Liquid Chromatography- Mass Spectrometry Method Development and its Validation for the Estimation of Favipiravir and Remdesivir in the Rat Plasma

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ABSTRACT

Introduction: Liquid Chromatography Mass Spectrometry (LC-MS/MS) is an exceedingly sensitive and specific analytical technique that can precisely determine the identities and concentration of compounds within our sample.

Objective: A selective, sensitive, and rapid bio-analytical technique has been developed and it is validated by using Liquid chromatography – Mass spectrometry (LC-MS/MS) process for the estimation of favipiravir and remdesivir in rat plasma.

Methods: An isocratic mode using the analytical column of waters symmetry C18 150 mm x 4.6 mm, 3.5 µm, and the mobile phase acetonitrile and buffer in the ratio of 30+70. The method was validated with a linearity range 2-40 ng/mL of both favipiravir and remdesivir. The % CV values of intraday, interday precision and accuracy were found to be within the acceptance criteria. The % recovery of favipiravir was 96.5% and remdesivir was 99.6% respectively.

Results: The validation of the method has been done successfully in parameters like accuracy, linearity, recovery, precision, pharmacokinetic studies and stability in rat plasma using LCMS/MS. Stability of the selected drugs present in all conditions like benchtop, wet extract, auto sampler stability, and freeze thaw.

Conclusion: This technique gives better results of precision, accuracy, recovery, precision, pharmacokinetic studies, and stability in rat plasma. The proposed technique was validated as per the guidelines set by International Conference on Harmonization (ICH).

Key Words: Favipiravir, HPLC, LC-MS, Remdesivir, Validation, Stability

INTRODUCTION

Gilead Sciences has developed Remdesivir as an antiviral drug by the name GS-5734. This drug requires bio activation within the cells. Remdesivir contain various basic molecular characteristics, this is an analogue of adenosine nucleotide, it comprises the RNA synthesis and as a result, it acts against RNA viruses.

The validation method of UHPLC-MS/MS of remdesivir and its GS-441524 quantification has been reported in human plasma in the literature. The evaluation of analyte stability has been done in detail. This method has been successfully validated and it states that the sample thermal inactivation is the best choice to improve biosafety.

For treating Nipah virus in African green monkey model the remdesivir shows a practicable and which is having great effect. This model explained by performing the experiments by infection with a fatal dose of the virus to the animals which are selected for this purpose with one dose of remdesivir daily for 12 days. The results showed that all the treated animals are saved compared to the untreated animals.

This drug is potent which inhibits selectively influenza viral RNA polymerase, also active against various strains and subtypes of viruses of influenza all subtypes and strains of influenza viruses including the ones sensitive to marketed M2 inhibitors and neuraminidase.

Favipiravir, initially used for treating against the SARS-CoV-2 in Wuhan, and as the virus spread to European countries, it has received emergency approval for use this drug received approval for emergency use in some other countries and it received DCGI approval for various levels of Covid-19 infections. There are various clinical trials...
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going on to assess the drug’s efficiency in managing the Covid-19.⁷

Favipiravir (T-705) has been discovered initially during the assessment of chemical agents for their antiviral activity and it is effective in treating the influenza virus and reported in the chemical library of Toyama chemicals. Favipiravir has been obtained with various modifications done on pyrazine moiety of T-1105 chemically.⁸⁻⁹ Favipiravir exhibits useful anti-viral activities with other RNA viruses’ also.¹⁰

The literature review suggests that a few methods for evaluating Favipiravir and Remdesivir had been published. However, have developed a method for the validation of these drugs in rat plasma by using LCMS/MS has been carried out in this paper. The newly developed process has been effectively validated using the guidelines set by International Conference on Harmonization (ICH).¹¹⁻¹³

MATERIALS AND METHODS

Instrumentation
Chromatography has been carried out with waters 2695 HPLC containing degasser, column oven, high speed autosampler, and SCIEX QTRAP 5500 mass spectrometer for providing a compact and with class Empower-2 software.

Reagents and chemicals
The reference sample was provided as Favipiravir and Remdesivir samples from Biocon, Bangalore. The chemicals like acetonitrile, methanol used are of HPLC grade and procured from Merck chemical supplier, Mumbai. Though out the process the water used of HPLC grade which is obtained from Milli-Q water purification system.

