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Rapid determination of remdesivir (SARS-CoV-2 drug) in human plasma for therapeutic drug monitoring in COVID-19-Patients

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ABSTRACT

To tackle the harmful consequences of the widespread COVID-19 pandemic, a broad-spectrum anti-viral drug remdesivir (RDV) has gained the utmost attention recently due to its promising application in treating COVID-19 patients. However, a fast and sensitive analytical methodology is important to monitor RDV drug profile in human plasma for pharmacokinetics (PK) and therapeutic drug monitoring (TDM). In this study, we demonstrate an improved vortex-assisted salt-induced liquid-liquid microextraction (VA-SI-LLME) technique coupled with UHPLC-PDA and UHPLC-MS/MS for rapid determination of RDV in human plasma. This technique involves simple one-step protein precipitation with hydrochloric acid and subsequent extraction with acetonitrile for analysis. Under the optimal VA-SI-LLME conditions (500 μL of acetonitrile with 2.5 g ammonium sulfate under 2 min vortex extraction), method validation results indicated an excellent correlation coefficient of 0.9969 for UHPLC-PDA (monitored at 254 nm) and 0.9990 for UHPLC-MS/MS (monitored at electrospray ionization with + ion mode transitions of *m/z* 603.1→*m/z* 402.20 and *m/z* 603.1→ *m/z* 199.90). The detection and quantification limits were 1.5 and 5 ng/mL for UHPLC/PDA, and 0.3 and 1 ng/mL for UHPLC-MS/MS, respectively. The developed method showed excellent extraction recoveries between 90.79–116.74 % and 85.68–101.34 % with intraday and interday precision ≤ 9.59 for both methods. These results proved that the developed method is a simple, fast, and sensitive analytical method that can be applied as a standard analytical tool for PK and TDM studies of RDV in clinical trials during the current worldwide outbreak.

1. Introduction

Coronavirus disease 2019 (COVID-19) [1] caused by severe acute respiratory coronavirus-2 (SARS-CoV-2) in humans was first reported in Wuhan city of China in December 2019. Later, SARS-CoV-2 was spread worldwide and infecting millions of people, and the world health organization (WHO) has announced the outbreak as a global pandemic since the number of infected people is still increasing day by day. As of 16th December 2020, the total (global) coronavirus cases were approximately 73,806,583 with reported deaths of 1,641,635 [2]. The fact being that SARS−COV-2 spread effectively through multiple spreading routes such as direct contact, airborne, fomite, and oral ingestion. The unexpected emergence of SARS−COV-2 significantly affects human health and lives and also affects the world economy. After the severe acute respiratory syndrome (SARS) and the middle east respiratory syndrome (MERS), SARS−COV-2 is the third member of the coronavirus family to cause illness in humans in the last few decades [2,3]. The infection of SARS-CoV-2 marks several symptoms such as mild cough, simple fever, cough, fatigue, diarrhea, hemoptysis, pneumonia, multiple organ failure, and finally leads to death [4,5]. Aged people with pre-existing cardiovascular or respiratory diseases are more vulnerable to COVID-19 and high risk for severe health complications [6,7]. COVID-19 infected patients are treated with common antibiotics, anti-parasitic, antiretroviral, anti-inflammatory drugs, and convalescent

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plasma therapy under intrusive and non-intrusive oxygen support [8–12]. Due to the lack of an effective therapeutic agent or anti-viral medication to target the coronavirus, COVID-19 remains uncontrolled with high death rates and has become a massive challenge around worldwide. Hence, it is essential to investigate the safe and effective treatment for COVID-19 patients. The availability of efficient anti-viral drugs with positive advantages would solve a severe unmet medical need to treat COVID-19 patients.

