Research Article

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Rapid sensitive bioscreening of remdesivir in COVID-19 medication: Selective drug determination in the presence of six co-administered therapeutics

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Abstract: The widespread coronavirus 2019 (COVID-19) pandemic, attributed to the severe acute respiratory syndrome coronavirus-2, has resulted in global lockdowns and excess mortality. Remdesivir (RM) is the first and only antiviral drug that the US Food and Drug Administration (FDA) has approved so far for COVID-19. The treatment protocol involves multidrug combinations, basically depending on RM, in addition to antimicrobials, antipyretics, corticosteroids, and anticoagulants. This study develops and validates sensitive and selective RM screening in spiked human plasma in the presence of commonly co-administered drugs. Hydroxychloroquine, azithromycin, paracetamol, dexamethasone, and anticoagulants (rivaroxaban and edoxaban) have been detected simultaneously with RM in the same biological matrix. Separation has been efficiently achieved by simple reversed phase HPLC with dual detectors. Diode array detector and fluorimetric detection have been used to compare their sensitivity and selectivity. Both assays have been validated according to bioanalytical FDA validation parameters. Chromatographic separation and quantitation of RM along with concomitant drugs instantly bioscreen COVID-19 multiple therapy medication in 10 min run time. Furthermore, the proposed in vitro study takes the lead for prospective testing of possible drug–drug interactions that alter the pharmacokinetic profiles of drugs.

Keywords: remdesivir, SARS-CoV-2, combination therapy, human plasma, HPLC-DAD, HPLC-FD

1 Introduction

The coronavirus 2019 (COVID-19) pandemic is a highly contagious disease caused by a newly discovered strain of the coronavirus (severe acute respiratory syndrome coronavirus-2 [SARS-CoV-2]). It was first reported in Wuhan, Central China, due to abnormal pneumonia cases identified by the end of 2019, but the progress of the disease was extremely rapid that in less than four months, the World Health Organization declared it as a pandemic in March 2020 [1].

The major peculiar aspect concerning SARS-CoV-2 is its non-specific symptoms, which are highly confused with influenza and common cold [2]. The other disturbing fact is its unpredictable prognosis as it can cause none-to-mild respiratory tract symptoms in a majority of patients who will recover with only supportive treatment or can cause fatal consequences such as pneumonia and acute respiratory distress syndrome. Serious pathological complications may occur, leading to death in elderly people and patients with diabetes, chronic respiratory and cardiovascular disorders, cancer, and immune-deficient patients [3].

Recommendations to prevent the spread of COVID-19 include handwashing hygiene, wearing facemasks, and social distancing. However, effective vaccination, side by side, with therapeutic treatments indicated for viral eradication is ultimately targeted [4].

Gilead Sciences first developed remdesivir (RM; GS-5734) as an Ebola virus treatment [5]. The IUPAC name for RM is 2-ethylbutyl (2S)-2-[[[(2R,3S,4R,5R)-5-(4-amino pyrrolo[2,1-f][1,2,4]triazin-7-yl)-5-cyano-3,4dihydroxyoxolan-2-yl]methoxyphenoxy phosphoryl]amino]propanoate (Figure 1) [6]. It is a nucleotide analog prodrug, which requires bioactivation into GS-441524 (its active form) and further intracellular phosphorylation to the active nucleoside triphosphate metabolite, which compromises the viral RNA
synthesis. RM has a wide activity against numerous RNA viruses [6]. For example, it was tested against the Nipah virus, Lassa fever virus, Junin virus, respiratory syncytial virus, Middle East respiratory syndrome coronavirus, and feline infectious peritonitis [5,7]. Numerous clinical trials investigated the RM effect on SARS-CoV-2 and its potential application for COVID-19 treatment. Meanwhile, it became the only antiviral drug approved by the Food and Drug Administration (FDA) for COVID-19 as it interferes with one of the key enzymes required for the RNA virus replication, thus preventing its multiplication [8,9].

