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A combined assay for quantifying remdesivir and its metabolite, along with dexamethasone, in serum

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Background: As global confirmed cases and deaths from coronavirus disease 2019 (COVID-19) surpass 100 and 2.2 million, respectively, quantifying the effects of the widespread treatment of remdesivir (GS-5734, Veklury) and the steroid dexamethasone is becoming increasingly important. Limited pharmacokinetic studies indicate that remdesivir concentrations in serum decrease quickly after dosing, so its primary serum metabolite GS-441524 may have more analytical utility.

Objectives: We developed and validated a method to quantify remdesivir, its metabolite GS-441524 and dexamethasone in human serum.

Methods: We used LC-MS/MS and applied the method to 23 serum samples from seven patients with severe COVID-19.

Results: The method has limits of detection of 0.0375 ng/mL for remdesivir, 0.375 ng/mL for GS-441524 and 3.75 ng/mL for dexamethasone. We found low intra-patient variability, but significant inter-patient variability, in remdesivir, GS-441524 and dexamethasone levels.

Conclusions: The significant inter-patient variability highlights the importance of therapeutic drug monitoring of COVID-19 patients and possible dose adjustment to achieve efficacy.

Introduction

Coronavirus disease 2019 (COVID-19), which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has resulted in the death of over 2.2 million people worldwide as of 15 October 2020.[1] Two of the most promising treatments for reducing morbidity and mortality are the antiviral drug remdesivir (GS-5734, Veklury) and the steroid dexamethasone. Remdesivir is an adenosine nucleotide prodrug of the monophosphate GS-441524, the primary metabolite measured in human serum.[2,3] In host cells, GS-441524 is phosphorylated into the active triphosphate metabolite, which inhibits the RNA polymerase activity of coronaviruses. While remdesivir has not been proven to significantly reduce mortality, it can reduce the duration of hospital stay in severe cases of COVID-19.[4–6] Quantifying GS-441524 is useful for understanding the pharmacokinetics of remdesivir, as previous methods have shown that remdesivir is rapidly metabolized within 24 h to levels below the limit of quantification.[7,8]

Dexamethasone, a glucocorticoid, is the first COVID-19 treatment found to reduce mortality in clinical trials.[9] Dexamethasone reduces the transcription of several inflammatory agents, potentially decreasing the severity of the innate inflammatory pathways that can lead to organ failure and death.[10]

Current NIH guidelines recommend the use of both remdesivir and dexamethasone in severe COVID-19, making their simultaneous quantification important.[11] Low levels of remdesivir due to drug–drug interactions or pharmacogenomic variation may partially explain the mixed clinical efficacy of the drug in various trials.[12,13] Thus, in an attempt to improve the breadth and analytic sensitivity of published methods for therapeutic drug monitoring,[14] we developed and validated an LC-MS/MS assay that simultaneously quantifies remdesivir, its primary metabolite GS-441524 and dexamethasone.

Methods

Chemicals

We purchased reference standards for remdesivir and GS-441524 (both 98% purity) from Aobious, for dexamethasone (≥98% purity) from Cayman

Chemical and for the internal standard dapivirine-d11 (96% chemical purity, <98% isotopic purity) from Santa Cruz Biotechnology. All solvents were HPLC-grade. We purchased water from Aqua Solutions, Inc., acetonitrile (ACN) and methanol (MeOH) from Honeywell Burdick and Jackson, and DMSO from Fisher Scientific. The drug-free serum we used is a product from UTAK Laboratories.

Stock and intermediate solutions

Remdesivir, dexamethasone and dapivirine-d11 were prepared at 1 mg/mL in MeOH and GS-441524 was prepared at 1 mg/mL in DMSO. All stock solutions were stored at −80°C. Intermediate mixes were prepared with 1:1 MeOH: H_2O (v/v).

Sample analysis

We used a protein precipitation method followed by evaporation. Similar methods have been published elsewhere.[14–18] To 50 μL of serum we added 100 μL of 7.5 μg/mL dapivirine-d11 in 1:1 MeOH: H_2O (v/v). We precipitated the proteins by adding 600 μL of cold 1:1 MeOH:ACN followed by vortex mixing and centrifugation (2800 **g** for 10 min). We evaporated the supernatant and reconstituted it in the same volume of H_2O.

We injected 10 μL of extract into an LC-MS/MS system (Agilent LC 1260-AB Sciex API 5500, Agilent Technologies, Santa Cruz, CA, USA and AB Sciex, Foster City, CA, USA) and used positive electrospray ionization in multiple reaction monitoring mode. We separated the analytes using an Agilent Poroshell 120 EC-C18 column (3 × 50 mm, 2.7 μm particle size) with gradient elution. H_2O and ACN were mobile phase A and B, respectively (Table 1).