There has been an immediate urge to develop the treatment for the widespread COVID-19 to lessen its adverse effects in humans. Several drugs such as Darunavir/cobicistat, Baricitinib, chloroquine, Tocilizumab, Remdesivir, Ruxolitinib, Lopinavir/ritonavir, Favipiravir, Hydroxychloroquine [13], Interferon beta-1b, Azithromycin [14], Ribavirin, and Corticosteroids drugs are under investigation for the potential treatment of COVID-19. Amongst, remdesivir (RDV) served as the utmost beneficial drug because of its anti-viral efficiency *in vitro* and *in vivo* studies. RDV is a small molecule and, acting as a therapeutic agent against the Ebola virus in rhesus monkeys, which has been developed by the Gilead Sciences biopharmaceutical company [15]. The anti-viral activity of RDV has been proven for SARS and MERS RNA viruses [16]. Based on the observed activity of RDV towards SARS-CoV-2 clinical trials have been initiated, and the recent studies on RDV showed *in vitro* inhibition of SARS-CoV-2 [17,18]. The replication process of COVID-19 virus can be reduced on drug administration by inhibiting the RNA-dependent polymerase, thus, proving the efficacy of RDV [19–22]. The European Medicines Agency (EMA) has not yet approved the COVID-19 treatment with RDV, but on the other hand United States Food and Drug Administration (US FDA) has marked its authentication and has been several ongoing studies and trials on the patients suffering from COVID-19 [21]. For targeting the SARS-CoV-2 in the COVID-19 patients, 200 mg dosage of RDV was prescribed on the first day, followed by 100 mg dose for the next nine days through route IV administration was proposed by Gilead. The RDV pharmacodynamics (PD), pharmacokinetic (PK), and therapeutic drug monitoring (TDM) studies are essential in the emergency COVID-19 pandemic situation. Consequently, researchers aimed to develop analytical methods to monitor the RDV for the above applications.

To date, only a few studies have been reported for the quantification of RDV in plasma [23–27]; however, these methods are laborious and time-consuming. Therefore, it is crucial to develop a simple, fast, sensitive, and accurate method for the monitoring of RDV in human plasma samples for PK and TDM applications during COVID-19 treatment. The main objective of this work is to develop a sensitive and robust analytical approach with minimal sample preparation process that can be applied as a routine tool for monitoring the dose of RDV in human plasma for the PK and TDM studies. For the first time, an improved vortex-assisted salt-induced liquid-liquid microextraction (VA-SI-LLME) technique coupled with ultra-high-pressure liquid chromatography with photodiode array (UHPLC-PDA) and tandem mass spectrometric technique (UHPLC-MS/MS) was developed for rapid quantification of RDV in human plasma. Several factors affecting the VA-SI-LLME method were carefully examined, including the type of extraction solvent, solvent volume, extraction time, salt type, and amount, and also analytical method was validated based on FDA guidelines, which can be applied for the routine monitoring of RDV in human plasma for PK and TDM studies.

2. Experimental

2.1. Materials and methods

2.1.1. Chemicals and materials

Remdesivir (RDV) (99 % purity) was obtained from MedKoo Biosciences, Inc. Morrisville, USA. RDV standard was stored in a dark place at −20 °C refrigerator to avoid degradation. LCMS grade solvent acetonitrile (ACN) and methanol were acquired from J.T. Baker

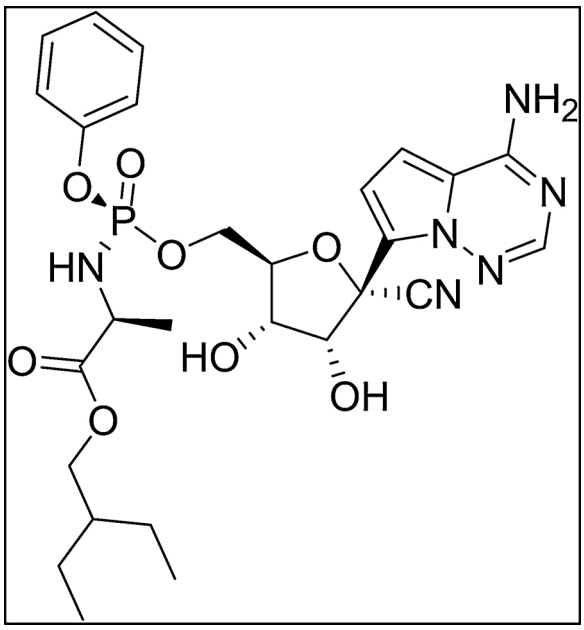

Fig. 1. Chemical structure of Remdesivir (RDV) drug.

Chemical Co. (Phillipsburg, NJ, United States). HPLC grade isopropanol and acetone were obtained from ECHO Chemicals CO., LTD (Taiwan). Dimethyl-sulfoxide (DMSO), tetrahydrofuran, ammonium sulfate ($(NH_4)_2SO_4$), sodium chloride (NaCl), sodium sulfate (Na_2SO_4), magnesium sulfate ($MgSO_4$), were bought from Xilong Scientific Co. Ltd (Shanghai, China). Hydrochloric acid (HCl 37 %) and formic acid were acquired from Fisher Chemical (Leicestershire, UK). Ultrapure water utilized in this work was prepared using an Uniss Pure water system (from Taiwan). Drug-free blank human plasma samples were purchased from Merck Chemicals and used for method development, validation, and recovery studies.