Although the FDA granted RM an “emergency use authorization” for severe COVID-19 since May 1, 2020, the drug assay has seldom appeared in the literature [10–14]. Similarly, the literature on its pharmacokinetics (PK), pharmacodynamics, and therapeutic drug monitoring (TDM) is insufficient. For further clinical investigations and high-throughput monitoring, there is an urgent need for a simple and valid bioanalytical method for RM quantification in the human plasma matrix in minimal time, with least sample preparation complexes, and with mean affordability. RM has been assayed using ultra performance liquid chromatography and liquid chromatography–mass spectrophotometry. Although the reported assays [10–14] estimated RM and its metabolite in high sensitivity, unaffordability and high cost remain as drawbacks, especially in developing countries. The urgent need for treatment has resulted in trying different experimental agents that already existed [15]. Thus, COVID-19 patients receive multiple therapy medications from different categories to help control the viral replication and the overall condition of the patients.

Solidarity concomitant protocol includes chloroquine or hydroxychloroquine (HY), which demonstrated antiviral activity for SARS-CoV-2 [16] besides its immune modulating [17] and potential antithrombotic effect [18]. The antibiotic
azithromycin (AZ) also proved to have in vitro antiviral, immune modulating, and anti-inflammatory functions [6]. Paracetamol (PR) in case of fever, corticosteroid such as dexamethasone (DX) to decrease the inflammatory response [19], and anticoagulants to decrease the tendency of thrombosis was demonstrated in almost all COVID-19 patients [20–22]. Chemical structures of these investigated RM co-administered drugs are shown in Figure 1.

The aim of this study is to develop a simple, selective, and economic analytical tool for the analysis of the only approved COVID-19 antiviral drug RM in plasma in the presence of the most commonly used drugs in corona virus treatment: HY, AZ, PR, DX, and anticoagulants such as rivaroxaban (RX) and edoxaban (EX), using apixaban (AX) as internal standard (IS), as they may affect RM selective determination especially for further PK studies in future.

2 Materials and methods

2.1 Instrumentation and chromatographic conditions

An Agilent 1,200 series high-performance liquid chromatography (HPLC) system, Agilent Technologies, Santa Clara, CA, USA was used with diode array detector (DAD) and fluorescence detector (FD), operated by Agilent ChemStation Software, USA. The HPLC system was supplied with an automatic injector, a quaternary pump, and a vacuum degasser. The separations were done on a reversed phase Agilent C18 (150 mm × 4.6 mm, 3 µm) column at 25°C. Gradient elution was applied using water acidified with orthophosphoric acid (A) (pH 4) and acetonitrile (B): from 0 to 4 min, the A:B ratio remained (70:30, v/v), the mobile phase changed linearly till it reached A:B (45:55, v/v) at 6 min, and the run was completed with this ratio till the end. An equilibrium time of 5 min with the initial mobile phase ratio was performed between runs. Mobile phase (filtered using a 0.45 µm membrane filter) of 1 mL min⁻¹ flow rate and 20 µL injection volumes were applied all over the runs. All runs were measured using both the DAD at 240 nm and the FD at \( \lambda_{	ext{ex/em}} \) 245/390 nm.

2.2 Materials and reagents

RM (99.38%), RX (99.60%), EX (99.07%), and AX (99.65%) were purchased from Selleck USA, and HY (≥98%, Sigma-Aldrich, Spain), AZ (99.5%, supplied by Amriya Pharmaceutical Industries, Egypt), and PR (99.8%, supplied by Pharonia Pharmaceuticals, Egypt) were used in this study. HPLC-grade acetonitrile, methanol, and orthophosphoric acid (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) were also used. High-purity, double-distilled water was also used in this study. Pooled drug-free human plasma was obtained from the Alexandria blood bank, Attarin, Alexandria.

