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Validation of LC-MS/MS methods for determination of remdesivir and its metabolites GS-441524 and GS-704277 in acidified human plasma and their application in COVID-19 related clinical studies

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ABSTRACT

Remdesivir (RDV) is a phosphoramidate prodrug designed to have activity against a broad spectrum of viruses. Following IV administration, RDV is rapidly distributed into cells and tissues and simultaneously metabolized into GS-441524 and GS-704277 in plasma. LC-MS/MS methods were validated for determination of the 3 analytes in human plasma that involved two key aspects to guarantee their precision, accuracy and robustness. First, instability issues of the analytes were overcome by diluted formic acid (FA) treatment of the plasma samples. Secondly, a separate injection for each analyte was performed with different ESI modes and organic gradients to achieve sensitivity and minimize carryover. Chromatographic separation was achieved on an Acquity UPLC HSS T3 column (2.1 × 50 mm, 1.8 μm) with a run time of 3.4 min. The calibration ranges were 4–4000, 2–2000, and 2–2000 ng/mL, respectively for RDV, GS-441524 and GS-704277. The intraday and interday precision (%CV) across validation runs at 3 QC levels for all 3 analytes was less than 6.6%, and the accuracy was within ±11.5%. The long-term storage stability in FA-treated plasma was established to be 392, 392 and 257 days at −70 °C, respectively for RDV, GS-441524 and GS-704277. The validated method was successfully applied in COVID-19 related clinical studies.

1. Introduction

Nucleoside analogs are a class of small-molecule antivirals which can directly inhibit viral transcription and replication by targeting the viral RNA-dependent RNA polymerase (RdRp) [1]. A novel small molecule nucleotide analog prodrug, remdesivir (RDV/GS-5734), was identified as a broad-spectrum antiviral with activity against Ebola Virus (EBOV) and a number of other RNA viruses [2]. As shown in the conversion scheme in Fig. 1, RDV is a single diastereomer monophosphoramidate prodrug of a nucleoside analog, GS-441524, that is intracellularly metabolized into a pharmacologically active triphosphate, GS-443902, which inhibits viral RNA polymerases [3,4] and has broad spectrum activity against members of the filoviruses (eg, EBOV [2,5–7], MARV), coronaviruses (eg, SARS-CoV [8–10], MERS-CoV [11,12]), and paramyxoviruses (eg, respiratory syncytial virus [RSV] [1], Nipah virus [NiV] [13], and Hendra virus [14]). These viruses are single-strand RNA viruses that share a similar replication mechanism in which the function of RdRp is essential [15].

Since the outbreak of the severe acute respiratory coronavirus 2 (SARS-CoV-2, COVID-19) in December 2019, it has become a worldwide pandemic. RDV was first found to have activity against SARS-CoV-2 in in vitro testing [16], and then showed clinical improvement against COVID-19 in its compassionate use for patients with severe symptoms from COVID-19 infection [17]. In a NIAID supported, randomized, controlled clinical trial to evaluate the safety and efficacy of the investigational antiviral remdesivir in hospitalized adults diagnosed with coronavirus disease 2019 (COVID-19) that took place in multiple locations globally, remdesivir was proved to be superior to placebo in shortening the time to recovery in adults hospitalized with Covid-19 and evidence of lower respiratory tract infection [18]. . Currently, there are multiple clinical trials with RDV at multiple sites at different geographic locations to access its effectiveness against broader patient populations. In these clinical studies, accurate determination of the prodrug RDV and its major metabolites, GS-441524 and GS-704277, in human plasma is critical for appropriate characterization of the pharmacokinetics (PK)

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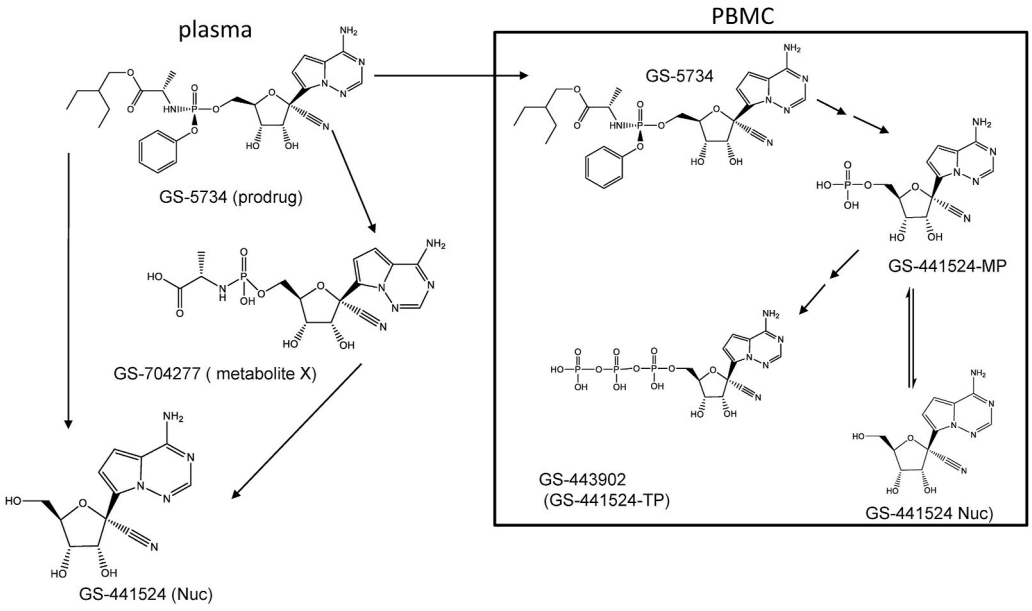

Fig. 1. The conversion scheme of RDV to GS-704277 and GS-441524 in plasma as well as RDV's permeation into PBMCs.

Table 1
Preparation of primary stock solutions.

Analyte	Primary stock I concentration (mg/mL)	Solvent	Primary stock II concentration (mg/mL)	Solvent	Correction Factor
RDV	2.263	ACN:DMSO at 50:50 (v:v)	2.276	ACN:DMSO at 50:50 (v:v)	0.984
GS-441524	2.213	ACN:DMSO at 50:50 (v:v)	2.115	ACN:DMSO at 50:50 (v:v)	0.756
GS-704277	2.925	ACN:DMSO at 50:50 (v:v)	2.910	ACN:DMSO at 50:50 (v:v)[a]	0.973
[$^{13}C_3$]-GS-5734 (GS-465124)	1.109	ACN:DMSO at 50:50 (v:v)	N/A	N/A	0.980
[$^{13}C_3$]-GS-441524 (GS-441285)	1.217	ACN:DMSO at 50:50 (v:v)	N/A	N/A	0.972
[$^{13}C_3$]-GS-704277 (GS-829466)	1.549	ACN:DMSO at 50:50 (v:v)	N/A	N/A	0.782

[a] If necessary stock A solution of GS-704277 can also be prepared in water.

and pharmacodynamics (PD) of RDV and its metabolites. To our knowledge, currently there is only one publication by Avataneo et al. [19] on the validation of a bioanalytical method for remdesivir and GS-441524 quantification in human plasma. The paper mentioned the stability issues regarding RDV and GS-441524, noting the lack of stability of RDV in plasma at room temperature (RT) and 4 °C within 24 h. Furthermore, no degradation was observed for GS-441524 after heat treatment of the plasma. However, very limited experimental details were provided, and it was not clear if both RDV and GS-441524 were present in the stability QC samples tested. The intermediate metabolite, GS-704277, that could be important in understanding the stability of RDV and GS-441524, was not mentioned. Furthermore, the authors used a 2-in-1 method, but did not address carryover issues, especially for the less polar RDV. We observed, however, early in the development of methods for determination of RDV and its metabolites in plasma (of rat, dog, monkey and human), temperature-dependent and pH-dependent stability shown by experimental data. Furthermore, degradation of RDV always led to observable increases in GS-441524 and GS-704277 and degradation of GS-704277 always led to observable increases in GS-441524. The conversion scheme of RDV to the intermediate metabolite, GS-704277 and the stable metabolite (parent), GS-441524 in plasma is shown in Fig. 1. Though instability is expected for such a prodrug that is designed to convert *in vivo* to an active metabolite, in a bioanalytical method development this instability issue must be addressed to ensure the precision, accuracy and robustness of the method. Moreover, since GS-441524 and GS-704277 are much less polar than RDV, it is challenging to address the carryover issue for RDV if the same LC gradient is used for all 3 analytes, or even for two analytes (RDV and GS-441524). The carryover issue for RDV need to be addressed separately. In this paper, we present the method development and validation of an LC-MS/MS method for determination of RDV and its major metabolites GS-441524 and GS-704277 in acidified human plasma, as well as the method's application in clinical studies.