Following the 14 calibration points, we injected two blanks and then low (0.6 ng/mL remdesivir, 12 ng/mL GS-441524 and 24 ng/mL dexamethasone), mid (12 ng/mL remdesivir, 120 ng/mL GS-441524 and 120 ng/mL dexamethasone) and high (120 ng/mL remdesivir, 1200 ng/mL GS-441524 and 1200 ng/mL dexamethasone) quality controls (QCs). Passing runs consisted of three sets of QCs within 15% accuracy and 15% precision, run at the beginning, middle and end of a sample batch.

Data analysis

We analysed the data using AB Sciex Analyst 1.6.3 and AB Sciex MultiQuant 2.1. We used the two most abundant fragment ion transitions and retention time to confirm peak identity. The area of the reference drugs over the area of dapivirine-d11 was used for quantification. We used 1/x weighted linear regression for the calibration curves.

Table 1. Gradient profile of the chromatographic method

Total time (min)	Flow rate (μL/min)	A (%)	B (%)
0.3	550	95	5
0.35	550	70	30
1	550	30	70
1.3	700	10	90
3.3	700	10	90
3.4	750	0	100
4.4	750	0	100
4.5	550	95	5
10	550	95	5

Method validation

We evaluated the linearity, sensitivity, precision, accuracy, matrix effect, recovery, dilution effect, injection repeatability, carry-over and specificity of the method. We also tested the stability of unextracted samples using this method. On three separate days, we ran a calibration curve (0.015–135 ng/mL for remdesivir, 0.15–1350 ng/mL for GS-441524 and 0.15–1350 ng/mL for dexamethasone). Passing criteria included an r value ≥0.95 and ≥75% of calibration points within ±20% accuracy. The limit of detection (LOD) was defined as the lowest concentration at which the signal to noise ratio was ≥3. The lower limit of quantification (LLOQ) was the lowest point with a signal to noise ratio ≥10 that maintained an r value ≥0.95.

We tested precision and accuracy by thrice preparing and running five replicates of spiked drug-free serum at the low, mid and high QC levels alongside a calibration curve. We assessed the intra- and inter-day imprecision via coefficient of variation (CV) and the intra- and inter-day accuracy via relative error (RE).

We tested the matrix effect by spiking low, mid and high QC levels into H_2O and comparing the concentrations with the same levels spiked into drug-free serum and then extracted. We tested recovery by spiking low, mid and high QC levels into extracted matrix and comparing the concentrations with the same levels spiked into drug-free serum and then extracted.

Due to the high matrix enhancement effect observed in remdesivir, we also analysed the matrix effect of remdesivir in five replicates of six patient samples that did not have detectable levels of remdesivir. We spiked remdesivir into the six patient samples and the drug-free serum at the three QC levels and compared the precision of the matrix effect within the replicates and between the seven serum types.

Due to the low quantitative range of remdesivir in the assay, we tested the effect of diluting samples with concentrations above the upper limit of quantification (ULOQ). We extracted five replicates of samples spiked 20-fold higher than the three QC levels and then diluted these samples 20-fold with extracted matrix blank. We assessed the precision (CV) and accuracy (RE) of these 15 samples to determine the dilution effect.

We tested injection repeatability by injecting a vial of low, mid and high QC levels five times in a row and assessing the CV. We tested carry-over by injecting the high QC once, twice and then thrice, each time preceded and followed by three matrix blank injections.

For specificity, we ran drug-free serum spiked with 20 common cold, flu and antiviral drugs at low and high levels of the drug's therapeutic range, or the therapeutic range for a similar drug if no published therapeutic range was found (Table 2). A peak was considered to interfere with the target analyte if it fell within 0.2 min of the established retention time. We also analysed specificity of the patient samples obtained.

We assessed the stability of unextracted samples at room temperature, in an ice bath (kept at approximately 2°C) and after three freeze-thaw cycles. For all three treatments, GS-441524 and dexamethasone were run at the low, mid and high QC levels. Remdesivir was run at elevated levels (2000, 1000 and 200 ng/mL) to replicate the higher remdesivir levels in patients immediately following infusion and then samples were diluted post-extraction with extracted matrix. All levels were run in triplicate and analytes were considered stable if the final timepoint concentration was within 20% of the original concentration. For the room temperature and ice bath experiments, we spiked drug-free serum with the analytes, capped the tubes and placed them in the appropriate storage area. We extracted at 0, 1, 2, 4, 6 and 8 h. In addition to the 8 h stability experiments, we also conducted a room temperature stability experiment of 48 h. We spiked remdesivir at the higher concentrations described earlier and spiked GS-441524 and dexamethasone at 20-fold higher than the QC levels. Following extraction at 0, 8, 24, 36 and 48 h, we diluted the samples 20-fold with extracted matrix. For freeze-thaw stability, samples were spiked as described above, frozen at −80°C and then thrice removed, thawed and returned to the freezer. To control for stability at −80°C, split samples were