2.2. Standard solutions preparation

RDV stock solution (5000 μg/mL) was prepared by dissolving RDV drug powder using the mixture of DMSO:MeOH (30:70 v/v). The chemical structure of RDV is displayed in Fig.1. Working standard, calibration standard, and quality control solutions were prepared using methanol by diluting the RDV stock solution. The RDV working standard solutions for calibration and quality controls were prepared using methanol in concentrations of 100, 10, 1, 0.1, 0.01 μg/mL. 1, 2.5, 5, 7.5, 10, 25, 50, 75, 100, 250, 500, 1000, and 5000 ng/mL sample solutions were prepared freshly by spiking calibration standard solutions into the blank human plasma samples for method calibration.

2.3. VA-SI-LLME process

The graphical representation of the VA-SI-LLME process was displayed in Fig. 2. In the typical VA-SI-LLME extraction procedure, 50 μL of human plasma sample was pipetted into a clean 5 mL glass test tube, and 2.0 mL of 0.1 N HCl was added into the sample. The glass tube was then vortexed for 30 s; subsequently, 2.5 *g* of $(NH_4)_2SO_4$ and 500 μL of ACN as an extraction solvent were added into the sample solution. After that, the mixture solution was vortexed for 2 min and centrifuged at 6000 rpm for 5 min by maintaining the centrifuge temperature at 4 °C. After centrifugation, the upper layer (supernatant layer) was collected into a fresh glass tube using a 0.5 mL glass syringe. Then, the extraction solvent was dried under nitrogen by a concentration evaporator. The final residue was re-dissolved using 100 μL of methanol and shifted into an HPLC vial for the analysis.

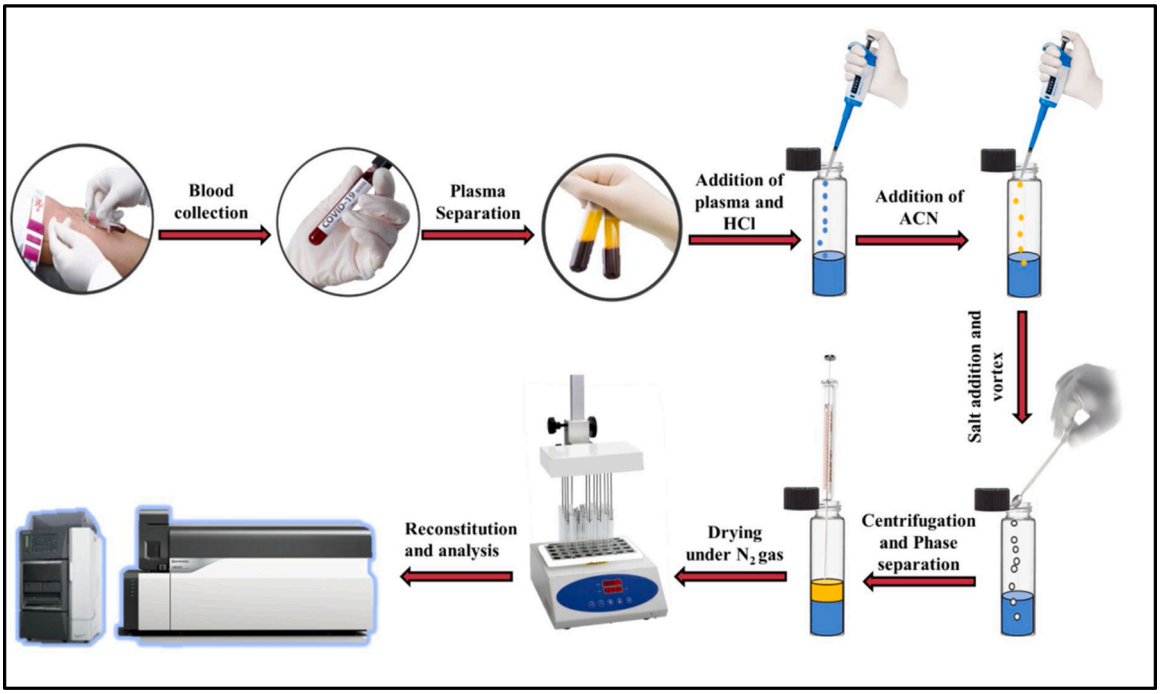

Fig. 2. Graphical representation of vortex-assisted salt-induced liquid-liquid microextraction (VA-SI-LLME) process.

