating. This method can be employed for the simultaneous estimation of assay and related substance of remdesivir in bulk and injectable dosage forms at quality control laboratories of pharmaceutical industries.

Keywords: Remdesivir, UPLC.

INTRODUCTION

Remdesivir (REM) is a nucleoside analog used to treat RNA viral infections including management of COVID-19. It is available in the form of injection and powder for injection. Remdesivir is a phosphoramidate prodrug of 1′-cyano-substituted nucleoside analogue. It inhibits viral replication by competing with endogenous nucleotides for incorporation into replicating viral RNA *via* RNA dependent RNA polymerase [1].

Remdesivir was first described in literature in 2016 and was intended to target emerging pathogenic RNA viruses [2]. Its use in case of ebola infections was described in 2019 [3]. Remdesivir activity against the Coronaviridae family was first examined in 2017 [4] leading to considerable interest in remdesivir as a possible treatment for COVID-19 [5,6]. Severe acute

respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of coronavirus disease 2019 (COVID-19), which is a respiratory disease that is capable of progressing to viral pneumonia and acute respiratory distress syndrome (ARDS). Like other RNA viruses, SARS-CoV-2 depends on an RNA-dependent RNA polymerase (RdRp) enzyme complex for genomic replication, which can be inhibited by a class of drugs known as nucleoside analogues [7]. Remdesivir was granted an FDA Emergency Use Authorization (EUA) [8] for use in adults and children aged 12 years and over weighing at least 40 kg with suspected or confirmed COVID-19 in a hospital setting with an $SpO_2 \le 94\%$. The U.S. Food and Drug Administration (FDA) has subsequently revised an Emergency Use Authorization (EUA) to permit the emergency use of VEKLURY for treatment of suspected or laboratory confirmed coronavirus disease 2019 (COVID-19) in hospitalized pediatric patients

weighing 3.5 kg to less than 40 kg or hospitalized pediatric patients less than 12 years of age weighing at least 3.5 kg [9,10].

Several publications related to the estimation of content of remdesivir and its metabolite in human plasma by LC-MS/MS are available in literature [11-16] however, there is a need of stability indicating analytical method for simultaneous estimation of assay and related substances from bulk and pharmaceutical dosage forms. Further review of literature indicates a lack of UPLC method that can quantify remdesivir and its forced degradation products accurately using ultra performance liquid chromatographic (UPLC) procedure. The UPLC procedures are short and help quick analysis of the samples that is key to providing access of the medicines to the patients in case of pandemic situations where demand rise is steep and sudden. The delay in analysis due to separate assay and related substances method coupled with high run time for related substances method causes delay in release of product leading to shortage of drug product in market leading to loss of precious human lives.

Since, the covid pandemic situation is still evolving with no surety that future variants of coronavirus will be less fatal, this research work was carried out to minimize the product release time by reducing the analysis time by developing a short and cost-effective test procedure. To the best of our knowledge, there is no stability indicating UPLC method for the simultaneous estimation of assay and related substances of remdesivir from bulk and pharmaceutical dosage forms in public domain. Hence, an attempt is made to develop a simple, rapid, mass compatible stability indicating UPLC method for simultaneous estimation of assay and related substances from bulk and pharmaceutical dosage forms and validate the developed method according to the ICH guidelines for validation of analytical procedures [17].

EXPERIMENTAL

HPLC grade ammonium acetate, HPLC grade acetonitrile and all other chemicals were obtained from Merck Chemical Division, Mumbai, India. HPLC grade water was used throughout the study. Commercially available remdesivir for injection drug product was used for this study and impurities were procured from contract laboratories. Waters acquity UPLC high class separation module equipped with the photodiode array detector/TUV detector was used. Empower software was used for data acquisition and processing.