Table 2. Drugs and their concentrations investigated in the specificity experiment

	Drug	Low (ng/mL)	High (ng/mL)
A	Dextromethorphan	10	50
	Tenofovir	10	50
	Codeine	10	50
	Diphenhydramine	10	50
	Dolutegravir	10	50
	Ibuprofen	10 000	50 000
	Aspirin	10 000	50 000
B	Cortisone	100	500
	Rilpivirine	100	500
	Prednisone	100	500
	Emtricitabine	100	500
	Prednisolone	100	500
	Pseudoephedrine	100	500
C	Cortisol	1000	5000
	Hydroxychloroquine	1000	5000
	Cabotegravir	1000	5000
	Darunavir	1000	5000
	Efavirenz	1000	5000
	Dapivirine	1000	5000
	Acetaminophen	1000	5000

stored at −80°C in parallel with the freeze-thaw experiment and sampled at each thaw cycle (0, 1, 2 and 4 h).

Patient sample testing

We obtained 26 remnant serum samples for up to 6 days from seven hospitalized patients seen at Zuckerberg San Francisco General Hospital between 23 September 2020 and 30 September 2020. These patients were confirmed to be positive for SARS-CoV-2 using routine molecular diagnostic methods. As part of their medical care, they were treated with remdesivir, dexamethasone or both. The Institutional Review Board (IRB) of the University of California, San Francisco, approved the use of remnant samples and review of medical and pharmacy records without consent. The samples were de-identified prior to delivery to the testing laboratory. As a condition of the IRB approval, results of the drug levels were not made known to either the patient or attending medical staff.

COVID-19 restrictions meant that we did not always have access to patient samples immediately following sample collection. Samples were stored for 2–5 days in the refrigerator and then for up to a month at −20°C before being extracted. This delay in receiving samples meant that we did not implement a sample inactivation procedure, as we would not have been able to inactivate immediately following sample collection.

Results

Method development

We developed a 10 min method to quantitatively analyse remdesivir (GS-5734), its primary plasma metabolite GS-441524 and dexamethasone in serum using LC-MS/MS. For the three target analytes and the chosen internal standard (dapivirine-d11), we analysed two mass spectral transitions (Figure 1). We used the most abundant transition as the quantifier ion and the other transition as the qualifier ion (Table 3).

Method validation

We validated the method using a 13-point calibration curve and three QC levels spanning the low, mid and high ends of the linear range.

Range and linearity

For remdesivir, the LOD and LLOQ were 0.0375 ng/mL and the ULOQ was 135 ng/mL. For GS-441524, the LOD and LLOQ were 0.375 ng/mL and the ULOQ was 1350 ng/mL. For dexamethasone, the LOD and LLOQ were 3.75 ng/mL and the ULOQ was 1350 ng/mL. The average linearity coefficients of determination for remdesivir, GS-441524 and dexamethasone were 0.998, 0.996 and 0.997, respectively.

Precision and accuracy

In assessing precision, all three analytes had average within- and between-run CVs <8%. Precision was lower at the low QC level than at the mid and high QC levels. In assessing accuracy, all three analytes had average within- and between-run REs <14%. See Table 4.

Matrix effect and recovery

The matrix greatly enhanced the remdesivir signal. At low, mid and high QC levels, the matrix effect was 601.7%, 787.5% and 634.5%, respectively. The CV at each level was <12%. The matrix effect was less pronounced for GS-441524 and dexamethasone. The matrix effect of GS-441524 at the low, mid and high QC levels was 2.1%, −2.2% and −2.4%, respectively. The CV at each level was <4%. The matrix effect of dexamethasone at the low, mid and high QC levels was 14.7%, −10.6% and −15.8%, respectively. The CV at each level was <12% (Table 5).

The precision of the remdesivir matrix effect was similar within the five replicates and between the six patient samples and drug-free serum sample. The CV at the low, mid and high QC levels was 3.0%, 4.5% and 2.0%, respectively. The precision of the five replicates for each QC level was <8% for all seven serum types (Table 6).

The recovery of remdesivir at the low, mid and high QC levels was 86.7%, 87.1% and 85.6%, respectively. The CV at each level was <6%. The recovery of GS-441524 at the low, mid and high QC levels was 85.3%, 92.7% 88.9%, respectively. The CV at each level was <5%. The recovery of dexamethasone at the low, mid and high QC levels was 88.4%, 86.4% and 82.1%, respectively. The CV at each level was <8% (Table 5).

Dilution effect

Dilution did not significantly affect the method precision and accuracy, as CV across the three QC levels was <10% and RE was <12% (Table 7).

Injection repeatability

The CV for each QC level was <4% for all analytes.