2. Experimental

2.1. Chemicals and reagents

RDV, GS-441524, and GS-704277and respective internal standards (ISs) [^{13}C3]-GS-5734 (GS-465124), [^{13}C3]-GS-704277 (GS-829466) and [^{13}C3]-GS-441524 (GS-441285) reference materials were provided by Gilead Sciences (Gilead, Foster City, CA). GS-5734, GS-704277, GS-441524, GS-829143, GS-829466, and GS-828840 are light-sensitive, and therefore, all procedures were performed under yellow light. Pooled and individual human plasma, hemolyzed, and lipemic human plasma, and human whole blood (all with K_2EDTA as anticoagulant) were obtained from Bioreclamation (Bioreclamation IVT, West Berry, NY). HPLC grade water, methanol, dimethyl sulfoxide (DMSO),

Table 2
LC gradients for RDV, GS-441524 and GS-704277.

RDV			GS-441524			GS-704277			Flow (mL/min)
Time (min)	% Phase A	% Phase B	Time (min)	% Phase A	% Phase B	Time (min)	% Phase A	% Phase B	
0	40	60	0	100	0	0	100	0	0.5
0.1	40	60	0.3	100	0	0.2	100	0	0.5
1.6	25	75	2.3	40	60	2	35	65	0.5
1.7	0	100	2.4	0	100	2.1	0	100	0.5
2.6	0	100	3.3	0	100	3	0	100	0.5
2.62	40	60	3.32	100	0	3.02	100	0	0.5
3.4	40	60	4.3	100	0	4	100	0	0.5

Mobile Phase A: 10 mM ammonium formate in 5% methanol, pH 2.5.
Mobile Phase B: 100% methanol.

ammonium hydroxide, hydrochloric acid, formic acid (FA; reagent grade, 88%), and ammonium formate were obtained from Fisher Scientific (Hampton, NH).

2.2. Analytical equipment

The following analytical equipment was used in this bioanalytic method validation: Vacuum Degasser, DGU-20A5R, Shimadzu Corp.; Solvent Delivery System LC-30AD, Shimadzu Corp.; Autoinjector, SIL-30ACMP, Shimadzu Corp.; Column Switching Unit/Oven, CTO-30A, Shimadzu Corp.; Mass Spectrometer, Triple Quadrupole MS (API 5000), AB Sciex.

2.3. 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 solutions was calculated using a corresponding correction factor (factor required to convert the mass of reference material weighed to the mass of the analyte free base or free acid that it contains) for the reference standard provided in the certificate of analysis. Table 1 lists the concentrations, solvent and correction factors for primary stock solutions preparation. Stock solutions were stored at −20 °C and protected from light.

2.4. Preparation of Working Solutions, calibration standards and QC samples

IS Working Solutions: The appropriate amounts of GS-829143/GS-829466/GS-828840 stock solution was added to a volumetric flask. The volumetric flask was filled to volume with methanol:water:FA at 50:50:0.1 (v:v:v), mixed well, and stored in an appropriate reagent bottle. Solutions were stored at approximately −20 °C until analysis.

Standard Spike-In Solution: The appropriate volume of stock solution was added with the appropriate volume of acetonitrile:dimethyl sulfoxide at 50:50 (v:v) to make a standard spike-in solution with GS-5734/GS-441524/GS-704277 concentrations of 160/80/80 (μg/mL).

2.5. Sample preparation

2.5.1. Solution, solvent and acidic plasma preparation

A nominally "20%" FA solution was prepared by mixing FA (88%) and water at 1:4 (v:v). Dissolution solvent was prepared with methanol:water:20%FA ratio to be at 50:50:0.1 (v:v:v). Reconstitution Solvent was prepared with10 mM ammonium formate in methanol:water (15:85, v:v). FA acidified plasma (FA-treated plasma) was prepared with pooled K_2EDTA Human Plasma:20% FA Solution at 100:8 (v:v).

2.5.2. Calibration standards and quality control samples for method validation

Calibration standards were prepared in pooled FA-treated plasma fortified with the first stock solutions of RDV, GS-441524, and GS-704277 to yield concentrations of 4/2/2, 8/4/4, 40/20/20, 80/40/40, 400/200/200, 800/400/400, 3600/1800/1800, 4000/2000/2000 ng/mL (RDV/GS-441524/GS-704277). QC samples were prepared in FA-treated plasma from different stock solutions of RDV, GS-441524, and GS-704277 at five concentration levels: 4/2/2 (lower limit of quantification, LLOQ); 12/6/6 (low quality control, LQC); 200/100/100 (low middle quality control, Low MQC); 1600/800/800 (high middle quality control, High MQC); and 3200/1600/1600 ng/mL (high quality control, HQC). Calibration standards and QC samples were stored at −70 °C until use, except that freshly prepared calibration standards were used for assessments of bench-top, freeze/thaw cycle, and long-term frozen storage stability of RDV, GS-441524, and GS-704277 in FA-treated plasma.

2.5.3. 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.

2.5.4. 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 pretreatment, 50 μL aliquots of plasma samples, calibration standards, or QC samples were added to separate wells of an appropriately labeled 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 Carry-over Blank wells were spiked with 50 μL of methanol:water:FA at 50:50:0.1 (v:v:v). 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:water at 15:85 (v:v). The collection plate was vortex-mixed for approximately 1

Table 3
Mass spectrometric Multiple Reaction Monitoring conditions.

Compound	TIS[a] Ionization Mode	Dwell Time (msec)	Declustering Potential (V)	Collision Energy (eV)	Collision Exit Potential (V)	Mass Transition (*m/z*)
GS-5734	+	150	110	23	15	603.3 → 402.2
GS-829143 (IS)	+	150	110	23	15	606.3 → 288.2
GS-441524	+	150	95	19	15	292.2 → 202.2
GS-828840 (IS)	+	150	95	19	15	295.1 → 205.2
GS-704277	–	200	−100	−29	−15	441.1 → 150.1
GS-829466 (IS)	–	200	−100	−29	−15	444.1 → 150.1

[a] TIS: turbo ion spray.

Table 4
Mass Spectrometer Settings for RDV, GS-441524, and GS-704277and their respective ISs.

Mass Spectrometer Settings	RDV/GS-829143 (IS)	GS-441524/GS-828840 (IS)	GS-704277/GS-829466 (IS)
Source Temperature (TEM):	650 °C	650 °C	650 °C
Collision Gas (CAD):	8 psig N_2	8 psig N_2	9 psig N_2
Curtain Gas (CUR):	35 psig N_2	35 psig N_2	35 psig N_2
Ion Source Gas 1 (GS1):	55 psig N_2	55 psig N_2	50 psig N_2
Ion Source Gas 2 (GS2):	55 psig N_2	55 psig N_2	65 psig N_2
Ion Spray Voltage (IS):	4500 V	−4500 V	4500 V
Entrance Potential (EP):	10 V	10 V	−10 V
Scan duration:	2.5 min	2.5 min	2.5 min

min at medium speed and plate was centrifuged at approximately 3000 rpm for 5 min prior to sequential injection of plate well reconstituted samples onto an LC-MS/MS system.

2.6. Bioanalytical method development

2.6.1. Plasma stability test

During method development, the individual stability of RDV, GS-441524, and GS-704277 at the LQC (12, 6, 6 ng/mL) and HQC concentrations (3200, 1600, 1600 ng/mL) in 20% FA-treated pooled K_2EDTA human plasma was compared with that in untreated pooled K_2EDTA human plasma. Analyte:IS peak area ratios ($n = 3$) after incubation at either room temperature (RT) or 4 °C were determined by LC-MS/MS for assessment of stability. Stability was further confirmed during method validation as described below.