Favipiravir Standard Stock Solution (80 ng/mL)
Weigh 8 mg of Favipiravir and diluted with a diluents in a 100 mL volumetric flask. Further dilute 0.1 mL to 100 mL with diluent.

Remdesivir Standard Stock Solution (80ng/mL)
Weigh 8 mg of Remdesivir standard in a 100 mL flask and diluted it with a diluent. Further dilute 0.1ml of above solution in 100 mL volumetric flask with diluent.

Preparation of Standard Solutions (20 ng/mL of Favipiravir and 20 ng/mL of Remdesivir)
In a centrifuged tube 500 µl of Favipiravir and Remdesivir stock standard solutions are taken. Dilute to volume with Plasma, acetonitrile and diluent.

Preparation of Linearity solution
These solutions are prepared with concentrations from 2 nanogram to 40 nanogram per mL of Favipiravir and Remdesivir prepared in a similar way as above. Centrifuge at 4000 RPM for 15 – 20 min. collect the supernatant solution in LC vial and inject into the chromatograph.

Extraction procedure
The treated and centrifuged plasma samples are labelled as per the time intervals. 200 µL sample of plasma is add with 500 µL diluent and shake it well. Further add 300 µL of Acetonitrile for precipitation of proteins and thoroughly mix it in a vortex cyclo mixture. It is centrifuged at about 4000 RPM for about 15 – 20 min, and the supernatant solution is collected in a HPLC vial and injected into a chromatograph.

Buffer Preparation
Transfer 1ml of formic acid into a1lt water. Filter by using 0.45µ membrane paper.

Methodology for Analysis
The Linearity solution, the blank, and sample solutions are injected into the chromatograph and their chromatograms have been recorded. The peak areas due to Favipiravir and Remdesivir are measured. The linearity curve obtained from the equation express the concentration of these compounds present in the sample of plasma.

RESULTS AND DISCUSSIONS

System suitability
The instrument efficiency is determined by performing an analysis with a set of standard and reference ones prior to the analytical process. The percentage of cumulative variation (% CV) for Favipiravir and Remdesivir was found to be 0.31 and 0.29 and area ratio of ISTD has been observed to be 0.59 %, and 0.44 %. Therefore, the suitability of the system has passed.

Specificity and screening of biological matrix
It was observed that in the samples blank rat plasma there were no interfering peaks of Favipiravir and Remdesivir or ISTD at retention times. The interfering peaks response in the standard (STD) Blank at the analyte retention time should be ≤ 20.00 % of that in LLOQ (Lower limit of quantification). Response of peaks that interfere at retention time in the STD Blank and the ISTD should be ≤ 5.00 % of that in LLOQ. About 80 % of the lots of the matrix (except heparinized, haemolysed and lipemic matrix lots) must be in the acceptance criteria with intended anticoagulant.
**Sensitivity**
The % CV for Favipiravir and Remdesivir was found to be 1.16 % and 0.71 %, % CV accuracy was 99.3 % and 99.4 %. Hence the sensitivity was passed.

**Matrix effect**
The matrix effect was determined for rat plasma constituents over ionization of the analyte and compared the post-extracted plasma standard MQC (Medium quality control) samples’ response (20 ng/ml of Favipiravir and 20 ng/ml of Remdesivir) (n = 6) with that of the analyte from pure samples at the equivalent concentrations. The intended method of the matrix effect has been assessed with chromatographically screened plasma of the rat.

Precision (% CV) for Favipiravir was found to be 0.39 % and 0.77 % for respectively at HQC (High quality control) and LQC (Low quality control). Precision (% CV) for Remdesivir was found to be 0.36% and 0.69 % respectively at HQC and LQC. The percentage mean accuracy of the back calculated concentrations of LQC, and HQC samples that are prepared with lots of different biological matrix must be within the 85.00-115.00 %. This shows that matrix effect on ionisation of the analyte has been shown to be in the limit of acceptance. Figure 1 shows the Matrix Effect Chromatogram of LQC.

**Linearity**
Over concentration range of 2-40 ng/mL the standard curves was linear for Favipiravir and Remdesivir. The correlation coefficient has been found to be 0.992 for Remdesivir and it is 0.9901 for Favipiravir. The calibration curves are found to be linear. By using ratio of peak area of the analyte and that of IS the samples have been quantified. The ratios of the peak area are plotted against concentrations of the plasma. The calibration standards’ peak area ratios were found to be proportional to the concentrations. Linearity results of Favipiravir were shown in table 1 and for Remdesivir were shown in Table 2. Figures 2, and 3 shows the plot of calibration for the concentration vs Area ratio of Favipiravir and Remdesivir respectively.

