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DEVELOPMENT AND VALIDATION OF LIQUID CHROMATOGRAPHY, MASS SPECTROMETER METHODS FOR DETERMINATION OF REMDESIVIR AND ITS METABOLITE GS-441524 IN PLASMA AND THEIR APPLICATION IN COVID-19 RELATED CLINICAL STUDIES

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ABSTRACT

A simple method based on liquid chromatography coupled to triple quandrupole mass spectroscopy detection using 50µL of plasma was developed and fully validated for quantification GS-41524. of remdesivir and its active metabolites GS-Remdesivir is a Phosphoramidate prodrug designed to have activity against a broad spectrum of viruses. RDV is rapidly distributed in to cells and tissues and simultaneously metabolised in to GS-441524 in plasma. LC-MS methods were validated for determination of 2analytes in human plasma that involved two key aspects to guarantee their precision, accuracy and robustness. Chromatographic separation was achieved on an Acquity UPLC HSS T3 column [2.1×50mm, 1.8µm] with a run time of 3.4min. The calibration ranges were 4-4000, 2-2000, respectively for RDV, GS-441524. Precision evaluated at 2.5,400 and 4000µg for Remdesivir and 12.5, 2000µg\ml for GS-441524 were lower than 14.7% and accuracy was in the 89.6-110.2% range. The validated method was successfully applied on covid-19 related clinical studies. A simple precipitation was carried out using 75µL of methanol containing the internal standard remdesivir.

KEYWORDS: Covid-19, GS-441524, Remdesivir, LC-MS, Plasma.

INTRODUCTION

The current pandemic coronavirus disease {covid-19} has developed interest in developing treatment in order to decrease impact on human life many pharmacologic treatment have been proposed against the server acute respiratory syndrome coronavirus 2 {SARS-CoV-2} in case in the pandemic there are currently on approved effective therapeutic agents available for the treatment of covid. 19 the most promising therapy appeared to be remdesivir.

Remdesivir is a board-spectrum small molecule antiviral drug that has demonstrated activity against RNA viruses in different families its therapeutic efficacy has been primary found against Ebola virus in rhesus monkey.

However remdesivir is a prodrug of a parent adenosine analong called GS-441524, both of which being metabolized in human cell into an active nucleoside triphosphate .GS-441524 is the major circulating metabolite of remdesivir No $EC_{5\ 0}$ has been published for GS-441524 on SARS-CoV-2 virus but this metabolite appeared to be more potent than the parent drug .when activated both seem to inhibit RNA dependent RNA polymerase form coronavirus targeting the viral genome replication process.

Until now remdesivir treatment is not European medicine agency {EMA}approved but has been recently approved by US food and drugs administration {FDA}. it is still being tested in on-going randomized tails or in compassionate use for patients with severe covid-19.

Only IV rout is actually available for remdesivir its main metabolism in human after IV administration is likely to predominantly in urine (74%) the predominant form being GS-441524 (49%) followed by remdesivir and other metabolise not currently clearly identified.

After 3-225 mg single dose IV in fusion over 2hours in humans it has been shown that remdesivir GS-441524 presented a linear PK profile. After 150-225mg two hours IV infusion 8 human subjects, remdesivir peak plasma concentration observed at the end of infusion were 2280µg/l and 4421.3µg/l, respectively. Half-life was approximately 1hour this short value leading to in not accumulation following multiple ones daily administration, GS-441524 reached steady state by day four and accumulated around two fold compared to single administration in accordance with its longer half-life around 24H.

For the treatment of covid19 in order to target exposer of the virus in plasma and in cells, in passions with impaired renal function, even if remdesivir is not cleared and changed in urine

to any substantial extent, its metabolite GS-441524 is found in great amount in urine and its plasma concentration may theoretically increase. Since esterase's are present in great quantity in whole blood, stability of remdesivir and that of its metabolite were also investigated in sample tubes in two different sampling tubes and in different storage conditions. Nucleoside analogs or a class of small molecules antivirals which can directly inhibit viral transcription and replication by targeting the viral RNA dependent, RNA polymerise.

