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Rapid and ecofriendly UPLC quantification of Remdesivir, Favipiravir and Dexamethasone for accurate therapeutic drug monitoring in Covid-19 Patient’s plasma

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ABSTRACT

Keywords: Favipiravir, Remdesivir, Dexamethasone, Plasma, UPLC, Co-administered

Innovative therapeutic protocols to the rapidly spreading coronavirus disease (COVID-19) epidemic is highly required all across the world. As demonstrated by clinical studies, Favipiravir (FVP) and Remdesivir (REM) are new antiviral medicines that are effective against COVID-19. REM is the first FDA approved antiviral medicine against COVID-19. In addition to antivirals, corticosteroids such as dexamethasone (DEX), and anticoagulants such as apixaban (PX) are used in multidrug combinations protocols. This work develops and validates simple and selective screening of the four medicines of COVID-19 therapeutic protocol. FVP, REM, DEX, and PX as internal standard in human plasma using UPLC method by C18 column and methanol, acetonitrile, and water acidified by orthophosphate (pH = 4) in a ratio of (15: 35: 50, by volume) as an eluate flowing at 0.3 mL/min. The eluent was detected at 240 nm. The method was linear over (0.1–10 μg/mL) for each of FVP, REM, and DEX. The validation of the UPLC method was assessed in accordance with FDA guidelines. The method can detect as low as down to 0.1 μg/mL for all. The recoveries of the drugs in spiked human plasma ranged from 97.67 to 102.98 percent. Method accuracy and precision were assessed and the drugs showed good stability. The method was proven to be green to the environment after greenness checking by greenness profile and Eco-Scale tool.

1. Introduction

Since the coronavirus disease 2019 (COVID-19) began as a fast spreading epidemic, it has claimed the lives of up to 4.6 million people globally through September 2021 [1]. Its severe acute respiratory syndrome, which affects the lower respiratory tract and can lead to mortality [2]. Many vaccines that were just approved failed to stop the life-threatening pandemic. This could be related to the inadequacy and unavailability of vaccination and mutation, as well as a lack of alternative acceptable therapeutic approaches [3]. As a result, repurposing the utilization of commercially available antiviral medicines like Favipiravir (FVP), and Remdesivir (REM) are viewed as a viable and effective approach [4,5].

To combat the clinical signs of patients infected with the severe acute respiratory syndrome coronavirus 2, several therapy options have been attempted. Although the clinical signs of COVID-19 pneumonia are similar to those of a common upper respiratory tract infection, a chest CT scan offers a higher degree of specificity [6].

But serious pathological problems, which can lead to mortality, can develop in people with diabetes, chronic respiratory and cardiovascular disorder, cancer, and immune-deficient patients and elderly [7].

The importance of the determination of Favipiravir and Remdesivir levels in human plasma in the determination of COVID-19 is related to the current use of it in some countries’ protocols until now, such as India, Egypt, and Russia protocols [8]. The activity of Favipiravir as an antiviral against severe acute respiratory syndrome and its wide therapeutic safety margin is indicated by a wide CC50/EC50 ratio for high doses. Due to the wide range of illustrated severe symptoms provided by COVID-19, different strategies for managing symptoms were included in the protocol of the coronavirus in different countries. The use of those drugs mainly depended on their efficacy to reduce the severe symptoms accompanied by COVID-19, such as Dexamethasone and anticoagulants.

FVP, or 6-fluoro-3-hydroxy-2-pyrazine carboxamide, was produced by Toyama Chemical Company in Japan as an anti-influenza drug
REM is a prodrug of an adenosine triphosphate (ATP) analogue. According to preliminary findings REM seemed to accelerate recovery in hospitalised individuals with severe COVID-19. It was the first medicine to be approved for emergency use by the US Food and Drug Administration (FDA) to treat COVID-19 patients who were hospitalised. Antivirals work by inhibiting RNA polymerase, which prevents coronavirus replication. Dexamethasone, a synthetic corticosteroid analogue, which is a potent anti-inflammatory drug that is used to treat various immune and inflammatory diseases. Anticoagulants to decrease the tendency of thrombosis were demonstrated and prescribed in almost all COVID-19 patients. To our knowledge, various analytical methods for determining FVP, REM, and DEX individually have been proposed, including liquid chromatography, electrochemical methods, and spectrofluorometric methods. The chemical structures of these investigated REM co-administered drugs are shown in Fig. 1.

