Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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A validated UHPLC-MS/MS method for simultaneous quantification of some repurposed COVID-19 drugs in rat plasma: Application to a pharmacokinetic study

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ABSTRACT

Since the emergence of Corona virus disease (COVID-19) in 2019, a number of medications have been developed and tried to combat the pandemic. In the present study, we develop a LC-MS/MS approach to detect and quantify certain COVID-19 candidate drugs in rat plasma, including Hydroxychloroquine, Favipiravir, Oseltamivir, and Remdesivir. The analytes were separated using Ultra High-Pressure Liquid Chromatography (UHPLC) over a 13-minute run on a C_{18} column. The extraction solvent for the (QuEChERS) quick, easy, cheap, effective, rugged and safe method was methanol, while the clean-up phase was primary secondary amine (PSA). Satisfactory recoveries were achieved for all compounds ranging from 82.39 to 105.87 %, with standard deviations smaller than 15.7. In terms of precision, accuracy, linearity, matrix effect, and stability, the method was validated according to US FDA criteria. The Limit of Detection (LOD) was determined to be between 0.11 and 10 ppb. The approach was further developed for a modest pharmacokinetic research in laboratory rats, and thus can be suitable for therapeutic drug monitoring in clinical cases under the same treatment.

1. Introduction

Large-scale attempts have been made to find medications that can prevent or treat COVID-19 since the global breakout of the pandemic [1]. Several national and international research groups have been working on the development and availability of therapies for SARS-CoV-2 (the virus causing COVID-19) variations and other pandemic viruses. Antiretroviral medications, as an initial therapy for COVID-19 sufferers, were found to be advantageous according to the reported data. They have accelerated clinical cure times, shortened hospital stays, postponed and reduced the need for mechanical and invasive ventilation and decreased fatality rates [2]. Nevertheless, the majority are being studied in preclinical and clinical trials.

Several medicines are now indicated for treating COVID-19-positive hospitalized patients. Remdesivir is a monophosphoramidate prodrug (RNA-dependent RNA polymerase inhibitor) that was created to treat hepatitis C and Ebola. It is the first FDA-approved treatment for adults and some paediatric COVID-19 patients who are sick enough to require hospitalization [3]. Hydroxychloroquine, an antimalarial medication with immunomodulatory and antiviral properties, could be an effective prophylactic strategy against COVID-19 [4,5].

The neuraminidase enzyme inhibitor Oseltamivir is a first-line antiviral medication used primarily in hospitals [6]. As reported by a recent study [7], the combination of Hydroxychloroquine and Oseltamivir was the most efficacious antiviral medication regarding duration of treatment, with an average COVID-19 patient survival rate of 83 %.

Favipiravir, an RNA-dependent RNA polymerase inhibitor, was approved officially in Japan for the treatment of influenza and could be a promising candidate for COVID-19 [8] during home isolation for mild to moderate cases [9]. Clinical trials with moderate and severe patients are currently being conducted to determine the efficacy of combining these repurposed medicines in COVID-19 [10–13].

To determine the best dose for achieving the desired outcome on SARS-CoV-2, pharmacokinetic investigations are required. Consequently, scientists have concentrated their efforts on finding medicines that can hinder the infection's most severe symptoms, as well as developing selective and sensitive analytical procedures for detection of drugs in biological matrices, such as tissues and fluids. Several analytical approaches for the detection and quantification of antiviral drugs now being explored for COVID-19 treatment have been developed in the previous decade. [14]

A literature analysis revealed a few chromatographic approaches for determining the antiviral medicines under evaluation in the treatment of SARS-CoV-2 using either protein precipitation with HPLC-MS/MS

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[15–17] or acid treatment with LC-MS/MS in human biological samples [18].

For the creation of medications, bioequivalence investigations, therapeutic drug monitoring, toxicological analysis and determination in biological matrices are critical. However, there are two frequent analytical issues that arise on employing chromatographic methods in drug investigation; these are ion suppression (the loss of ion intensity of the target analyte) and matrix interferences. Lipids are the most common source of matrix interferences in plasma, and they will accumulate on the analytical column, resulting in ion suppression [19,20]. Accordingly, several sample preparation approaches may be explored to produce higher quality outcomes and avoid possible interferences. The classic liquid–liquid extraction process is being phased out in favor of miniaturized alternatives. This is due to the necessity for analytical processes that can analyze data quickly and efficiently without consuming a lot of solvents [21].

The (QuEChERS) quick, easy, cheap, effective, rugged and safe technique was used to extract drugs in this investigation. This approach was originally designed to detect pesticide residues in foods [22–25]. Its use has recently been extended to a wide variety of matrices and analytes [26]; pharmaceuticals [27], benzodiazepines [28], antibiotics [29], antivirals [30] and pollutants [31] can also be extracted by QuEChERS from biological samples like urine, blood, plasma or food products.

The main goal of this study is to establish a sensitive approach for simultaneous quantitation of majorly repurposed drugs for SARS-CoV-2, such as Hydroxychloroquine, Favipiravir, Oseltamivir, and Remdesivir in rat plasma samples using UHPLC-MS/MS preceded by a miniaturized QuEChERS sample pretreatment. The experiment was accomplished in 13 min with a small amount of sample and using limited quantity of chemical reagents. This approach has been thoroughly verified in accordance with FDA bioanalytical validation criteria [32] and tested in a short pharmacokinetic investigation in live rats.

2. Experimental

2.1. Materials

2.1.1. Pure standards

Entecavir monohydrate (used as internal standard (IS)), Hydroxychloroquine sulphate, Favipiravir, Oseltamivir phosphate and Remdesivir were graciously provided by Pharmed healthcare (Cairo, Egypt), IPCA lab (Mumbai, India), Honour lab limited (Mumbai, India), EIPICO (Cairo, Egypt) and Apex pharma (India); respectively and were labelled as having purity not less than 98.5 %. Fig. 1 shows the chemical structures of the investigated analytes and the utilized internal standard.