Since the outbreak of the severe acute respiratory coronavirus 2 in December, it has become a worldwide pandemic. Remdesivir was first found to have activity against SARS COV-2 in vitro testing and then showed clinical improvement against covid-19 in its compassionate use for passions with severe symptoms from covid-19 infection. Remdesivir was proved to be superior to placebo in shortening in the time to recovery in hospitalised with covid-19 and evidence of lower respiratory tract infection. Currently there are multiple clinical trials with RDV at multiple site at geographic locations to access its effectiveness against broader patient populations in these clinical studies accurate determination of the prodrug and RDV and its major metabolites GS-441524, in human plasma in critical for appropriate characterisation of the pharmacokinetic and pharmacodynamics of RDV and its metabolites. Validation of a bio analytical method for RDV and GS-441524 quantification in plasma. Determination of RDV and its metabolites in plasma, temperature dependent in pH dependent stability shown by experimental data, degradation of RDV always led to observable increases in GS-441524. Though instability is expected for such a pro drug that is designed to convert in vivo to an active metabolite, in bio analytical method development this instability issue must be address to ensure the precision, accuracy and robustness of the method. Moreover since GS-441524 is much polar than RDV, it is challenging to address the carryover issue for RDV if the same LC gradient is used for all three analytes are even for two analytes. The carryover issue for RDV need to be address for separately. In this paper we present the method development and validation of an LC –MS method for determination of RDV and its major metabolites GS-441524 in acidified human plasma as well as the methods application in clinical studies.

MATERIALS AND METHODS

Chemicals and reagents

The reference standards of remdesivir GS-441524 and remdesivir-$^{13}C_6$, used as internal standard (IS) were purchased from Alsachim (Illkirch, France). Formic acid and methanol

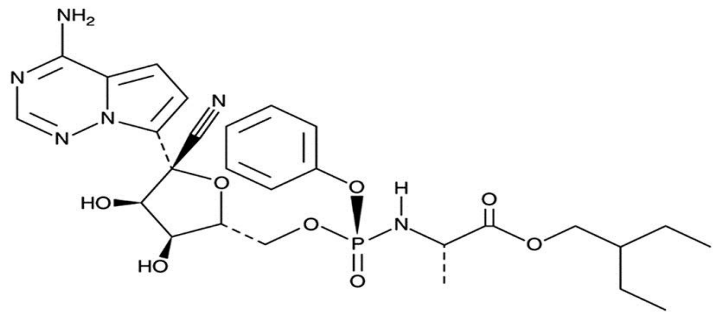
were high-performance liquid chromatography (HPLC) grade while acetonitrile was mass spectrometry (LC/MS) grade and were obtained from Merck (Saint-Quentin Fallavier, France). Zinc sulfate ($ZnSO_4$) was obtained from Prolabo (Paris, France) and prepared at a concentration of 1 M in ultra-pure water (18 MΩ) obtained by ultrafiltration with a Q-Pod (Millipore Corp., Molsheim, France). Formate buffer containing 10 mM ammonium formate in 0.1% formic acid was prepared in ultra-pure water and stored after each analysis at +4 °C for a maximum of one week. Blank plasma was obtained from the Etablissement Français du Sang.

Working solutions, calibration standards and quality controls

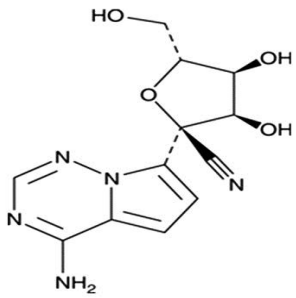
Stock solutions of remdesivir, remdesivir-$^{13}C_6$ and GS-441524 were prepared in methanol at a concentration of 1 mg/mL. Working solutions for calibration standards (CS) were obtained by dilution of the stock solution of remdesivir and GS-441524 in methanol to obtain solutions at 10, 1, 0.1, and 0.01 µg/mL for remdesivir and five times more concentrated for the metabolite. Working solution for quality control (QC) preparation was also prepared at the same concentrations by dilution in methanol of another 1 mg/mL stock solution prepared separately. IS was diluted in methanol to obtain a working solution at 0.2 µg/mL.

CS used for calibration curve were prepared each day by spiking with appropriate volumes of the previously mentioned working solutions in 50 µL blank plasma in order to obtain the following concentrations: 1, 5, 10, 25, 50, 100, 250, 500, 1000, and 5000 µg/L for remdesivir, and 5, 25, 50, 125, 250, 500, 1250, and 2500 µg/L for GS-441524, with a zero CS.

