**Development and validation of a UHPLC-MS/MS method for the quantification of the prodrug remdesivir and its metabolite GS-441524: a tool for clinical pharmacokinetics of SARS-CoV-2/COVID-19 and Ebola virus disease.**

**Authors:** Valeria AVATANEO1•, Amedeo DE NICOLÒ1, Jessica CUSATO1, Miriam ANTONUCCI1, Alessandra MANCA1, Alice PALERMITI1, Catriona WAITT2,3, Stephen WALIMBWA3, Mohammed LAMORDE3, Giovanni DI PERRI1,4 and Antonio D’AVOLIO1,4.

1 Laboratory of Clinical Pharmacology and Pharmacogenetics#, Amedeo di Savoia Hospital, Department of Medical Sciences, University of Turin, Turin, Italy.

2 Department of Molecular and Clinical Pharmacology, University of Liverpool, Liverpool, United Kingdom.

3 Infectious Diseases Institute, Makerere University College of Health Sciences, P.O. Box 22418, Kampala, Uganda.

4 CoQua Lab, Turin, Italy.

# PHASE I AIFA, UNI EN ISO 9001 and 13485 Certificate Laboratory; Certificate No. IT-64386 and DM/17/154/S; Certification for: “DESIGN, DEVELOPMENT AND APPLICATION OF DETERMINATION METHODS FOR CLINICAL ANALYTES AND DRUGS, ALSO WITH REFERENCE TO IN VITRO DYAGNOSTICS. PHARMACOGENETIC ANALYSES.” and "DESIGN AND DEVELOPMENT AND APPLICATION OF QUANTIFICATION AND DETECTION METHODS OF CLINICAL ANALYTES AND DRUGS, FINALIZED TO THE PRODUCTION OF IN VITRO DIAGNOSTICS" www.tdm-torino.org

•**Corresponding Author:** Valeria Avataneo (BSc, MSc)

Tel. +39.011.4393867, Fax: +39.011.4393996; e-mail: valeria.avataneo@unito.it.

**Short Running Title: Remdesivir and GS-441524 plasma quantification.**

**Number of Figures and Tables:** 1 and 4

**Synopsis**

**Background:** Remdesivir has received significant attention for its potential application in the treatment of COVID-19 caused by SARS-CoV-2. Remdesivir has already been tested for Ebola virus disease treatment and found to have activity against SARS and MERS coronaviruses. Remdesivir core is constituted by GS-441524 which interferes with RNA-dependent-RNA-polymerases alone. In non-human primates, following intravenous administration, remdesivir is rapidly distributed into peripheral-blood-mononuclear-cells, and converted within two hours to the active nucleoside triphosphate form, while GS-441524 is detectable in plasma up to 24 hours. Nevertheless, remdesivir pharmacokinetics and pharmacodynamics in humans are still unexplored, highlighting the need of a precise analytical method for remdesivir and GS-441524 quantification.

**Objective:** The validation of a reliable UHPLC-MS/MS method for remdesivir and GS-441524 quantification in human plasma.

**Methods:** Remdesivir and GS-441524 standards and quality controls were prepared in plasma from healthy donors. Sample preparation consisted of protein precipitation, followed by a dilution and injection in the QSight 220 UHPLC-MS/MS system. Chromatographic separation was obtained through an Acquity HSS T3 1.8µm 2.1x50mm column, with a gradient of water and acetonitrile with 0.05% of formic acid. The method was validated following EMA and FDA guidelines.

**Results:** Analytes stability has been evaluated and described in detail. The method successfully fulfilled the validation process and it was demonstrated that, when possible, sample thermal inactivation could be a good choice in order to improve biosafety.

**Conclusion:** This method represents a useful tool for studying remdesivir and GS-441524 clinical pharmacokinetics, particularly during the current COVID-2019 outbreak scenario.