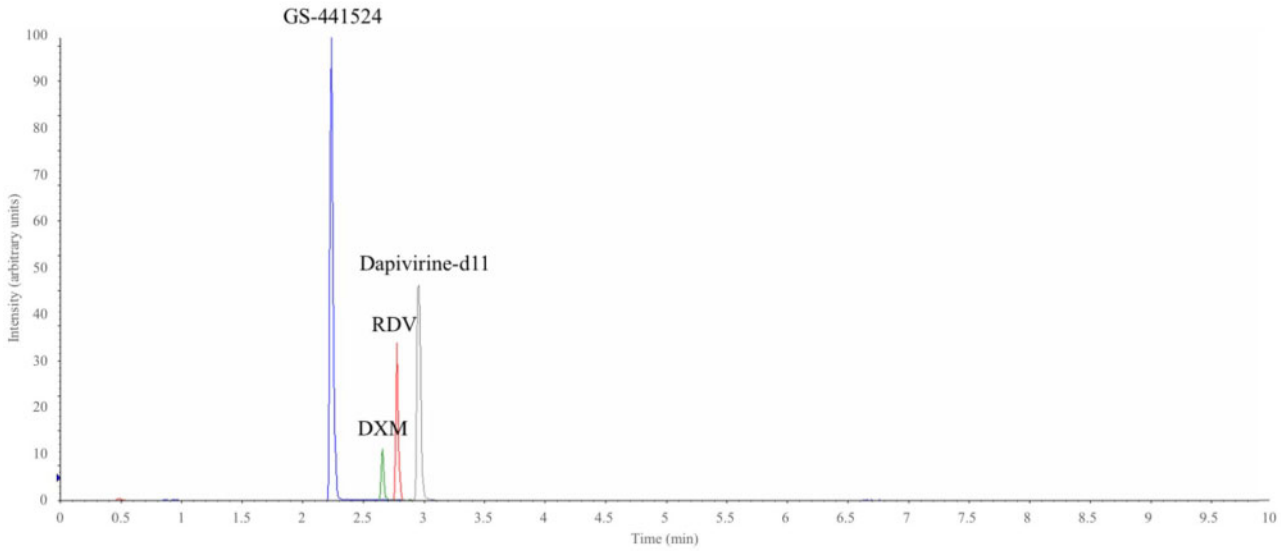

Figure 1. Chromatogram of remdesivir (RDV), GS-441524, dexamethasone (DXM) and dapivirine-d11 from a mid QC injection. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Table 3. MS parameters for each analyte in the panel

ID	Q1 mass (Da)	Q3 mass (Da)	Time (ms)	Declustering potential (V)	Exit potential (V)	Collision energy (V)	Collision exit potential (V)
Remdesivir-1	603.5	200	40	71	12	43	20
Remdesivir-2	603.5	403.2	40	11	12	21	20
GS-441524-1	292	147	40	56	12	39	18
GS-441524-2	292	163	40	91	12	39	18
Dexamethasone-1	393.5	147	40	76	12	25	12
Dexamethasone-2	393.5	326.1	40	81	12	13	24
Dapivirine-d11-1	341.5	168	40	121	12	12	43
Dapivirine-d11-2	341.5	152.2	40	121	12	12	14

Table 4. Within- and between-run precision and accuracy

		Within-run			Between-run		
Analyte	Parameter	low QC	mid QC	high QC	low QC	mid QC	high QC
Remdesivir	precision (CV)	5.57%	3.51%	2.29%	4.11%	2.92%	1.86%
	accuracy (RE)	4.40%	5.22%	7.29%	4.51%	6.99%	5.32%
GS-441524	precision (CV)	5.95%	3.62%	2.87%	3.89%	2.14%	1.82%
	accuracy (RE)	7.12%	6.59%	7.17%	3.02%	4.62%	7.72%
Dexamethasone	precision (CV)	4.84%	2.06%	1.42%	4.09%	2.14%	2.42%
	accuracy (RE)	1.96%	0.07%	6.94%	3.51%	4.53%	7.95%

Carry-over

No signal above the threshold signal-to-noise ratio of three was detected in any of the matrix blank samples for GS-441524 or dexamethasone. The remdesivir signal was observed, but decreased after one matrix blank injection.

Specificity in spiked samples

No interfering signal for remdesivir, GS-441524 or dexamethasone was detected at the correct retention time after running low and high concentrations of 20 other common cold, flu and antiviral drugs.