2.4. Instrument parameters

2.4.1. UHPLC-PDA method parameters

RDV analysis was performed using a Shimadzu UHPLC Nexera-I series 2040C 3D system consisting of a quaternary pump (Tokyo, Japan). An ACE C18 column (150 × 4.6 mm, 3 µm) brought from Advanced Chromatography Technologies Ltd (Aberdeen, Scotland) was utilized for the separation of RDV. The mobile phase A used was 0.05 % (v/v) formic acid in ultrapure water, and mobile phase B was 100 % ACN with isocratic elution (A: B) 52:48 % with the total analysis time of 10 min. The column flow rate was set at 0.5 mL/min with 35 °C as the ideal column temperature, and the injection volume was 5 µL. RDV was monitored at 254 nm absorbance in the PDA detector.

2.4.2. UHPLC-MS/MS parameters

The UHPLC parameters utilized were the same as mentioned in UHPLC-PDA method parameters section 2.4.1. The RDV was also simultaneously monitored using a Shimadzu UHPLC-MS/MS system model LCMS-8045 (Tokyo, Japan) equipped with triple quadrupole with an electrospray ionization (ESI) source. The ESI positive ion mode was used to detect RDV with the scheduled multiple reaction monitoring (MRM) mode by the protonated RDV molecule used as precursor ion. The ESI ion source heating block temperature was set at 300 °C, DL temperature was set at 250 °C, the interface temperature was set at 300 °C, the heating gas flow rate at 10 L/min, the nebulizing gas flow rate at 3 L/ min, and drying gas flow 10 L/min for the RDV analysis. The ion transitions of RDV were *m/z* 603.1→*m/z* 402.20 and *m/z* 199.90, dwell time 100 msec for both ions, collision energy (CE) are -17 and -42, Q1 pre-bias (V) -22.0 and Q3 pre-bias (V) -15.0 and -20.0 were used to monitor.

3. Results and discussion

Various factors affecting the extraction recoveries in the developed VA-SI-LLME procedure, including extraction solvent type, solvent volume, extraction time, salt type, and salt amount, were assessed utilizing RDV standard solutions.

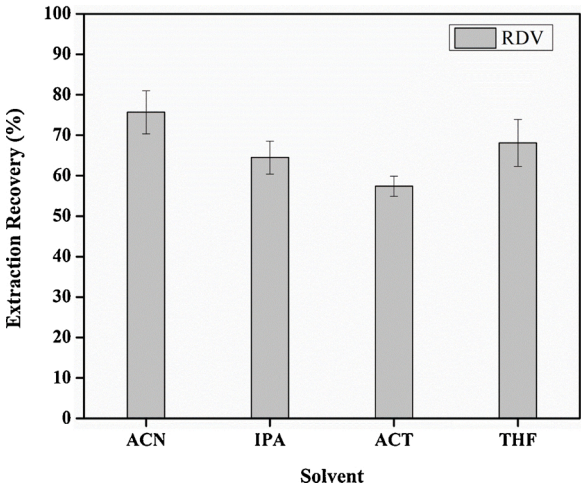

Fig. 3. Effect of extraction solvent type on the Remdesivir (RDV) extraction recoveries. Conditions: 50 µL of human plasma sample (spiked at 100 ng/mL), 2.0 mL 0.1 N hydrochloric acid (HCl); extraction solvent, Acetonitrile(ACN) /Isopropanol(IPA) /Acetone(ACT) /Tetrahydrofuran (THF); volume of extraction solvent - 0.5 mL; extraction time - 120 s; salty type-Ammonium Sulfate ($(NH_4)_2$ SO_4); salt amount - 2.5 g.

3.1. Effect of extraction solvent

The extraction solvent opting is the core aspect of the equilibrium dependent extractions. The essential criteria for selecting extraction solvent in the developed VA-SI-LLME are as follows i) maximum extraction ability towards target analytes, ii) the density of the solvent should be lower than water, iii) miscibility with the aqueous phase, iv) solvent must also to possess the efficiency to precipitate proteins in the plasma sample to achieve cleaner extract after extraction. The initial experiment trials were carried out with water-miscible solvents such as acetonitrile (ACN), isopropanol (IPA), tetrahydrofuran (THF), and acetone (ACT) [28]. The results achieved are presented in Fig. 3. ACN showed maximum extraction recovery for the RDV drug when compared

