

Original Research Article

Development and Validation of an HPLC-MS/MS method for the determination of Remdesivir in Rat Plasma

Abstract

Background: Remdesivir is an excellent drug for the medication of covid-19. So, it is an interesting method to develop a novel and reliable MS/HPLC strategy for establishment of Remdesivir.

Objective: To develop a new rapid and sensitive HPLC-MS/MS method for the estimation of Remdesivir in rat plasma using D₄-Remdesivir.

Method: Separation was carried on inertsil ODS column (150x4.6mm, 3.5 μ m) using a isocratic elution with a buffer containing 1mL of tri ethyl amine in 1Lit of water and the mixture of two components like Buffer and Acetonitrile in the ratio of 50:50 as mobile phase with 1mL/min flow rate at ambient temperature.

Results: Analysis was performed within 10 minutes over a good linear concentration range from 1ng/mL to 20ng/mL ($r^2 = 0.999$) for Remdesivir. Precision and recovery study results were within the acceptable limit. An electro spray ionization source was used to study Remdesivir. This method has been successfully applied, exploring Remdesivir with its internal standard (D₄-Remdesivir) was extracted from rat plasma using liquid - liquid extraction.

Conclusion: This paper focuses on the consistent evaluation of key bio-analytical validation parameters, including accuracy, precision, sensitivity, selectivity, and standard curve, quantification limits, range, recovery, and stability. In the case of chromatographic methods used in bio analysis, these validation parameters are defined, along with examples of validation methodology.

Key words: HPLC-MS/MS, Remdesivir, development, validation, rat plasma.

1. INTRODUCTION

Remdesivir, also known as Veklury, is a broad-spectrum antiviral drug developed by Gilead Sciences [1, 2]. It's given as an injection into a vein [4]. Remdesivir was licenced or authorised for emergency use to treat COVID 19 in about 50 countries during the 2020 COVID-19 pandemic. The World Health Organization's (WHO) updated recommendations [5] in November 2020 contain a conditional recommendation against using remdesivir for COVID-19 therapy. Remdesivir was first developed to treat hepatitis C [5, 6], then tested for Ebola virus disease [7, 8] and Marburg virus infections [9, 10] before being investigated as a COVID-19 post-infection drug. Raised blood levels of liver enzymes are the most common side effect in healthy volunteers (a sign of liver problems). Nausea is the most common side effect in people who have COVID 19 [11, 12]. Liver inflammation and an infusion-related reaction with nausea, low blood pressure [13], and sweating [14, 15] are potential side effects.

The aim of this study is to investigate, develop, and authorise a specific and delicate MS/HPLC strategy for the determination of remdesivir plasma in rats, as well as to assess the pharmacokinetics of these drugs following intravenous administration of test extracts in rats.

2. MATERIALS AND METHOD

2.1 Chemicals and Materials

Zyodus Cadila, Ahmedabad, provided Remdesivir ($C_{27}H_{35}N_6O_8P$) and D_4 -Remdesivir (Internal Standard, $C_{27}H_{31}D_4N_6O_8P$) with purity levels of 99 percent. Merck (India) Ltd., Worli and Mumbai, India, provided acetonitrile (LCMS Grade, 99.99 purity), water (Milli Q), and tri ethyl amine (HPLC grade, 99.0 percent). All of the other reagents and components were of AR quality and readily available.

2.2 Instruments & Conditions

For the development of a bio analytical assay, an HPLC device (Waters alliance e-2695 model) was connected to a mass spectrometer QTRAP 5500 triple quadrupole instrument (SCIEX). Using an inertsil ODS (150x4.6 mm, 3.5 μ m) column, chromatographic separation was achieved on an isocratic model at room temperature. The mobile step was a mixture of tri ethyl amine (0.1%) and acetonitrile (50:50v/v) with a flow rate of 1.0mL/min. The injection volume was 10L, and the total run time was 10 minutes. The study was carried out on a QTRAP 5500 triple quadrupole mass spectrometer with a positive ion electrospray ionisation interface. (Remdesivir's internal standard.) Following are the working parameters of mass spectrometry after optimization: Ion spray voltage 5500V; temperature source 550°C; drying gas temperature 120-250°C; collision gas -Nitrogen; pressure 55psi; drying gas flow stream-5mL/min; Declustering potential 40V; Entrance potential 45V; Exit potential 15V; Capillary voltage 5500V and Dwell time 1sec Table 1 displays the instrumentation specifications in detail.