Screening of mobile phase, column and wavelength of detection: The RP-UPLC method was developed by varying different chromatographic conditions including buffer, pH, mobile phase ratio, gradient, solvents, columns, flow rate, column oven temperature and diluent based on established method development strategies [18,19]. The mobile phase and stationary phase (column) were selected based on physico-chemical properties of remdesivir. The pK_a of remdesivir is 10.23 (strongest acidic) and 0.65 (strongest basic) with a log P value of 2.01 [10]. Based on the pK_a value, acidic mobile phase with pH 3.5 was selected to have sharp peak with consistent retention time. Remdesivir and its impurities (total 10 impurities) are having different polarities. The method was optimized

to separate all the known impurities from remdesivir and each other by using gradient mode separation and dual wavelength detection. The optimized method showed good separation of impurities but some blank peaks were found interfering. This interference was eliminated by using ghost buster cartridge. Remdesivir and its impurities were separated within 8.5 min with resolution of more than 4.0 for closest eluting peaks.

Screening of diluent and optimization of sample preparation: Remdesivir is insoluble in water and soluble in acetonitrile. Hence, mixture of water and acetonitrile in the ratio of 50:50 was selected as initial diluent and remdesivir was found to be completely dissolved in diluent. The peak shape of impurity-1 and impurity-2 was found to be distorted in the above diluent whereas the peak shapes of other impurities and remdesivir was sharp. To improve the impurity peak shape, sample was initially dissolved in small amount (5% of total volume) of acetonitrile and then diluted with mobile phase A (buffer: acetonitrile 90:10 v/v), which yielded a clear solution and good peak shape for all the impurities and remdesivir. For drug product, the lyophilized powder was reconstituted with 19 mL of water as per the instructions of use and further diluted with mobile phase A to get 0.1 mg/mL of remdesivir.

Methodology

Buffer: Dissolved about 0.77 g of ammonium acetate in 1000 mL of water and adjust the pH to 3.50 ± 0.05 with trifluoroacetic acid. Filtered through 0.22 μ membrane filter.

Mobile phase A: Degassed mixture of buffer and acetonitrile in the ratio of 90:10 v/v.

Mobile phase B: Degassed mixture of buffer and acetonitrile in the ratio of 10:90 v/v.

Diluent: Mobile phase A was used as diluent.

Chromatographic condition: Column: Waters Acquity BEH C18 (50 mm × 2.1 mm, 1.7 μ); Flow rate: 0.35 mL/min; Column oven temperature: 35 ℃; Injection volume: 2 μL; Wavelength of detection: 245 nm for assay, related substances and 270 nm for impurity-3 and impurity-10; Sampling rate: 10 pts/s; In-line filter: Ghost buster cartridge (Welch, 30 mm length, 2.1 mm internal diameter). The gradient program details are shown in Table-1.

TABLE-1
GRADIENT PROGRAM

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Curve
0	100	0	Initial
0.5	100	0	6
1.2	86	14	6
3.0	70	30	6
6.0	60	40	6
8.0	0	100	6
10.0	0	100	6
11.0	100	0	6
15.0	100	0	6

Preparation of standard solution: Accurately weighed 20 mg of remdesivir reference standard was dissolved in 10 mL of acetonitrile and diluted to 200 mL with diluent, mixed well to get a concentration of 0.1 mg/mL of remdesivir.

Preparation of sensitivity solution: Pipetted out 100 μL of the above standard solution into a 200 mL volumetric flask, diluted to volume with diluent and mixed well to get a concentration of 0.05 μg/mL of remdesivir.

Preparation of sample solution for assay and related substance from bulk drug: Accurately weighed 20 mg of remdesivir drug substance into a 200 mL volumetric flask, added 10 mL of acetonitrile. Dissolved and diluted to volume with diluent, mixed well to get a concentration of 0.1 mg/mL of remdesivir.

Preparation of sample solution for assay and related substance from injectable dosage form (100 mg/vial): Five drug product vials were taken and reconstituted each vial with 19 mL of water to yield a nominal concentration of 5 mg/mL of remdesivir. Combined the entire contents of all 5 vials in a suitable container (pooled sample). Pipetted 2 mL of pooled sample and transferred into a 100 mL volumetric flask, then diluted to volume with diluent and mixed well to get a 0.1 mg/mL of remdesivir in a final solution.