The importance of determination of Favipiravir, Remdesivir and Dexamethasone levels in human plasma is related to their current use for management of COVID-19 disease in many countries’ protocols, remdesivir received a EUA in May 2020 on the basis of preliminary results from ACCT (Adaptive COVID-19 Treatment Trial) and full FDA approval in October 2020. Dexamethasone, already approved by the FDA for various indications, has been shown to reduce COVID-19 mortality among hospitalized patients requiring oxygen alone or mechanical ventilation. Current clinical guidelines from the National Institutes of Health include both remdesivir and dexamethasone as treatments for some adults hospitalized with clinically suspected or laboratory-confirmed SARS-CoV-2 infection.

Since SARS-CoV2 is an RNA virus, remdesivir and favipiravir, both broad-spectrum RNA polymerase inhibitors, were repurposed for

![Fig. 1. Chemical structure of Favipiravir (FVP), Remdesivir (REM), Dexamethasone (DEX) and Apixaban (PX).](image-url)
treated COVID-19 patients. A new research. Co-administration of Favipiravir and the Remdesivir Metabolite GS-441524 Effectively Reduces SARS-CoV-2 Replication in the Lungs of the Syrian Hamster Model. mBio. 13. e0304421. https://doi.org/10.1128/mbio.03044–21.) published in 2022 proved that the co administration of remdesivir and favipiravir more efficiently reduced virus load in hamster lungs than did single administration also efficiently inhibited lung inflammation in the infected animals [41]. In addition to antivirals, corticosteroids such as dexamethasone are used in multidrug combinations protocols. Development of sensitive and selective methods for simultaneous determination of the three drugs is very important as they can be used in pharmacokinetic studies and monitoring of therapeutic doses when they are co administered, or as a greener and more cost and time saving method than the published ones for determination of each of the three drugs if taken individually.

Due to the worldwide spread of COVID-19 infection, hence the lack of vaccinations, and no methods were reported for concurrent analysis of FVP, REM, and DEX in human plasma. That encourages us to develop a highly sensitive, simple and eco-friendly analytical method for the analysis of the co-prescribed drugs in the COVID-19 protocol in pure form. In addition, application of the method to human plasma is vital for drug monitoring in ICU hospitalized patients. Over and above, the freeze– thaw cycle showed that the drugs were stable when stored at –8 °C and –20 °C. The developed method is the first developed one, and it has the advantages of being sensitive, robust and selective.

The greenness of analytical techniques [42–47] is now regarded as a prerequisite since it ensures safety for both individuals and the environment by limiting the use of carcinogenic solvents or replacing them with more environmentally friendly ones [48].

2. Experimental

2.1. Apparatus

UPLC Dionex Ultimate 3000 (Germany) instrument with Dionex Ultimate 3000 SR (pump (HPG-3400), Diode array detector (DAD-3000RS), column compartment (TCC-3100), and Auto sampler (WPS-3000). The software was Chromeleon, USA. The UPLC system was supplied with an automatic injector, a quaternary pump, and a vacuum degasser. The separations were done on a reversed phase Agilent, version 7.2. It was BEH C18 (150 mm × 2.1 mm, 1.7 μm) at analytical that was used for separation and quantification.

2.2. Reagents and materials

The HPLC grade methanol, acetonitrile, and orthophosphoric acid were bought from (Riedel-dehaen, Sigma-Aldrich, Germany).

While deionized water (SEDICO Pharmaceuticals Co., Cairo, Egypt).