Table 1. Optimized Liquid Chromatography and Mass Spectroscopic Conditions

LC parameters		MS parameters	
UPLC	Waters Acquity	MS	Sciex QTRAP 5500

Isocratic step mobile	ACN: tri ethyl amine 0.1% in water 50:50 v/v	Ionization source	Drying gas: N ₂ gas Drying flow rate: 5 ml/min Pressure: 55 psi
	Flow level: 1 ml/min		Source temperature: 550°C
	Injection volume: 10 µl		Capillary voltage: 5500V
Waters symmetry C ₁₈	150mm length	Collision cell gas	Nitrogen with high purity
	4.6 mm ID	Mode	MRM
	3.5 µm PS		
Analyte	Remdesivir	Remdesivir MRM transitions	CE– 15V
Internal standard	D ₄ -Remdesivir	D ₄ -Remdesivir MRM transitions	CE – 15V

Abbreviations:

CE-Collision energy, MRM- Multi reaction monitoring transitions, v/v-volume/volume, ml/min-milli litter per min, m/z-mass to charge ratio. LLQC-Lower limit quality control sample, LQC-Limit of quantification, MQC-Middle quality control, HQC-High quality control.

3. EXPERIMENT

3.1 Stock Preparedness, Calibration and Quality Control Specimens

Stock solutions of 10 ng/mL Remdesivir dissolved in 0.1 percent tri ethyl amine and 50:50 v/v Acetonitrile, linearity ranging from 1 to 20 ng/mL Remdesivir. Calibration and quality control specimens were made by diluting the working solutions described above and mixing them with blank plasma. The concentrations of Remdesivir in eight calibration specimens were 1, 2.5, 5, 7.5, 10, 12.5, 15, 20 ng/mL, and QC specimens were prepared in the same way, with final concentrations of 1 ng/mL (LLOQ), 5 ng/mL (LQC), 10 ng/mL (MQC), and 15 ng/mL (MQC) (HQC). Both specimens were held at -20°C until they were fully analysed and then returned to room temperature.

3.2 Preparation of Solution for Plasma Samples

For sample making, aliquots of 200µl of rat plasma specimens were spiked with 500 µl internal standard (IS) working solution. Subsequently, 300µl of Acetonitrile vortex were mixed for 15minutes, the samples were centrifuged at 5000 rpm for 15 minutes, and supernatant managed solution was divided, collected and filtered via 0.45µ nylon syringe filter into a vial and injected into the HPLC system.

3.3 Validation of Bio analytical Method

3.3.1 Selectivity, Matrix Effect and Recovery

Selectivity was tested by examining rat plasma specimens from 6 heaps of different rats to look for obstruction from unknown specimens during Remdesivir and IS retention time. The peak zone proportion in post extracted plasma samples from 6 separate drug free plasma samples and slick recovery samples was compared to determine the effect matrix for remdesivir. The trials were conducted in triplicate with six different lots of plasma at MQC

levels, with a reasonable accuracy (percent CV) of 15%. The degree of recovery was determined by comparing highlights of separate guidelines to non-extricated peak areas of standards. Remdesivir extraction efficiencies were determined by analysing six times at each concentration of QC.

3.3.2 Dilution Integrity and Carry over

By spiking matrix above the ULOQC with analyte concentration and diluting this test with a blank matrix, dilution integrity was demonstrated. The analyte retained by the chromatographic device during the injection of a sample that occurs in subsequent blank or unknown samples is referred to as carry over.

3.3.3 Precision and Accuracy

It was evaluated at a lower quantification limit (LLOQ), low quality control (LQC), medium quality control (MQC), high quality control (HQC) levels by replication analysis of quality control specimens (n=6). The level of CV should be less than 15%, except for LLOQ, where it should be less than 20%.