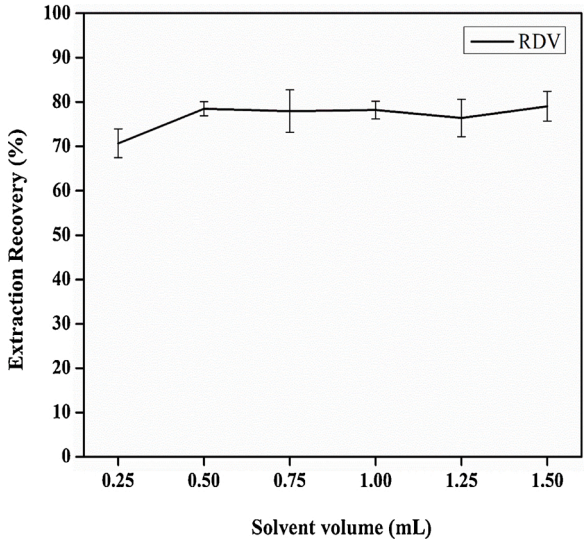

Fig. 4. Effect of extraction solvent volume on the Remdesivir (RDV) extraction recoveries. Conditions are the same as in Fig. 2. Except solvent volume (0.25, 0.5, 0.75, 1.0, 1.25, 1.5 mL).

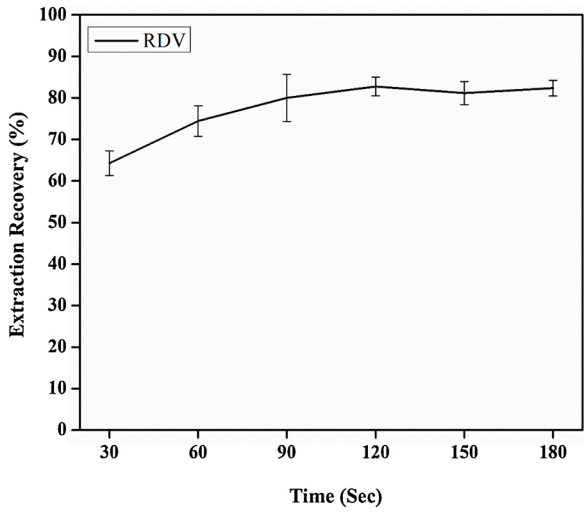

Fig. 5. Extraction time effect on the Remdesivir (RDV) extraction recoveries. Conditions are the same as in Fig. 2. Except extraction time (30, 60, 90, 120, 150, and 180 s).

to THF, IPA, and ACT. The reason is that the ACN indicated high extraction efficiency towards RDV is due to its polarity and high miscibility in water, and more efficient in providing the salting-out effect along with higher protein precipitation capacity from human plasma [29]. The other solvents THF, IPA, and ACT, were showed less protein precipitation and low extraction recoveries. Therefore, ACN was utilized as an ideal solvent for extraction and consequent experiments.

3.2. Effect of extraction solvent volume

The solvent volume is the essential aspect in the liquid phase microextraction techniques because it directly affects the extraction recoveries, and the volume should be adequate to extract all the analytes from the sample. The influence of extraction solvent (ACN) volume on the extraction recoveries of RDV was investigated by examining various ACN volumes, including 0.25, 0.5, 0.75, 1.0, 1.25, and 1.5 mL, and the acquired results were displayed in Fig. 4. The extraction recovery was increased by an increase in the ACN volume from 0.2 to 0.5 mL, and the

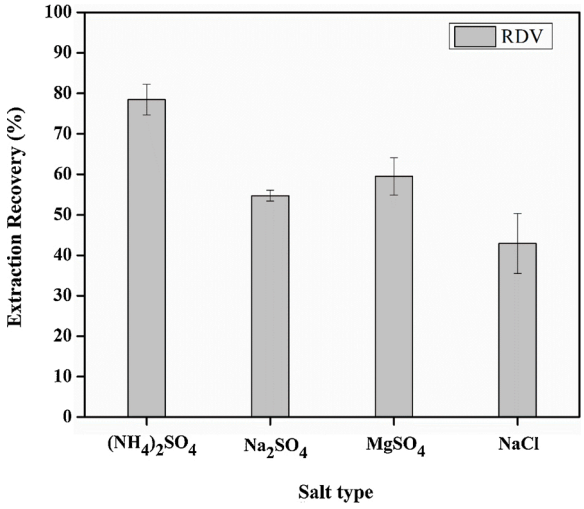

Fig. 6. Effect of salt type on the Remdesivir (RDV) extraction recoveries. Conditions are the same as in Fig. 3. Except for salt type Ammonium Sulfate ($(NH_4)_2$ SO_4), Magnesium Sulfate ($MgSO_4$), Sodium Sulfate (Na_2SO_4), and Sodium Chloride (NaCl).