2.6.2. 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). Table 2 lists optimized gradients for each of the analyte that was injected separately and the combined mobile phase flow rate.

2.6.3. Mass spectrometric conditions

Ionization and detection of RDV, GS-441524, and GS-704277 and their 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. Negative $(M - H)^+$ ions were monitored for GS-704277 in Multiple Reaction Monitoring (MRM) mode. Quantitation was performed using parent → product ion (*m/z*). The Mass spectrometric Multiple Reaction Monitoring conditions are listed in Table 3. The source-dependent parameters maintained for the 3 analytes were as shown in Table 4. Analyst® software version 1.4.1 was used for LC-MS/MS parameter control and data collection.

2.7. Bioanalytical method validation

Validation of the method for determination of RDV, GS-441524, and GS-704277 in FA-treated plasma was done following the FDA and EMA guidelines [20,21]. The calibration and linearity, precision and accuracy, dilution linearity, selectivity, matrix effect, injection carryover, extraction recovery, effect of hemolysis, and effect of lipemia were evaluated. Experiments were also conducted to evaluate the stability of RDV, GS-441524, and GS-704277 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, and GS-704277 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.

2.7.1. 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 ($1/x^2$) 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 (r^2) 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.

2.7.2. 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.

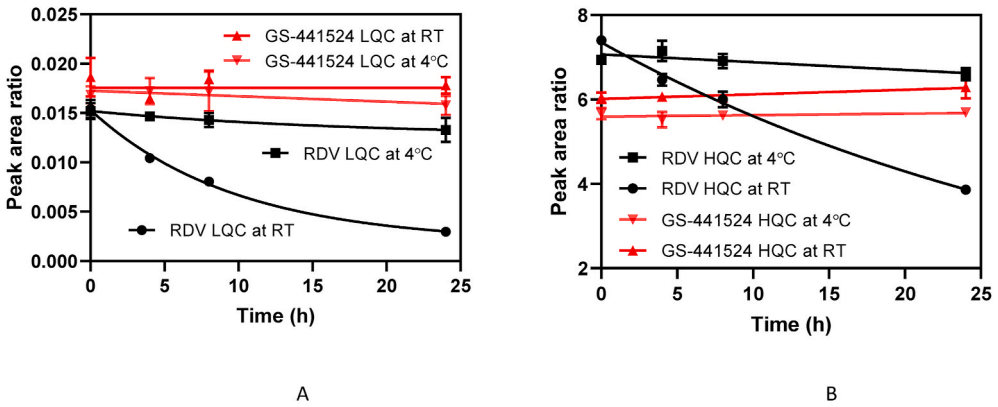

Fig. 2. RDV and GS-441524 stability in untreated human plasma measured by peak area ratio, RDV/[13C3]-RDV and GS-441524/[13C3]-RDV. A: at LQC concentration [RDV]/[GS-441524] = 12/6 ng/mL; B: at HQC concentration [RDV]/[GS-441524] = 1600/800 (ng/mL).

Interday precision and accuracy were determined by analyzing six replicate aliquots of all QC concentrations over three independent precision and accuracy runs. The observed mean, %CV and %RE were calculated at the QC levels for all three analytical runs. The acceptance criteria both for intraday and interday precision and accuracy runs required the %CV value to be ≤ 15.0% and the %RE value of the mean to be equal to or within ±15.0% of nominal, except for LLOQ QC samples, for which the acceptable %CV value was ≤20.0% and the %RE of the mean was equal to or within ±20.0% of nominal.

2.7.3. 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, and GS-704277 (10 000/5000/5000 ng/mL, respectively) and 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%.

2.7.4. 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.

2.7.5. 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 ng/mL, n = 3) for RDV/GS-441524/GS-704277 and at one concentration (400/200/200 ng/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 serve 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.

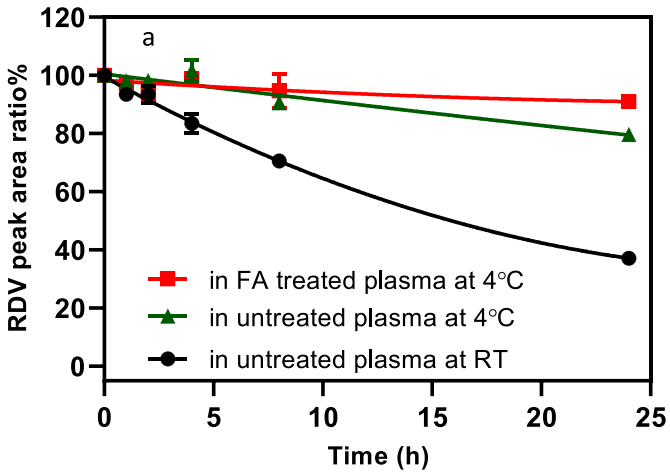

Fig. 3A. RDV (4000 ng/mL) conversion to GS-441524 in untreated human plasma measured by a. RDV level using peak area ratio, RDV/[$^{13}C_3$]-RDV.

In addition to the normal matrix, the effects of lipemic FA-treated plasma and 5% hemolyzed FA-treated plasma on the assay performance were examined at two analyte concentrations (12/6/6 and 3200/1600/1600 ng/mL, n = 3) for RDV/GS-441524/GS-704277 and at one concentration (400/200/200 ng/mL, n = 3) for their ISs. One lot of lipemic matrix and one lot of hemolyzed matrix were evaluated. For the results from the lipemic and hemolyzed 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%.

2.7.6. 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.

2.7.7. 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 ng/mL, n = 5) for RDV/GS-441524/GS-704277. 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 unlabeled

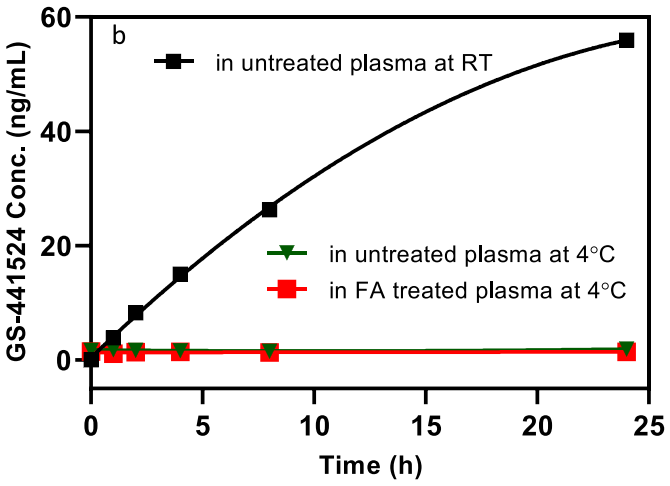

Fig. 3B. The correspondingly resulted GS-441524 concentration (ng/mL).

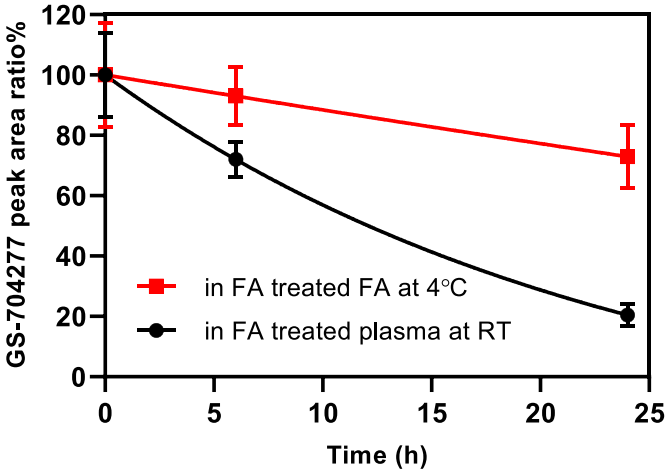

Fig. 3C. GS-704277 (6 ng/mL) stability in FA treated human plasma measured by GS-704277 level using peak area ratio, GS-704277/[$^{13}C_3$]-GS-704277.

analyte. The recovery of the analytes in this assay was evaluated by comparing the mean peak areas from the analyte added to and recovered from the biological matrix (extracted samples) to the peak areas from the sample extracts spiked at the nominal analyte concentrations (post-extract spiked samples). The %CV of the results for the three concentrations tested could not exceed 20.0%.