1. **Introduction**

Remdesivir has received significant attention for its potential application in the treatment of COVID-19 caused by SARS-CoV-2, a zoonotic pathogen that emerged in 2019. Remdesivir is a monophosphoramidate prodrug of an adenine nucleotide analogue: its core is a 1′-cyano-substituted adenine C-nucleoside ribose analogue (GS-441524) linked to another small molecule through an ester bond, which confers it a better penetration within cells.1, 2 From 2015, remdesivir was tested in healthy volunteers during Phase I clinical trials and it then entered Phase II studies in the context of Ebola virus disease.3, 4 It soon revealed activity against SARS and MERS coronaviruses (CoVs).5, 6 Notably, CoVs are positive-sense, single-stranded RNA viruses that infect a wide range of animal hosts. In humans they are known to cause upper respiratory tract infections and pneumonia.6 Due to GS-441524 effectiveness in interfering with the activity of viral RNA-dependent RNA-polymerases and inhibiting viral RNA synthesis, remdesivir is being developed for COVID-19, with encouraging preliminary data.7-9 Concerning its metabolism, Warren *et al.* demonstrated that, upon intravenous (IV) administration of a 10 mg kg−1 dose in rhesus monkeys, remdesivir exhibited a short plasma half-life (t1/2 = 0.39 hours) with rapid systemic elimination followed by the appearance of transient systemic levels of a key intracellular intermediate alanine metabolite and more persistent levels of GS-441524 (detectable for over 24 hours in plasma). Thereafter, remdesivir is rapidly distributed into peripheral blood mononuclear cells (PBMCs), and is converted within two hours into the active nucleoside triphosphate form.1

However, information about remdesivir pharmacokinetics (PK) and pharmacodynamics (PD) in humans are inadequate and no therapeutic or toxic ranges have been reported, and this is partially due to the small number of patients who were treated with remdesivir. Therapeutic Drug Monitoring (TDM), consisting in the measurement of drugs concentration in biological fluids in order to optimize drug posology avoiding toxic effects or therapeutic failures is already well established in several areas, such as for the HIV treatment, and could be useful even in this context.10-12 Therefore, both for PK studies and for a possible future TDM purpose, there is the emerging need for a reliable analytical method for remdesivir and its metabolite GS-441524 quantification in human plasma. Here we present the first Ultra-High Performance Liquid Chromatography coupled to tandem Mass Spectrometry (UHPLC-MS/MS) method, validated according to FDA and EMA guidelines, for both remdesivir and GS-441524 determination.

1. **Material and Methods**
   1. ***Chemicals***

HPLC grade acetonitrile (ACN) and methanol (MetOH) were purchased from VWR Chemicals (Radnor, Pennsylvania); Mass grade H2O (MilliQ) was produced with Milli-DI system coupled with a Synergy 185 system by Millipore (Milan, Italy); dimethyl sulfoxide (DMSO) and 6,7-dimethyl-2,3-di(2-pyridyl)quinoxaline (QX; purity 98.5%, used as Internal Standard - IS) were purchased from Sigma-Aldrich Corporation (Milan, Italy). Blank plasma from healthy donors was supplied by the Blood Bank of *Città della Salute e della Scienza* of Turin (Italy).

Remdesivir (purity 98.3%) and its metabolite GS-441524 (purity 98%) were kindly donated by CoQua Lab (Turin, Italy). All powders were stored at -20°C in the dark, in order to prevent any possible degradation.

* 1. ***Stock solutions, Internal Standard, Standards, and Quality Controls***

Remdesivir and GS-441524 stock solutions (1 mg/mL) were prepared in a mixture of DMSO:MetOH 50:50 (v:v) while QX stock solution (1 mg/mL) in a mixture of H2O:MetOH 5:95 (v:v).

Remdesivir and GS-441524 stock solutions were stored at -80°C in the dark until use (with expiry date 6 months later), while QX stock solution was stored at 4°C (with expiry date 1 year later).

Series of aliquots of the highest standard (STD) sample of the calibration curve, STD 9, and quality controls (QCs) were prepared by independently spiking blank plasma with stock solutions and then stored at -80°C. The same calibration ranges and QC concentrations were used both for remdesivir and GS-441524, in accordance with few information reported in literature: STD 9 = 1000 ng/mL, QC H (high) = 800 ng/mL, QC M (medium) = 100 ng/mL, QC L (low) = 10 ng/mL and STD 1 (the lowest point of the calibration curve) = 3.91 ng/mL.13 An overview of all the concentrations is reported in Table S1.

All other standards (8 to 1) of the calibration curve were prepared by serial 1:1 dilutions of STD 9.