Table 5. Matrix effect and recovery and their precision

	Remdesivir	CV	GS-441524	CV	Dexamethasone	CV
Matrix effect low QC	601.69%	11.28%	2.06%	3.38%	14.74%	11.73%
Matrix effect mid QC	787.48%	9.46%	−2.23%	2.49%	−10.60%	3.29%
Matrix effect high QC	634.46%	8.14%	−2.41%	2.44%	−15.78%	3.92%
Matrix effect mean	674.54%	9.63%	−0.86%	2.77%	−3.88%	6.31%
Recovery low QC	86.69%	5.95%	85.32%	4.73%	88.38%	7.53%
Recovery mid QC	87.06%	4.72%	92.72%	2.88%	86.38%	2.16%
Recovery high QC	85.64%	1.90%	88.91%	2.01%	82.11%	2.77%
Recovery mean	86.46%	4.19%	88.98%	3.21%	85.62%	4.15%

Table 6. Precision of remdesivir matrix effect within and between six patients and drug-free serum

	Low QC (CV)	Mid QC (CV)	High QC (CV)
Patient 1	4.5%	0.7%	3.4%
Patient 2	2.3%	1.0%	1.6%
Patient 3	3.2%	3.3%	5.6%
Patient 4	0.9%	1.6%	7.6%
Patient 5	4.1%	2.6%	0.8%
Patient 6	3.4%	2.6%	1.7%
Drug-free serum	2.3%	1.6%	3.9%
CV between all serums	3.0%	4.5%	2.0%

Table 7. Dilution effect following 20-fold dilution using extracted matrix blank

		Low QC	Mid QC	High QC
Remdesivir	precision (CV)	4.6%	8.9%	9.6%
	accuracy (RE)	2.5%	3.1%	1.2%
GS-441524	precision (CV)	5.2%	5.4%	7.6%
	accuracy (RE)	7.4%	2.4%	9.4%
Dexamethasone	precision (CV)	3.0%	6.9%	5.8%
	accuracy (RE)	11.7%	1.9%	6.7%

Stability

At room temperature, the unextracted sample stability of remdesivir at all three concentrations was less than 80% after 1 h (Table 8). Over 8 h, remdesivir concentration decreased by approximately two-thirds and the 200 ng/mL sample was below the LOD after 48 h (Table 9). Concentrations of 1000 and 2000 ng/mL remained quantifiable after 48 h. Conversely, GS-441524 and dexamethasone remained stable over 48 h at room temperature. When the samples were stored in an ice bath, the three analytes remained stable at all measured concentrations for the duration of the experiment (8 h). The analytes remained stable after two freeze-thaw cycles (Table 10). After the third freeze-thaw, low concentrations of remdesivir decreased by 26% from the original concentration. GS-441524 and dexamethasone remained stable through all three freeze-thaw cycles. In the split samples stored at −80°C during the freeze-thaw experiment, all three analytes remained stable for the duration of the experiment (4 h).

Patient sample testing

Remdesivir, GS-441524 and dexamethasone were quantified in 23 samples obtained from seven COVID-19 patients. Remdesivir was given in six of the seven patients (Table 11). We did not observe remdesivir or GS-441524 in samples of the patient that did not receive remdesivir (3A–3E) as well as in a sample collected from a patient prior to remdesivir administration (7A). Dexamethasone was given in five of seven patients. We did not observe dexamethasone in the two patients that were not administered dexamethasone (5 and 6), verifying the specificity of our method in clinical samples.

After their administration remdesivir and dexamethasone were <LOD in some samples, while GS-441524 was quantified in all relevant samples.

Discussion

Although several vaccine candidates are currently in late stage trials, SARS-CoV-2 will likely remain an important human pathogen for the foreseeable future and optimized treatment regimens will be critical. Thus far, only remdesivir (an antiviral) and dexamethasone (a steroid) are approved for the treatment of COVID-19. The data on remdesivir have been mixed, leading to interest in therapeutic drug monitoring, which may lead to more efficacious dosing and improved patient care. In this paper, we describe the development and validation of a rapid, sensitive, specific, accurate and precise method to analyse the two most important small molecule treatments currently in widespread use for COVID-19.

Method development

We initially developed a protein precipitation method without dilution to maintain a high sample concentration in the final extract. Our protein precipitation attempts suppressed the signal significantly, so we tried solid phase extraction (SPE). While SPE improved sensitivity, we then pursued a protein precipitation method that employs dilution to cut down on extraction cost and time. We modified a previously published protein precipitation method by