2.1.2. Reagents:

All solvents were LC-MS grade: acetonitrile, methanol and water were all acquired from Supelco (Germany), and formic acid was 99 % pure (Carlo Erba, France). Phenomenex provided primary secondary amine (PSA) (Sepra^{TM} (50 μm, 70A) bulk packing) (USA). Fisher chemical provided anhydrous magnesium sulphate (UK). El-Nasr pharmaceutical chemicals co. provided sodium chloride (Abu-Zaabal, Cairo). El-Nile company for pharmaceuticals and chemical industries provided rat plasma from adult male Sprague-Dawley rats (Cairo, Egypt). Greiner-Bio-One GmbH provided vacutainer EDTA tubes (vacuette K3E) (Germany).

2.2. Methods

2.2.1. Apparatus

- Gradient Chromatography was performed on a Nexera LC-2040C (Shimadzu, Japan) with Shim-pack GISS C_{18} column (150 mm × 2.1 mm i.d, 1.9 μm) (Shimadzu, Japan) protected by a Shim-pack GISS C_{18} guard column (10 mm × 2.1 mm i.d, 1.9 μm) (Shimadzu, Japan).
- ZX3 Advanced Vortex mixer (F20230176, Alfa medical Westbury, China)
- Mechanical Shaker (Heidolph, Germany)

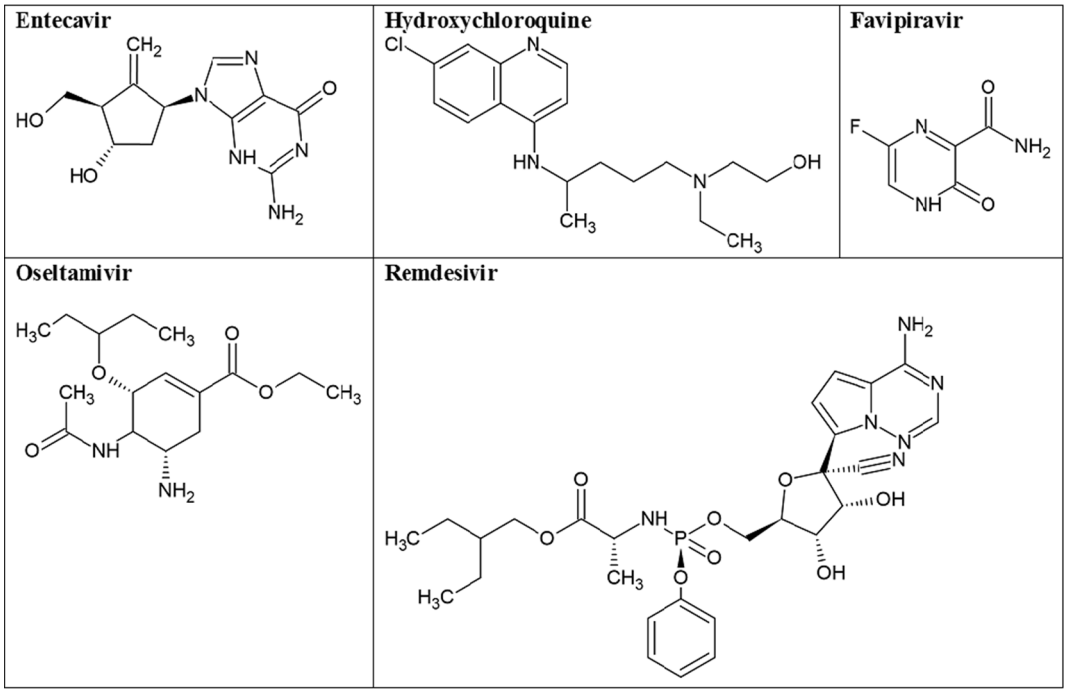

Fig. 1. Chemical structure of the studied compounds and the utilized IS.

- Z 36 HK Super High Speed Refrigerated Centrifuge (Hermle Labortechnik, Germany)

2.2.2. Chromatographic conditions:

Gradient elution was performed with Entecavir as the IS and mobile phase (A): water and (B): acetonitrile, both with 0.1 % (v/v) formic acid at a flow rate of 0.25 mL/min. The eluent was 80 % A and 20 % B for the first minute; after that, the proportion of eluent B climbed linearly to 100 % B in 6 min, then 100 % B till 8 min. 80 % A was continued for 5 min to return the column back to its original form. 1-μL sample injection was performed, and the temperature of autosampler was set at 4 °C, while column temperature was 45 °C. The overall run time was 13 min, including the stabilization period.

2.2.3. Mass spectrometric conditions:

The mass spectrometric analyses were carried out using a Shimadzu MS-8045 Triple Quadrupole Mass Spectrometer with an electrospray ion (ESI) source in the multiple reaction monitoring (MRM) mode to collect both the precursor and product ion spectra for analyte identification and confirmation. All of the compounds were polarised in the ESI positive mode. Data from the Shimadzu equipment was processed using Lab Solutions software. Interface temperature: 300 °C, Desolvation temperature: 250 °C, Heat block temperature: 400 °C, Drying gas flow: 10 L/min, Nebulizing gas flow: 3 L/min, Heating gas flow: 10 L/min. Table A.1 lists the mass operating parameters used in this study for detection.

2.3. Solutions and standards

Working solutions for Entecavir (IS), Hydroxychloroquine, Favipiravir, Oseltamivir, and Remdesivir were made by diluting standard stock solutions (1.0 mg/mL) in methanol to create a final concentration of (1.0 μg/mL) for all drugs except for Favipiravir working solution, with a concentration of (10 μg/mL). Final concentrations of (5, 10, 20, 30, 70, 100 ng/mL) for Hydroxychloroquine, (50, 100, 200, 500, 700, 1000 ng/mL) for Favipiravir, (5, 10, 20, 50, 70, 100 ng/mL) for Oseltamivir, and (10, 20, 30, 50, 70, 100 ng/mL) for Remdesivir were obtained by serial dilution from the working solutions. The mobile phase was used to carry out the dilutions. For analysis or validation, working solutions were freshly prepared. When not in use, all solutions were kept at −20 °C.