2.3. Samples

Rameda Company (Cairo, Egypt) graciously provided FVP, REM, and DEX authentic samples as gifts, with claimed purity of 99.95 % ± 1.24, 99.98 % ± 1.57 and 99.87%± 1.07, respectively. EVA Pharma (Giza, Egypt) graciously provided PX as a gift with a purity claim of 101.28 % ± 1.04. The plasma samples were provided by the National Egyptian Blood Bank and stored at –20 °C until used.

2.4. Standard solutions

- Working standard solutions (100 μg/mL) were prepared by accurately and separately transferring 10 mL of each drug into a 100 mL volumetric flask and completing the volume with methanol.

2.5. Chromatographic conditions

The separations were done on a reversed phase BEH C18 (150 mm × 2.1 mm, 1.7 μm) column at 25 °C. Elution was applied using methanol, acetonitrile and water acidified with orthophosphoric acid (pH 4) as a mobile phase in the following ratio: (15: 35: 50, by volume) respectively. The injection volume was 5 μL, while 0.3 mL/min was used as the flow rate at room temperature. The drugs were detected at 240 nm. Using PX as an internal standard. Using an internal standard corrects for the loss of analyte during sample preparation and analysis, provided that it is selected appropriately.

But using apixaban, especially after trying more than 12 drugs such as baclofen, mepivacine, and other drugs, only 3 of them give good separation with our drugs, but we choose apixaban due to its value as an anticoagulant and also its role in the COVID-19 protocol.

2.6. Analytical curves

In three different series of 10 mL volumetric flasks, different aliquots of each drug were accurately transferred from their corresponding working solutions: 1 mL of PX (100 μg/mL), and 1 mL of thawed plasma were accurately added to each sample, and methanol was used to complete the volume. The solutions were mixed using a vortex, centrifuged at 4500 rpm for 10 min, filtered through a 0.45 μm Millipore syringe filter, and used for analysis under the specified chromatographic conditions.

The integrated peak areas of FVP, REM, and DEX/peak area of PX (peak area ratio) were used to establish the analytical curves and regression equations in the method for the three drugs. Each analytical curve was constructed over a range of 0.1–10 μg/mL.

3. Results and discussion

The Smart and green UPLC method was developed for determining FVP, REM, and DEX in human plasma with minimal impact on the environment. The developed UPLC method has the advantages of effectively separating several analytes with low solvent consumption and a relatively simple sample preparation procedure to achieve good resolution with high accuracy and reproducibility. From the literature survey discussed previously, no UPLC or TLC methods were published for the simultaneous quantitation of these co-prescribed drugs in human plasma samples, so it is important to develop methods that are able to determine the concentrations of FVP, REM, and DEX at their C concentration (is the maximum serum concentration) and around it in plasma, hence it was reported that the C of favipiravir is 51.5 µg/mL, t max is 0.5 h, and the clinical PX dose is 1600 mg twice daily on day 1, 600 mg twice daily from day 2, while the C of remdesivir is in the range of 0.057 µg/mL to 4.420 µg/mL, t max is 0.68 h and the clinical dose is 200 mg loading dose on day 1, followed by 12 days of 100 mg. Also, dexamethasone C max is 64.4 µg/mL, t max is 2 h and the clinical dose is 1–1.5 mg/m².

The C of each of the studied drugs was within its corresponding linearity range making the proposed method applicable for therapeutic drug monitoring of these drugs in hospital COVID-19 patients and their simple determination in a single chromatographic run at their C or even at lower concentrations.

3.1. Method development and optimization

To achieve the best separation for FVP, REM, DEX, and PX, several chromatographic parameters, especially the mobile phase composition, were tuned.
The factors affecting the proposed UPLC method were adjusted, including:

3.1.1. Stationary phase selection

The C18 (250 mm 4.6 mm), C8 (250 mm 4.6 mm), and C18 (150 mm 2.1 mm) columns were all tested. The C18 (150 mm 2.1 mm) column provided the best separation and minimal baseline noise with the shortest run time. While the C8 column did not result in the best retention or separation of REM, the C18 UPLC column was the best choice because it attained the required separation in 3.65 min.