* 1. ***UHPLC-MS/MS analysis***

A Perkin Elmer LX-50 ® UHPLC system coupled with Triple Quadrupole QSight 220 ® (Perkin Elmer, Milan, Italy) was used for the chromatographic analysis. Chromatographic separation was obtained on an Acquity® HSS T3 1.8μm 2.1x50mm column (Waters, Milan, Italy), protected by a physical filter [“Frit”, 0.2 µm, 2.1 mm] (Waters, Milan, Italy) precolumn, at 40°C using a column thermostat. The gradient elution was obtained by using two different mobile phases: phase A (H2O + formic acid 0.05%) and phase B (ACN + formic acid 0.05%) (table 1). Positive electrospray ionization (ESI+) was used for all the analytes. The quantification MRM traces (m/z) were: 603.15>200 for remdesivir, 292>163 for GS-441524 and 313.2>78.05 for QX. All instruments settings are detailed in Table 2.

* 1. ***Sample extraction protocol***

The extraction procedure consists of a low-cost and rapid protein precipitation: briefly, 100μl of IS working solution (H2O:MetOH 70:30 v:v added with QX at the concentration of 100ng/mL) and 600μl of precipitant solution, consisting in a mixture of MetOH:ACN 50:50 (v:v), are added to a volume of 50μl of plasma sample/calibration standard/quality control (QC). After being vortexed for 30 seconds, samples undergo centrifugation (21000 x *g* for 10 minutes at 4°C); then, 300µL of the supernatant is diluted with 600μl of pure water, mixed and injected (8 μl) into the UHPLC system.

* 1. ***Specificity, selectivity, accuracy, precision and limit of quantification/detection***

Six inter-day validation sessions were performed, as stipulated by FDA and EMA guidelines.14-16 Accuracy and inter-day imprecision were evaluated performing the quantification in duplicate of the three different QC samples during each validation session; intra-day imprecision was evaluated in five intra-day replicates. Inter-day and intra-day imprecision were expressed as the relative standard deviation (RSD) at each QC concentration. Integration was performed considering peak areas for each analyte.

Specificity and selectivity were evaluated using six individual sources of the blank plasma matrix, individually analyzed and evaluated for interferences. Also the extent of any interference caused by possible co-administered medications has been investigated: briefly, an aliquot of blank plasma was spiked with fourteen antiretroviral drugs currently used for the treatment of HIV (amprenavir, atazanavir cobicistat, darunavir, dolutegravir, efavirenz, elvitegravir, etravirine, lopinavir, maraviroc, nevirapine, raltegravir, rilpivirine and ritonavir) and analyzed.15 The absence of detectable interfering peaks at the analytes retention times were considered as lack of interference.

The Upper Limit Of Quantification (ULOQ) corresponds to STD 9, the highest calibration standard, for both the analytes; Lower Limits Of Quantification (LLOQ), were the lowest concentration of analytes in a sample which can be quantified reliably, with a deviation from the nominal concentration (measure of accuracy) and RSD (measure of precision) lower than 20% and with a signal-to-noise ratio higher than 5.15 On the other hand, limit of detection (LOD) was considered as the lower dilution of LLOQ which yielded a signal-to-noise ratio higher than 3.

In order to ensure a good coverage, even in case of therapeutic regimens different from what adopted against Ebola virus (such in the case of CoVs), the defined calibration range was used to quantify a standard higher than the ULOQ, spiked at the concentration of 3000 ng/mL for both analytes.

* 1. ***Recovery and extraction efficiency***

Recovery (REC) was evaluated during six validation sessions at high, medium and low concentrations by comparing peaks areas from extracted QCs (pre-spiked) with those obtained by the direct injection of a chemical mix containing both the drugs and the IS at the same concentrations as the QCs (rec).14 The extraction efficiency (EE) was measured by comparing the peak areas of pre- and post-spiked samples.

* 1. ***Matrix effect and IS-normalized matrix effect***

Separate plasma samples from six healthy donors were used for the preparation of standards and for the evaluation of matrix effect (ME). The ME was calculated by comparing the signal from the analysis of a post-extraction spiked samples (post-spiked) at high, medium and low QC levels with the ones from the direct injection of the same concentration of analytes without matrix, as described by Taylor and in FDA guidelines (post-extraction addition method).14, 17

The IS-normalized matrix (IS-nME) effect was calculated as described by De Nicolò *et al.*18-20

* 1. ***Stability and impact of thermal inactivation***

As a preliminary experiment, the photostability of the analytes was tested: three concentrations of “rec” were considered (rec H, rec M, and rec L) and analyzed by keeping two aliquots for each level, one in the dark and the other under the light, on the bench top for 4 hours (a large excess of the maximum time requested by the extraction protocol).