2.3 General procedure

2.3.1 Standard stock solutions

Stock solutions (1,000 µg·mL⁻¹) of RM, HY, AZ, PR, DX, AX, RX, and EX were separately prepared in methanol. AX solution, chosen as an IS, was further diluted with methanol to prepare a working solution of 150 µg·mL⁻¹. The stock solutions were stable for at least 30 days, stored at 4°C protected from light.

2.3.2 Calibration graphs for RM analysis in spiked human plasma samples

Six working standards of RM were prepared from RM standard stock solution (1,000 µg·mL⁻¹) in methanol, starting from 0.2 µg·mL⁻¹ (for HPLC-DAD) or 0.1 µg·mL⁻¹ (for HPLC-FD) to 150 µg·mL⁻¹. In six Eppendorf tubes, each 250 µL human plasma was spiked separately with 25 µL of each working solution and 25 µL of IS working solution (AX) followed by 500 µL of acetonitrile to achieve concentrations in the range of 0.02 (for HPLC-DAD) or 0.01 (for HPLC-FD) to 15 µg·mL⁻¹ plasma. After vortex mixing for 2 min, centrifugation was done for 15 min in a cooling centrifuge (−4°C) at 15,000 rpm. The supernatant was then filtered by a 0.45 µm syringe adapter. A 20 µL volume was then injected in triplicate and chromatographed. In addition, quality controls (QCs) were prepared in the same way, 0.02 (for HPLC-DAD) or 0.01 (for HPLC-FD), 0.06 (for HPLC-DAD), or 0.03 (for HPLC-FD), 5 and 15 µg·mL⁻¹ plasma to be lower limit of quantification (LLOQ), low QC (LQC), mid QC (MQC), and high QC (HQC), respectively. The ratios of peak areas of analyte/IS against corresponding drug concentrations were plotted for calibration graph construction.

3 Results and discussion

3.1 Optimization of chromatographic separation

All factors that may affect the separation and resolution of RM in the presence of the other investigated drugs were optimized.
3.1.1 Stationary phase selection

Different columns have been tried such as C8 (250 mm × 4.6 mm), C18 (150 mm × 4.6 mm), and C18 (250 mm × 4.6 mm). The best separation and baseline noise with short run time were achieved using the Agilent C18 (150 mm × 4.6 mm) column. The C8 column did not give the required retention or separation of RM, while the long C18 column increased the analysis time. So, the best choice was the C18 short column as it achieved the required separation within 10 min, and it is a cheap and readily available column in all laboratories unlike monolithic columns or other next-generation packed stationary phases.

3.1.2 Mobile phase optimization

Organic modifiers such as methanol, ethanol, and acetonitrile in different ratios were tried, and acetonitrile gave the best results of resolution, baseline noise, and peak symmetry. Isocratic trials were first attempted to try to reach a simple mobile phase ratio that separates RM within reasonable retention times and also away from the plasma co-eluting peaks or the other co-administered drugs. However, none of these trials succeeded in resolving RM from the other drugs. Introducing methanol to acetonitrile as organic solvents in different ratios was also tried to reach the required separation goal, but it did not succeed as expected. Thus, gradient programming was adopted. After numerous trials, the optimum choice was to start with (70:30, v/v) acidified water and acetonitrile, respectively, till 4 min, and then linearly switched to (45:55, v/v) till 6 min, to complete with the latter ratio till the end of the run.

3.1.3 pH optimization

Phosphate, acetate, and formate buffers at different pH values (pH 3.0–7.0) were tried to select the optimum aqueous phase. The pH value itself did not have a significant effect on RM peak. However, using buffers resulted in distortion of the baseline especially after applying the gradient programming, and the loss of peak symmetry was constantly observed during the trials. Using acidified water at an intermediate acidic pH 4 gave a clear baseline with minimal noise. Formic acid and acetic acid were tried as water acidifiers, but they caused huge solvent front peak that interfered with the analysis, unlike "orthophosphoric acid" that resulted in a clean chromatogram.