System suitability criteria: Blank in single, sensitivity solution in single, standard solution in 5 replicate injections. The signal to noise (S/N) ratio for remdesivir peak in sensitivity solution should not be less than 10. The % RSD for the peak area of remdesivir from five replicate standard injections should be not more than 0.73%, theoretical plates should not be less than 50,000 and tailing factor should not be more than 1.5. The duplicate injections of sample solution were made upon successful demonstration of system suitability.

Analytical method validation: The analytical method validation for the simultaneous estimation of assay and related substance of remdesivir in bulk and injectable dosage form was conducted. Analytical method validation parameters such as system suitability, specificity (matrix interference and forced degradation), precision (method precision and intermediate precision), linearity, accuracy, limit of detection (LOD), limit of quantitation (LOQ), solution stability and robustness were performed according to ICH guidelines [17] to demonstrate the suitability of the method for the simultaneous estimation of assay and related substances of remdesivir.

System suitability: System suitability was demonstrated by injecting 2 μL of sensitivity solution in single, standard solution in 5 replicates.

Specificity: The specificity of the analytical method was evaluated by injecting blank, placebo, individual known impurities and sample spiked with all the known impurities to demonstrate that the method has the ability to quantify the analyte in the presence of components, which may be expected to be present *e.g.*, degradation products and excipients [17].

Forced degradation studies: Drug product was subjected to acid, base, peroxide, metal ion, thermal and photolytic stress conditions to induce deliberate degradation of drug product. Degraded samples were injected into a UPLC equipped with PDA detector.

Method precision/repeatability: Precision (repeatability) was demonstrated by analyzing six replicate preparations of remdesivir drug substance (bulk) and remdesivir powder for injection drug product. The assay and related substances of 6 replicate sample preparations are calculated.

Intermediate precision/ruggedness/reproducibility: Intermediate precision was demonstrated by analyzing six replicate preparations of remdesivir drug substance (bulk) and remdesivir powder for Injection drug product by different HPLC, different column on different days. The assay, related substance, SD, % RSD for assay and related substances of 12 replicate preparations (n = 6 for method precision and n = 6 for intermediate precision) were calculated.

Accuracy: The accuracy of the analytical method for assay was demonstrated by spiking known amount of remdesivir standard in placebo at 50%, 100% and 150% of nominal test concentration in triplicate. The accuracy for the related substances method was also demonstrated by spiking all the known impurities in sample from LOQ to 200% of limit (considering 2.0% for impurity-1 and 1.0% for rest of the impurities (impurities 2 to 10) as nominal level of impurities). The amount added, amount recovered, recovery, % mean recovery and % relative standard deviation was calculated at each level.

Linearity: Linearity of the analytical method was evaluated by plotting peak area and concentration of remdesivir in the range of LOQ to 150% of the nominal test concentration and LOQ to 200% of nominal limit (2.0% for Imp-1 and 1.0% for rest of the 9 impurities) to demonstrate that the absorbance is directly proportional to the concentration of analyte in the sample. The correlation coefficient (CC), regression coefficient (R^2), intercept and slope were calculated from the regression line.

Limit of detection (LOD) and limit of quantitation (LOQ): LOQ was demonstrated at reporting threshold (0.05%) and LOD at 0.02% for remdesivir and its all the known impurities. Signal to noise ratio was calculated for all the peaks. Also, the LOQ solution was injected 6 times and % RSD was calculated.

Solution stability: The standard and sample solutions were injected at different interval up to 24 h by keeping the solution at 5 ºC. After 24 h, standard solution area differed from initial by 1.1% and sample by 0.1% and solutions are considered stable for 24 h at 5 ºC.

Robustness: Robustness of the analytical method was investigated under a variety of conditions by making deliberate changes in chromatographic parameters. During the robustness study effect of composition of buffer and acetonitrile in the mobile phase A (90:10 ± 2% absolute) and mobile phase B (10:90 ± 2% absolute), flow rate (0.35 ± 0.05 mL/min), column temperature (35 ± 5 ºC) and buffer pH 3.5 ± 0.2 was evaluated. The system suitability criteria of signal to noise ratio, %RSD, tailing factor and theoretical plates were evaluated in all the robustness conditions. Spiked sample was injected in each robustness condition and resolution between impurities was evaluated.