Stability was assessed by maintaining single aliquots of the QCs in the following conditions: 24 hours bench-top at room temperature (RT), 24 hours at 37°C, 24 hours at +4°C, 24 hours at -20°C and 1, 2, 4, 5 and 7 months at -80°C. Three freeze-thaw cycles were monitored. Furthermore, in order to measure the processed sample stability, extracted samples were maintained 24 hours and 7 days in the autosampler at +10°C.15 All the above-mentioned tested conditions were compared with “freshly” extracted QCs, which had been stored at -80°C since preparation.

Finally, in the context of biosafety, the effect of thermal inactivation on analytes has been studied: based on the study by Rabenau and colleagues concerning SARS-CoV, which demonstrated that heat treatment at 56°C over 30 minutes reduced the virus titer below the detection limit, three aliquots of QCs, coming from -80°C, have been directly put and maintained at 58°C for 38 minutes.21 Considering also the importance of disulphide bonds for the maintenance of enzymes conformation (considering in this case plasma esterases) and the impact of heat on disulphide bonds, three aliquots of QCs that underwent thermal inactivation were then kept 24 hours bench-top at RT and finally compared with “freshly” extracted QCs.22, 23

1. **Results**
   1. ***Specificity and selectivity***

Mean retention times for the considered analytes were 0.98 min for GS-441524, 1.67 min for remdesivir and 1.72 min for QX, the IS (Figure 1). Blank plasma, alone and spiked with antiretroviral drugs, presented no interfering peaks at the analytes retention times (Figure S1).

* 1. ***Accuracy, imprecision, ULOQs, LLOQs and LODs***

Accuracy and imprecision values for each analyte at the three QC levels are summarized in Table 3. All these parameters fitted FDA and EMA guidelines. The ULOQ coincides with STD 9 for both remdesivir and GS-441524 (1000 ng/mL), the LLOQ value for both the analytes was 0.98 ng/mL while the LOD values were 0.24 ng/mL for remdesivir and 0.98 for GS-441524 (Figure S2).

Calibration curves had a good fit with linear through zero regression models, with a *1/X* weighting factor, to ensure high accuracy at low concentrations. Determination coefficients (r2) of calibration curves were all above 0.998.

The defined calibration range revealed able to quantify the highest standard (3000 ng/mL for both remdesivir and GS-441524), without requiring a pre-dilution step, and with a deviation from the nominal concentration lower than 20%.

* 1. ***Recovery, extraction efficiency, matrix effect and IS-normalized matrix effect***

All the parameters fitted FDA and EMA guidelines and are detailed in Table 3. Mean values were, as follows: REC was 71% (RSD 6%) for remdesivir and 102% (RSD 7%) for GS-441524; EE was 67% (RSD 9%) for remdesivir and 105% (RSD 10%) for GS-441524; ME was 6% (RSD 4%) for remdesivir and -2% (RSD 12%) for GS-441524; IS-nME was -5% (RSD 4%) for remdesivir and -6% (RSD 8%) for GS-441524.

* 1. ***Stability and impact of thermal inactivation***

Any photodegradation was observed for remdesivir or GS-441524. All results obtained from the stability tests are reported in Table 4. Both remdesivir and GS-441524 remained stable in QCs conserved at -80°C for over four months; moreover, remdesivir revealed to be stable in the stock solution for at least 10 months (GS-441524 stock solution was not tested yet). Nevertheless, remdesivir, when dissolved in plasma, was found to be unstable at RT and +4°C, even for 24 hours; on the contrary, in extracted plasma samples remdesivir is stable for up to 7 days in the autosampler (10°C).

Finally, the inactivation of three QC levels by keeping at 58°C for 38 minutes did not have a significant impact on analytes concentrations (mean degradation observed was 7% for remdesivir and none for GS-441524); interestingly, the QCs that were previously stressed by heat and then kept at RT for 24 hours showed only a mean 26% degradation for remdesivir (no degradation was observed for GS-441524).