QC samples were prepared in bulk in blank plasma at concentrations of 1.0 (lower limit of quantification, (LLOQ)), 2.5, 400 and 4000 µg/L for remdesivir and 5.0 (LLOQ), 12.5, 125, 2000 µg/L. Stock and working solutions and plasma QCs were stored at −20 °C.



(A) Remdesivir



(B) GS-441524

Analytical equipment

The following analytical equipment was used in this bio analytic method validation: Vacuum Degasser, DGU-20A5R, Shimadzu Corp.; Solvent Delivery System LC-30AD, Shimadzu Corp.; Auto injector, SIL30ACMP, Shimadzu Corp.; Column Switching Unit/Oven, CTO-30A, Shimadzu Corp.; Mass Spectrometer, Triple Quandrupole MS (API 5000), AB Scitex.

Preparation of primary stock solutions

For test articles, two primary stock solutions from independent weightings by two different scientists were prepared and were verified to be within 5.0% of each other. For ISs, one primary stock solution was prepared. The concentration of each stock solution was calculated using a corresponding correction factor (factor required to convert the mass of reference material weighed to the mass of the analytic free base or free acid that it contains) for the reference standard provided in the certify cite of analysis.

Sample preparation

Fifty µL of plasma were precipitated with 75 µL methanol containing IS and 5 µL of $ZnSO_4$ 1 M. After vortex mix for 15 s, samples were left at +4 °C for 10 min, and then centrifuged (10 min, 18000 *g*). Supernatant was transferred into injection vials for analysis (injection volume 20 µL).

LC-MS/MS system and conditions

Chromatography was performed on a Dionex Ultimate 3000 pump (ThermoFisher, Les Ulis, France) using a Kinetex® 2.6 µm Polar C18 100A LC column (100 × 2.1 mm i.d.) (Phenomenex, Le Pecq, France) preheated at 30 °C. The elution was performed with a gradient of 10 mM sodium formate buffer in 0.1% formic acid (A) and acetonitrile (B) starting from 0% of (B) to 100% in 2 min, and hold for 1 min to 100%. The equilibration time between two consecutive runs was set at 2 min. The total run time was 5 min. The mobile phase was used at a constant flow rate of 0.5 mL/min. Compounds were detected by a TSQ Endura triple-quadrupole mass spectrometer (ThermoFisher) equipped with an electrospray ionization (ESI) source set in a positive mode with ion spray potential at +3.5 kV. Capillary temperature was set at 350 °C. Nitrogen (Nitrox UHPLCMS 18, nitrogen generator; Domnick Hunter, Villefranche sur Saone, France) was employed as sheath gas at 35 arbitrary pressure units. The argon gas collision-induced dissociation was used with a pressure of 1.5 mTorr. Data were collected in multiple reactions monitoring (MRM) mode. The ion transitions (and corresponding collision energies) used to monitor

compounds were *m*/*z* 603.3 → *m*/*z* 200.0 (35%) and *m*/*z* 229.0 (23%) for remdesivir, *m*/*z* 292.2 → *m*/*z* 173.1 (24%) and *m*/*z* 147.1 (29%) for GS-441524 and *m*/*z* 609.3 → *m*/*z* 206.0 (33%) for remdesivir-$^{13}C_6$. Data acquisition was performed using Xcalibur and LC-Quan softwares (both ThermoFisher).

Clinical samples

Within 30 min of the blood collection, human blood samples were processed by centrifugation at ~1500 g (3000 rpm) for 10 min at 4 ◦C to obtain plasma. Next, 500 µL of each plasma sample was immediately transferred into a corresponding clean polypropylene tube containing 40 µL of the 20% FA solution and mixed well. Immediately thereafter and within 1 h (h) of blood collection, the polypropylene tubes were placed upright on dry ice prior to transfer to a − 70 ◦C freezer for storage prior to shipping. These clinical study FA-treated plasma samples were then kept frozen at − 70 ◦C during shipping and storage until analysis**.**