RESULTS AND DISCUSSION

A novel stability indicating RP-UPLC method was developed and subjected to validation challenges in accordance to ICH guidelines [17]. The system suitability results (Table-2) met the predefined acceptance criteria throughout the study. No

System suitability parameter	Acceptance criteria	Results	Conclusion
	TABLE-2		
	SYSTEM SUITABILITY RESULTS		
S/N of remdesivir from sensitivity solution	NLT 10	19.2	Success
% RSD of peak area of remdesivir	NMT 0.73%	0.2	Success
Tailing factor for remdesivir	NMT 1.5	1.2	Success
Theoretical plates for remdesivir	NLT 50000	110294	Success
% RSD of RT of remdesivir	NMT 0.5%	0.03	Success

interference was observed at the retention time of remdesivir from blank, placebo, impurities. All the known impurities were found well separated from remdesivir and each other with a resolution greater than 4.0 between any closest pairs. Peak purity was found passing for remdesivir peak in the spiked sample and in all the forced degradation condition samples confirming that no impurity co-elutes with remdesivir peak (Fig. 1). Remdesivir degrades in acidic, basic and free radical stress

conditions predominantly forming Impurity-1, 2, 4. All the degradants were well separated from remdesivir peak and also with each other, thus indicating the method is specific and stability indicating (Tables 3 and 4). The RSD for the six replicate assay results from method precision experiment found to be 0.2% and RSD for individual known and total impurities were 0.0% indicating the method is precise for the determination of assay and its related substances. The overall RSD
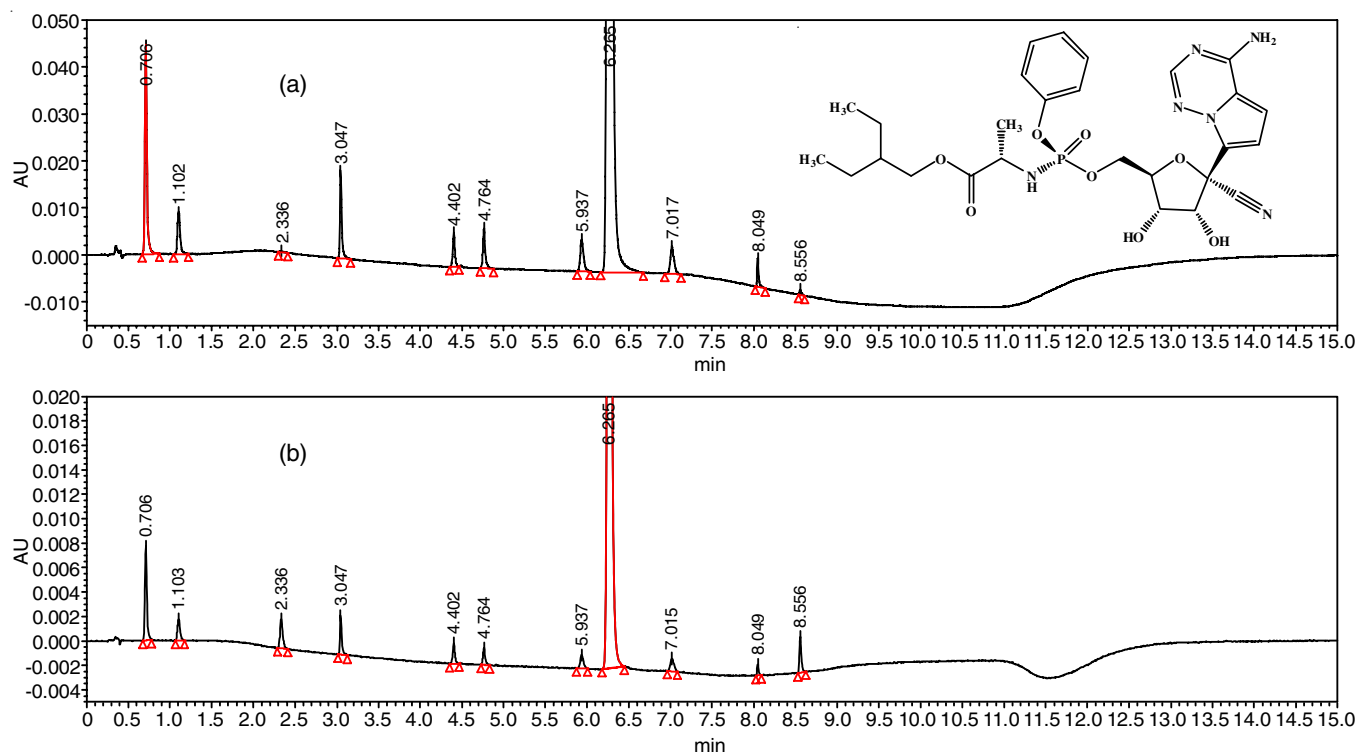