2.3.1. Spiked and real rat plasma samples

The standard working solution (1 μg/mL) was spiked in exact amounts (5–100 μL) into 50 μL of blank rat plasma. Hydroxychloroquine concentrations were (5, 10, 20, 30, 70, 100 ng/mL), Favipiravir concentrations were (50, 100, 200, 500, 700, 1000 ng/mL), Oseltamivir concentrations were (5, 10, 20, 50, 70, 100 ng/mL), and Remdesivir concentrations were (10, 20, 30, 50, 70, 100 ng/mL). Entecavir at a concentration of 50 ng/mL was utilized in all measurements. Hydroxychloroquine and Oseltamivir had QC levels of (5.0, 50.0, and 100.0 ng/mL), whereas Favipiravir had QC levels of (50, 500, 1000 ng/mL) and Remdesivir had QC levels of (10.0, 50.0, and 100.0 ng/mL).

Except for Oseltamivir, which was administered orally, samples were extracted from rats after injection of the examined mixture intraperitoneally. Blood was taken from the rat's venous sinus while it was immobilised, the neck was lightly scruffed, and the eye was left to protrude. Dorsally, medially or laterally, a capillary tube was inserted where blood was allowed to flow. Biological samples were placed in vacutainers containing EDTA and kept at −20 °C until analysis. Freshly manufactured QC samples and calibration standard samples were used.

2.4. Sample preparation

A minor QuEChERS process was used to vortex 50.0 μL of rat plasma (blank or spiked) in an eppendorff tube with 700 μL of methanol. Vortexed for another 5 min after adding 0.05 g sodium chloride and 0.1 g anhydrous magnesium sulphate. For 20 min, the tube was centrifuged at a high speed (6000 rpm). The acetonitrile-containing upper layer was then transferred to a clean eppendorff containing 60 mg anhydrous magnesium sulphate and 7 mg PSA, which was vortexed for 1 min and centrifuged for another 20 min at 6000 rpm. For UHPLC-MS/MS analysis, the pure extract was evaporated to dryness and then reconstituted in 1-mL mobile phase.

2.5. Method validation

The assay was fully validated in accordance with US-FDA criteria [32].

2.5.1. Linearity

Six analyte calibration standards were generated in blank rat plasma as mentioned before, and each was analyzed in five replicates. Peak area ratios of the analytes of interest to the IS versus their relative concentrations were used to create calibration curves for each analyte.

Departure from true concentrations should be less than 20% at the (LLOQ) and less than 15% at other concentrations with variation coefficients less than 20% and 15%; respectively [32].

2.5.2. Accuracy and precision

Five replicates of three distinct concentrations (n = 15) in rat plasma were analyzed, along with calibration standards made independently from the quality control (QC) samples, to determine accuracy. At the three QC levels stated above under spiked samples, accuracy was assessed as percentage recoveries, while intraday (n = 9) and interday (n = 9) precision were calculated as R.S.D %. At the lower limit of quantification (LLOQ), both precisions should be less than 20%, and at other concentrations, less than 15% [32].

2.5.3. Specificity and selectivity

Six blank rat plasma samples from six discrete sources were prepared and chromatograms at LLOQ and in blank plasma were compared to ensure that the assay was free of potential interfering chemicals.

Compounds co-eluting with the analyte or IS should not have peak areas more than 20 % of the analyte peak area at the LLOQ or 5% of the IS area. For LLOQ samples, departure from true concentrations should be less than 20 % [32].

2.5.4. Matrix effect and absolute recovery

The matrix effect was assessed by comparing the average peak areas of Hydroxychloroquine, Favipiravir, Oseltamivir, and Remdesivir spiked in rat plasma samples post-extraction to the analytes in pure solvent. They were calculated and expressed as recovery %. Absolute recovery was estimated by comparing the average peak areas of the studied drugs in spiked rat plasma samples at five different concentrations to the analytes' peak areas in pure solvent at the same concentrations.

2.5.5. Stability

The stability of Hydroxychloroquine, Favipiravir, Oseltamivir, and Remdesivir at LQC (5, 50, 5, 10 ng/mL respectively) and HQC (100, 1000, 100, 100 ng/mL) was evaluated. Samples were determined by comparing newly generated spiked rat samples to those exposed to varied stability conditions. The sample's short-term stability was tested at 4 °C for 6–12 h, as well as after 48 h in the autosampler at 4 °C. At −20 °C, the stability was tested after three freeze–thaw cycles. After two weeks of freezing at −20 °C, the samples were checked for long-term stability. When 90–110 % of the fresh sample's ratio is found in stock and working solutions, analytes are termed stable, and when 85–115 % of the initial concentration is recovered in biological extracts, they are also considered stable [32].

2.6. Animal treatment with pharmaceuticals

After approval of the Research Ethics committee at the Faculty of Pharmacy, Ain Shams University, all procedures were performed in accordance with relevant laws and institutional guidelines for the care and use of laboratory animals [33]. A short pharmacokinetic investigation in male adult healthy rats (8 weeks, 150–200 g, n = 7) was supported by the analytical approach presented in this work. Rats were kept in cages made of polystyrene at the animal facility of Ain Shams University's Faculty of Pharmacy, under constant humidity and temperature conditions. They were given an adequate amount of drinking water, however, they were denied food for one day prior to the experiment and were acclimatized to the animal facility habitat for some days before the study began. Rats were denied food to reduce variability in investigatory parameters as the presence of food in the digestive tract may reduce the absorption of drugs.

Except for Oseltamivir, which was given orally in this trial, other medications were given intraperitoneally. Hydroxychloroquine's dose was 6.5 mg/kg, Favipiravir was given at 8 mg/kg, Oseltamivir's dose was 10 mg/kg, and Remdesivir was given at 50 mg/kg. Blood samples were taken at various time intervals (0.5, 1, 2, 4, 6 and 8). Then, they were transferred to anticoagulant tubes and centrifuged for 10 min at 4° C at 4,000 rpm for plasma separation, after which the plasma was withdrawn and kept at −20 °C for analysis.

3. Results and discussion:

Several analytical approaches for detection and quantification of antiviral drugs presently being evaluated for COVID-19 treatment in biological fluids and tissues have been proposed in the recent decade, including sample pretreatment and detection procedures [14]. The current study proposes a sensitive UHPLC-MS/MS method for quantitative assessment of Hydroxychloroquine, Favipiravir, Oseltamivir, and Remdesivir using the QuEChERS procedure for extraction in rat plasma, which can be extended for therapeutic drug monitoring in COVID-19 patients on the same treatment.