maximum extraction recovery was obtained at 0.5 mL of ACN (Fig. 4). The extraction recovery of RDV almost remained similar when further increasing ACN volume because the collected extract was reconstituted to 100 µL for analysis. Volumes less than 0.5 mL ACN showed significantly less phase separation and not achieved the complete extraction due to the insufficient ACN volume for the extraction of RDV. Therefore, 0.5 mL of ACN was selected as the optimum solvent volume for further investigation.

3.3. Effect of extraction time

The distribution of analytes between aqueous and organic phases mainly depends on extraction time; hence extraction time is the main parameter in all the LLME techniques, particularly in this VA-SI-LLME technique. Various studies have reported that vortex agitation enhancing the extraction recoveries of target analytes from aqueous sample solution to the organic extraction phase [30,31]. In this work, the effect of vortex times were investigated in terms of extraction time, such as 30, 60, 90, 120, 150, and 180 s. As indicated in Fig. 5, the extraction recoveries were raised with vortex time from 30 to 120 s and achieved the maximum extraction recovery of RDV at 120 s. This indicates that the transfer of RDV to extraction solvent reached a maximum within a short vortex time; further increase in the vortex time does not show any significant improvement on RDV extraction recoveries. Therefore, the vortex time of 120 s was selected as the outmost time and utilized for further investigations.

3.4. Effect of salt type and salt amount

The addition of salt to the sample solution can increase the ionic strength of the sample solution, thereby reduces the solubility of target analytes in the aqueous solution phase by means of the salting-out effect. Therefore, it leads to the efficient transfer of analytes into the organic extraction phase to achieve maximum preconcentration. Moreover, salt addition into the sample is also to reduce matrix interferences, especially in biological and food samples [32,33]. In this work, the influence of different kind of salt addition into the sample solution, including ammonium sulfate ($(NH_4)_2SO_4$), magnesium sulfate ($MgSO_4$), sodium sulfate (Na_2SO_4), and sodium chloride (NaCl) were examined. The obtained results were shown in Fig. 6, and the results indicated that ammonium sulfate showed higher extraction recovery than other salts due to the nature of ammonium ions having a higher tendency to

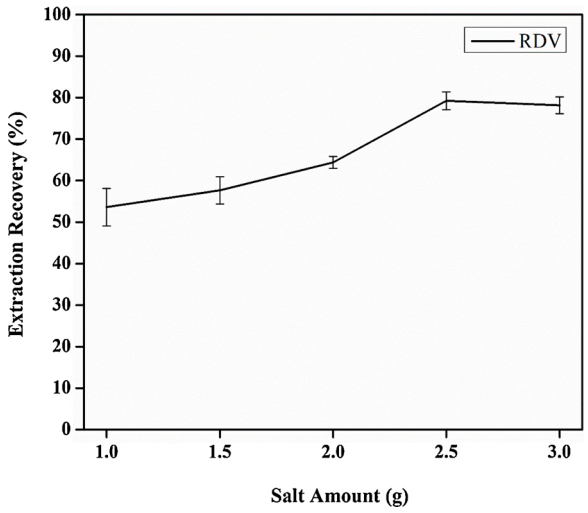

Fig. 7. Effect of Salt amount on the Remdesivir (RDV) extraction recoveries. Conditions are the same as in Fig. 3. Except salt amount (1.0, 1.5,2.0,2.5, and 3.0).

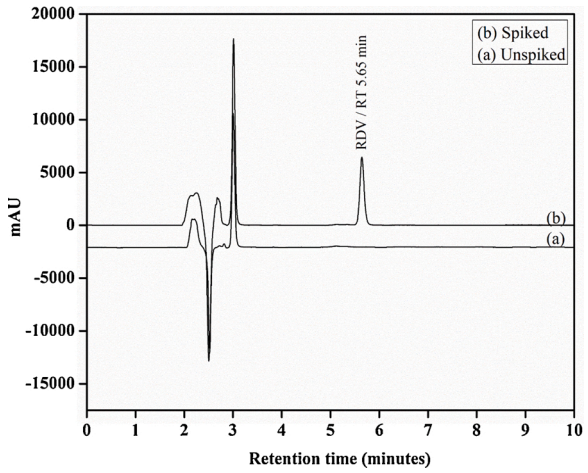

Fig. 8. Ultra-high-performance liquid chromatographic-photodiode array (UHPLC-PDA) chromatograms, blank (unspiked) human plasma sample a), and spiked (at 500 ng/mL) human plasma sample b).

salting-out effect than sodium, potassium or magnesium ions. On this basis of results, ammonium sulfate has opted for further studies.