**LOD and LOQ**
Table 3 shows the LOD and LOQ results of the compounds. Limit of detection (LOD) and limit of quantification (LOQ) have been determined separately with a method of calibration curve. The compound’s LOD and LOQ have been calculated by progressive injection of standard solutions of lower concentrations with the developed LC-MS method. The concentration of LOD for Favipiravir is 0.2 ng/ml and the S/B value is 7. The concentration of LOQ for Favipiravir is 2.2 ng/ml and the S/B value is 27. The LOD concentrations for Remdesivir are 0.2 ng/ml the S/B value is 5. The LOQ concentration for Remdesivir is 2.2 ng/ml the S/B value is 22. The chromatograms for MQC and blank are shown in figures 4 and 5 respectively.

**Precision and accuracy**
The estimation of precision and accuracy has done by analysis of six replicates with Favipiravir and Remdesivir at four kinds of QC levels. The determination of the inter-assay precision was done by the analysis of four levels QC samples with four different runs. For accepting the data the criteria include, the accuracy in the range 85–115% from actual value and the precision in the range of ±15% RSD except for LLQC, where it should be in the range of 80–120% accuracy and <20% RSD.

**Recovery of analyte**
The IS and the drug recovery has been carried out at three levels of concentrations like high, medium and low quality control. By comparing the response in the replicate samples with the responses of neat standard solutions the recovery is determined. The recovery of the analyte from sample matrix is (extraction efficiency) done by comparing the response of the analyte from the amount of added analyte with that calculated from the sample matrix. Because of the basic properties of Favipiravir and Remdesivir, the extraction was done with Acetonitrile solvent.

The results of the recoveries of the analyte with the experiments with the spiked compounds are 85.1 % - 90.5 % and for IS 84.25 %. At each QC level % CV of recovery and for ISTD should be ≤ 15.00 %. Overall mean recovery of % CV for all QC levels should be ≤ 20.00 %. Recovery plots for Favipiravir and Remdesivir in Rat plasma were shown in figures 6 and 7 respectively.

**Ruggedness on precision accuracy**
The % CV for Favipiravir and Remdesivir was found to be 0.07-1.27 %. The LLOQ QC should be in the range of 80.00-120.00 %. The % mean accuracy of LQC, MQC and HQC samples should be in the range of 85.00-115.00 %, and in case of samples of LLOQ QC, it should be in the range of 80.00-120.00 %. Hence the Ruggedness on precision accuracy was passed.

**Ruggedness on reinjection reproducibility**
The % CV for Favipiravir and Remdesivir were found to be 0.12 % - 1.89 %. The % mean accuracy of samples of LQC, MQC, and HQC should be in the range of 85.00-115.00 % and in case of samples of LLOQ QC it should be in the range of 80.00-120.00 %. Hence the Ruggedness on reinjection reproducibility was passed.

**Bench Top Stability**
The % CV of HQC and LQC, mean accuracy for Favipiravir and Remdesivir were found to be 0.09% and 0.38%, 98.2
The % CV of the HQC, MQC, LQC, and the mean accuracy for Favipiravir were found to be 0.06%, 98.3 %, 0.62 %, 98.2 %, 1.80 %, and 98.5 % and for Remdesivir were found to be 0.60%, 98.8%, 0.62 %, 96.6 %, 1.22%, 98.4 %. Hence the Auto Sampler Stability was passed.

Freeze Thaw
The % CV and the mean accuracy for Favipiravir was observed to be 0.08 %, 98.2 % and 0.28%, 98.6% and for Remdesivir was found to be 0.11 %, 98.8 % and 0.17 %, 98.5 %. The % mean stability for LQC and HQC samples should be in the range of 85.00-115.00 %. Hence the freeze thaw stability was passed.

Wet Extract
The % CV and the mean accuracy for Favipiravir was found to be 0.10 %, 98.2 % and 0.24 %, 98.6 % and for Remdesivir was 0.08 %, 99.8% and 0.17 %, 99.5 %.

The % mean stability of samples of LQC and HQC should be in the range of 85.00-115.00 %. Hence the stability of the wet extract was passed.