3.1.2. Mobile phase optimization

Organic modifiers such as methanol, ethanol, and acetonitrile in different ratios and combinations were tried, and a mixture of acetonitrile and methanol was the best organic modifier that produced promising results.

Different results of acetonitrile and methanol along with water were tried, observing optimum chromatographic resolution, separation, and peak symmetry.

Where the ratio of (15: 35: 50, by volume) of methanol, acetonitrile, and water, respectively. This resulted in a promising separation, but still, the plasma and FVP were not well resolved.

3.1.3. pH optimization

Formate, acetate, and phosphate buffers with varying pH values (pH 3.0–7.0) were tested. The pH value had no effect on drug peaks. However, the use of buffers resulted in baseline distortion, and the loss of peak symmetry was constantly observed during the trials. The use of acidified water at an intermediate acidic pH of 4 resulted in a clean baseline with low noise and symmetrical drug peaks. As water acidifiers, formic acid and acetic acid were attempted, but they caused tailing of the REM and DEX peaks, unlike “orthophosphoric acid,” which produced excellent and symmetrical peaks with minimum noise in the chromatogram.

3.1.4. Flow rate optimization

Different flow rates were studied from 0.2 to 0.5 mL/min. The selected optimal rate was 0.3 mL/min, owing to reasonable drug retention time without loss of separation and peak symmetry.

3.1.5. Optimization of detection wavelengths

Different wavelengths (220, 240, 245, and 300 nm) were tried for scanning, where scanning at 240 nm resulted in the best results for all drugs and the highest sensitivity of all drugs.

Finally, the optimum conditions for the mobile phase used for the concurrent determination of FVP, REM, and DEX using PX as an internal standard in plasma were a mixture of methanol, acetonitrile, and acidified water (pH = 4) (15: 35:50, v/v). The chromatograms for the plain and spiked plasma with FVP (tR = 2.05), DEX (tR = 3.08), REM (tR = 3.65), and PX (tR = 2.5) were shown in Figs. 2 and 3, respectively.

3.2. Method validation

The method described above was validated following the Food and Drug Administration’s “Guidance for Industry Bioanalytical Method Validation” [49].

3.2.1. Range of linearity

The calibration plots were constructed using the chromatographic conditions that were specified. For FVP, REM, and DEX, good correlation coefficients were developed over the concentration range of 0.1–10 µg/mL for all.

The following regression equations were observed:

For FVP: A1 = 0.1143x + 0.0053, r = 0.9999

For REM: A2 = 0.1406x - 0.0030, r = 0.9999, For REM.

For DEX: A3 = 0.1065x + 0.0337, r = 0.9999, For DEX.

Where, A represents the peak area at 240 nm, x represents the concentration in µg/mL and r is the correlation coefficient. This confirms the good linearity of the developed method. Regression and analytical parameters are listed in Table 1.

Moreover, the lowest concentration with a precision ≤ 20% (expressed as % RSD) was determined for each drug as listed in Table 1.

3.2.2. Accuracy and precision

The precision and accuracy were determined intra and inter-daily by analysis of three replicates of (0.1, 1, 3, 7 µg/mL) for FVP and REM, and (0.1, 1, 4, 7 µg/mL) for DEX. Acceptable results of precision (% RSD ≤ 15%) were obtained as listed in Table 2.

3.2.3. Selectivity

Eight plasma samples were prepared and analyzed by the produced method to detect the interference of the plasma constituents with FVP, REM, DEX, and PX at their retention times. Figs. 2 and 3 indicate that the plasma matrix doesn’t interfere with any of FVP, REM, DEX, and PX.

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Fig. 2. UHPLC chromatogram of plasma.
3.2.4. System suitability parameters
Symmetry factors, selectivity, and resolution parameters were investigated as system suitability parameters. The results, which were within acceptable limits, are summarized in Table 3.

3.2.5. Extraction recovery
FVP, REM, DEX, and PX recoveries from plasma were estimated from the relation extraction recovery = (the mean peak areas of the drugs in spiked plasma samples / mean peak areas of pure drugs in methanol).