1. **Discussion and Conclusion**

We report the first, to the best of our knowledge, published method for remdesivir and GS-441524 quantification using a highly precise quantitative technology, UHPLC-MS/MS. The validation procedure here reported suggests that it is feasible to perform TDM for remdesivir and GS-441524, that could then be applied to identify therapeutic and/or toxic ranges, in order to individualize dosing, avoid toxicity and minimize the risk for therapeutic failures. This assay is important because it could be applied to clinical research, not only for COVID-19, but also for Ebola virus disease. In 2019, interim analysis of the PALM clinical trial comparing four therapeutics for Ebola virus (Zaire) outbreak found lower mortality rates for two monoclonal antibody products (mab114, REGN-EB3) and these drugs have been prioritized over remdesivir.24 However, remdesivir remains relevant as an investigational therapeutic for other Ebola strains (Sudan virus, Bundibugyo virus) and for Marburg virus disease where therapies are currently lacking.

The marked remdesivir degradation observed only in the presence of unextracted plasma may be due to intense residual activity of esterases, probably inhibited by the low temperature when stored at -20 and -80°C. This phenomenon may explain the relatively low REC and EE (around 70%): in pre-spiked samples, remdesivir is possibly degraded by plasma esterases, as already demonstrated for artesunate in the context of malaria, while this reaction does not occur in post-spiked samples, where the plasma does not contain its proteins anymore, and degradation does not occur in chemical mix spiked with the drug.25 Another confirmation of this phenomenon comes from the observation that, after thermal inactivation, QCs are more stable if kept at RT for 24 hours, probably because plasma residual esterases loss their original conformation when excessively stressed by heat. These findings have implications for sample collection, transportation, storage and biosafety when processing for TDM or PK evaluation of remdesivir in tropical countries, where ambient temperatures may be high and access may be limited to a cold-chain for sample transportation and storage. In this study, we observed that, if samples undergo thermal inactivation immediately after withdrawal and they are then stored in a freezer (maybe -20°C could be enough), a large part of degradation could be avoided.

Importantly, in PBMCs the nucleoside triphosphate represents the predominant metabolite and it tends to accumulate (with a t1/2 of 14 hours). Consequently, a parallel method for the intracellular quantification of remdesivir and of the triphosphate active form in the near future is assured, following an already tested protocol.26-28

In conclusion, although this method wasn’t applied to real-life samples yet (due to a couple of factors: remdesivir is in Phase II evaluation and the number of treated patients is still low, to date, in Italy), it represents the first step in order to ensure a useful tool for the study of remdesivir PK.

1. **Acknowledgments:** A special thanks to Dr. Massimo Tempestilli (Spallanzani Hospital, Rome) for his kind support. This research is part of the EDCTP2 programme supported by the European Union (RIA2018EF-083).
2. **Funding:** This research is part of the EDCTP2 programme supported by the European Union (RIA2018EF-083).
3. **Transparency declarations:** none to declare.
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**Figure legends:**

**Figure 1:** overlaid chromatograms of GS-441524, remdesivir (RDV) and QX (the internal standard) with respective retention times, obtained from the injection of a STD 9, the highest point of the calibration curve.

**Figure 1.tif**

**Table 1:** chromatographic gradient; phase A: H2O + 0.05% formic acid; phase B: acetonitrile + 0.05% formic acid.

|  |  |  |  |
| --- | --- | --- | --- |
| Minutes | % Phase A | % Phase B | Flow (mL/min) |
| 0.00 | 95 | 5 | 0.4 |
| 0.30 | 95 | 5 | 0.4 |
| 0.35 | 70 | 30 | 0.4 |
| 1.50 | 30 | 70 | 0.4 |
| 1.80 | 10 | 90 | 0.4 |
| 2.80 | 10 | 90 | 0.4 |
| 2.90 | 95 | 5 | 0.4 |
| 4.00 | 95 | 5 | 0.4 |

**Table 2:** mass parameters and instrumental settings; IS: internal standard.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **General instrumental settings** | | | | |
| **Drying Gas Temperature** | | 130°C | | |
| **HSID Temperature** | | 270°C | | |
| **Nebulizer Gas** | | 350 | | |
| **ElectroSpray V1 Positive** | | 5000 | | |
| **Source Temperature** | | 350°C | | |
| **Multipole 1 RF** | | 370 | | |
| **Collision Pressure** | | 410 | | |
| **Analyte-specific parameters** | | | | |
|  | **REMDESIVIR** | | **GS-441524** | **QX (IS)** |
| **Quantification trace (m/z)** | 603.15 > 200 | | 292 > 163 | 313.20 > 78.05 |
| Collision Energy | -53 | | -32 | -50 |
| Entrance voltage | 15 | | 43 | 30 |
| Collision Cell Lens 2 | -116 | | -64 | -80 |
| **Secondary ion trace (m/z)** | 603.15 > 318 | | 292 > 147 | 313.20 > 246.15 |
| Collision Energy | -28 | | -50 | -50 |
| Entrance voltage | 12 | | 2 | 30 |
| Collision Cell Lens 2 | -104 | | -80 | -80 |
| **Ionization** | ESI + | | ESI + | ESI + |