2.7.8. Stability assessment

2.7.8.1. Benchtop stability. Benchtop ice bather stability of RDV, GS-441524, and GS-704277 in FA-treated K_2EDTA human plasma was tested to evaluate analyte stability in the matrix in an ice bath during sample handling and processing. Stability was determined at two concentrations (12/6/6 and 3200/1600/1600 ng/mL for RDV/GS-441524/GS-704277). The samples were stored in an ice bath for 8 h prior to extraction. The determined concentration at each level could not exceed ±15.0% RE from the nominal concentration, and the %CV of the determined concentrations at each level could not exceed 15.0%.

2.7.8.2. Freeze/thaw stability. Freeze/thaw stability was tested to evaluate the stability of RDV, GS-441524, and GS-704277 in FA-treated K_2EDTA human plasma after five freeze/thaw cycles. Stability samples at two concentrations (12/6/6 and 3200/1600/1600 ng/mL for RDV/GS-441524/GS-704277) were frozen at −20 °C or −70 °C (for a minimum of 24 h for the first cycle and a minimum of 12 h for the other cycles) and thawed in an ice bath. After the completion of the fifth cycle, the samples were analyzed. The determined concentrations at each level could not exceed ±15.0 %RE from the nominal concentration, and the % CV of the determined concentrations at each level could not exceed 15.0%.

2.7.8.3. Processed sample stability. Processed sample stability was tested to ensure that the integrity of the processed samples from an analytical run would be maintained if those samples were stored for the specified time interval prior to injection. Processed sample stability was determined at 4 °C. All replicates of the low and high QC samples (PSS QCs) of a valid run were kept refrigerated. When evaluating the processed sample stability, the PSS QCs were injected to the LC-MS/MS system along with newly extracted calibration standards and quality control samples.

2.7.8.4. Whole blood stability. Stability of RDV, GS-441524, and GS-704277 in K_2EDTA human whole blood was evaluated to ensure the stability of the analyte during the sample collection process. Human blood was pre-incubated at 37 °C for approximately 20 min. RDV, GS-441524, and GS-704277 were spiked into pre-incubated K_2EDTA whole blood at 12/6/6 and 3200/1600/1600 ng/mL for RDV/GS-441524/GS-704277 in triplicate within 4 h of collection. The spiked whole blood stability samples were incubated at 37 °C for 10 min to reach equilibrium. The spiked whole blood stability samples were then transferred to plastic culture tubes and then held in an ice bath for 0, 1, 2, and 4 h before centrifugation in a refrigerated (4 °C) centrifuge for approximately 10 min at 1600 *g*. A total of 500 μL of each resulting plasma was spiked into corresponding a plastic culture tube and 40 μL of formic acid solution was added to each, and the tubes were vortex-mixed well. Aliquots of the samples were subjected to the standard sample processing procedure, and stability for each analyte was evaluated using analyte-to-IS peak area ratio as a function of ice bath storage time of the spiked whole blood samples.

2.7.8.5. Analyte stock solution stability. Solution stability for each analyte was tested to evaluate analyte stability in the stock solutions that were used to prepare calibrations standards, QCs, and other validation samples. Stock solution storage stabilities in either acetonitrile:dimethyl sulfoxide at 50:50 (v:v) or in water was evaluated by comparing the response of a stock kept at −20 °C to the response of a freshly prepared solution (from powder or sealed ampule) as a reference solution. The reference solution must be used within one day of its preparation. Similarly, stability of a stock solution stored at ambient temperature was determined by comparing its response initially to the response of a freshly prepared reference stock, and later to the response to the reference stock stored at −20 °C when verification of its stability at −20° had been confirmed for the specified duration. The solution maintained in the freezer or the freshly prepared stock solution served as the reference for the ambient temperature stock solution stability evaluation. In order for the solution to be considered stable, the %CV of responses from replicates determination (n = 3) of both the test and reference solutions could be no greater than 15.0% and the %Diff between the mean responses of the test and reference solutions could be no more than ±10.0%.

2.7.8.6. Long-term storage stability in matrix. Long-term storage stability was evaluated to ensure RDV, GS-441524, and GS-704277 in FA-treated K_2EDTA human plasma was stable after storage at −20°C or −70°C. The stability samples were initially analyzed once to verify that the samples were prepared correctly. . For the verification assessment, the %CV of the calculated concentrations at each level could not exceed 15.0% and %RE calculated for the mean of the determinations using the observed and nominal concentration values had to be within ±15.0%.

Table 5
Bioanalytical method validation results summary.

Analyte Name	GS-5734	GS-441524	GS-704277
Internal Standard (IS)	GS-829143	GS-828840	GS-829466
Analytical Method Type	LC-MS/MS		
Extraction Method	Protein Precipitation		
Sample Volume	50 μL		
QC Concentrations	4, 12, 200, 1600, and 3200 ng/mL	2, 6, 100, 800, and 1600 ng/mL	2, 6, 100, 800, and 1600 ng/mL
Standard Curve Concentrations	4, 8, 40, 80, 400, 800, 3600, and 4000 ng/mL	2, 4, 20, 40, 200, 400, 1800, and 2000 ng/mL	2, 4, 20, 40, 200, 400, 1800, and 2000 ng/mL
Lower Limit Of Quantitation	4 ng/mL	2 ng/mL	2 ng/mL
Upper Limit Of Quantitation	4000 ng/mL	2000 ng/mL	2000 ng/mL
Mean Recovery of Analyte (%)	97.4	105.4	81.1
Mean Recovery of IS (%)	NA		
LLOQ QC Intraday Precision Range (% CV)	4.9 to 7.3	7.0 to 9.3	4.2 to 10.4
LLOQ QC Intraday Accuracy Range (% RE)	−2.5 to 9.5	−8.0 to 5.5	−17.5 to −9.0
Analytical QC Intraday Precision Range (%CV)	1.2 to 4.8	2.3 to 6.6	1.6 to 4.6
Analytical QC Intraday Accuracy Range (%RE)	−1.6 to 11.5	−1.9 to 10.0	−10.3 to −1.9
LLOQ QC Interday Precision (%CV)	7.7	9.8	9.2
LLOQ QC Interday Accuracy (%RE)	2	0	−12.5
Analytical QC Interday Precision Range (%CV)	2.3 to 3.8	3.5 to 5.3	2.1 to 3.8
Analytical QC Interday Accuracy Range (%RE)	0.0 to 9.5	−0.6 to 8.0	−9.8 to −3.5
Dilution Integrity	10000 ng/mL diluted 20-fold	5000 ng/mL diluted 20-fold	5000 ng/mL diluted 20-fold
Selectivity	≤20.0% LLOQ for analyte; ≤ 5.0% for IS	≤20.0% LLOQ for analyte; ≤ 5.0% for IS	≤20.0% LLOQ for analyte; ≤ 5.0% for IS
FA-Treated 2% Hemolyzed Plasma Test	No impact on assay performance		
FA-Treated 5% Hemolyzed Plasma Test	No impact on assay performance		
Lipemic Plasma Test	No impact on assay performance		

For subsequent stability timepoint evaluations, the same acceptance criteria for precision (%CV) and accuracy (%RE) that were used in the initial verification assessment were applied.

2.7.8.7. Gamma-ray irradiation stability in matrix. Gamma-ray irradiation stability was evaluated to ensure RDV, GS-441524, and GS-704277 in FA-treated K_2EDTA human plasma were stable after being subjected to Gamma-ray irradiation, used as a means of destroying virus species such as Ebola. The stability samples were first analyzed once to verify that the samples were prepared correctly, as described for long-term storage stability. Two sets of the stability samples were then shipped frozen to the NIH NIAID Integrated Research Facility to perform gamma-ray irradiation, where one set of the stability samples was not irradiated and served as the control, whereas the other set of the stability samples was subjected to the gamma-ray irradiation of minimum required dose of 5 Mrad which is sufficient to inactivate EBOLA virus and coronavirus in a sample with 1×10^6 focus forming units [FFU]/mL [22]. Both sets of samples were then shipped back to QPS, LLC for analysis. Acceptance criteria to demonstrate adequate stability were that the %CV and%RE calculated for the mean of the determinations using the observed and nominal concentration values must be within ±15.0%.