3.1.4 Flow rate optimization

The flow rate was studied from 0.7 to 1.5 mL·min⁻¹. The selected optimal rate was 1 mL·min⁻¹, owing to reasonable retention drug time without loss of separation and peak symmetry.

3.1.5 Optimization of detection wavelengths

RM and AX (IS) were quantified using the DAD at 240 nm to achieve the maximum sensitivity for RM assay. Other co-drugs were monitored at the same detection wavelength. As RM has a native fluorescent nature, fluorescence detection was tried and compared to the DAD detection. Fluorescence detection offered higher selectivity and sensitivity compared to DAD. The best λ excitation was found to be 245 nm that gave the highest fluorescence intensity for RM with an acceptable corresponding emission at 390 nm, and none of the co-administered drugs had fluorescent intensity at the chosen λex and λem except the IS drug AX. Thus, as observed, HPLC-FD counts more selective for RM determination in plasma matrix with a wider range of possible interferences.

All system suitability parameters (retention time, capacity factor, selectivity, resolution, asymmetry, and plates efficiency) have been calculated before the optimization of chromatographic parameters. They are within the acceptable limits as shown in Table 1.

3.2 Optimization of sample extraction

Protein precipitation was used to prepare the samples. Generally, protein precipitation can be considered a simple and eco-friendly method as it reduces the use of excess solvents as well as extra practical steps unlike the liquid–liquid or the liquid–solid extractions. Cooling centrifugation was applied for samples to ensure complete protein precipitation. Acetonitrile gave clearer supernatant compared to methanol, so it was selected as the preferred precipitating agent. There was no need to add strong acids, or pH adjustment, or evaporating the solvent to dryness. The acetonitrile:plasma ratio (2:1) was optimum to reach a clear supernatant with the targeted sensitivity in plasma without any additional treatment steps. Prepared sample solutions were filtered to ensure the samples are completely clear before injection into the HPLC system. An approximate volume of 0.1 mL of the sample was used for the filter saturation and discarded. The rest of the sample was put into the HPLC vials and 20 µL was injected into the HPLC system.
at the chosen anticoagulant. Besides the fact that it is a coacting drug, AX showed a good fluorescence intensity at the chosen λex and λem for the assay of RM, so it can be detected by both DAD and FD. In addition, it had optimum separation among the drugs of interest within the 10 min analysis time. No other drug showed equivalent properties. AX was the optimal choice as an IS.

### 3.3 IS selection

Besides the fact that it is a co-administrated drug (an oral anticoagulant), AX showed a good fluorescence intensity at the chosen λex and λem for the assay of RM, so it can be detected by both DAD and FD. In addition, it had optimum separation among the drugs of interest within the 10 min analysis time. No other drug showed equivalent properties. AX was the optimal choice as an IS.

### 3.4 Method validation

The FDA validation parameters for bioanalytical methods were assessed [23].

#### 3.4.1 Linearity and range

The range in which the method is linear was done by plotting RM peak areas/IS peak area against RM corresponding concentrations. The proposed method was linear in ranges as stated in Table 2. The regression equation parameters and limits of detection and quantitation presented in Table 2 prove the acceptable linearity of the proposed method by using both detectors.

The proposed HPLC method offered LLOQ of 0.02 μg·mL⁻¹ (for HPLC-DAD) and 0.01 μg·mL⁻¹ (for HPLC-FD) in human plasma for RM, which proves appropriate sensitivity of the method. In addition, the reported Cmax of RM following a single 2 h intravenous infusion of RM solution formulation ranges from 0.0575 μg·mL⁻¹ (in case of a small dose of 3 mg) to 4.4213 μg·mL⁻¹ plasma (in case of a dose of 225 mg). The linearity range of the proposed method covers this range and starts from lower concentration than the Cmax. Thus, this method is sensitive enough for the quantitation of RM for...
Figure 2: Representative HPLC-DAD chromatograms of extracted plasma samples spiked with RM (15 μg·mL⁻¹ plasma) and the co-administered drugs (10 μg·mL⁻¹ plasma) showing: (a) AZ; (b) AX, RX, and EX; (c) DX and HY; and (d) PR all with IS (AX).
PK and TDM studies required for a new chemical entity like RM.