Dry Extract
The % mean accuracy of the back calculated concentration of samples of LQC and HQC should be in the range of 85.00-115.00 %. The % CV of samples of LQC and HQC should be ≤ 15.00 %. The % mean stability of samples of LQC and HQC should be in the range of 85.00-115.00 %.

The % CV and mean accuracy in different intervals of time for Favipiravir and for Remdesivir were passed. It indicates the Dry Extract stability.

Short term
Short term stability of Favipiravir and Remdesivir were shown in Table 4 and Table 5. The % CV of samples of HQC, LQC and MQC samples for Favipiravir was 0.66, 4.18 and 1.04 and for Remdesivir was 0.09, 0.26 and 0.13. Mean accuracy for Favipiravir was 95.99 %, 95.88 % and 96.98 % and for Remdesivir was 81.52 %, 88.78 % and 85.76 %.

Pharmacokinetic Studies
Table 6 shows the Pharmacokinetic studies of the compounds. For isolation of Favipiravir and Remdesivir from rat plasma liquid-liquid extraction technique has been used. To carry out this, plasma sample (200µl) (respective concentration) was added into labeled polypropylene tubes and swirl briefly and add 0.3 ml of Acetonitrile and vortexed for 10 min and centrifuge this at 20 °C at 4000 rpm. The supernatant liquid from these samples was taken into a label vial tube and evaporate the solvent to dry at 40 °C. The samples have been reconstituted with a 500 µl of diluent and briefly vortexed and transferred for injection into a auto sampler vials.

The sample of Favipiravir and Remdesivir was injected into the rat body and samples collected at various time intervals like 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4 and 5 h in six different rats. After that samples are prepared as per test method and injected into a chromatographic system and recorded values. At 2 h Favipiravir and at 1 h Remdesivir reaches the maximum result.

The study of pharmacokinetics of Favipiravir and Remdesivir has been carried out in healthy South Indian male subjects (n = 6). The written informed consent was taken from all the volunteers and the protocol has been approved by the local Independent Ethics Committee. The single dose of Favipiravir tablet (200 mg) and Remdesivir injection (100 mg) was given orally to the volunteers and collected the samples of blood at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4 and 5 h post-dose. An aliquot of 5 mL blood was collected at each point of time in a K2 EDTA vacutainer tubes. Additionally, in order to check the possible interferences from plasma the predose sample was collected. To obtain the plasma the samples were collected, centrifuged, and stored at −70±10 °C. The samples of plasma were spiked with IS and it is processed at four concentrations along with QC samples. The parameters of pharmacokinetics of Favipiravir and Remdesivir have been calculated with WinNonlin (Version 5.2) software. The stability of these samples was established using incurred sample reanalysis (ISR). For this ISR purpose two samples from each subject were selected (10 samples in total) near the Cmax and elimination phase in pharmacokinetic profile. These samples were considered stable and the % difference should not be higher than 20.

CONCLUSION
The main objective of the work presented here is to develop a simple, sensitive, cost-effective, and rugged method for determination of Favipiravir and Remdesivir in LC-MS with internal standards Favipiravir-D₆ and Remdesivir-D₆. The proposed work suggests less run time in comparison with other work articles. The total run time in chromatographic studies is 8.0 min and with a retention time for Favipiravir and Remdesivir respectively at 2.241, and 5.098 min. This method has been validated for Favipiravir and Remdesivir over a dynamic linear range of 2-40 ng/mL and with correlation coefficient of r² 0.999. The intra-batch and inter-batch precision (% CV) across five levels (LLOQ, ULOQ, LQC, MQC, and HQC) is less than 11.15. These can be validated as per the guidelines given by USFDA.
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Conflict of Interest:

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Contribution:

First author has developed methods and validated the proposed methods. Second author guided in entire work (preparing manuscript). Third author helped in experimental work and literature gathering.