3.2.6. Drug stability in biological fluid
The stability of FVP, REM, and DEX drugs in plasma samples was assessed by evaluation of bench top stability and freeze–thaw stability:

3.2.6.1. Bench top stability. At the start of the day, three concentrations which were LLQC (lower limit of quantitation), LQC (lower quality control), MQC (middle quality control), and HQC (higher quality control) concentrations were (0.1, 1, 3, and 7) for FVP, REM (0.1, 1, 4 and 7) for DEX. Table 4.

**Table 1**
Validation parameters for the proposed UPLC method, for determination of REM, FVP and DEX in spiked human plasma.

| Parameters | UPLC |
|-----------|------|
| Spiked human plasma samples | | |
| | FVP | REM | DEX |
| | | | |
| Calibration range (µg/mL) | 0.1–10 | 0.1–10 | 0.1–10 |
| **Slope** ± SE | 0.1143 ± 0.0005 | 0.1065 ± 0.0005 | 0.1406 ± 0.0013 |
| **Intercept** ± SE | 0.0053 ± 0.0033 | 0.0337 ± 0.0034 | -0.0030 ± 0.0002 |
| Analytical curves | | | |
| Correlation coefficient | 0.9999 | 0.9999 | 0.9999 |
| Accuracy (RSD %)* | 100.92 ± 2.14 | 100.67 ± 1.19 | 100.28 ± 1.73 |
| LLOQ (µg/mL) | 0.1 | 0.1 | 0.1 |
| ULOQ (µg/mL) | 10 | 10 | 10 |

**Table 4**
Validation parameters for the proposed UPLC method, for determination of REM, FVP and DEX in spiked human plasma.

**Fig. 3.** UHPLC chromatogram of mixture of plasma, FVP, PX (IS), DEX and REM using a developing system of methanol- acetonitrile- acidified water, pH 4 with H3PO4 (15:35:50, by volume).
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Table 2

Intra and inter assay precision and accuracy of LLOQ, LQC, MQC and HQC of spiked plasma sample.

| Concentration (µg/mL) | Intra-day | Inter-day |
|-----------------------|-----------|-----------|
|                        | Recovery % | RSD %     | Recovery % | RSD %     |
| FVP                   | 102.33     | 0.21      | 100.61     | 0.48      |
| LQC                   | 101.72     | 1.51      | 100.09     | 1.35      |
| MQC                   | 98.82      | 0.54      | 97.34      | 0.39      |
| HQC                   | 100.95     | 0.85      | 97.46      | 1.82      |
| REM                   | 101.12     | 0.19      | 97.74      | 1.79      |
| LQC                   | 98.78      | 0.63      | 93.97      | 0.74      |
| MQC                   | 101.87     | 0.36      | 97.09      | 0.88      |
| HQC                   | 101.25     | 0.12      | 94.42      | 0.85      |
| DEX                   | 101.96     | 1.02      | 102.46     | 1.62      |
| LQC                   | 100.97     | 0.65      | 95.15      | 1.61      |
| MQC                   | 100.25     | 1.15      | 99.24      | 1.16      |
| HQC                   | 100.06     | 0.27      | 96.83      | 0.97      |

Table 3

Parameters of system suitability of the developed UPLC method for the determination of the proposed drugs.

| Parameters | Plasma | FVP | PX | DEX | REM | (Srivastava, 2011) |
|------------|--------|-----|----|-----|-----|--------------------|
| Capacity factor (K') | 0.80 | 1.10 | 1.67 | 2.07 | 2.69 |                     |
| Symmetry factor | 1.05 | 1.20 | 1.00 | 1.12 | 1.06 | 1.00 – 1             |
| Resolution (Rs) | 1.53 | 2.74 | 1.73 | 2.5 | R greater than 1.5 | Rs greater than 1 |
| Selectivity (α) | 1.38 | 1.52 | 1.24 | 1.30 | 1.24 | 1.00 – 1             |
| N           | 856    | 2085 | 2153 | 2844 | 3189 |                     |
| HETP(cm)    | 0.0175 | 0.0072 | 0.0069 | 0.0053 | 0.0047 | ——                  |

(N) the number of theoretical plates.

