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Eco-friendly stability-indicating HPTLC micro-determination of the first FDA approved SARS-CoV-2 antiviral prodrug Remdesivir: Study of degradation kinetics and structural elucidation of the degradants using HPTLC-MS

Amira H. Abo-Gharam, Dina S. El-Kafrawy *

Pharmaceutical Chemistry Department, Faculty of Pharmacy, University of Alexandria, Elmessalah, 21521, Alexandria, Egypt

ABSTRACT

The worldwide spread coronavirus (covid-19) pandemic caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) represents a global health crisis. The world was forced to face a great challenge to control and overcome this health disaster through various containment measures including efficient vaccination side by side with effective medication. Remdesivir (RMD) is the first FDA approved antiviral agent for treatment of covid-19 pandemic and hence regarded as the first-in-class medication of this highly contagious respiratory disease. The current study represents the first stability indicating HPTLC method for the estimation of RMD in bulk form and pharmaceutical formulation. The method employed TLC silica gel aluminum plates 60 F254 as stationary phase and green mobile phase composed of ethyl acetate and ethanol (96: 4, v/v) with densitometric detection at 245 nm. Comprehensive validation of the adopted method was accomplished according to the ICH guidelines regarding linearity, ranges, detection and quantification limits, precision, accuracy and robustness. The developed method offered a neat separation of the drug in presence of pharmaceutical excipients as well as in presence of acidic, alkaline, neutral hydrolytic, oxidative and photolytic degradants. Additionally, structural elucidation of alkaline and hydrolytic oxidation degradation products was carried out using HPTLC-MS. Furthermore, for the first time the acidic and alkaline degradation kinetics of RMD were studied and its degradation rate constants and half-lives were calculated. Moreover, greenness appraisal of the developed method as well as comparison with previously published stability indicating HPLC methods were performed using analytical Eco-scale, GAPI and AGREE metrics.

1. Introduction

In the current century, the outbreak of coronavirus disease (covid-19) represents the most serious pandemic health crisis. Since the start of covid-19 pandemic in December 2019, over 414 million people worldwide have got the infection with this severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) and over 5.8 million deaths were recorded globally because of this disease up to February 16, 2022 (WHO, February 17, 2022. https://www.who.int/covid-19).

Coronavirus is one of the fierce RNA complex viruses causing a highly contagious respiratory disease with symptoms ranging from
mild respiratory disorders to more severe complications like pneumonia, acute respiratory distress or even multiorgan failure and death (Chen et al., 2020). Albeit the universal containment actions taken and the effective vaccination worldwide to control this pandemic, there is a continuously rising need for effective treatment to overcome this health catastrophe (Jamrozik and Selgelid, 2020). Remdesivir (RMD) (Fig. S1) (in supplementary file 1) is the first antiviral drug that has gained the FDA Emergency Use Authorization (EUA) on October 22, 2020 for treatment of covid-19 infection in hospitalized adults and pediatric patients aged 12 years old (U.S. Food and Drug Administration FDA, 2020a, U.S. Food and Drug Administration FDA, 2020b). RMD was first developed by Gilead Sciences Biopharmaceutical company for treatment of Ebola and Marburg viral infections (Eastman et al., 2020). It also possesses activity against the Middle East Respiratory Syndrome Coronavirus (MERS-CoV), SARS-CoV and SARS-CoV-2 (Warren et al., 2016; Gordon et al., 2020a, 2020b). RMD is a protide (a nucleotide analog prodrg), which is first hydrolyzed and dephosphorylated in plasma then intracellularly converted to its active triphosphate metabolite which interferes with the action of viral RNA-dependent RNA polymerase and hence inhibits viral RNA production (Sheahan et al., 2017; Eastman et al., 2020; Gordon et al., 2020c). RMD is lately contraindicated in covid-19 patients with liver or renal dysfunction (WHO, January 14, 2022). Nausea, low blood pressure and liver inflammation are reported as possible side effects of RMD (European Medicines Agency EMA, 2020; U.S. Food and Drug Administration FDA, 2020).