Table 8. Short-term stability of unextracted samples

Analyte	Treatment	Spiked	0 h	1 h	Stability	2 h	Stability	4 h	Stability	6 h	Stability	8 h	Stability
Remdesivir	room	200	233.1	151.8	65.1%	174.1	74.7%	118.1	50.6%	90.2	38.7%	63.5	27.2%
	temperature	1000	1117.4	779.6	69.8%	846.1	75.7%	629.6	56.3%	325.0	29.1%	307.3	27.5%
		2000	2116.7	1584.7	74.9%	1442.6	68.2%	1383.0	65.3%	931.5	44.0%	749.0	35.4%
	ice bath	200	227.4	235.3	103.5%	185.4	81.5%	208.1	91.5%	196.9	86.6%	199.1	87.5%
		1000	1161.0	1189.3	102.4%	989.0	85.2%	1150.4	99.1%	1184.0	102.0%	1230.5	106.0%
		2000	2238.4	2560.2	114.4%	1969.6	88.0%	2035.2	90.9%	1938.8	86.6%	2263.8	101.1%
GS-441524	room	6	5.3	5.3	100.6%	5.8	109.8%	5.6	106.9%	6.2	118.2%	5.6	105.9%
	temperature	120	119.5	125.7	105.2%	129.5	108.3%	125.9	105.3%	132.9	111.2%	135.0	113.0%
		1200	1132.8	1179.2	104.1%	1198.9	105.8%	1180.8	104.2%	1201.5	106.1%	1192.1	105.2%
	ice bath	120	117.0	118.2	101.0%	104.6	89.4%	105.0	89.7%	115.6	98.8%	117.3	100.3%
		1200	1227.6	1269.2	103.4%	1080.9	88.0%	1092.3	89.0%	1126.0	91.7%	1207.9	98.4%
Dexamethasone	room	24	23.4	21.3	91.2%	22.3	95.4%	24.8	106.1%	26.7	113.9%	24.6	105.0%
	temperature	120	116.7	106.0	90.8%	121.0	103.7%	120.8	103.5%	128.0	109.7%	125.1	107.2%
		1200	1299.4	1018.5	78.4%	1213.3	93.4%	1266.7	97.5%	1218.1	93.7%	1249.3	96.1%
	ice bath	120	110.6	113.1	102.3%	97.1	87.8%	130.4	117.9%	115.1	104.0%	108.9	98.5%
		1200	1295.5	1342.0	103.6%	1077.0	83.1%	1184.4	91.4%	1060.5	81.9%	1161.5	89.7%

All concentrations in ng/mL.

Table 9. Long-term stability of unextracted samples

Analyte	Spiked	0 h	8 h	Stability	24 h	Stability	36 h	Stability	48 h	Stability
Remdesivir	200	160.3	46.1	28.7%	5.3	3.3%	0.2	0.1%	<LOD	0.0%
	1000	971.9	332.1	34.2%	23.6	2.4%	5.6	0.6%	1.5	0.2%
	2000	1882.2	738.5	39.2%	67.8	3.6%	16.4	0.9%	3.7	0.2%
GS-441524	6	7.8	7.5	96.1%	8.3	106.0%	7.7	98.7%	8.4	107.7%
	120	112.8	119.6	106.0%	113.5	100.6%	121.1	107.3%	129.1	114.5%
	1200	1116.6	1059.2	94.9%	1081.9	96.9%	1123.4	100.6%	961.2	86.1%
Dexamethasone	6	6.6	5.3	81.2%	7.0	107.0%	5.5	83.6%	6.5	99.8%
	120	99.9	87.8	88.0%	105.7	105.8%	91.6	91.7%	106.3	106.5%
	1200	1037.5	1067.9	102.9%	996.5	96.1%	1077.2	103.8%	951.0	91.7%

All concentrations in ng/mL.

simplifying the solvent and mobile phase composition and eliminating an additional 3-fold dilution step during final extract reconstitution.[14] Thus, dilution is employed in our method only during the protein precipitation process itself. This allowed us to run samples at a higher concentration. At the same time we achieved a lower LLOQ with a newer generation platform. Increased sensitivity may allow us to quantify remdesivir much longer after its dosing despite its reported short half-life.[7,8]

As a trade-off, we must dilute post-extraction with extracted matrix blank to quantify high levels of remdesivir in samples submitted for analysis within 1 h of remdesivir dosing. Samples requiring such treatment represent a small fraction of those typically available for analysis. In such cases though, our method demonstrated high precision and accuracy following sample dilution as shown in Table 7. The ULOQ of the method for remdesivir can be effectively extended to 2.7 μg/mL with the dilution step we apply in these samples.[7,8]

Method validation

The method demonstrated reproducible linearity over a wide dynamic range (ULOQ/LLOQ = 3600 for remdesivir and GS-441524, and 360 for dexamethasone) without interference from other common cold drugs that may be used concurrently with remdesivir and dexamethasone. All precision CVs and accuracy REs were below 15%, the cut-off recommended by the FDA and CLSI.[19,20] The CV for injection repeatability was also below the established cut-off. Due to remdesivir signal carry-over following high QC injections, we recommend running blank injections following each patient sample injection, unless they are anticipated to contain low levels of remdesivir, such as samples taken more than 1 h after remdesivir dosing.[7,8] When we began developing the method, stable isotope-labelled remdesivir was not widely available, so we used dapivirine-d11, a different isotope-labelled antiviral with a similar retention time.