3.1. Optimization of chromatographic conditions

It was a challenge to establish a selective and sensitive approach for simultaneous assessment of Hydroxychloroquine, Favipiravir, Oseltamivir, and Remdesivir due to their polarity differences. This approach an UHPLC C_{18} column (150 mm × 2.1 mm i.d, 1.9 μm) to boost separation speed, provide better resolution, eliminate the potential impact from a

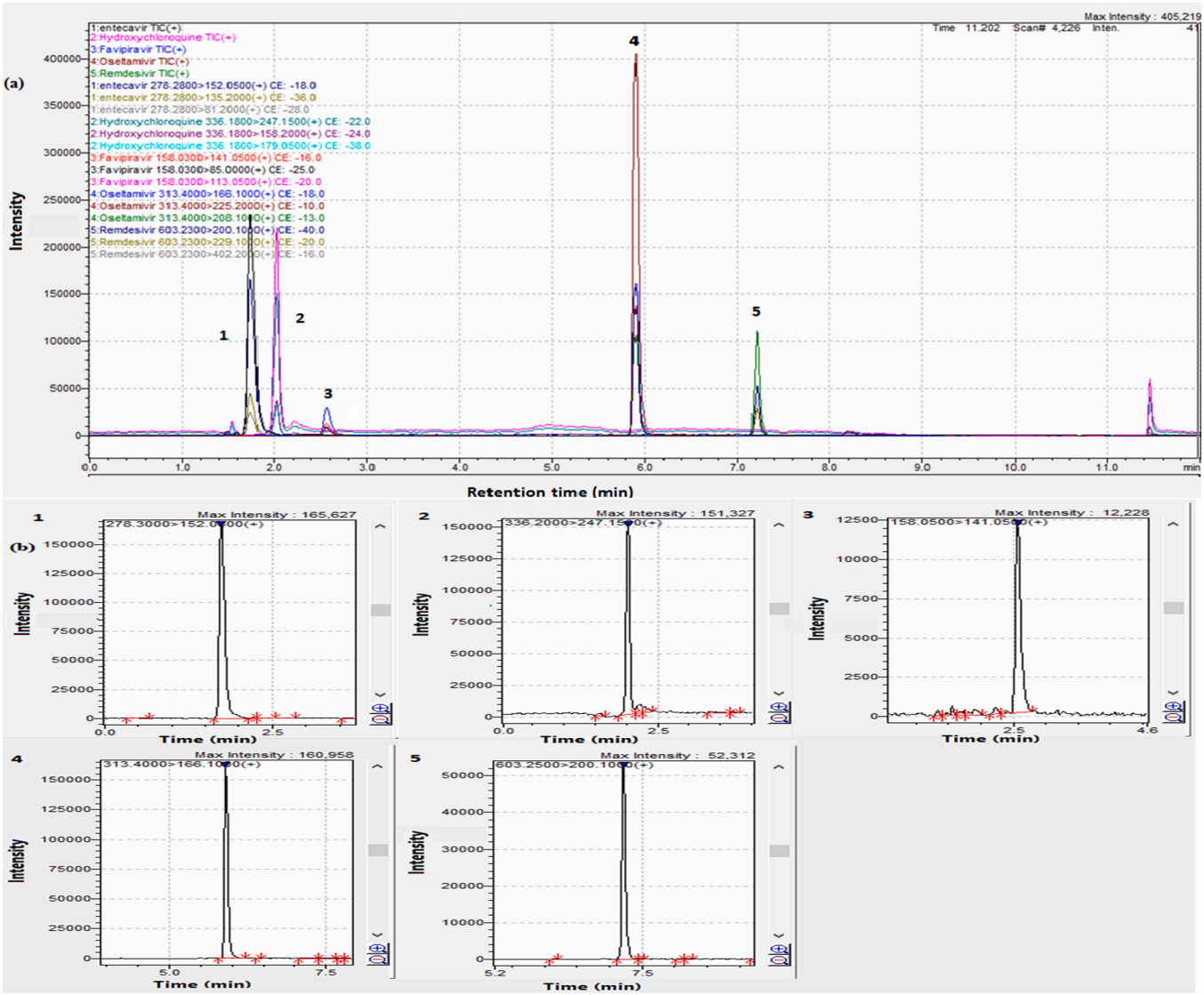

Fig. 2. (a) TIC chromatograms, (b) Extracted ion chromatograms of 1.(50 ng/mL) Entecavir, 2. (20 ng/mL) Hydroxychloroquine, 3. (200 ng/mL) Favipiravir, 4. (20 ng/mL) Oseltamivir and 5. (30 ng/mL) Remdesivir.

complicated matrix and improve efficiency of analysis which results in fast method development in addition to using fewer solvents. Methanol: water and acetonitrile: water; both with formic acid were tested, however, acetonitrile: water with formic acid was the mobile phase of choice as it produced lower back pressure in addition to its better separation without ghost peaks, unlike methanol. 0.1 % (v/v) formic acid was more preferred than acetic acid for its better ionization efficiency. Gradient elution was performed with flow rate 0.25 mL/min to obtain a shorter run time of 13 min as shown in Fig. 2a, Fig. 2b and Fig. 3 for total ion, extracted ion and MRM chromatograms of the studied analytes; respectively.

3.2. Optimization of MS parameters

The response for Hydroxychloroquine, Favipiravir, Oseltamivir, Remdesivir, and Entecavir (IS) was significantly better when ESI was operated in positive ion mode than when it was run in negative ion mode as all the investigated drugs had higher affinities for protonation forming abundant protonated molecules (precursor ions) which were then fragmented in the collision cell into product ions with high intensities. The multiple-reaction-monitoring (MRM) technique of detection was used to achieve excellent selectivity and sensitivity by using the most prevalent product ions for each compound. Table 1 shows the MRM ion transitions for analytes and IS, while Fig. 4 shows the product ion mass spectra.