Reviewing the literature reveals few methods for the determination of RMD, most of these methods adopted HPLC or UPLC techniques coupled with MS/MS detectors (Alvarez et al., 2020; Avataneo et al., 2020; Habler et al., 2021; Hu et al., 2021; Pasupuleti et al., 2021; Xiao et al., 2021). HPLC-UV and direct spectrophotometric (Bulduk et al., 2021), spectrofluorimetric (Elmansi et al., 2021) and electrochemical (Tkach et al., 2021) determinations of RMD were also reported.

Regarding stability indicating assays of RMD, four HPLC methods were reported employing either UV detection (Ibrahim et al., 2021), diode-array detection (Hamdy et al., 2021; Ramakrishna Reddy et al., 2021; Surabbi and Jaina, 2021) or fluorescence detection (Hamdy et al., 2021). Concerning HPTLC technique, a single recent analytical report was published dealing with the direct determination of RMD and favipiravir in pharmaceutical formulations and human plasma (Noureldeen et al., 2022). So far, the scientific database lacks any analytical report tackling the stability indicating HPTLC determination of RMD, also no previous reports have attempted the study of the degradation kinetics of RMD under different stress conditions.

The present study illustrates a novel fully validated, eco-friendly, stability indicating HPTLC method for the micro-determination of RMD in bulk form, pharmaceutical formulation as well as in presence of its stress degradation products. Furthermore, a preliminary structural elucidation of the resulted degradants was performed using HPTLC-MS. Additionally, for the first time the degradation kinetics of the drug were studied under acidic and alkaline stress degradation conditions. Finally, a comprehensive appraisal of the method's eco-friendliness was carried out using Analytical Eco-scale (AES) (Galuszka et al., 2012), Green Analytical Procedure Index (GAPI) (Plotka-Wasyłka, 2018) and Analytical Greenness (AGREE) (Pena-Pereira et al., 2020) metrics. Besides, a thorough comparison of the method's greenness with other previously published stability indicating HPLC procedures (Ibrahim et al., 2021; Hamdy et al., 2021; Ramakrishna Reddy et al., 2021; Surabbi and Jaina, 2021) and the only reported HPTLC direct determination of RMD (Noureldeen et al., 2022) was also illustrated using the more inclusive GAPI and AGREE metrics.

The current study represents the first ecologically benign, fast and simple stability indicating HPTLC determination of RMD which could be considered as a green, more economic and faster alternative to the previously published stability indicating HPLC procedures (Ibrahim et al., 2021; Hamdy et al., 2021; Ramakrishna Reddy et al., 2021; Surabbi and Jaina, 2021). Hence, it is more eligible for the routine analysis of RMD in quality control laboratories as well as for testing the drug purity and stability.

# 2. Experimental

## 2.1. Instrumentation

Spots of the samples were applied using Cammag microliter syringe under nitrogen stream with the aid of a Cammag Linomat IV sample applicator (Switzerland). The application was performed on precoated TLC silica gel aluminum plates 60 F254 (20 × 10 cm, 200 μm thickness, Merck, Darmstadt, Germany). Development was carried out in a Cammag twin trough glass chamber (20 × 20 cm) saturated with the mobile phase for 15 min at room temperature. Densitometric scanning was achieved using Cammag TLC scanner III controlled with WinCATS software (V 3.15 CAMAG).

## 2.2. Materials and reagents

Pure RMD (99.38%) was bought from Selleck USA. HPLC grade absolute ethanol (Fisher Scientific, Loughborough, UK), ethyl acetate, sodium hydroxide and 30% hydrogen peroxide (El-Nasr Pharmaceutical Chemicals Co., Qalubiya, Egypt) and hydrochloric acid 37% (Merck, Darmstadt, Germany) were used in this work. The pharmaceutical preparation used in the current investigation was Remdesivir® concentrate solution for IV infusion containing 100 mg RMD per 20 mL vial (Eva pharma, Giza, Egypt, B.N. 2105598A).