3. Results and discussion

3.1. Bioanalytical method development

3.1.1. Investigation of plasma acidification to stabilize concentrations of GS-5734, GS-441524, and GS-704277

The stabilities of GS-5734, GS-441524, and especially the stability of GS-704277 in FA-treated pooled human plasma was evaluated and compared with the stabilities in untreated plasma. Fig. 2 shows RDV and GS-441524 stabilities in untreated human plasma measured by peak area ratio, RDV/[$^{13}C_3$]-RDV, and GS-441524/[$^{13}C_3$]-RDV at both room temperature (RT) and 4 °C. At the respective LQC concentrations for RDV and GS-441524 (12 and 6 ng/mL, both in the same sample), stability issues were observed. After 24 h RVD had decreased >80% at RT and 13% at 4 °C, while GS-441524 decreased 4% and 6% respectively (Fig. 2A). At the respective HQC concentrations for RDV and GS-441524 (1600 and 800 ng/mL), a similar pattern was observed: After 24 h RVD had lost more than 48% at RT and 5% at 4 °C, while GS-441524 increased 5% and 0.4%, respectively (Fig. 2B). Based on these results, conditions were sought:

1). To prevent potential conversion of RDV to GS-704277 and GS-441524;
2). To prevent potential conversion of GS-704277 to GS-441524.

The effect of adding FA to human plasma on the stability of RDV and GS-441524 is shown in Fig. 3A and Fig. 3B, in which RDV alone was spiked at 4000 ng/mL into blank pooled K_2EDTA plasma, and aliquots were withdrawn at the indicated times after the sample was incubated at room temperature and analyzed by the developed method, in which the IS was added when the sample was extracted. As shown in Fig. 3A, in 24 h at 4 °C, RDV peak area ratio (RDV:[^{13}C3]-RDV) decreased only 9% with FA treated plasma, whereas the peak area ratio decreased by more than 20% for the untreated plasma sample, and at RT the peak area ratio decreased by more than 60%. For the same samples, Fig. 3B shows the observed GS-441524 concentrations initially at or near zero at time 0, after 24 h at 4 °C were 1.43 ng in FA-treated plasma and 1.90 in untreated plasma, but after 24 h at RT had significantly increased to 56 ng/mL. Fig. 3C shows the stability of GS-704277 (6 ng/mL) in FA-treated human plasma at 4 °C and RT measured by GS-704277/[$^{13}C_3$]-GS-704277 peak area ratio observed upon analysis of the sample by the developed method. The data show that GS-704277 is only moderately stable in FA-treated plasma at 4 °C (<10% decrease in ~8 h) and less table at RT (~10% loss in ~2 h). Therefore, for accurate determination of GS-704277 itself, a FA-treated plasma sample should be analyzed after storage for 8 h or less at 4 °C and less than 2 h at RT. Also, depending on relative concentrations of GS-704277 and GS-441524 in a sample, accuracy of determinations of GS-441524 could be affected by its generation from GS-704277.

The stability study results demonstrate the need for stabilization of clinical samples upon collection. Results from plasma stability studies of RDV, GS-704277, and GS-441524 further confirmed the need for FA as a stabilizing agent to prevent conversion of RDV to GS-704277 and conversion of existing or newly formed GS-704277 to GS-441524 during the sample collection, storage and analysis processes. Human plasma samples with K_2EDTA as anticoagulant (K_2EDTA plasma) were treated immediately upon collection as described above. Such acidification was a suitable balance of inhibition of endogenous esterase activities, reagent acceptability for clinical sites, and prevention of acid-related plasma sample gelling; it and had been successfully used for similar prodrugs [23] As a prodrug, RDV was designed to be subject to hydrolysis by endogenous esterases [24], and previous work had shown that known esterase inhibitor dichlorvos was effective in minimizing esterase

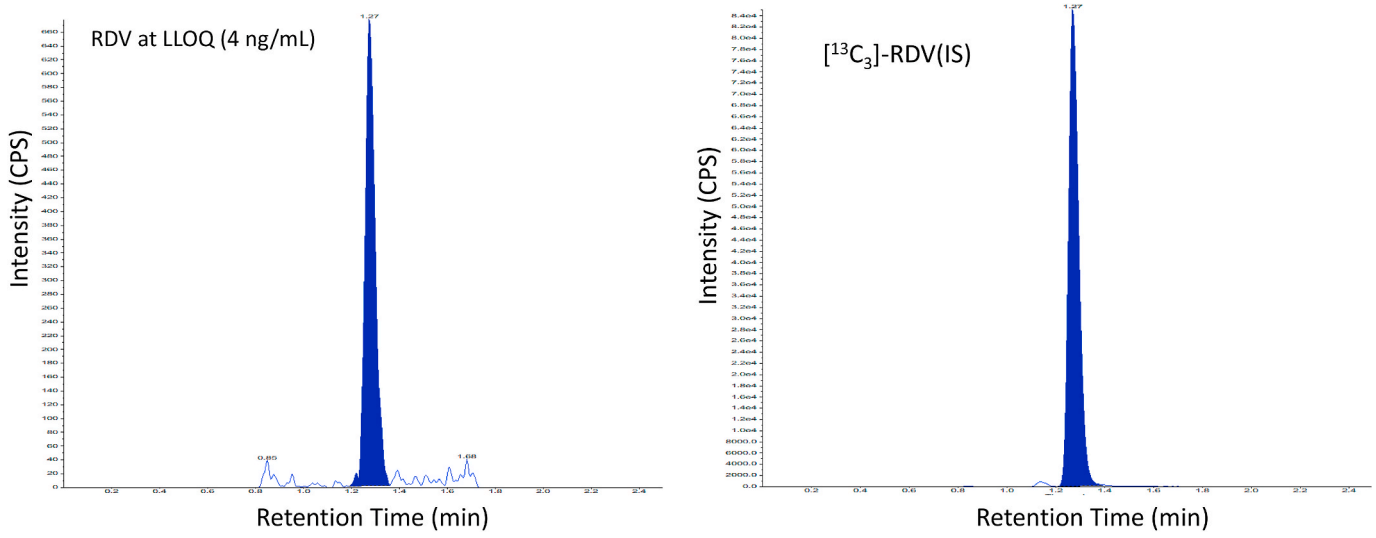

Fig. 4A. chromatogram for RDV of LLOQ at 4 ng/mL and IS [$^{13}C_3$]-RDV.