(HETP) The height equivalent to a theoretical plate can be calculated when N and the column length (L) are known HETP = L/N.

Table 4

Extraction recovery results of the studied drugs in spiked human plasma by UPLC method.

| Analyte | Concentration (µg/mL) | Recovery % ± SD * |
|---------|-----------------------|-------------------|
| FVP     | 0.1                   | 82.77             |
|         | 1                     | 88.58             |
|         | 3                     | 89.41             |
|         | 7                     | 89.96             |
| Mean ± SD | 78.68 ± 3.79        |                   |
| REM     | 0.1                   | 86.96             |
|         | 1                     | 80.99             |
|         | 3                     | 89.83             |
|         | 7                     | 87.75             |
| Mean ± SD | 86.83 ± 4.39        |                   |
| DEX     | 0.1                   | 89.10             |
|         | 1                     | 83.84             |
|         | 4                     | 87.51             |
|         | 7                     | 89.31             |
| Mean ± SD | 87.44 ± 2.89        |                   |

*Average of 3 determinations.

3.2.6.2. Freeze-thaw stability (FTS). Spiked plasma samples were frozen for 24 h and then allowed to melt at room temperature by using three concentrations (low, medium, and high). The freeze–thaw cycle was carried out three times before being examined for significant changes. Table 5 shows that after three cycles, there was no significant change in sample concentrations.

3.3. Greenness evaluation

The absence or limited use of hazardous chemicals, the reduction of waste, and the reduction of energy consumption are all characteristics of green analysis. The greenness profile of the new method was calculated using the National Environmental Method Index (NEMI) [50]. The usage of non-persistent, bio-accumulative, and toxic solvents has an impact (PBT). Acetonitrile and methanol were used as solvents in the described process, and both were PBT in minor concentrations. Furthermore, the pH of the mobile phase was around 8, therefore it was not deemed corrosive. In addition, the waste generated per sample for UPLC was 3 mL. It was deemed an eco-friendly green approach after passing all three quadrants of the greenness profile (Table 6). The obtained results reveal that the proposed method is environmentally benign.

The analytical Eco-Scale is proposed as a more comprehensive technique to assess the greenness of analytical methodology. It is based on allocating penalty points to analytical process parameters that include reagents amount, occupational hazards, waste, and energy using the following equation (analytical Eco-Scale score = total penalty) [43]. The analytical process is termed “outstanding green analysis” if the score is greater than 75. Table 6 shows that the developed UPLC technique scored 83 on the Eco-Scale, suggesting that it is ecologically friendly.

4. Conclusion

For the first time, a new, easy, green, and highly sensitive UPLC approach for monitoring FVP, REM, and DEX utilizing PX anticoagulant as an internal standard has been created. The proposed method was regarded as green and environmentally benign, and it may be used in quality control laboratories by removing carcinogenic and environmentally toxic solvents frequently used in chromatographic procedures. It was validated and used satisfactorily for estimating the examined components in pure form and plasma samples. It provided a rapid and validated UPLC analytical method for quantifying FVP, REM, and DEX in the presence of the anticoagulant PX as an internal standard for simple therapeutic drug monitoring and possible pharmacokinetic studies in
COVID-19 patients’ plasma.

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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Table 6

| Reagent | Analytical Eco-scale | NEMI |
|---------|---------------------|------|
| Methanol | 6 | |
| Acetoneitrile | 4 | |
| Water | 0 | |
| Orthophosphoric acid | 1 | |
| Instrument UPLC | 0 | |
| Energy: UPLC - 0.1 kW h per sample | 3 | |
| Occupational hazards | 3 | |
| Waste (1–10 mL) | 17 | |
| Total penalty points | 83 | |

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Further reading

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