3.3. Sample preparation development

One of the analytical problems in chromatographic analysis is sample pretreatment in order to achieve high throughput with improved separation without affecting the analytical column. One of the most favorable methods for high-speed, high-efficiency analysis is to miniaturize the apparatus and the sample preparation process. To precipitate plasma proteins, samples were extracted using a minimal "QuEChERS" technique with a minimum amount of plasma (50 μL) and a minimum amount of organic solvent "methanol" (700 μL). Reproducible clean samples with high recoveries and appropriate selectivity for all analytes were obtained using this approach. Acetonitrile, methanol and a combination of both in equal ratios were studied as extraction solvents. As indicated in Fig. 5, methanol was found to be the most efficient in extraction as it had good solubility with the target analytes, poor solubility with the impurities in addition to its volatility and clean extract resulting in the highest extraction yield. Primary secondary amine (PSA) and C_{18} were examined as adsorbents in the clean-up process. Remdesivir was significantly retained using C_{18} adsorbent, with a recovery of 0%, and hence was not considered in the clean-up stage; instead, PSA was utilized. The amount of magnesium sulphate used was also optimized, with 0.06 g yielding the best results (see Fig. 5).

3.4. Method validation

3.4.1. Selectivity

By evaluating both blank rat plasma samples from six distinct sources and blank plasma treated with the drugs at LLOQ, selectivity was demonstrated. There was no evidence of endogenous matrix components interfering with the retention times of the compounds examined. Fig. A1 shows chromatograms of blank rat plasma to illustrate this.

3.4.2. Linearity and sensitivity

As shown in Supplementary Fig. A2, linearity was assessed by building calibration curves of peak area ratios of the analytes to the IS against their relative concentrations. All analytes had linear ranges with regression coefficients greater than 0.999 for all compounds, 5–100 ng/mL for Hydroxychloroquine, 50–1000 ng/mL for Favipiravir, 5–100 ng/mL for Oseltamivir, and 10–100 ng/mL for Remdesivir. Table 2 shows that the detection and quantitation limits ranged from 0.11 to 10 ppb and 0.36 to 33.33 ppb; respectively. The LLOQ within 20% accuracy and precision defines sensitivity. It was also assessed using the signal to noise ratio (S/N).

3.4.3. Accuracy and precision

Percent recovery was used to assess accuracy by comparing measured and real concentrations. QC samples at 5, 50, 100 ng/mL for Hydroxychloroquine, 50, 500, 1000 ng/mL for Favipiravir, 5, 50, 100

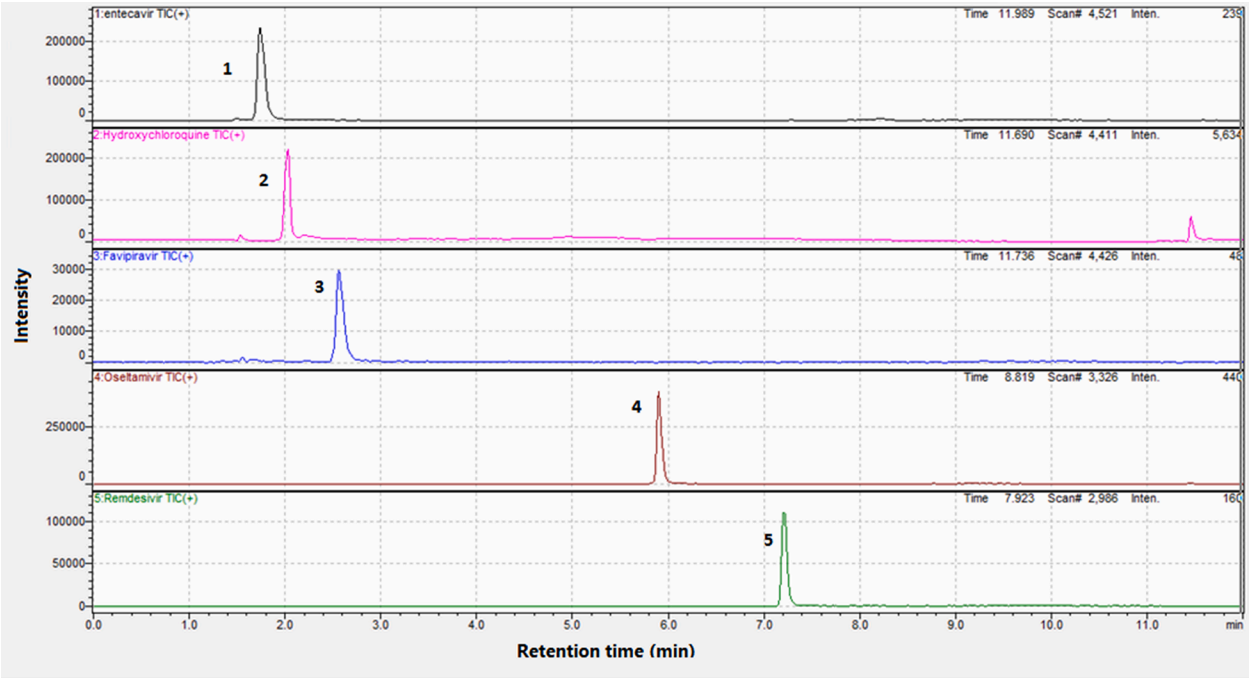

Fig. 3. MRM chromatograms of 1. (50 ng/mL) Entecavir, 2. (20 ng/mL) Hydroxychloroquine, 3. (200 ng/mL) Favipiravir, 4. (20 ng/mL) Oseltamivir and 5. (30 ng/mL) Remdesivir.

Table 1
Detection parameters and retention time of the analytes and IS.

Analyte	Retention time (min)	Precursor (*m/z*) (Qualifier ion)	Product (*m/z*)	Dwell time (msec)	Q1 pre Bias (V)	CE	Q3 pre Bias (V)
Entecavir (IS)	1.73	278.28	**152.10***	50	10	18	29
			135.05		10	35	25
			81.00		21	10	22
Hydroxychloroquine	2.07	336.18	**247.15***	50	16	22	26
			158.20		12	24	29
			179.05		12	38	18
Favipiravir	2.57	158.03	**141.05***	50	10	16	26
			85.00		10	25	15
			113.05		17	20	20
Oseltamivir	5.91	313.40	**166.10***	50	11	18	30
			225.20		11	10	23
			208.10		15	13	22
Remdesivir	7.21	603.23	**200.10***	50	22	40	21
			229.10		22	20	23
			402.20		22	16	19

* Quantifier ions; ions used for quantification (target ions) in bold.