## 2.3. General procedure

### 2.3.1. Chromatographic conditions

Application of an accurate volume of 5 μL of each sample was performed with the aid of a Cammag microliter syringe. The application settings were 7 mm from the margin and 10 mm from the bottom of the plate with bandwidth 5 mm and inter-band spaces 4 mm. Fifteen milliliters of a mobile phase composed of mixture of ethyl acetate and ethanol (96: 4) were put in the glass chamber. The plates were ascendingly developed over approximately 9.8 cm after saturation of the chamber with mobile phase for 15 min at ambient temperature (25 ± 2 °C). The developed plates were then removed, air-dried and scanned densitometrically at 245 nm.
2.3.2. Construction of the calibration graph

Stock solution of RMD was prepared by dissolving 100 mg of the pure drug powder into 100 mL of 50% aqueous ethanol yielding 1000 µg/mL stock solution which is then stored at 4 °C. Into a set of 5-mL volumetric flasks, aliquots of stock solution corresponding to 30–500 µg were transferred and then diluted to the mark with the same solvent. Triplicate bands of 5 µL from each working solution were applied on the TLC plate in the form of bands to get a final concentration range of 30–500 ng/spot of RMD. The plates were then developed under the aforementioned chromatographic conditions. The calibration graph was constructed relating the average peak areas of the cited drug to its relevant concentrations and the linear regression equation was calculated.

2.3.3. Assay of Remdesivir® concentrate solution for IV infusion

Accurate volume of 2 mL of Remdesivir® concentrate solution was transferred to 10 mL volumetric flask and completed to mark with 50% aqueous ethanol to reach a final concentration of 1000 µg/mL RMD (stock sample solution). Portions of the stock sample solution were transferred to 5 mL volumetric flasks and made up to volume with 50% aqueous ethanol to reach its concentration range. The working sample solutions were then treated as under “General Procedure”. Calculation of recovery values was performed using concurrently prepared standard solutions (external standard method). The percentage recovery and standard deviation were then calculated.

2.3.4. Forced degradation studies of RMD

Forced degradation studies of RMD were conducted according to ICH guidelines under different conditions as acidic, alkaline, neutral hydrolysis, oxidative and photolytic conditions (ICH, 2003).

2.3.4.1. Acidic and alkaline degradations. Into 5 mL volumetric flasks, 0.5 mL RMD stock solution was separately treated with 1 mL 1 M HCl or 1 mL 0.003 M NaOH. The solutions were then kept protected from light for 30 min at room temperature. After the specified time, the solutions were neutralized by adjusting the pH to 7.0 by adding 1 M NaOH or 0.003 M HCl, respectively and then diluted to volume with ethanol to obtain a final concentration of 100 µg/mL RMD.

2.3.4.2. Neutral hydrolysis degradation. A volume of 2 mL of distilled water was added to 0.5 mL RMD stock solution into 5 mL volumetric flask and then protected from light in a boiling water bath for 6 h. After the specified time, the flask was cooled then the volume was completed to the mark with ethanol to obtain a final concentration of 100 µg/mL RMD.

2.3.4.3. Oxidative degradation. This degradation experiment was performed by transferring 0.5 mL RMD stock solution into 5 mL volumetric flask then treated with 1 mL of 30% v/v H2O2 and placed protected from light in a thermostat-controlled water bath at 80 °C for 30 min. After the specified time, the flask was cooled and the volume was completed to the mark with ethanol to reach a final concentration of 100 µg/mL RMD.

2.3.4.4. Photolytic degradation. RMD stock solution (1000 µg/mL) was exposed to UV radiation at 254 nm for 16 h in a transparent flask. After that, a portion of 0.5 mL was transferred to a 5 mL volumetric flask and diluted to volume with 50% aqueous ethanol to obtain a final concentration of 100 µg/mL RMD.