Both GS-441524 and dexamethasone exhibited matrix effects within 15% and recovery between 82% and 93%. More

Table 10. Freeze-thaw stability of unextracted samples compared with non-thawed vials kept in −80°C

Analyte	Treatment	Spiked	Timepoint 0	Freeze-thaw 1	Stability	Freeze-thaw 2	Stability	Freeze-thaw 3	Stability
Remdesivir	freeze-thaw	200	172.7	138.2	80.0%	151.1	87.5%	127.2	73.6%
		1000	955.9	804.0	84.1%	895.7	93.7%	807.6	84.5%
		2000	1899.2	1867.6	98.3%	1762.2	92.8%	1642.7	86.5%
	−80°C	200	172.7	157.2	91.0%	161.0	93.2%	152.0	88.0%
		1000	955.9	964.4	100.9%	889.7	93.1%	876.3	91.7%
		2000	1899.2	1988.4	104.7%	1780.3	93.7%	1742.4	91.7%
GS-441524	freeze-thaw	120	111.4	116.0	104.2%	122.8	110.3%	126.6	113.7%
		1200	1137.6	1291.5	113.5%	1272.9	111.9%	1172.1	103.0%
	−80°C	120	111.4	121.0	108.7%	121.5	109.1%	131.1	117.7%
		1200	1137.6	1231.4	108.2%	1226.7	107.8%	1289.7	113.4%
Dexamethasone	freeze-thaw	120	129.7	126.7	97.7%	125.9	97.1%	137.0	105.6%
		1200	1024.1	1130.7	110.4%	1111.2	108.5%	992.9	97.0%
	−80°C	120	129.7	134.8	104.0%	136.6	105.3%	138.9	107.1%
		1200	1024.1	1139.4	111.3%	1132.2	110.6%	1079.6	105.4%

All concentrations in ng/mL.

Table 11. Patient data for seven COVID-19 patients receiving remdesivir, dexamethasone or both

			Remdesivir (RDV)				Dexamethasone (DXM)		
Sample ID	Gender	Age (years)	dose given	hours between drug administration and sample collection	RDV conc. (ng/mL)	GS-441524 conc. (ng/mL)	dose given	hours between drug administration and sample collection	DXM conc. (ng/mL)
1A	male	78	100 mg	17.50	<LOD	129.44	6 mg	19.13	<LOD
1B			100 mg	18.17	<LOD	153.03	6 mg	19.95	<LOD
1C			100 mg	17.53	<LOD	169.05	6 mg	19.22	<LOD
1D			100 mg	18.11	<LOD	156.47	6 mg	20.07	<LOD
1E			N/A	41.47	<LOD	70.58	N/A	43.42	<LOD
1F			N/A	89.30	<LOD	27.23	N/A	91.25	<LOD
2A	male	52	100 mg	6.33	3.39	116.51	6 mg	19.75	8.34
2C			100 mg	5.60	3.51	98.99	6 mg	18.10	4.12
2D			100 mg	6.33	<LOD	90.08	6 mg	19.90	2.73
3A	female	73	N/A	N/A	<LOD	<LOD	6 mg	not known	159.65
3B			N/A	N/A	<LOD	<LOD	6 mg	18.78	111.70
3C			N/A	N/A	<LOD	<LOD	6 mg	19.53	68.54
3D			N/A	N/A	<LOD	<LOD	6 mg	20.92	<LOD
3E			N/A	N/A	<LOD	<LOD	6 mg	19.90	<LOD
4A	male	49	100 mg	14.77	27.30	435.62	6 mg	9.08	<LOD
4B			100 mg	15.20	44.09	597.45	6 mg	9.22	<LOD
5A	male	60	100 mg	20.15	<LOD	184.09	N/A	N/A	<LOD
5B			100 mg	21.12	<LOD	204.44	N/A	N/A	<LOD
6A	male	76	100 mg	6.08	<LOD	73.92	N/A	N/A	<LOD
6B			100 mg	11.15	<LOD	73.93	N/A	N/A	<LOD
7A	male	41	N/A	N/A	<LOD	<LOD	N/A	N/A	<LOD
7B			200 mg	5.33	128.00	156.76	6 mg	5.30	64.17
7C			100 mg	4.30	2.64	152.98	6 mg	4.30	76.58

N/A, not applicable (dosing was stopped prior to the previous sample collection or medication was not administered at all).

importantly, the precision measured for these two parameters at all QC levels is within 15% CV. The same was observed for the recovery and precision of recovery for remdesivir. A significant enhancement of the signal was observed for remdesivir in serum. The specific serum component that causes this enhancement was not investigated in our study. However, we observed high precision of the matrix effect within and between six patient samples and the drug-free serum. Previously published methods did not report the same enhancement of the signal most likely because of the substantial dilution (45-fold) employed during protein precipitation.[8,14]

Consistent with previous reports, we confirmed that remdesivir is unstable at room temperature,[8,14] even after only 1 h, but cold storage allows for stability of at least 8 h (Table 8). We found remdesivir degradation under 20% after 8 h in an ice bath at approximately 2°C, but others have shown degradation of 65%–83% at 4°C after 24 h, so an ice bath or refrigerator may only be a suitable option for the short-term.[8,14] The freeze-thaw stability of remdesivir is unsettled in the literature[8,14] and we demonstrated stability after two freeze-thaw cycles (Table 10). We found GS-441524 and dexamethasone were stable at all tested conditions and following three freeze-thaw cycles, as others have found (Tables 8–10).[8,14,21]

Patient sample testing

Using samples obtained from seven patients with severe COVID-19, we demonstrated the utility of our validated method in measuring both remdesivir and dexamethasone in clinical samples.