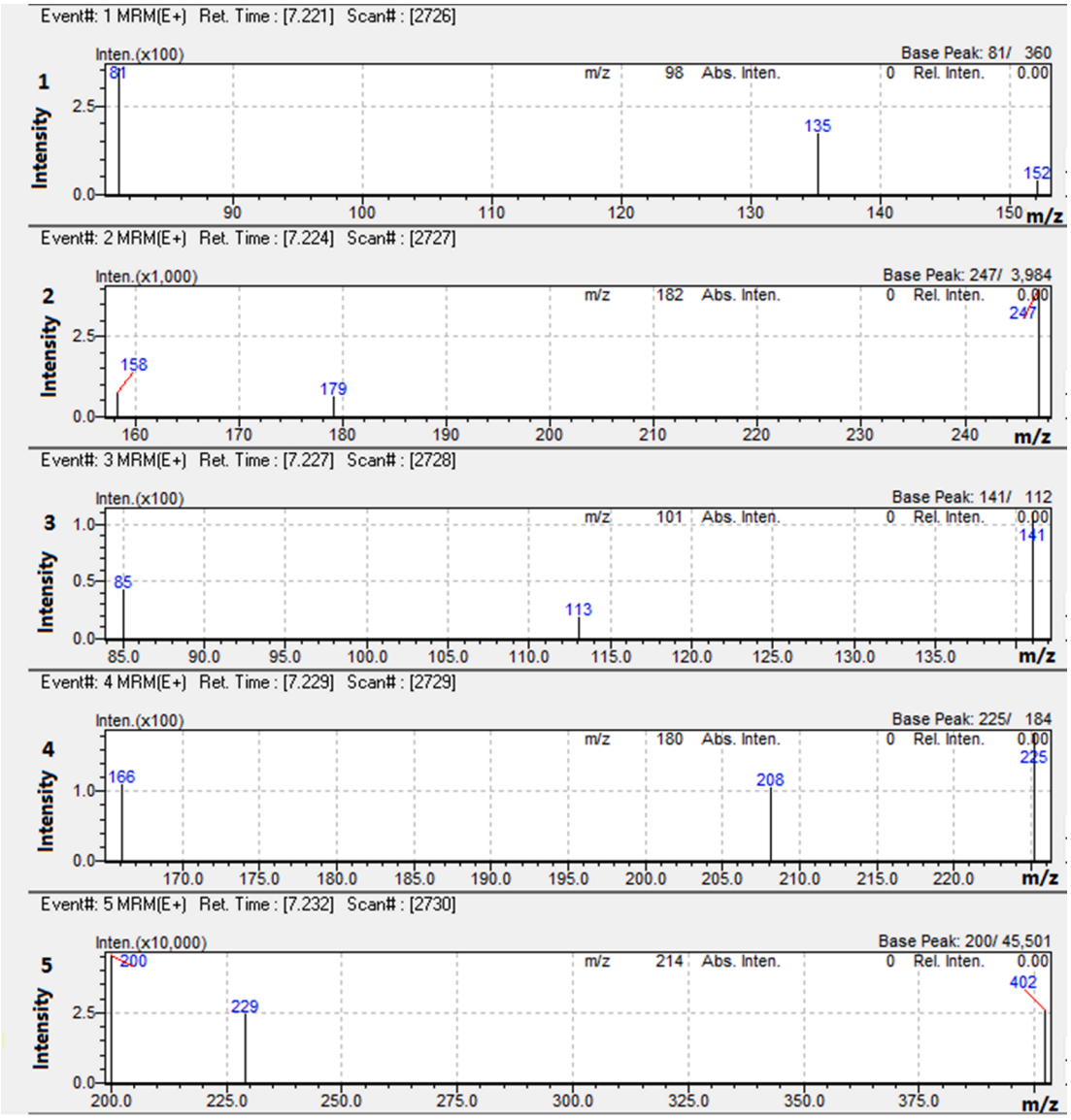

Fig. 4. Product ion mass spectra of 1. (50 ng/mL) Entecavir, 2. (20 ng/mL) Hydroxychloroquine, 3. (200 ng/mL) Favipiravir, 4. (20 ng/mL) Oseltamivir and 5. (30 ng/mL) Remdesivir.