The salt amount is an outmost parameter in the salting-out LLPE techniques. In this work, the effect of salt amount on extraction recoveries was studied from 1 to 3.0 g. The RDV extraction recovery was increased with rising the salt amount from 1 to 2.5 g as displayed in Fig. 7, and this is due to the increase in the salt amount increases the ionic strength in the sample solution which results in the enhances the extractant volume recovery, and also decrease the solubility of RDV drug in the aqueous phase. Further increase in the salt amount higher than 2.5 g showed no effect on extraction recoveries (Fig. 7). The reason is that increases in salt amount subsequently increase the sample solution's viscosity, which lowers the transfer of RDV to the organic phase. Therefore, 2.5 g of ammonium sulfate was chosen as the optimum concentration.

3.5. Analytical method validation studies

The developed VA-SI-LLME technique coupled with UHPLC-PDA and UHPLC-MS/MS method for fast monitoring of RDV in the human plasma sample, was validated under the optimized conditions. The analytical

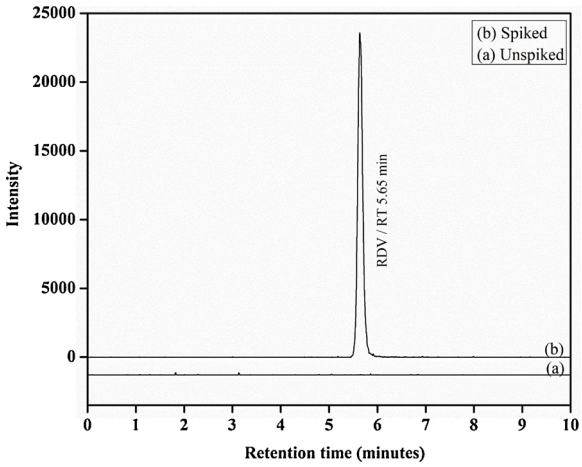

Fig. 9. Ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC–MS/MS) chromatograms, blank (unspiked) plasma sample a), and spiked (at 50 ng/mL) human plasma sample b).

parameters including, linearity, recovery, precision, detection limit (LOD), and lower quantification limit (LLOQ) were evaluated and presented as follows.

3.5.1. Specificity and carryover

The mean retention time of RDV was 5.64 min for spiked human plasma samples in both UHPLC-PDA and UHPLC-MS/MS methods, and the blank and spiked human plasma samples chromatogram were displayed in Figs. 8 and 9. The column carryover study was investigated by injecting a blank sample after the injection of a higher spiked concertation sample, and results indicated no carryover or interference peaks observed.

3.5.2. Linearity of the method

The linearity (method calibration curve) study for RDV was carried over the concertation between 5–5000 ng/mL for VA-SI-LLME with UHPLC-PDA, and 1–5000 ng/mL for UHPLC-MS/MS, respectively. RDV quantitation was carried out by plotting the RDV peak area against the concentration obtained after VA-SI-LLME. The obtained linear equations were displayed in Table 1. The correlation coefficients were 0.9969 for UHPLC-PDA, and 0.9990 for UHPLC-MS/MS.

3.5.3. Lower quantification limit (LLOQ) and detection limit (LOD)

The LLOQ and LOD for the RDV were calculated from the linear equation as follows

$$LLOQ = \frac{10 \times \sigma}{S} \longrightarrow \quad (1)$$

$$LOD = \frac{3.3 \times \sigma}{S} \longrightarrow \quad (2)$$

Where σ = standard deviation

S = slope of analyte obtained from the method calibration curve

The LLOQ was achieved at a lower concentration of 1 ng/mL for UHPLC-MS/MS and 5 ng/mL for UHPLC-PDA. The obtained LOD were 0.3 and 1.5 ng/mL, as showed in Table 1.