Fig. 1. Chromatograms of spiked sample containing impurity 1 to 10 at 245 nm (a) and 270 nm (b)

Name	Chemical name	RT (min)	RRT	Resolution	Peak purity		Results
					Purity angle	Purity threshold	
	TABLE-3						
	SPECIFICITY RESULTS						
Impurity-1	Trihydroxy cyano triazine	0.712	0.11	–	0.376	0.678	Pass
Impurity-2	Phosphate impurity	1.114	0.18	8.1	1.860	2.578	Pass
Impurity-3	Phenol	2.344	0.37	21.5	3.366	5.010	Pass
Impurity-4	Remdesivir acetonide	3.058	0.49	14.9	0.796	1.262	Pass
Impurity-5	Amide analog	4.415	0.70	32.7	1.555	2.297	Pass
Impurity-6	Butyl impurity	4.777	0.76	7.3	1.618	2.356	Pass
Impurity-7	Rp Isomer	5.959	0.95	18.8	1.901	2.782	Pass
Remdesivir	Remdesivir	6.290	1.00	4.5	0.045	0.281	Pass
Impurity-8	S-Isomer at CN	7.046	1.12	10.1	2.889	3.380	Pass
Impurity-9	Ethyl butyl impurity	8.062	1.28	18.7	1.817	2.738	Pass
Impurity-10	Ethylbutyl ester impurity	8.570	1.36	14.3	1.623	2.561	Pass

Name of the stress sample	Peak purity of remdesivir peak			Assay (%)	Total degradation (%)
	Purity angle	Purity threshold	Results		
Sample as such (Unstressed)	0.053	0.314	Pass	101.0	0.32
Acid degradation	0.057	0.308	Pass	48.9	52.3
Base degradation	0.051	0.360	Pass	85.7	11.5
Oxidation	0.052	0.471	Pass	99.8	0.81
Thermal degradation	0.049	0.317	Pass	99.8	0.33
Photolytic degradation	0.046	0.308	Pass	98.7	0.46
Metal ion degradation	0.045	0.320	Pass	98.3	3.07

TABLE-4
FORCED DEGRADATION RESULTS

for the assay and impurities from 12 results (n = 6 from method precision and n = 6 from intermediate precision) found to be 0.6% and 5.1%, respectively for bulk drug and 0.3% and 9.9% for injectable dosage form indicating the method is precise, repeatable, rugged and reproducible when analyzed on different days using different column and UPLC (Tables 5 and 6).