After accomplishing the different treatments, A volume of 5 µL of each of the stress degraded solutions was spotted three times on the TLC plate to obtain a final concentration of 500 ng/spot, and the plates were developed as described before. The densitograms of the degradation solutions were compared to standard solution of the same concentration of pure RMD to assess the decrease in the peak area and inspect the formation of any new degradation peaks. Percentage of degradation was quantified by computing the decrease of the drug peak area. Structure elucidation of the formed degradants were done through hyphenation of HPTLC with mass spectrometry (HPTLC-MS). Their identification was accomplished through the HPTLC-MS-Interface Advion Plate Express™ automated TLC plate reader, NY (USA), Nawah Research Center, Cairo, Egypt. The degradant bands were eluted from the TLC plate via a semi-automatic piston. MS analysis was accomplished on Advion Compact Mass Spectrometer (CMS), NY (USA). Data acquisition was carried out in negative electrospray ionization mode (ESI) in the mass range 100–700 Da.

2.3.5. Degradation kinetic studies

2.3.5.1. Acidic degradation. 1 mL volumes of different concentrations of HCl solutions (0.2, 0.5 and 1.0 M) were separately mixed with 0.5 mL RMD stock solution in a series of 5 mL volumetric flasks. The solutions were left at room temperature for different time intervals (0.25, 0.50, 0.75 and 1.00 h). Then, each flask was separately neutralized to pH 7.0 with its corresponding concentration of NaOH to obtain a final concentration of 100 µg/mL RMD.

2.3.5.2. Alkaline degradation. Into a series of 5 mL volumetric flasks, aliquots of 0.5 mL RMD stock solution were separately mixed with 1 mL volumes of different concentrations of NaOH solutions (0.001, 0.003 and 0.005 M) at room temperature. At different time intervals (0.25, 0.50, 0.75 and 1.00 h), each flask was separately neutralized to pH 7.0 with its equivalent concentration of HCl to get a final concentration of 100 µg/mL RMD.

Five microliters of each of the degraded solutions under different stress conditions were spotted (in triplicate) and then analyzed using the aforementioned chromatographic conditions. Percentage degradation was determined by calculating the decrease in the RMD peak area in each analyzed sample compared to a standard RMD densitogram.

3. Results and discussion

3.1. Optimization of the HPTLC densitometric method

Our main goal is the development of a novel, simple and eco-friendly stability indicating HPTLC method for the quantitative estimation of RMD. So, several trials were conducted to separate the cited drug along with its degradants of different stress conditions. Concerning the optimization of the mobile phase composition, many attempts were done to select an eco-friendly solvent system.
which is the most important factor in the HPTLC method development. Although the application of nonpolar solvents such as toluene, n-hexane, chloroform or benzene in the mobile phase system is a common tactic to achieve good separation, these nonpolar compartments were omitted in this study because of their recognized environmental threats. We started our trials with the greenest mobile phase ever, ethanol and water in different ratios, but unfortunately it was resulted in distorted RMD peak with high $R_f$ value. Water was replaced with ethyl acetate which is regarded as an environmentally benign nonpolar solvent having the least environmental burden compared to other nonpolar options. The mobile phase mixture of ethanol and ethyl acetate in different ratios showed a symmetrical peak of RMD with different $R_f$ according to their ratio. The optimization of the ratio of mobile phase components was carefully studied.

![HPTLC densitograms](image)

**Fig. 1.** HPTLC densitograms of a standard solution of 500 ng/spot RMD (a), 500 ng/spot RMD after acidic (b), alkaline (c), oxidative (d), neutral (e) and photolytic (f) stress conditions at 245 nm.
to resolve the degradants from the investigated drug without the need for adding any hazardous solvent such as glacial acetic acid or ammonia, even in a minute amount. The mobile phase system consisting of ethyl acetate and ethanol (96: 4) resulted in efficient separation of the cited drug along with its degradation products. Thus, it was selected as the optimum mobile phase system where RMD appeared as a well-defined peak with $R_f$ value of 0.40 ± 0.02.