In the 17 samples collected after remdesivir administration, detectable levels of remdesivir are generally observed up until about 6.5 h post-dosing. The levels of remdesivir observed in these early timepoint samples are very low except for the sample collected from patient 7 (7B) after the loading dose of 200 mg was administered. Beyond 7 h, remdesivir is below our LOD except in patient 4. These results are consistent with the limited pharmacokinetic studies reported for remdesivir where detectable levels of remdesivir were observed only until about 10 h post-dosing in COVID-19 patients. The estimated half-life for remdesivir in these studies was 0.8 h.[7,8]

Similar to the two previous studies that have reported remdesivir levels in COVID-19 patients ($n = 3$), the metabolite GS-441524 was observed at higher levels and with a longer half-life than remdesivir in the 17 serum samples from six patients in our study.[7,8] It is notable that in each of the six patients the observed GS-441524 levels in their samples fall within a narrow range (within 20% of the mean). We were also able to demonstrate the slow decrease of GS-441524 levels after ending remdesivir administration (1D–1F) consistent with the reported half-life of 24 h for GS-441524.[7,8] More interestingly, there is significant variability in the GS-441524 level between patients. For example, even though the sampling timepoints relative to remdesivir administration are roughly the same for patients 1 and 4, patient 4 has an about 4× higher level of GS-441524 than patient 1. The remdesivir levels observed in patient 4 are also remarkably high at 14 h post-dosing compared with all the other patients. This may suggest possible differences in the rate at which patients metabolize remdesivir, which may have important implications for the duration at which an efficacious dose of the active metabolite of remdesivir is maintained in patients.

Recent data from the WHO's SOLIDARITY trial that remdesivir neither improved mortality nor time to recovery differed from that in the Adaptive COVID-19 Treatment Trial (ACTT-1) where remdesivir shortened the time to recovery.[5,6] Drug–drug interactions and pharmacogenomic differences in drug metabolism are well established for several other drugs including other antiretrovirals.[22] Only about 65%–74% of patients treated with remdesivir have significant positive clinical outcomes.[5,23] The cause of this variability in outcomes may be multifactorial, with pharmacogenomics likely to be an important consideration in designing a more effective dosing regimen for some COVID-19 patients. *In vitro* studies suggest that remdesivir is a substrate for drug metabolizing enzymes CYP2C8, CYP2D6 and CYP3A4, all of which have reported pharmacogenomic variants.[24] Further studies that use this novel method of therapeutic drug monitoring and assess pharmacogenomics may result in more optimal dosing and better outcomes with remdesivir.

Similarly, we did not observe dexamethasone in patient samples when it is not given or prior to the first dexamethasone dose, supporting the specificity of our assay for dexamethasone in clinical samples. There is also significant variability in dexamethasone levels observed across patients, as previously observed.[25,26] Sampling timepoints relative to dexamethasone dosing are very similar across patients 1, 2 and 3, and yet their dexamethasone levels differ by up to 20-fold. Moreover, our results for patients 2 and 3 clearly demonstrate dexamethasone auto-induction via CYP3A4.[27] For both patients 2 and 3, the dexamethasone levels progressively decrease with prolonged administration. Our findings suggest that pharmacogenomic differences and auto-induction should be considered in optimizing dexamethasone dosing regimens in patients with severe COVID-19.

Conclusions

In summary, we developed and validated an LC-MS/MS serum assay that simultaneously quantifies the two most important therapeutics administered in severe cases of COVID-19: remdesivir and dexamethasone. All validation parameters established for remdesivir, GS-441524 and dexamethasone in our method adhere to the guidelines established by the FDA and CLSI for laboratory developed tests employing LC-MS/MS.[19,20] We confirmed previous reports that GS-441524 clears more slowly than remdesivir and therefore may increase the analytical utility of the assay.[7,8] Application of our method to 23 serum samples obtained from seven COVID-19 patients demonstrated the specificity of our assay and its wide dynamic range in quantifying these three analytes in clinical samples. To our knowledge, our method is the first to combine multiple COVID-19 therapeutics in a single LC-MS/MS assay and is also the largest study to date to examine clinical samples. The variability of levels observed for these three analytes across patients underscores the importance of therapeutic drug monitoring for these medications and indicates that more comprehensive pharmacokinetic and pharmacogenomic studies for both remdesivir and dexamethasone in larger cohorts are warranted.

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Transparency declarations

None to declare.

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