3.3.4 Stability

The area response of the analyte in the stability samples was compared to the region response of the specimen prepared from fresh stock solution to determine stock solution stability. Six replicates of each dose were used in plasma stability experiments at different concentration levels of LQC and HQC. According to the USFDA's guidelines, analyte was considered steady if the change was less than 15%. The stability of spiked rat plasma samples stored at room temperature was tested for 24 hours (bench top stability). For 24 hours, the stability of spiked rat plasma deposited in an auto sampler at 2-8 °C (auto sampler stability) was tested. The auto sampler's stability was determined by comparing extract plasma samples injected immediately with samples re-injected at 2-8 °C for 24 hours after storage in the auto sampler. The freeze-thaw durability was determined by comparing freshly spiked quality control samples with durability samples frozen at -30°C and thawed three times. The freeze-thaw stability test used six aliquots in each concentration spectrum in the LQC and HQC. For long-term stability monitoring, the concentration obtained after 24 hours was compared to the initial concentration.

4. RESULTS

4.1 Bio analytical Method Development

In this step, the ESI has the most intense reaction over the chemical ionisation by atmospheric pressure (APCI) mode. Remdesivir has been subjected to the MRM mode in order to quantify its ions. As compared to ion-negative mode, Remdesivir has a strong positive ion response mode.

We evaluated different buffers with Acetonitrile as the mobile phase in different ratios for isocratic and gradient mode to obtain the best chromatographic conditions. At each trial, the mobile phase composition was tweaked to improve peak shape and achieve reasonable retention times. Finally, the mobile step was chosen to be 0.1 percent tri ethyl amine and ACN in isocratic mode at 50:50 v/v ratios because it provided the best response of the drugs. We used different stationary phases in the optimization process, such as C18, C8, and CN-propyl. Using an inertsil ODS column with dimensions of 150mmx4.6mm, 3.5 connected to a PDA detector, we were able to obtain a strong peak shape of Remdesivir from various trials. Flow rates in the mobile process were set to 1 mL/min. Using the above parameters, we calculated Remdesivir's retention time to be 5.219 minutes. Six replicate

injections yield 0.59 percent CV, indicating that the suggested technique is very specific. According to USFDA guidelines, the method in development has been validated.

4.2 Validation of Bio analytical Process

4.2.1 Matrix Effect and Recovery

Different sources of rat plasma tested for Remdesivir had no significant effect. The following findings show that the matrix effect on analyte ionisation and internal specifications were both within reasonable limits. Remdesivir recovery in rat plasma was assessed at low, medium, and high concentration levels are 5, 10 ng/mL and 15 respectively. It is obvious that Remdesivir's extraction efficiency is good (Table 2).

Table 2. Results of Matrix Variability and Recovery (%) of Remdesivir in Plasma

Analyte	Matrix	Matrix factor bias (%)		% CV	% Recovery		
		LQC	HQC		LQC	HQC	%CV
Remdesivir	Plasma	97.01	90.81	1.73	97.18	98.97	3.07

4.2.2 Linearity, Consistency and Precision

The region at its high proportions in adjustment norms was relative under focus. The range of linearity for Remdesivir of this method was 1-20ng/mL. The curves of calibration appeared over the linear concentration range and correlation coefficient was found to be beyond 0.9993 for Remdesivir at different QC levels. Linearity and correlation results of Remdesivir are shown in table 3 and 4 and the calibration curve shown in Fig 1.

Table 3. Linearity results of Remdesivir

Linearity	Remdesivir conc. (ng/ml)	Remdesivir area response ratio
1	1.00	0.111
2	2.50	0.244
3	5.00	0.486
4	7.50	0.714
5	10.00	0.986
6	12.50	1.203
7	15.00	1.463
8	20.00	1.877
Slope	0.0940	
Intercept	0.01637	
CC	0.99935	