Sample processing

Prior to analysis, all frozen clinical study samples, calibration Standards, and QC samples were thawed and allowed to equilibrate in an ice bath, and then vortex-mixed for approximately 1 min before pipetting. Samples were kept in an ice-bath during the processing steps. For sample processing and pre-treatment, 50 µL aliquots of plasma samples, calibration standards, or QC samples were added to separate wells of an appropriately labelled 96-well extraction plate. 50 µL of IS was spiked into the Blank + IS, Calibration Standard, QC (and system suitability test (SST) sample and study sample, if applicable) wells. Blank and Carryover Blank wells were spiked with 50 µL of methanol. The plate was capped and centrifuged for about 1 min at 1000 rpm and vortex-mixed for approximately 1 min at medium speed. 500 µL of methanol was spiked into all wells. The plate was capped and vortex-mixed for approximately 5 min at high speed and centrifuged for approximately 10 min at 3000 rpm. 200 µL of the supernatant was transferred from the preparation plate to the collection plate. The collection plate was evaporated to dryness in a 40 ◦C bath under nitrogen stream. All samples in the collection plate were reconstituted with 400 µL of 10 mM ammonium formate in methanol. The collection plate was vortex-mixed for approximately.

Plasma stability test

During method development, the individual stability of RDV, GS-441524, at the LQC (12, 6, 6 mg/mL) and HQC concentrations (3200, 1600, 1600 mg/mL) in 20% FA-treated pooled

K2EDTA human plasma was compared with that in untreated pooled K2EDTA human plasma. Analytes peak area ratios (n = 3) after incubation at either room temperature (RT) or 4 ◦C were determined by LCMS/MS for assessment of stability. Stability was further confirmed during method validation as described below.

Liquid chromatographic conditions

The chromatographic analysis was performed using an Acquity UPLC HSS T3 column (2.1 × 50 mm, 1.8 µm, waters, Milford, MA).

Mass spectrometric conditions

Ionization and detection of RDV, GS-441524,and its respective ISs were carried out on an API-5000 triple quadrupole mass spectrometer (AB-Sciex, Toronto, Canada), equipped with Turbo Ion Spray® MS/MS detection. Positive (M + H) + ions were monitored for both RDV and GS-441524 in Multiple Reaction Monitoring (MRM) mode. Quantitation was performed using parent → product ion (m/z). The Mass spectrometric Multiple Reaction Monitoring conditions are listed. The source dependent parameters maintained for the 3 analytes.

Bio analytical method validation

Validation of the method for determination of RDV, GS-441524, in FA-treated plasma was done following the FDA and EMA guidelines. The calibration and linearity, precision and accuracy, dilution linearity, selectivity, matrix effect, injection carryover, extraction recovery, effect of haemolysis, and effect of lipemia were evaluated. Experiments were also conducted to evaluate the stability of RDV, GS-441524, in FA-treated plasma samples stored in wet ice, carried through freeze/thaw cycles, and following long-term storage (− 20 ◦C and − 70 ◦C). RDV, GS-441524, stability was further assessed in human whole blood and in processed samples. To accommodate the possible need for decontamination of samples from virus-infected individuals (e.g., Ebola), stability to standard procedures using gamma-ray exposure known to inactivate such viruses both on the tube exterior and within the tube interior contents was also assessed. Means, standard deviations, and values of %CV (Coefficient of Variation) and %RE (Relative Error) were calculated by standard statistical calculations, and except where specifically stated, the nominal and the observed concentrations were used for calculation of %RE. Unless otherwise stated, %Diff of a determined value from a reference value was calculated as the [(determined value) - (mean reference value)]/(mean reference value) and expressed as a percentage.

Calibration and linearity

The linearity of the method was determined by analysis of standard plots associated with an eight-point standard calibration curve. Eight non-zero calibration standards were analyzed in each of the three precision and accuracy batches. Peak area ratios of analyte: IS obtained from MRM analysis of the chromatograms from the calibration standards and their corresponding nominal concentrations were utilized for the construction of calibration curves, using weighted (1/x2) linear least squares regression. Back-calculations were made from the curve equations to determine the concentration of each analyte in each individual calibration standard sample. A correlation coefficient (r2) greater than 0.99 was required for each the calibration curve to be acceptable. The lowest standard on the calibration curve was to be accepted as the lower limit of quantitation (LLOQ), at which the analyte response (peak area ratio) was required to be at least five times greater than response at the same retention time from drug free (blank) extracted plasma. In addition, the analyte peak of the LLOQ sample needed to be identifiable, discrete, and reproducible, and have a mean precision (%CV) not greater than 20.0% and mean accuracy (RE %) within 80.0–120.0% of its nominal concentration. The deviation of the mean back calculated concentrations of individual standards other than the LLOQ standard needed to be within ±15.0% of the corresponding nominal concentrations.