### 3.4.2 Accuracy and precision

Accuracy and precision were validated at LLOQ, LQC, MQC, and HQC concentrations \((n=6)\) on the same day and over three different days. All percentage recoveries and deviations values did not exceed 20% for the LLOQ and 15% for other QC samples, which indicates validity of the method’s accuracy and precision (Table 3).

### 3.4.3 Recovery

Signals of LQC and HQC concentrations were compared with those from the plasma samples spiked with equivalent RM standard concentrations without extraction. Mean extraction recovery of RM was 96.56 ± 2.50% (RSD%) and that of AX (IS) was 95.50 ± 1.98% (RSD%). These extraction recoveries were sufficient and confirm successful extraction from human plasma after the simple sample pretreatment step used.

### 3.4.4 Selectivity

Chromatograms showing blank plasma and plasma spiked with only the IS (AX) are shown in Figure A1, where no evidence of interfering peaks was noticed throughout the analysis. In addition, the enhanced sensitivity using the FD can be observed. Six different blank plasma samples obtained from different sources were also analyzed by the proposed method and showed the same results (lack of interference).

### Table 4: Stability tests of RM in human plasma \((n=6)\)

| Stability                  | QC samples | HPLC-DAD          | HPLC-FD          |
|----------------------------|------------|-------------------|-------------------|
|                             |            | Mean % recovery ± % RSD | % E<sub>r</sub> | Mean % recovery ± % RSD | % E<sub>r</sub> |
| Short-term room temperature | LQC        | 102.56 ± 1.85      | 2.56             | 98.50 ± 1.55         | −1.50            |
|                            | HQC        | 100.72 ± 1.70      | 0.72             | 100.99 ± 1.00        | 0.99             |
| Freeze–thaw (3 cycles at −70°C) | LQC | 105.42 ± 1.99      | 5.42             | 104.99 ± 0.89        | 4.99             |
|                            | HQC        | 101.55 ± 0.50      | 1.55             | 102.59 ± 1.20        | 2.59             |
| Post-preparative 5°C       | LQC        | 99.70 ± 1.05       | −0.30            | 99.12 ± 0.75         | −0.88            |
|                            | HQC        | 101.71 ± 1.09      | 1.71             | 101.69 ± 1.27        | 1.69             |
| Long term 45 days at −70°C | LQC        | 99.48 ± 1.32       | −0.52            | 98.70 ± 0.98         | −1.30            |
|                            | HQC        | 102.56 ± 1.17      | 2.56             | 102.96 ± 1.86        | 2.96             |

![Figure 3](image-url): Overlaying spectra illustrating peak purity of RM obtained from spiked plasma using the proposed (a) HPLC-DAD and (b) HPLC-FD methods.
The aspect of taking into consideration the common drugs administered for coronavirus patients and the ability of the method to selectively separate and quantify RM in their presence adds a lot to the selectivity of the method and gives much greater reliability of the results. This is especially needed in case of further PK studies required to be applied on COVID-19 patients who were administrated with RM while taking other medications. Chromatograms in Figure 2 show plasma samples spiked with RM (15 μg·mL⁻¹ plasma) and the co-administered drugs (10 μg·mL⁻¹ plasma) where they were all separated from RM. In order to be able to monitor all co-administered drugs using the proposed method, the DAD must be used as shown in Figure 2. For better selectivity and sensitivity, FD can be used using the proposed mobile phase and column. Calculated parameters for each chromatogram are illustrated in Table A1.