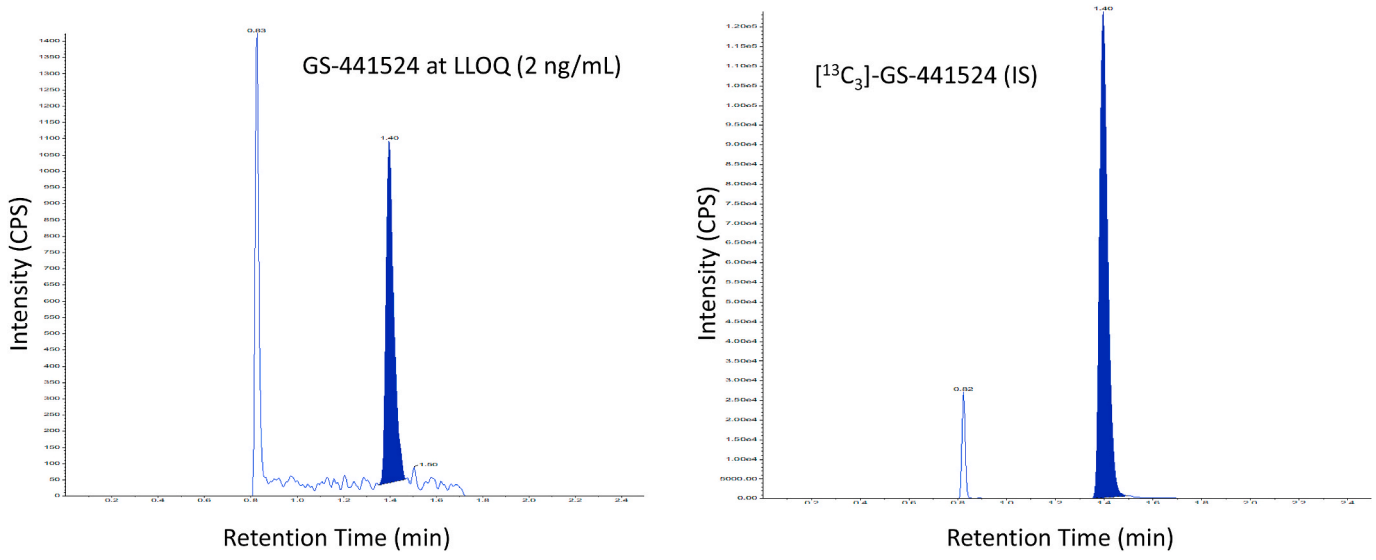

Fig. 4B. chromatogram for GS-441524 of LLOQ at 2 ng/mL and IS [$^{13}C_3$]-GS-441524.

hydrolysis of RDV in animal plasma samples. However, the toxicological effects of dichlorvos [25]. Precluded its use as a stabilizer at many clinical sites. Moreover, the specificity of dichlorvos or another esterase inhibitor toward multiple esterases that might be present in a sample was a concern. The previous success with FA addition for ester prodrug stabilization, and observations that both the final sample pH and the concentration of the added FA can impact the onset of gelling (or coagulation) of plasma led to experiments that showed that acidification of human K_2EDTA plasma to a pH of $<$4.7 caused gelation after $\leq$24 h, whereas acidification to a pH of 5.3 showed no gelation. Furthermore, both the concentration and the corresponding volume of added acid needed to achieve a plasma pH of 5.3 was important: Addition 40 μL of 20% aqueous FA to 500 μL of plasma resulted in no gelling, whereas addition of lower volumes of higher FA concentrations caused time-dependent gelling, and addition of a very low volume of full-strength (88%) FA caused instantaneous gelling. Therefore, upon collection at clinical sites, plasma samples were treated with 20% FA, and calibration standards and QC samples used for method validation and sample analysis were also prepared in FA-treated human K_2EDTA plasma.

3.1.2. LC-MS/MS conditions optimization for each individual anlayte

GS-5734, GS-441524, and especially GS-704277 are polar compounds that are hard to retain on a reversed phase column. Hydrophilic interaction chromatography (HILIC) was tested, which typically runs at very high organic mobile phase. However, we found that GS-5734 and GS-441524 could not be retained under HILIC condition with multiple types of columns tested, while GS-704277 always got very broad retention without a reasonable peak. An Acquity UPLC HSS T3 (C18,1.8 μm) column, whose stationary phase was designed to be aqueous mobile phase compatible and to retain and separate small, water-soluble polar organic compounds [26], was selected based on both the preliminary assessments and our previous experience with a variety of related nucleoside drugs and prodrugs. Though standards and QCs were prepared as 3-in-1 solutions and one protein precipitation extraction was conducted, 3 separate injections for GS-5734, GS-441524, and GS-704277 from the same processed sample plate were performed with 3 separate organic gradients as shown in Tables 2 and 3 for the following considerations:

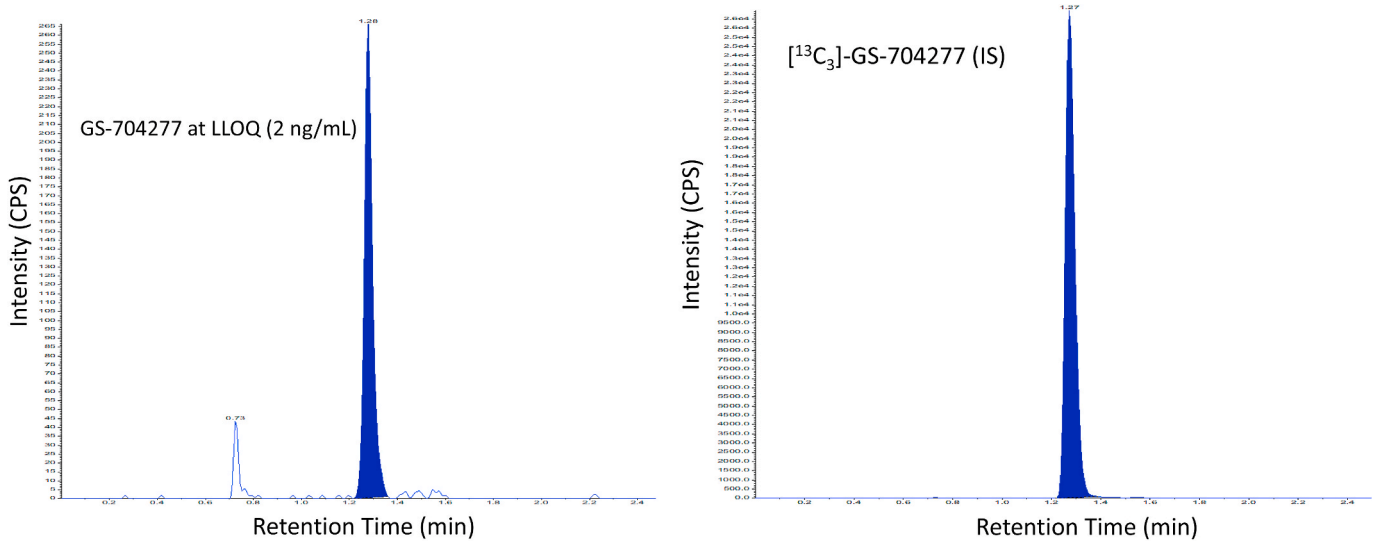

Fig. 4C. chromatogram for GS-704277 of LLOQ at 2 ng/mL and IS [$^{13}C_3$]- GS-704277.

1). Different MRM polarity mode (positive vs negative) for different analytes. GS-704277 could have shared the same injection with GS-441524 if they could use the same polarity of MRM. However, GS-704277 showed much higher sensitivity (4–5 folds) with negative mode detection. For GS-5734, we found that the sensitivity at both positive mode and negative mode were similar. However, GS-441524 had very low sensitivity at negative mode that would not meet the target LLOQ, thus it had to be in positive mode. Due to the fact that GS-704277 required negative mode for sensitivity, whereas GS-441524 required positive mode for sensitivity, the 3-in-1 assay required 3 separate injections for GS-5734, GS-441524, and GS-704277, respectively. In regard to instrument sensitivity, it was found that API-6500 did not have better, actually lower, sensitivity for GS-704277 than API-5000 at negative mode. At positive mode, API-5000 and API-6500 had the same sensitivity of GS-704277. We concluded that GS-704277 had the best sensitivity on API-5000 in negative MRM mode.

2). Different LC gradients. When keeping both GS-5734 and GS-441524 within the same chromatography (injection), the starting mobile phase had to contain very low organic solvent (5%) in order for GS-441524 to be retained by the column, which caused GS-5734 to have significantly higher and potentially unacceptable carryover. Separation of GS-5734 and GS-441524 into two separate LC gradients (injections) enabled use of a starting mobile phase with high organic solvent (60%) for GS-5734 chromatography, which minimized the carryover of GS-5734. GS-704277, due to its high polarity, required 100% aqueous running buffer initially, and then a gradual organic gradient that reached to 65% organic in 1.6 min, followed with 100% aqueous phase equilibration for 0.8 min.