TABLE-5
COMPARISON OF METHOD PRECISION AND
INTERMEDIATE PRECISION FOR BULK DRUG

Validation parameter	Replicates	Assay (%)	Imp-5 (%)	Total Imp (%)
Method precision	Set-1	98.9	0.05	0.05
	Set-2	100	0.05	0.05
	Set-3	100	0.05	0.05
	Set-4	101.2	0.05	0.05
	Set-5	100.2	0.05	0.05
	Set-6	99.7	0.06	0.06
Intermediate precision	Set-1	99.6	0.06	0.06
	Set-2	99.3	0.06	0.06
	Set-3	99.4	0.06	0.06
	Set-4	99.5	0.06	0.06
	Set-5	99.6	0.06	0.06
	Set-6	99.4	0.06	0.06
Mean (n = 12)		99.8	0.06	0.06
SD (n = 12)		0.5805	0.0051	0.0051
% RSD (n = 12)		0.6	5.1	5.1

Note: The results of Imp-1,2,3,4,6,7,8,9,10, Unknown impurities are below LOQ (< 0.05%).

The % mean accuracy of remdesivir at each level were ranging from 99.5% to 101.1% for assay with overall mean accuracy of 100.5%. The % overall mean recovery from LOQ to 200% for remdesivir for related substance concentration is found to be 99.8% and % overall mean recoveries of impurities (Impurity 1 to 10) are found to be in the range of 96.5% to 102.9%. The individual and mean recoveries for remdesivir and its impurities were found to be well within the acceptable range of 85% to 115% indicating that the method is accurate in the range of LOQ (0.05%) to 200% of specification limit of impurities and 50% to 150% of test concentration for assay (Table-7).

The method is linear from 0.05 μg/mL to 150 μg/mL for remdesivir. Linearity demonstrated from LOQ (0.05%) to 200% of specification limit for impurities. The correlation coefficient of regression line was found to be 1.000 for remdesivir and all the known impurities indicating that the method shows excellent linearity in the above stated range. Limit of detection and limit of quantification was demonstrated to be 0.02%w/w and 0.05% w/w, respectively for remdesivir and its impurities. The % RSD for 6 replicate injections of precision at LOQ is found to be between 0.9% and 3.4% for remdesivir and its impurities which is well within the 15% indicating method is precise at LOQ level (Table-8). The signal to noise ratio for remdesivir and all the known impurities in LOD, LOQ solutions are found to be greater than 3 and 10, respectively indicating that the method is sensitive to detect and quantify

TABLE-6
COMPARISON OF METHOD PRECISION AND INTERMEDIATE PRECISION FOR INJECTABLE DOSAGE FORM

Validation parameter	Replicates	Assay (%)	Imp-1 (%)	Imp-2 (%)	Imp-5 (%)	Imp-9 (%)	Total imp (%)
Method precision	Set-1	99.3	0.05	0.06	0.06	0.06	0.23
	Set-2	99.1	0.05	0.07	0.06	0.05	0.23
	Set-3	99.9	0.05	0.07	0.06	0.06	0.24
	Set-4	100	0.05	0.06	0.06	0.06	0.23
	Set-5	99.7	0.05	0.07	0.06	0.05	0.23
	Set-6	99.6	0.05	0.07	0.06	0.06	0.24
Intermediate precision	Set-1	99.6	0.05	0.07	0.07	0.06	0.25
	Set-2	99.3	0.05	0.06	0.07	0.06	0.24
	Set-3	99.4	0.05	0.06	0.07	0.06	0.24
	Set-4	99.5	0.05	0.06	0.07	0.07	0.25
	Set-5	99.6	0.05	0.06	0.07	0.06	0.32
	Set-6	99.4	0.05	0.07	0.07	0.06	0.25
Mean (n = 12)		99.5	0.05	0.07	0.07	0.06	0.25
SD (n = 12)		0.257	0	0.0052	0.0052	0.0051	0.0247
% RSD (n = 12)		0.3	0	7.4	7.4	8.5	9.9

Note: The results of Imp-3,4,6,7,8,10, Unknown impurities are below LOQ (<0.05%).