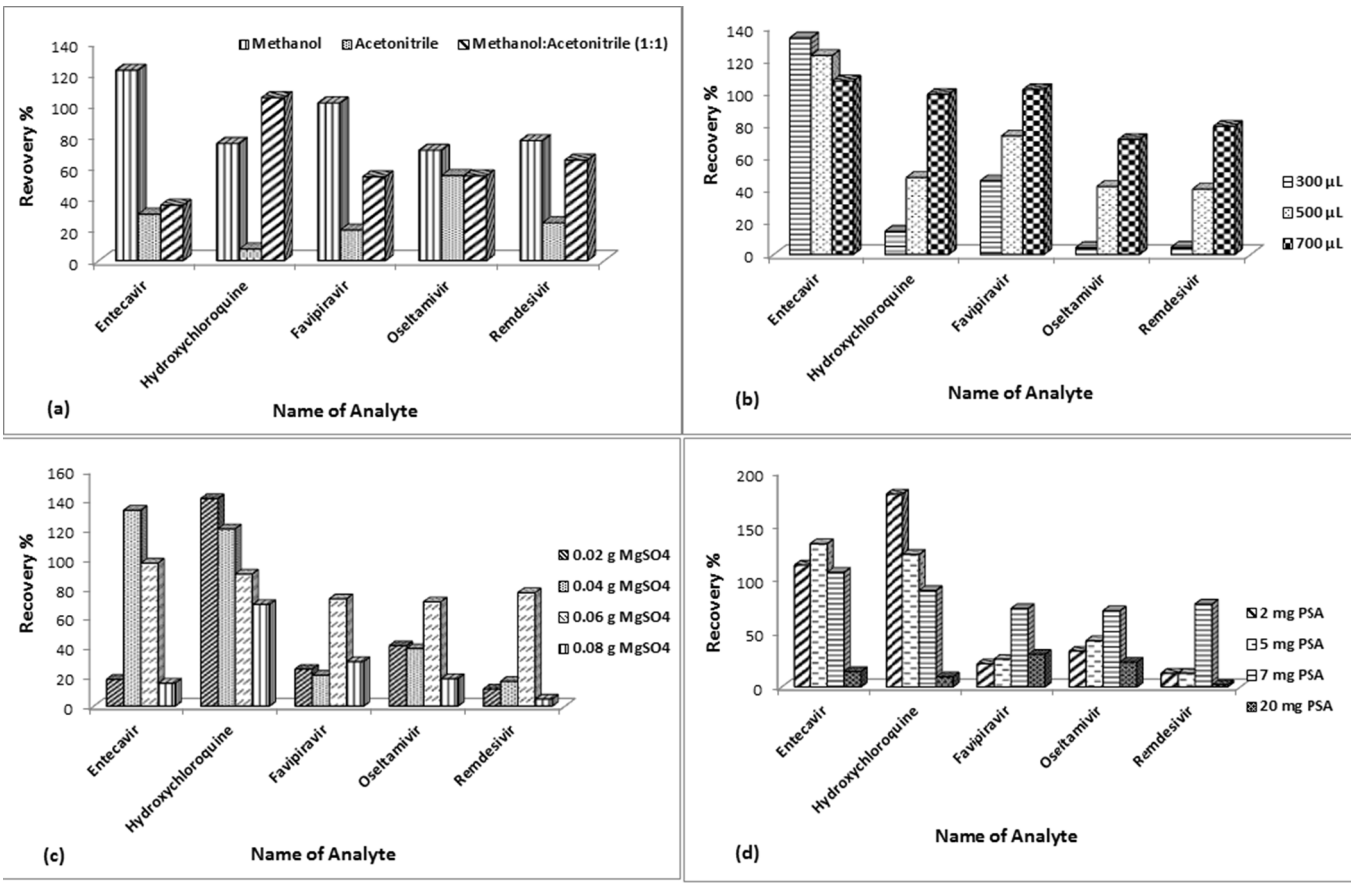

Fig. 5. Effect of clean-up step on the extraction efficiency of the compounds from spiked rat plasma using (a) different solvents, (b) different amounts of methanol, (c) different amounts of $MgSO_4$ and (d) different amounts of PSA.

Table 2
Validation parameters of the method for the studied analytes in rat plasma.

Parameter	Hydroxychloroquine	Favipiravir	Oseltamivir	Remdesivir
Linear range (ng/mL)	5–100	50–1000	5–100	10–100
Mean ± Standard deviation (S.D)	99.75 ± 0.75	98.01 ± 1.41	99.63 ± 1.31	100.04 ± 1.67
R.S.D*	0.75	0.14	1.31	1.67
Regression Equation	Y = 0.0277 X -0.0079	Y = 0.0003X + 0.021	Y = 0.0235X + 0.1993	Y = 0.0197X ± 0.1648
Correlation Coefficient (r)	0.9999	0.9998	0.9999	0.9999
Intraday precision (%R.S.D)	0.83	0.47	2.30	1.10
Interday Precision (%R.S.D)	0.60	0.85	0.52	0.55
Limit of Detection (L.O.D)** (ng/mL)	0.11	10.00	0.92	1.71
Limit of Quantitation (L.O.Q)*** (ng/mL)	0.36	33.33	3.07	5.72

LOD and LOQ are determined based on signal to noise ratio.
RSD*: relative standard deviation before
LOD** =3 × S/N.
LOQ***=10 × S/N.

ng/mL for Oseltamivir, and 10, 50, 100 ng/mL for Remdesivir were used to assess intraday and interday precision in rat plasma. RSD % was used to evaluate precision, whereas Table A.1 shows that the proposed approach has great accuracy in plasma samples, with RSD values of less than 2.34 % for all analytes in the QC samples.

3.4.4. Recovery

Peak area ratios from plasma samples treated according to the miniaturized extraction technique "QuEChERS" were compared to peak area ratios of the studied drugs' standard solutions in the mobile phase (which represented 100 % recovery) to evaluate absolute recoveries. Table 3 reveals that recoveries ranged from 82.39 % to 105.87 % for all analytes with standard deviations less than 15.7.

3.4.5. Matrix effect

The presence of co-eluting chemicals in the sample causes the matrix effect, which leads to alteration in the analytical assay. Matrix effect is studied to examine if there is any variation in ionization as a result of the matrix's various components. The peak area ratios of spiked samples post-preparation were used to determine the matrix effect (compared to those obtained from the measurement of pure standards with similar concentrations). All analytes showed ion enhancement at all concentrations except for Hydroxychloroquine and Entecavir (IS), which showed ion suppression. This might be due to competition between polar unretained matrix components and co-eluting Hydroxychloroquine and Entecavir ions in the sprayed solution, matrix interferences that bind to these analytes and cause them to co-precipitate,

Table 3
Absolute Recovery and matrix effect at five different concentration levels for the studied analytes in rat plasma using UHPLC-MS/MS (n = 15).

Name of Analyte	Absolute Recovery		Matrix effect			
	Spiked level (ng/mL)	Found (ng/mL)	Rec % *	Spiked level (ng/mL)	Found (ng/mL)	Matrix Effect (ME %)*
Entecavir (IS)	50	52.94	105.88	50	43.10	86.20
	50	53.89	107.78	50	48.99	97.98
	50	50.82	101.64	50	46.58	93.15
	50	55.55	111.1	50	45.75	91.50
	50	51.48	102.96	50	44.38	88.75
			Rec ± S.D	**105.87 ± 3.79**	**Rec ± S.D**	**91.52 ± 4.48**
Hydroxychloroquine	5	3.92	78.4	5	4.25	85.00
	10	8.00	80	10	8.49	84.90
	30	32.46	108.2	30	24.22	80.73
	70	76.33	109.04	70	58.18	83.11
	100	105.82	105.82	100	87.73	87.73
			Rec ± S.D	**96.31 ± 15.64**	**Rec ± S.D**	**84.29 ± 2.59**
Favipiravir	50	50.11	100.22	50	54.23	108.46
	100	91.93	91.93	100	112.52	112.52
	300	270.84	90.28	300	320.28	106.76
	500	558.65	111.73	500	578.2	115.64
	1000	851.30	85.13	1000	1195	119.5
			Rec ± S.D	**95.86 ± 10.40**	**Rec ± S.D**	**112.58 ± 5.20**
Oseltamivir	5	5.55	111	5	5.58	111.65
	20	17.89	89.45	25	28.66	114.64
	50	45.81	91.62	50	53.1	106.20
	90	74.53	82.81	75	87.31	116.41
	100	83.92	83.92	100	113.08	113.08
			Rec ± S.D	**91.76 ± 11.37**	**Rec ± S.D**	**112.40 ± 3.89**
Remdesivir	10	7.81	78.1	10	10.06	100.60
	30	25.04	83.47	30	31.04	103.47
	50	42.77	85.54	50	51.64	103.28
	70	56.34	80.49	70	78.38	111.97
	100	84.37	84.37	100	102.48	102.48
			Rec ± S.D	**82.39 ± 3.04**	**Rec ± S.D**	**104.36 ± 4.40**