3). Extraction Method. In consideration of the polarity of GS-704277 and GS-441524, the method utilized protein precipitation extraction of the analytes and ISs from FA-treated human plasma using methanol (sample:methanol = 50 μL:500 μL). After sample vortexing, 200 μL of the resultant supernatant was transferred from the preparation plate to the collection plate. The collection plate supernatant samples were evaporated to dryness in a 40 °C bath under nitrogen stream. The samples were then reconstituted in a solvent of 400 μL of 10 mM ammonium formate in methanol: water at 15:85 (v:v)

3.2. Bioanalytical method validation

The quantitation range for GS-5734/GS-441524/GS-704277 was validated from 4/2/2 to 4000/2000/2000 ng/mL using a 50 μL sample volume. The precision and accuracy of the method for determination of RDV/GS-441524/GS-704277 concentrations in FA-treated plasma were evaluated by analyzing QC samples at five concentration levels (LLOQ 4/2/2, LQC 12/6/6, MQC 200/100/100, HQC 1600/800/800, ULOQ 3200/1600/1600 ng/mL) within the calibration standard curve range. The results from three precision and accuracy batch runs are presented in Table 5, which show that the precision and accuracy of the method were within the aforementioned acceptance criteria. All the validation assessments (calibration and linearity, dilution linearity, selectivity, matrix effect, extraction recovery, effect of hemolysis, and effect of

Table 6
Stability data established in the method validation.

Analyte Name	GS-5734	GS-441524	GS-704277
Stock Solution Stability in Acetonitrile:Dimethyl Sulfoxide at 50:50 (v:v)	243 Days at −20 °C 8 h at RT	243 Days at −20 °C 8 h at RT	70 Days at −20 °C 6 h at RT
Processed Sample Stability	145 h at 4 °C	155 h at 4 °C	185 h at 4 °C
Benchtop Stability in Formic Acid-Treated Plasma	8 h in an Ice Bath	8 h in an Ice Bath	8 h in an Ice Bath
Freeze/Thaw Stability in Formic Acid-Treated Plasma	5 Cycles at −20 °C and −70 °C	5 Cycles at −20 °C and −70 °C	5 Cycles at −70 °C
Benchtop Stability in Whole Blood	4 h in an Ice Bath	4 h in an Ice Bath	4 h in an Ice Bath
Long-term Storage Stability in Formic Acid-Treated Plasma	392 Days at −70 °C 3 Days at −20 °C	392 Days at −70 °C 3 Days at −20 °C	257 Days at −70 °C
Gamma-Ray Irradiation Stability at Required Radiation Dose of 5 Mrad on Dry Ice for Virus load $< 1 \times 10^6$ $TCID_{50}$	No impact on assay performance	No impact on assay performance	Data negative bias (impact on assay performance)

lipemia) also passed the acceptance criteria, and the results are also presented in Table 5. These results are straightforward and are not discussed in further detail. Fig. 4A, Fig. 4B, and Fig. 4C show the chromatograms from an LLOQ sample for RDV, GS-441524, and GS-704277, respectively.

3.2.1. Carryover

Autosampler injection carryover was evaluated by injection of an extracted blank matrix sample, (containing neither analyte nor IS), immediately after the injection of the highest calibration standard (ULOQ) extract. The carryover was calculated as the peak area observed in of the carryover blank expressed as a percentage of the mean peak area of the lowest calibration standard determined in the same run. Ideally, the carryover value is <20% of the mean LLOQ analyte peak area. For 63 of 65 runs, the peak area of RDV observed in the first and second carryover blanks was less than 20.0% of the corresponding observed LLOQ peak area. For GS-704277, injection carryover for the two carryover replicates was 20.6% and 23.7% in Run 12, 25.5% and 22.5% in Run 15, 22.3% and 38.2% in Run 58. For GS-441524, injection carryover was 32.6% in Run 14. In the application of the method, based on the maximum level of carryover observed, each sample was assessed against that possible carryover amount. If a sample was deemed to have been potentially impacted by carryover, i.e., if the calculated carryover value from a preceding sample to a study sample is more than 0.05 (i.e., 5%), the affected sample would be re-analyzed. Carryover was less than 5.0% for the IS. The injection carryover of the IS met the acceptance criteria in all runs.

3.2.2. Stability assessment

Analyte stability is a function of the chemical properties of the analyte, the matrix in which the analyte is stored, and the storage conditions. Table 6 summarizes the analyte stability data established in the method validation. The assessment of stability of one analyte was always performed in the presence of the other two analytes to mimic clinical applications, and the tests evaluated the stability of the analyte in situations likely to be encountered during actual sample handling and analysis.

RDV, GS−441524, and GS−704277 stability in FA-treated K_2EDTA human plasma was demonstrated for 8 h in an ice bath and for five freeze/thaw cycles at−70°C. GS−5734 and GS−441524 stability in FA-treated K_2EDTA human plasma was demonstrated for five freeze/thaw cycles at −20 °C, for 3 days of long-term storage at −20 °C, for at least 392 days of long-term storage at −70 °C and upon sample exposure to gamma-ray irradiation. GS−704277 stability in FA-treated K_2EDTA human plasma was also demonstrated for at least 257 days of long-term storage at −70 °C. RDV, GS−441524, and GS−704277 stability was demonstrated in human whole blood in an ice bath for 4 h and in processed samples for 145, 185, and 155 h, respectively, at 4 °C. GS-5734 and GS-441524 stability was demonstrated in acetonitrile:dimethyl sulfoxide (50:50, v:v) for 243 days at −20 °C. GS-704277 stability was demonstrated in acetonitrile:dimethyl sulfoxide (50:50, v:v) for 6 h at ambient temperature and for 70 days at −20 °C. The quantitation range was 4/2/2 to 4000/2000/2000 ng/mL for GS-5734/GS-441524/GS-704277 using a 50 μL sample volume.

Freeze/thaw stability results for GS-704277 in formic acid-treated K_2EDTA human plasma after five freeze/thaw cycles at −20 °C did not meet the acceptance criteria in one run and its repeated run. For these failed tests, it was noticed that samples were gelling as they were being used for extraction. Freeze/thaw stability results for of GS-5734, GS-704277, and GS-441524 in formic acid-treated K_2EDTA human plasma after five freeze/thaw cycles at −70 °C met the acceptance criteria. Long-term storage stability tests at −20 °C were conducted due to the above failed 31-day long-term storage stability tests at −20 °C. The LQC and HQC for GS-704277 and the HQC for both GS-5734 and GS-441524 in formic acid-treated K_2EDTA human plasma did not met the acceptance criteria after long-term storage at −20 °C for at least 31 days in 3 out of 11 runs. For these failed tests, it was noticed that samples were gelling as they were being used for extraction. This sample gelling was only observed in the samples stored at −20 °C. Based on the data, it is recommended that study samples be stored at −70°C only.

4. 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. Which was applied to multiple clinical studies for application for use of RDV as treatment for COVID-19. Fig. 5A, Fig. 5B, and Fig. 5C show mean (n = 10) RDV, GS-441524 and GS-704277 plasma concentration-time profiles by cohort 8, semi-

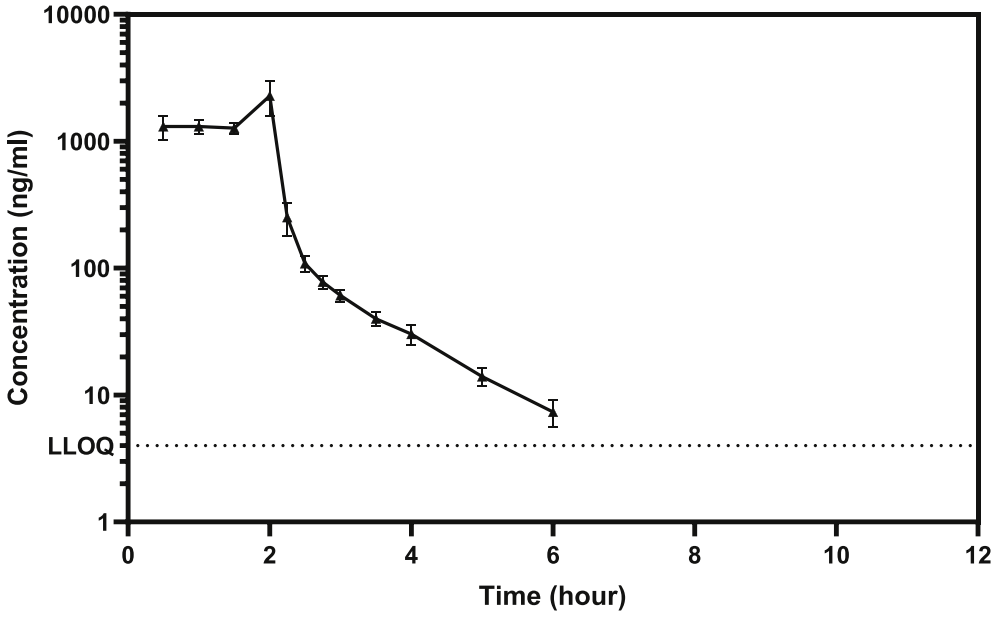

Fig. 5A. RDV mean (n = 10) plasma concentration-time profiles after GS-5734 150-mg intravenous injection over a 2-h period with lyophilized formulation from Gilead study GS-US-399-1812.