TABLE-7
ACCURACY FOR RELATED SUBSTANCE AND ASSAY METHOD

Component name	QL (0.05%) level	50% Level	100% Level	150% Level	200% Level	Overall mean recovery
Mean % recovery (n = 3 at each level) for RS method*						
Remdesivir	99.0	98.6	101.9	99.6	100.0	99.8
Imp-1	100.8	99.7	101.0	101.1	102.4	101.0
Imp-2	103.3	101.2	102.4	102.0	103.5	102.5
Imp-3	105.7	104.5	100.9	100.7	102.5	102.9
Imp-4	100.3	100.0	100.7	100.7	101.9	100.7
Imp-5	102.3	97.4	99.2	99.1	100.8	99.8
Imp-6	98.7	100.3	101.7	101.5	103.5	101.1
Imp-7	104.3	101.4	101.3	102.1	103.3	102.5
Imp-8	98.3	97.9	98.6	98.7	99.5	98.6
Imp-9	98.7	96.9	107.4	104.8	104.5	102.5
Imp-10	96.7	96.8	96.6	95.3	95.2	96.5
Mean % recovery (n = 3 at each level) for assay method*						
Remdesivir	–	99.5	101.1	101.0	–	100.5

*Accuracy level in % of spec limit for RS method and % of nominal concentration for Assay method

TABLE-8
LOD AND LOQ RESULTS

Name	S/N of LOD (Limit: ≥ 3)	S/N of LOQ (Limit: ≥ 10)	% RSD for precision at LOQ (n = 6) (Limit: NMT 15.0%)
Remdesivir	20.1	36.4	2.0
Impurity-1	15.4	66.9	3.3
Impurity-2	23.0	31.4	3.3
Impurity-3	2.9	12.9	5.5
Impurity-4	46.2	69.3	1.2
Impurity-5	15.6	24.5	2.7
Impurity-6	21.9	34.7	2.6
Impurity-7	17.9	28.2	1.4
Impurity-8	17.4	26.8	2.8
Impurity-9	20.3	32.1	3.7
Impurity-10	3.7	18.9	3.4

the presence of very low concentrations of impurities in the sample. The pre-defined system suitability criteria were met and all the impurities are well separated from remdesivir and each other for all the robustness conditions indicating that the deliberate change in the chromatographic parameters has no significant effect on chromatographic behaviour of the method, therefore the method is robust (Table-9). The standard and sample solutions were found to be stable for 24 h at refrigerated temperature. The range of the method is defined as LOQ (0.05%) to 150% of nominal test concentration for remdesivir and LOQ to 200% of specification limit for impurities based on the demonstrated linearity, accuracy and precision.

Conclusion

A novel stability indicating, mass compatible UPLC method has been developed for the simultaneous estimation of assay and the related substance of remdesivir in remdesivir bulk and injectable dosage forms. The developed method is capable of separating all the potential impurities within 15 min with superior resolution capabilities. The developed method is demonstrated to be simple, fast, economic, specific, stability indicating, accurate, linear, precise, rugged, sensitive, robust and high throughput. The same method can be extended for content uniformity of finished products. Hence, this method can be employed for the estimation of assay, content uniformity and related substances in bulk and finished products stability studies in quality control laboratories of pharmaceutical industries to enhance the access of the drug product during sudden demand stress due to Covid-19 pandemic. The developed method if employed in quality control laboratory has the potential to reduce the drug product release time significantly.

TABLE-9
ROBUSTNESS

Name of the condition	S/N of Sensitivity solution (Limit: NLT 10)	% RSD (Limit: NMT 0.73%)	Tailing factor (Limit: NMT 1.5)	Theoretical plates (Limit: NLT 50000)
Original condition (control)	19.2	0.2	1.2	110294
Increase in flow rate (+0.05 mL/min)	18.5	0.1	1.2	104180
Decrease in flow rate (-0.05 mL/min)	20.5	0.1	1.3	115324
Increase in column oven temp (+5 °C)	25.8	0.1	1.2	108079
Decrease in column oven temp (-5 °C)	18.3	0.1	1.2	111434
Increase in buffer pH (+0.2)	19.4	0.03	1.2	112763
Decrease in buffer pH (-0.2)	14.4	0.1	1.2	110811
Increase in organic phase (+2%) in MP-A & B	15.4	0.2	1.3	106644
Decrease in organic phase (-2%) in MP-A & B	25.4	0.1	1.2	140304

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