The HPLC peak purity of RM was also checked. The purity angle was within the purity threshold limit where the threshold value of the noise was not exceeded in the purity plot. In addition, the absorption and emission spectra of RM were recorded at different time intervals across its peak, which indicate the purity of the chromatographed RM peaks in the presence of the other drugs and in plasma (Figure 3).

3.4.5 Stability

Stability results of RM were obtained at LQC and HQC (n = 6) under different stability conditions explained in Table 4. In addition, the stock and working standard solutions of RM were stable for 30 days at refrigerator temperature and for at least 5 h at ambient temperature. The RSD% and E% obtained in Table 4 indicate good stability under all the studied conditions.

4 Conclusion

RM is the first antiviral drug to be approved by the FDA for treatment of the COVID-19 pandemic, and the emergency use of this medication especially for hospitalized patients requires more PK studies for RM and its interaction with other co-administered drugs. This method is the first HPLC-DAD and HPLC-FD method for the determination of RM in human plasma with adequate sensitivity owing to its Cmax. Achieved simple pre-treatment steps and short run time are two basic factors in high-throughput tool as a core of PK studies. Yet, the proposed method has considered the different drugs commonly prescribed in the solidarity treatment protocol. Future perspective of the present analytical procedure is the in-vivo drug monitoring for concomitant administration of RM with one or more of the therapeutic agents under investigation. Furthermore, full validation of the proposed methods ascertains their accuracy, reproducibility, and selectivity.

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Data availability statement: The files used to support the data findings of this study are available from the corresponding author on request.

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Appendix

Table A1: System suitability parameters calculated for all separated analytes

| Chromatogram | Analyte | Retention time (R<sub>t</sub>, [min]) | Asymmetry (A<sub>f</sub>) | Efficiency (plates/m) | Resolution (R<sub>s</sub>) | Selectivity (α) |
|--------------|---------|--------------------------------------|-------------------------|----------------------|---------------------------|-----------------|
| 1            | AZ      | 2.73                                 | 1.23                    | 4,416                | 17.53                     | 3.66            |
|              | AX      | 7.99                                 | 1.10                    | 15,960               | 3.88                      | 1.30            |
|              | RM      | 9.80                                 | 1.05                    | 20,136               | —                         | —               |
| 2            | RX      | 2.24                                 | 0.88                    | 13,615               | 22.32                     | 4.94            |
|              | AX      | 7.82                                 | 1.02                    | 19,708               | 1.95                      | 1.06            |
|              | EX      | 8.18                                 | 0.99                    | 20,818               | 3.35                      | 1.23            |
|              | RM      | 9.79                                 | 0.98                    | 22,569               | —                         | —               |
| 3            | HY      | 2.24                                 | 0.93                    | 13,415               | 15.03                     | 4.38            |
|              | DX      | 6.75                                 | 0.77                    | 8,367                | 2.44                      | 1.19            |
|              | AX      | 7.78                                 | 0.98                    | 10,684               | 3.89                      | 1.29            |
|              | RM      | 9.79                                 | 1.05                    | 22,339               | —                         | —               |
| 4            | PR      | 2.25                                 | 0.78                    | 10,699               | 22.40                     | 4.54            |
|              | AX      | 7.85                                 | 0.97                    | 12,090               | 4.05                      | 1.35            |
|              | RM      | 9.79                                 | 1.01                    | 21,928               | —                         | —               |
Figure A1: Representative HPLC chromatograms of (a) blank plasma (DAD, 240 nm), (b) blank plasma (FD, $\lambda_{ex/em}$ 245/390 nm), (c) treated blank plasma with IS (DAD, 240 nm), (d) treated blank plasma with IS (FD, $\lambda_{ex/em}$ 245/390 nm), (e) extracted plasma spiked at the LLOQ levels with IS (DAD, 240 nm), and (f) extracted plasma spiked at the LLOQ levels with IS (FD, $\lambda_{ex/em}$ 245/390 nm).