**Table 3:** overview of method validation parameters.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  | **IMPRECISION (RSD%)** | |  |  |  |  |
|  |  | **ACCURACY** | **INTRA-DAY** | **INTER-DAY** | **MEAN RECOVERY (RSD%)** | **MEAN EXTRACTION EFFICIENCY (RSD%)** | **MEAN MATRIX EFFECT (RSD%)** | **MEAN IS-NORMALIZED MATRIX EFFECT (RSD%)** |
| **REMDESIVIR** | HIGH QC LEVEL | 104% | 2% | 6% | 67% (6%) | 66% (7%) | 2% (2%) | -10% (1%) |
| MEDIUM QC LEVEL | 100% | 1% | 6% | 67% (8%) | 67% (11%) | -1% (3%) | -6,9% (3%) |
| LOW QC LEVEL | 87% | 5% | 6% | 78% (4%) | 67% (9%) | 16% (7%) | 3% (7%) |
| LLOQ | 118% | 10% | 12% |  |  |  |  |
| **Mean (RSD%)** | **102%** | **4.5%** | **7.5%** | **71% (6%)** | **67% (9%)** | **6% (4%)** | **-5% (4%)** |
| **GS-44154** | HIGH QC LEVEL | 96% | 2% | 3% | 104% (6%) | 99% (5%) | 5% (4%) | 1% (4%) |
| MEDIUM QC LEVEL | 102% | 6% | 4% | 99% (5%) | 105% (17%) | -3% (21%) | -9,2% (10%) |
| LOW QC LEVEL | 92% | 9% | 11% | 104% (10%) | 112% (9%) | -7% (10%) | -10% (10%) |
| LLOQ | 81% | 9% | 14% |  |  |  |  |
| **Mean (RSD%)** | **93%** | **6%** | **8%** | **102% (7%)** | **105% (10%)** | **-2% (12%)** | **-6% (8%)** |

**Table 4:** percentages of degradation of remdesivir (RDV) and GS-441524 in different conditions. Table A concerns aliquots of QCs maintained in different conditions and “stressed”. Table B concerns the processed samples and the stock solution stabilities.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **A** | **% of degradation** | **24h RT** | **24h 37°C** | **24h 4°C** | **24h**  **-20°C** | **2° freeze-and-thaw** | **3° freeze-and-thaw** | **1 month -80°C** | **2 months -80°C** | **4 months -80°C** | **5 months -80°C** | **7 months -80°C** | **Thermal inactivation of QCs (58°C for 38 min)** | **Thermal inactivation of QCs + 24h RT** |
| **RDV** | **H** | 93 | 99 | 17 | 0 | 6 | 6 | 0 | 4 | 0 | 0 | 0 | 9 | 26 |
| **M** | 95 | 100 | 17 | 0 | 10 | 4 | 8 | 0 | 0 | 7 | 0 | 5 | 24 |
| **L** | 100 | 100 | 22 | 2 | 5 | 8 | 16 | 2 | 0 | 0 | 0 | 4 | 27 |
| **GS-441524** | **H** | 0 | 0 | 1 | 0 | 1 | 0 | n/a | n/a | 0 | ongoing | ongoing | 4 | 0 |
| **M** | 5 | 5 | 1 | 0 | 8 | 0 | n/a | n/a | 0 | ongoing | ongoing | 0 | 1 |
| **L** | 0 | 0 | 0 | 0 | 4 | 0 | n/a | n/a | 0 | ongoing | ongoing | 0 | 0 |

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **B** | **% of degradation** | **Processed sample stability** | | **10 months in stock solution** |
| **24 hours AUTOSAMPLER (10°C) – post extraction** | **7 days AUTOSAMPLER (10°C) – post extraction** |
| **RDV** | **H** | 0 | 0 | 0 |
| **M** | 4 | 0 | 0 |
| **L** | 7 | 0 | 0 |
| **GS-441524** | **H** | 0 | 0 | ongoing |
| **M** | 0 | 0 | ongoing |
| **L** | 0 | 0 | ongoing |