Precision and accuracy

Precision and accuracy of the method were evaluated by analyzing QC sample replicates (n = 6) at five different nominal analyte concentrations across the standard curve range. Intraday precision and accuracy were determined by analyzing six replicate aliquots of the QC samples prepared at five concentrations (LLOQ QC, LQC, Low MQC, High MQC, and HQC) in each of the three precision and accuracy runs.

Dilution integrity

To ensure accurate measurement for samples with concentration above the upper limit of the standard curve or for samples with limited volume, dilution integrity needed to be established. The dilution test was conducted to ensure that samples with concentrations above the upper limit of the standard curve could be diluted with blank matrix without affecting the final calculated concentration. A FA-treated plasma sample was prepared at one concentration of RDV, GS-441524, diluted in five replicates at a dilution factor of 20 with pooled blank FA-treated plasma. For the dilution integrity results to be acceptable, the %RE

of the determined concentrations of the diluted samples after applying the dilution factor had to be within ±15.0% of the nominal value before dilution, and the %CV could not exceed 15.0%.

Selectivity

The selectivity of the method towards endogenous plasma matrix components was assessed by extracting and analyzing six different individual lots of FA-treated plasma (i.e., each lot from a single donor) with no added analyte or IS. For the selectivity test to be acceptable, none of the six individual lots could show an interference peak area at the retention time of the analyte that was >20.0% of the mean analyte peak area from the LLOQ (4/2/2 ng/mL, respectively) and none of the six individual lots could show an interference peak area at the retention time of IS that was >5.0% of the mean IS peak area.

Matrix effect

The matrix effect was determined in six different individual lots of FA treated plasma at two analyte concentrations (12/6/6 and 3200/1600/ 1600 mg/mL, n = 3) for RDV/GS-441524 and at one concentration (400/200/200 mg/mL, n = 3) for their ISs. The matrix effect was evaluated by comparing the ratio of peak areas of solutions in the presence of the matrix to the peak areas of solutions in the absence of the matrix, which server as reference samples. The %CV of the results for the mean IS-normalized matrix factor could not exceed 15.0% for it to be considered acceptable and consistent across the validated assay method range. In addition to the normal matrix, the effects of lip emic FA-treated plasma and 5% haemolysed FA-treated plasma on the assay performance were examined at two analyte concentrations (12/6/6 and 3200/ 1600/1600 mg/mL, n = 3) for RDV/GS-441524and at one concentration (400/200/200 mg/mL, n = 3) for their ISs. One lot of lip emic matrix and one lot of haemolysed matrix were evaluated. For the results from the lip emic and haemolysed plasma tests to be acceptable, the %RE of the five replicates needed to be within ±15.0% and the %CV could not exceed 15.0%.

Carryover

An extracted blank sample was inserted in the injection sequence after the highest calibration standard (ULOQ) from both the first and second set of calibration standards, and injection volumes (10 µL) were constant for all samples. Carryover was defined as minimal if the peak areas of the analyte observed in the first and second carryover blanks were less than 20.0% of the corresponding analyte peak area observed in the lowest calibration standard.

Protein precipitation recovery

The recovery test was conducted to evaluate the efficiency of the protein precipitation extraction process. Recovery was determined at three standard concentrations (12/6/6, 200/100/100, and 3200/1600/ 1600 mg/mL, n = 5) for RDV/GS-441524. The recovery test for the IS was not required since a stable isotope label was used and therefore, the results are expected to be similar to those of the unlabelled.