Average of 5 determinations*

or LC column overloading [20]. The intrinsic ionization efficiency of analytes is determined by both physical and chemical parameters (e.g., basicity and surface activity). Because biological sample matrices naturally include numerous endogenous species with high basicity and surface activity, the overall concentration of these species in the sample will rapidly approach levels where ion suppression is predicted. Table 3 summarizes the permitted ranges for mean recovery percentages and % RSD.

3.4.6. *Stability*

Table 4 summarizes the stability data, which shows that all compounds were stable in rat plasma held at ambient temperatures for up to 12 h. Reproducibility was checked where samples remained stable after 48 h in the autosampler. In rat plasma, samples were also stable for three freeze–thaw cycles (at −20 °C). Finally, long-term stability was tested, with all chemicals being stable in the final extract at −20 °C for up to 2 weeks. All of the analytes examined were stable in stock and working solutions for up to one month except for Hydroxychloroquine, which deteriorated and was no longer stable after one month.

Table 4
Stability data for Hydroxychloroquine, Favipiravir, Oseltamivir and Remdesivir by means of the proposed method.

Compound	Short term Stability					Autosampler Stability				
	Initial Conc.	After 6 h*	After 12 h*	Accuracy	% RSD	Initial Conc.	After 18 h*	After 24 h*	Accuracy	% RSD
Hydroxychloroquine	5	4.80	4.39	91.90	6.31	5	4.72	4.19	89.10	8.41
	100	107.5	113.48	110.49	3.83	100	96.36	93.42	94.89	2.19
Favipiravir	50	45.84	47.51	93.35	2.53	50	47.21	43.15	90.36	6.35
	1000	964.48	941.59	95.30	1.70	1000	948.28	921.05	93.47	2.06
Oseltamivir	5	4.52	4.67	91.90	2.31	5	4.29	4.72	90.10	6.75
	100	96.37	94.61	95.49	1.30	100	96.28	92.04	94.16	3.18
Remdesivir	10	9.07	9.31	91.90	1.85	10	9.19	10.55	98.70	9.74
	100	93.71	92.08	92.90	1.24	100	93.64	90.81	92.23	2.17
Compound	**Freeze and thaw Stability**					**Long term Stability**				
	Initial Conc.	**1st cycle***	**3rd cycle***	**Accuracy**	**% RSD**	**Initial Conc.**	**1st day***	**Last day***	**Accuracy**	**% RSD**
Hydroxychloroquine	5	4.41	4.34	87.50	1.13	5	4.26	4.45	87.10	3.08
	100	96.49	90.03	93.26	4.90	100	96.57	92.21	94.39	3.27
Favipiravir	50	46.81	54.79	101.60	11.11	50	46.91	45.81	92.72	1.68
	1000	972.61	1050.55	101.16	5.45	1000	967.71	935.81	95.18	2.37
Oseltamivir	5	4.64	5.42	100.60	10.97	5	4.51	4.32	88.30	3.04
	100	103.51	107.49	105.50	2.67	100	97.43	92.89	95.16	3.37
Remdesivir	10	9.62	9.45	95.35	1.26	10	8.92	8.61	87.65	2.50
	100	97.81	94.48	96.15	2.45	100	93.43	90.51	91.97	2.25

*: Average of 3 replicates

Table 5
Plasma concentrations and Pharmacokinetic parameters of the studied analytes in plasma samples obtained from male rats after administration of a single dose (n = 7).

Analyte (Dose in mg/kg)	*Plasma concentrations (ng/mL) at different time intervals						Cmax (ng/mL)	Tmax (h)	AUC**
	0.5 h	1 h	2 h	4 h	6 h	8 h			
Hydroxychloroquine (6.5 mg/kg)	295.74	356.57	930.10	1393.22	368.58	124.78	1393.22	4	2255.16
Favipiravir (8 mg/kg)	13099.24	15980.35	20335.90	31496.33	3197.78	960.39	31496.33	4	38852.27
Oseltamivir (10 mg/kg)	0.27	2.62	3.13	7.39	15.42	5.74	15.42	6	43.97
Remdesivir (50 mg/kg)	11.68	11.37	14.20	25.83	9.73	4.28	25.83	4	49.57

*: Average of 3 replicates.
AUC**: area under the curve.

3.5. Application of the method to the analysis of real samples

The suggested procedure's efficiency was demonstrated when it was used to quantify the target analytes in rat plasma samples for a minor pharmacokinetic research, as shown in Table 5 which shows the concentration profile of the analytes and pharmacokinetic parameters after intraperitoneal and oral treatment in rats (n = 7) during an 8-hour period. All compounds showed a progressive increase in plasma concentration, with the maximum achieved at 4 h except for Oseltamivir, which was delivered orally and reached its maximum concentration at 6 h. Favipiravir had the greatest peak concentration, followed by Hydroxychloroquine and Remdesivir, while Oseltamivir exhibited the least peak concentration.

4. Conclusion

Using a simplified QuEChERS extraction approach, we offer a unique, sensitive UHPLC/MS/MS method for quantification of some drug candidates for COVID-19 named Hydroxychloroquine, Favipiravir, Oseltamivir, and Remdesivir in rat plasma samples. The established method demonstrated high accuracy and reproducibility, allowing it to be employed in a short pharmacokinetic study in real rats as a reference for future clinical studies.

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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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Appendix A. Supplementary data

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