The choice of scanning wavelength was also crucial in order to attain the highest sensitivity for RMD determination as well as the clear appearance of its degradation products peaks. Upon examination of the UV spectrum of the studied drug, it possesses two absorption maxima at 245 and 275 nm where the former showed higher sensitivity. So, the plates were scanned at 245 nm for RMD

Fig. 1. (continued).
quantification. Optimization of all sample application parameters was also done. The optimum bandwidth was 5 mm with inter-band space of 4 mm. Symmetrical well-resolved peaks were noticed when samples spotting was preceded by the activation of the plates at 80 °C for 20 min and when plates' development was preceded by saturation of the chamber with the mobile phase for 15 min at ambient temperature. Typical HPTLC densitogram for the standard solution of RMD was illustrated in Fig. 1a.

The calculation of system suitability parameters was manually performed for the cited drug where RMD appeared at $R_f$ 0.40 with tailing factor 0.90 and capacity factor 0.60. these parameters confirm the satisfactory performance of the operating system.

3.2. Validation of the proposed method

The proposed HPTLC method was validated as per the International Conference on Harmonization (ICH) guidelines on validation of analytical procedures (ICH, 2005).
3.2.1. Linearity and concentration range

Linearity of the investigated drug was examined under the optimal conditions. Then, linear regression equation was derived using least-squares treatment of the measured peak areas. It was found that the measured peak areas of RMD were perfectly proportional to its relevant concentrations over a concentration range of 30–500 ng/spot (6–100 μg/mL). The linearity data are summarized in Table 1. Regression analysis demonstrates good linearity of the calibration graph as proved by high correlation coefficient value (0.9997) together with low RSD% value of the slope (not more than 1.20%).

3.2.2. Limit of detection and quantification

The signal to noise approach was used for calculation of limits of detection (LOD) and quantification (LOQ). LOD is defined as the concentration level that gives a signal-to-noise ratio of 3:1. Whereas, the ratio considered for LOQ is 10:1. The LOD and LOQ values of the analyte were calculated and presented in Table 1.

3.2.3. Accuracy and precision

The accuracy and within-day precision (repeatability) of sample application and measurement of peak area were evaluated using three different concentrations within the linearity range of the cited drug using triplicate determinations for each concentration on a single day. Likewise, the accuracy and between-day precision (intermediate precision) were assessed by analyzing the same three solutions using triplicate determinations repeated on three consecutive days. Table S1 (in supplementary file 1) presents the obtained analytical results where the percentage relative standard deviation (RSD %) and percentage relative error (E %) were found to be less than 2.00% proving the excellent precision and accuracy of the proposed method.

3.2.4. Robustness

To test the robustness of the developed method, insignificant intended variations were carried out to different method parameters such as the mobile phase composition (±2%), working wavelength (±2 nm), volume of the mobile phase (±3 mL), time from development to scan (0, 20, 40 and 60 min) and duration of the mobile phase saturation (±5 min) then the measured peak areas and R_f values were recorded. Good robustness of the suggested method was guaranteed through satisfactory RSD% values of peak areas (not exceeding 3%) along with small changes in R_f values of RMD, indicating the reliability of the proposed method during the routine work. Table S2 (in supplementary file 1) summarizes the robustness results of the proposed method.

3.2.5. Stability of solutions

To check the stability of RMD solutions during storage and analysis, the stock solution prepared in 50% aqueous ethanol was analyzed every day through its storage in the refrigerator for the next day. It was noticed that the stock solution was stable when kept refrigerated at 4 °C for at least 3 days. The stability of working solution in the same diluting solvent at room temperature was also assessed along 8 h (on the same day) where no change in the drug R_f and peak area was noticed, confirming the stability of the working solution.