3.5.4. Recoveries, method precision, and stability studies

Method precisions and recoveries for quality control samples are displayed in Table 2. The recoveries of the RDV were between 90.79–116.74 %, with intraday and interday precision $\leq$ 9.59 for UHPLC-PDA. The recoveries of the RDV were between 85.68–101.34 % with intraday and interday precision $\leq$ 9.39 for UHPLC-MS/MS.

The stability of RDV in human plasma samples were investigated,

Table 1
Analytical method validation parameters of the developed method for Remdesivir (RDV).

Analyte	Instrument used	Linearity range (ng/mL)	Linear equation	R^2	LOD (ng/mL)	LLOQ (ng/mL)	RSD (%)
RDV	UHPLC-PDA	5−5000	y = 316.31x + 950.41	0.9969	1.5	5	4.89
	UHPLC-MS/MS	1−5000	y = 426061x− 138,252	0.9990	0.3	1	2.66

Note: R^2- correlation coefficients, Lower quantification limit (LLOQ), and limit of detection (LOD).

Table 2
Relative recoveries and method precision studies by the developed method.

Analyte Name	Instrument Name	Spiked Human Plasma Sample				
		Spiked Conc. (ng/mL)	Found Conc. (ng/mL)	Recovery (%)	Intra-day Precision (RSD%)	Inter-day Precision (RSD%)
RDV	UHPLC-PDA	$LLOQ^1$	5.83	116.74	9.59	8.66
		Low^1	51.35	102.70	6.89	7.47
		Medium	453.95	90.79	5.83	4.61
		High	4720.54	94.41	6.90	8.64
	UHPLC-MS/MS	$LLOQ^2$	0.86	85.68	9.27	9.39
		Low^2	10.13	101.34	3.61	4.68
		Medium	453.96	90.79	4.38	3.59
		High	4909.00	98.18	2.85	3.79

RSD, relative standard deviation; Quality control samples levels: $LLOQ^1$, 5 ng/mL; Low^1 50 ng/mL; $LLOQ^2$, 1 ng/mL; Low^2 10 ng/mL; Medium, 500 ng/mL, High, 5000 ng/mL.

Table 3
Remdesivir (RDV) degradation (%) at different temperatures and days by the developed method.

Analyte	Instrument used	RDV degradation (%)					
		Spiked Conc. (ng/mL)	Day-1			Day-3	
			Room Temperature	4 °C	−20 °C	4 °C	−20 °C
RDV	UHPLC-PDA	Low^1	99.36	18.48	1.48	22.92	1.96
		Medium	99.01	16.53	0.66	24.88	0.69
		High	97.87	11.30	0.32	18.35	0.40
	UHPLC-MS/MS	Low^2	98.77	14.64	1.84	17.38	2.31
		Medium	97.54	11.17	0.65	16.89	0.74
		High	94.74	8.89	0.43	13.26	0.57

Note: Low^1 50 ng/mL; Low^2 10 ng/mL; Medium, 500 ng/mL, High, 5000 ng/mL.

and the results are showed in Table 3. Moreover, the RDV stock solution's stability was also examined, and results showed that it could be stable for at least two weeks if stored adequately at -20 °C. Although, RDV spiked human plasma samples are found to be unstable at room temperature or 4 °C if kept for more than 3 days, and it was highly stable at -20 °C. Therefore, the extraction solvent obtained after VA-SI-LLME from the plasma sample showed good stability until 3 days in HPLC autosampler temperature at 4 °C, and the RSD was less than 7.54 % for 3 days. The achieved analytical results from the method validation indicated good linearity, precision values and showed higher sensitivity. The results obtained from recovery studies indicated that the relative recoveries are in the acceptable range. Hence, the developed VA-SI-LLME coupled UHPLC-PDA and UHLC/MS/MS method is evident to be sensitive, fast, accurate, and reliable for the pharmacokinetics (PK) and therapeutic drug monitoring (TDM) studies of RDV in plasma samples during COVID-19 treatment.

4. Conclusion

An improved VA-SI-LLME based fast sample preparation technique coupled with UHPLC-PDA and UHPLC-MS/MS methods were developed and validated for the RDV related clinical studies in human plasma samples. This VA-SI-LLME process is simple, fast, low-cost, low-organic solvent consumption, and eco-friendly nature. The analytical method validation studies indicated that the developed technique achieved good extraction recoveries, showed decent linearity, higher sensitivity, and selectivity, and there was no carryover observed. The developed method can be useful for the pharmacokinetics (PK) or therapeutic drug monitoring (TDM) studies of RDV during clinical trials or COVID-19 treatment.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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