Calibration Curve

The response of the instrument with regard to the concentration was tested on six different calibration curves analyzed on six different days using the CS previously described. Quantitation was achieved by plotting the peak area ratios of remdesivir and metabolite to the IS versus concentration. The equation showing the lowest and most constant percentage total bias from nominal CS values was considered as the best-fit model. The back calculated concentrations of the CS should be within ±15% of the nominal value, except for the LLOQ for which it should be within ±20%, and at least 75% of the CS must fulfill this criterion. Concentration of analyte in the unknown samples was calculated from their peak area ratios and the calibration curve.

LLOQ and limit of detection (LOD)

The LLOQ was the lowest concentration of remdesivir and GS-441524 which can be measured with accuracy in the range 80–120% and a precision with a coefficient of variation (CV) of less than 20%. It was validated by analysis of a specific QC sample. Moreover, the signal of analyte for the LLOQ sample had to be at least 5 times the signal of blank sample. The limit of detection was evaluated as the lower concentration with a signal/noise ratio > 3.

Accuracy and precision

The accuracy (reported as percent of the nominal value) and precision (CV) of the assay were determined for the three QC samples and at the LLOQ. For the intraday assay, six replicates of each QC were processed the same day. For the inter-day assay, six replicates of each QC level were processed at three different days. The concentrations obtained were analyzed using analysis of variance (ANOVA), which separated the intraday and intraday standard deviation and the corresponding coefficients of variation (CV). Accuracy within the range 85–115% of the nominal values and a precision with a CV of ±15% was required.

RESULTS

LC-MS/MS analysis

The chromatogram obtained for remdesivir-$^{13}C_6$, remdesivir and GS-441524 in a blank sample, the low QC sample and T_{4h} of the pharmacokinetic of the patient. The retention time was 1.69 min for metabolite, 2.41 and 2.42 min for remdesivir and IS.

Clinical application

Plasma sample treatment with FA stabilized the analytes and improved the robustness of the method. This and the individually optimized LC gradient and ESI mode for each analyte ensured the success of the validation of the method. This was applied to multiple clinical studies for application for use of RDV as treatment for COVID-19. GS-441524 plasma concentration-time profiles by cohort 8. D. Xiao et al. Analytical Biochemistry 617 (2021) 114118 11 log or linear scale, upon 150-mg intravenous infusion of RDV in lyophilized formulation over a 2-h period from Gilead study GS-US-399- 1812. As expected, RDV plasma concentration exposure reaches to a mean Cmax of 2720 mg/mL immediately after infusion cessation and decreases rapidly to the LLOQ (4 mg/mL) at 5 h post dose. GS-441524, the stable metabolite, shows a mean Cmax of 148 mg/mL at 4 h and a long half-life of ~25 h. The intermediate metabolite, peaks at ~2 h with a mean Cmax of 230 mg/mL but drops to the LLOQ (2 mg/mL) at 10 h. The performance of the method was proved to be excellent in multiple RDV related clinical studies. In the first human study involving IV doses 200 mg RDV, or PTM (placebo to match), administered IV for the first day, followed by 100 mg RDV, or PTM, daily for 4, 9, and 13 days, in which 952 plasma samples were analysed in 35 runs, the overall %CV values from the results of duplicate analyses per run of each of 4 QC samples with concentrations. Recently, results of such plasma sample analyses were included in regulatory submissions that resulted in approvals in the US, Japan, and EU for use of remdesivir as treatment for COVID-19.

CONCLUSIONS

The LC-MS/MS bio analytical method for the determination of concentrations of RDV, GS− 441524 in FA-treated K2EDTA human plasma was validated successfully with respect to linearity, sensitivity, accuracy, precision, dilution, selectivity, hemolyzed plasma, lip emic plasma, batch size, recovery, matrix effect, and carryover. Since RDV can be hydrolysed to its metabolites in untreated human plasma samples, it was important to stabilize it by adding FA at the appropriate amount, concentration, and FA: plasma ratio upon sample collections.

This avoided overestimation of GS- 441524 concentrations especially when relatively high RDV concentrations were present in a sample, (e.g., typically 1–2 h after administration of RDV). As such, the stability of all the analytes in K2EDTA human plasma samples treated with FA solution has been established for processed sample stability, bench top stability in plasma, freeze/thaw stability in plasma, bench top stability in whole blood, and long-term frozen storage stability in plasma. In addition, the individually optimized LC gradient for each analyte avoids carryover issue that would happen when using a single LC gradient for all analytes.

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