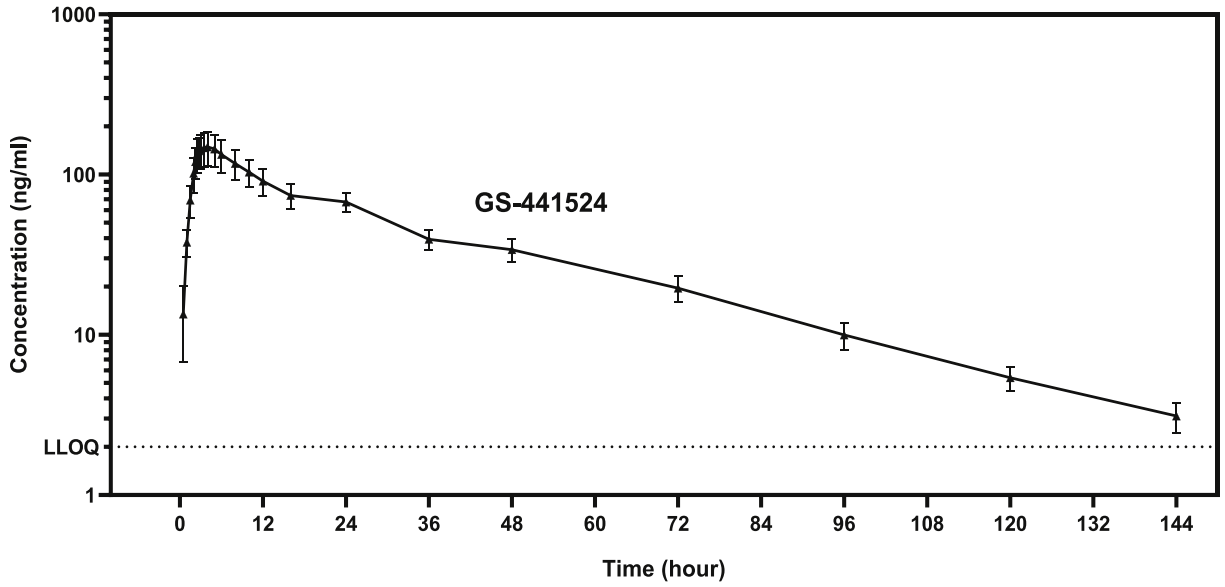

Fig. 5B. GS-441524 mean (n = 10) plasma concentration-time profiles after GS-5734 150-mg intravenous injection over a 2-h period with lyophilized formulation from Gilead study GS-US-399-1812.

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 [27]. As expected, RDV plasma concentration exposure reaches to a mean C_{max} of 2720 ng/mL immediately after infusion cessation and decreases rapidly to the LLOQ (4 ng/mL) at 5 h postdose. GS-441524, the stable metabolite, shows a mean C_{max} of 148 ng/mL at 4 h and a long half-life of ~25 h. GS-704277, the intermediate metabolite, peaks at ~2 h with a mean C_{max} of 230 ng/mL but drops to the LLOQ (2 ng/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 analyzed in 35 runs, the overall %CV values from the results of duplicate analyses per run of each of 4 QC samples with concentrations that spanned the calibration curve ranged from 3.6% to 6.5% for GS-5734, from 5.7% to 10.0% for GS-445134, and from 4.5% to 11.8% for GS-704277, and %RE values ranged from 0.9% to 8.0% for GS-5734, from −0.6% to 6.8% for GS-441524, and from 0.6% to 11.0% for GS- GS-704277. 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 (Veklury®) as treatment for COVID-19.

5. Conclusions

The LC-MS/MS bioanalytical method for the determination of concentrations of RDV, GS−441524, and GS−704277 in FA-treated K_2EDTA human plasma was validated successfully with respect to linearity, sensitivity, accuracy, precision, dilution, selectivity, hemolyzed plasma, lipemic plasma, batch size, recovery, matrix effect, and carryover. Since RDV can be hydrolyzed 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−704277 and 441524 concentrations,

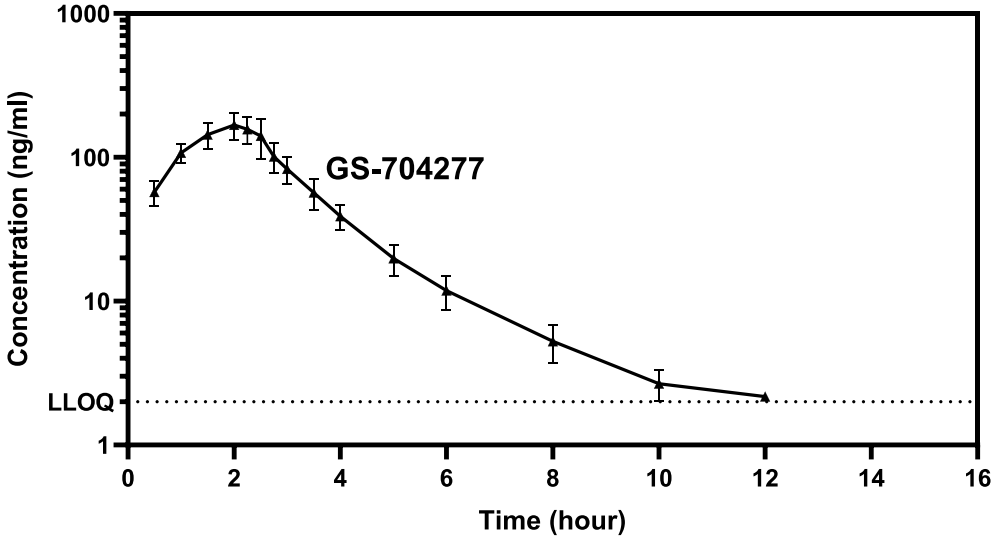

Fig. 5C. GS-704277 mean (n = 10) plasma concentration-time profiles after GS-5734 150-mg intravenous injection over a 2-h period with lyophilized formulation from Gilead study GS-US-399-1812.

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, benchtop stability in plasma, freeze/thaw stability in plasma, benchtop stability in whole blood, and long-term frozen storage stability in plasma. In addition, the individually optimized LC gradient for each analyte avoid carryover issue that would happen when using a single LC gradient for all analytes. Detecting GS−704277 in negative ion mode made better sensitivity than detecting it in positive ion mode, which is better for both GS−5734 and GS−441524. Overall, the validated method was precise, accurate, reproducible and robust enough for its application in multiple clinical studies that were the basis of a preliminary new drug application for RDV.

CRediT authorship contribution statement

Deqing Xiao: Writing - original draft, Writing - review & editing. **Kah Hiing John Ling:** Writing - review & editing, Supervision. **Thomas Tarnowski:** Writing - review & editing, Supervision. **Rita Humeniuk:** Writing - review & editing. **Polina German:** Supervision. **Anita Mathias:** Supervision. **Jasper Chu:** Methodology, Data curation. **Yuan-Shek Chen:** Methodology, Supervision. **Eric van Ingen:** Supervision, Writing - review & editing.

Declaration of competing interest

The authors have declared that no conflicts of interest exist.

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