Table 4. Co-rrelation results of Remdesivir

Validation parameter	Remdesivir		
	Low	Medium	High
QC Conc. (ng /ml)	5	10	15

Linearity range	1-20 ng/ml
Correlation (r^2)	0.9993

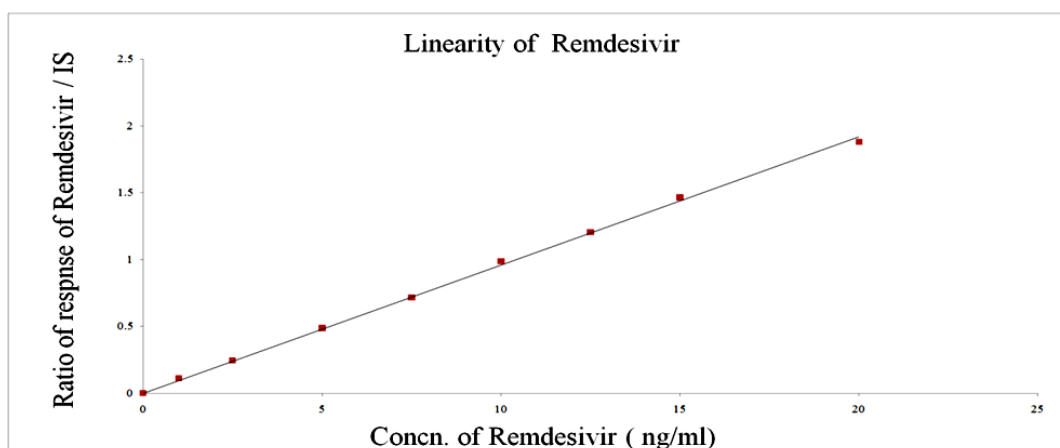


Fig. (1). Calibration plot of Remdesivir

The precision and accuracy were controlled by pooling all test results of different QC specimens. The exact results of quality control samples for Remdesivir were found to be 90.23-100.73%. And %CV of Remdesivir was <5% for all quality control samples at various concentrations. All the results of exactness and precision were within the quantification limit. **Table 5** shows the details of results.

Table 5. Precision and accuracy results of Remdesivir in rat plasma

Matrix	Sample	Remdesivir		
		Accuracy bias (%)	Precision RSD (%)	
			Intra-day	Inter-day
Plasma	LLOQC	0.51	0.82	0.76
	LQC	1.74	0.16	0.27
	MQC	0.38	0.33	0.26
	HQC	0.19	0.08	0.14

4.2.3 Dilution Integrity and Carry Over

Spiking the analyte matrix fixation over the ULOQC and diluting this specimen with blank matrix demonstrated dilution integrity. The purity of the dilution was tested at 2ULOQC (20ng/mL for Remdesivir). Remdesivir's percent CV was found to be within reasonable limits. Table 6 shows the specifics of the results.

Table 6. Results of Dilution Integrity

Analyte	ULOQC conc.	Calculated conc.	%CV
Remdesivir	20 ng/ml	20.13 ng/ml	3.74

Carryover is a form of system error that can affect the sample's calculated value. The following protocol was used to test sample carryover on a Waters Alliance-configured LC/MS system. The flow injection method was used to

conduct a machine blank injection volume of 10L for 0.1 percent tri ethyl amine and Acetonitrile (50:50) on the waters Z-spray triple quadrupole mass detector. We may conclude from this method that the proposed strategy's accuracy and precision were unaffected. Remdesivir sample carryover findings were LLQC (1.56 percent), ULQC (0.48 percent), and LLQC (3.74 percent), ULQC (0.69 percent) within reasonable limits. Table 7 provides the specifics of the carryover effects.

Table 7. Results of carry over

Concentration	% of carry over
	Remdesivir
Blank	0
LLOQC	1.56
ULOQC	0.48

4.2.4 Re injection Reproducibility

Reproducibility of the reinjection was checked during real-world sample analysis to ensure that the device operated after the hard product disintegrated due to instrument failure. Since the shift in levels at LQC and HQC was less than 2.0, the community was re-infused during genuine subject specimen investigation due to instrument malfunction, and samples were prepared and re-injected after 24 hours. Since the percent change at LQC and HQC levels was less than 2.0 percent, the batch could be re-injected after 24 hours.

4.2.5 Stabilities

The bench top stability of Remdesivir was investigated by a stock solution prepared and stored at room temperature for 18 hrs, in case of auto sampler stability the stock solution was stored for 24h in auto sampler at room temperature gives reliable stability behaviour under these conditions. Assessment of freeze thaw stability, the stock arrangement was stored for 24 h at $(-28\pm 5)^{\circ}\text{C}$, in wet extract stability, the stock solution was stored for 18h at $2-8^{\circ}\text{C}$, in dry extract stability, the stock was stored for 18h at $(-20\pm 3)^{\circ}\text{C}$. The short term stability shows stability of drugs was stored for 7 days at $(5\pm 3)^{\circ}\text{C}$, and in long term stability, the stock was stored for 28 days at $(-20\pm 3)^{\circ}\text{C}$ and inject into the HPLC. Compare the stability results of freshly arranged stock solution with stock solution made before 24h. From this we observed a % change of Remdesivir was 1.11%, it indicates that solutions are stable up to 24h.