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Table 1: Linearity Results of Favipiravir

| Final conc. in ng/ml | RES | Area response ratio |
|----------------------|-----|---------------------|
| 0                    | 0   | 0.0                 |
| 2.00                 | 0.241 | 0.118               |
| 5.00                 | 0.482 | 0.235               |
| 10.00                | 1.076 | 0.527               |
| 15.00                | 1.524 | 0.749               |
| 20.00                | 2.036 | 1.002               |
| 25.00                | 2.514 | 1.228               |
| 30.00                | 3.027 | 1.481               |
| 40.00                | 4.152 | 2.034               |
| Slope                | 0.0489|
| Intercept            | 0.01688|
| R² Value             | 0.9901|

Table 2: Linearity Results of Remdesivir

| Final conc. in ng/ml | RES | Area response ratio |
|----------------------|-----|---------------------|
| 0                    | 0   | 0.0                 |
| 2.00                 | 0.263 | 0.121               |
| 5.00                 | 0.548 | 0.253               |
| 10.00                | 1.182 | 0.548               |
| 15.00                | 1.623 | 0.752               |
| 20.00                | 2.152 | 1.001               |
| 25.00                | 2.635 | 1.227               |
| 30.00                | 3.159 | 1.461               |
| 40.00                | 4.276 | 1.974               |
| Slope                | 0.0490|
| Intercept            | 0.02232|
| R² Value             | 0.9992|
Table 3: LOD and LOQ Results

| Drug Name | LOD (S/B) Value | LOQ (S/B) Value |
|-----------|-----------------|-----------------|
| Favipiravir | 7               | 27              |
| Remdesivir | 5               | 22              |

Table 4: Short term Stability of Favipiravir

| Replicate No. | HQC | LQC | MQC | Nominal Concentration (ng/ml) | Analyte peak area |
|---------------|-----|-----|-----|-----------------------------|-------------------|
| 1             |      |     |     | 30.327                      | 2.923x10^5        |
| 2             |      |     |     | 10.318                      | 9.91x10^5         |
| 3             |      |     |     | 20.224                      | 9.91x10^5         |
| 4             |      |     |     |                             | 9.91x10^5         |
| 5             |      |     |     |                             | 9.91x10^5         |
| 6             |      |     |     |                             | 9.91x10^5         |
| n             | 6   | 6   | 6   |                             |                   |
| Mean          |     |     |     |                             | 2.941x10^5        |
| SD            |     |     |     |                             | 0.01928           |
| %CV           |     |     |     |                             | 0.66              |
| % Mean Accuracy |     |     |     |                             | 95.99%            |

Table 5: The short term Stability of Remdesivir

| Replicate No. | HQC | LQC | MQC | Nominal Concentration (ng/ml) | Peak area of the analyte |
|---------------|-----|-----|-----|-----------------------------|--------------------------|
| 1             |      |     |     | 30.472                      | 3.028x10^5              |
| 2             |      |     |     | 10.419                      | 1.066x10^5              |
| 3             |      |     |     | 20.362                      | 2.093x10^5              |
| 4             |      |     |     |                             |                         |
| 5             |      |     |     |                             |                         |
| 6             |      |     |     |                             |                         |
| n             | 6   | 6   | 6   |                             |                         |
| Mean          |     |     |     |                             | 3.025x10^5              |
| SD            |     |     |     |                             | 0.00281                 |
| %CV           |     |     |     |                             | 0.09                     |
| % Mean Accuracy |     |     |     |                             | 81.52%                  |

Table 6: Pharmacokinetic studies

| Time Intervals (H) | Favipiravir (ng/mL) | Remdesivir (ng/mL) |
|--------------------|---------------------|--------------------|
| 0.25               | 2.356               | 4.834              |
| 0.5                | 4.479               | 10.207             |
| 0.75               | 7.967               | 15.759             |

Table 6: (Continued)

| Time Intervals (H) | Favipiravir (ng/mL) | Remdesivir (ng/mL) |
|--------------------|---------------------|--------------------|
| 1                  | 13.748              | 18.389             |
| 1.5                | 16.386              | 13.658             |
| 2                  | 19.053              | 8.362              |
| 3                  | 12.331              | 3.986              |
| 4                  | 7.514               | 0                  |
| 5                  | 3.394               | 0                  |

Figure 1: Matrix Effect Chromatogram of LQC.

Figure 2: Plot of calibration for the concentration vs Area ratio of Favipiravir.

Figure 3: Plot of calibration for the Concentration vs Area ratio of Remdesivir.
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Figure 4: Chromatogram of MQC.

Figure 5: Chromatogram of Blank.

Figure 6: Recovery plot for Favipiravir in Rat plasma.

Figure 7: Recovery plot for Remdesivir in Rat plasma.