At room temperature Remdesivir was stable in plasma for different conditions. It was evaluated that, LQC, MQC and HQC levels continued freezing and defrosting of plasma specimens spiked with Remdesivir, didn't influence its stability. It was clear from long-term stability that Remdesivir was stable at a capability temperature of -30°C up to 24h. The overall stability results of Remdesivir was shown in **table 8**.

Table 8. Stability results of Remdesivir in plasma of rat under different storage conditions

Stability	Storage condition	Conc. level	Measured conc (ng/ml) (Mean \pm SD, n=6)	% RSD	% Recovery
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Bench top stability	18 hrs at room temperature	5	1.64±0.01	0.67	94.7
		10	3.412±0.008	0.23	98.5
		15	5.025±0.012	0.24	96.7
Auto sampler stability	24 hrs in auto sampler at room temperature	5	1.649±0.023	1.40	95.3
		10	3.458±0.027	0.78	99.9
		15	5.056±0.021	0.42	97.3
Long term stability	28 days at (-20±3)°C	5	1.45±0.005	0.33	83.7
		10	3.021±0.006	0.21	87.2
		15	4.555±0.009	0.20	87.7
Freeze thaw stability	24 hrs at (-28±5)°C then exposed to three freeze and thawed cycles	5	1.632±0.011	0.70	94.3
		10	3.419±0.010	0.3	98.7
		15	5.018±0.014	0.28	96.6
Wet extract stability	18 hrs at 2-8°C	5	1.63±0.0098	0.60	94.1
		10	3.411±0.0068	0.20	98.5
		15	5.039±0.011	0.19	97.0
Dry extract stability	18 hrs at (-20±3)°C	5	1.631±0.012	0.76	94.2
		10	3.39±0.006	0.17	97.9
		15	5.037±0.014	0.28	97.0
Short term stability	7 days at (5±3)°C	5	1.562±0.021	1.36	90.2
		10	3.307±0.004	0.13	95.5
		15	4.908±0.007	0.13	94.5

5. DISCUSSION

The optimized method showed good chromatographic separation and mass transitions with acetonitrile and 0.1% tri ethyl amine buffer (50:50 v/v) concentration because of its volatility as it required in mass spectrometry analysis. Symmetry C₁₈ (150mmx4.6mm, 3.5 μ) column at 30°C was used for column efficiency. The best fit for calibration curve of chromatographic response versus concentration is determined by linear regression model. The data of intra and inter-day precision and accuracy for Remdesivir values are within the acceptable limit. The extraction recovery was good, consistent and precise, reproducible with the optimized extraction procedure. The injector carry over test showed 0% carry over for the extracted blank samples followed by extracted ULOQC, LLOQC samples. Freeze and thaw stability studies frozen at -30°C and thawed three times showed acceptable limits 85-115%. The auto sampler sample stability 20°C for 70 hrs, each of LQC and HQC concentration levels showed mean % accuracy within limits 85-115%. Bench top stability using standard stock solutions of Remdesivir, D₄-Remdesivir was set aside on the bench up to 18 hrs and compared with newly prepared stock solutions each of LQC and HQC concentration levels. The % accuracy was found to be within limits 85-115%. Long term stability evaluation was performed for 28 days and compared with initial concentrations each of LQC and HQC concentration levels. The % accuracy was found to be within limits 85-115%.

6. CONCLUSION

A fast, sensitive, and cost-effective bio-analytical system for HPLC monitoring of Remdesivir in rat plasma has been developed and successfully located, and is applicable to Remdesivir bioavailability. In contrast to other methods of studying Remdesivir in rat plasma samples, the established method had additional advantages. The validation studies also revealed that the optimised HPLC system has precision, sensitivity, and linearity, as well as accuracy, precision, and stability, over the entire spectrum of intense plasma therapeutic concentrations.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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