3.2.6. Specificity and selectivity

Method’s specificity and selectivity were proved by the successful resolution and neat separation of the remained intact drug from its degradants’ peaks and by the separation of RMD peak from co-formulated excipients in marketed dosage form. Moreover, the peak purity and homogeneity of RMD in standard solution, degraded samples and dosage form solution were evaluated by means of HPTLC scanner. The specificity and selectivity of the proposed method was confirmed through the peak purity profiling. After recording the UV spectrum of the analyzed drug at several points across each peak by the HPTLC scanner, the software can assess the purity of the peaks through 2 main steps. At first, calculation of the correlation coefficient (r_s,m) between the spectra extracted at peak start and peak maximum and the correlation coefficient (r_e,m) between the spectra extracted at peak end and peak maximum is performed. Then, mathematical interpretation of the significance of the correlation values was carried out to check the purity of the specified peaks (Hewala et al., 2012). Fig. S2 (in supplementary file 1) shows superimposed UV spectra of standard RMD with remained intact RMD in forced degradation solutions and RMD in its marketed dosage form sample solution. Spots were found pure as the spectra extracted at

| Table 1 |
|---|

| Analytical parameters for the determination of RMD using the proposed HPTLC method. |
|---|
| Parameter | RMD |
| Wavelength (nm) | 245 |
| Linearity range (ng/spot) | 30–500 |
| [(μg/mL)] | [6–100] |
| Intercept (a) | 215.495 |
| Slope (b) | 6.826 |
| Correlation coefficient (r) | 0.9997 |
| S_a | 21.986 |
| S_b | 0.078 |
| RSD% of slope (S_b%) | 1.14 |
| S_a/S_b | 34.686 |
| LOD (ng/spot) | 8.33 |
| [(μg/mL)] | [1.67] |
| LOQ (ng/spot) | 27.77 |
| [(μg/mL)] | [5.55] |
different points across the absorption spectra were superimposed on one another. Table S3 (in supplementary file 1) illustrates the values of $r_{s,m}$ and $r_{e,m}$ of RMD spots from standard, degradation samples and Remdesivir® infusion sample solution and the calculated correlation coefficients values were found not less than 0.99948 confirming the purity of RMD spots with no overlapped peaks from neither degradation products nor co-formulated excipients.

Fig. 2. ESI-MS analysis of alkaline degradation product at $R_f$ 0.01 (a) and $R_f$ 0.03 (b) and oxidative degradation product at $R_f$ 0.75 (c) with their proposed structures.
3.3. Application of the proposed method on analysis of Remdesivir® concentrate solution

Upon the application of the proposed method, RMD was effectively quantified in its pharmaceutical formulation without any interfering peaks from the excipients (Fig. S3, in supplementary file 1). Calculation of recoveries was performed using external standard method. Acceptable accuracy and precision were ascertained from % recovery, SD and RSD% values (Table S4, in supplementary file 1). The drug content was found to be not less than 99.30%. Also, the low RSD% value (less than 1.60%) indicates the suitability of this method for the routine analysis of RMD in its pharmaceutical dosage form.

Additionally, a reference reversed phase HPLC method (Hamdy et al., 2021) was applied for the determination of RMD in its commercial product. Statistical comparison of the recovery data attained from the proposed method with those obtained from the reference method was performed using the Student’s t-test and F-test. The calculated values did not go beyond the tabulated value at the 95% confidence level (Table S4, in supplementary file 1) proving no considerable differences between the proposed HPTLC and reference HPLC method. Thus, our developed method can be applied to the analysis of RMD in its marketed dosage form with optimum and analogous analytical performance.

3.4. Stability-Indicating aspects and degradation hypothesis

Forced degradation experiments were performed on standard solution of RMD to produce the relevant degradation products. Acidic (1 M HCl), alkaline (0.003 M NaOH), neutral, oxidative (30% H2O2) and photolytic degradation experiments were conducted, and the resulting densitograms were compared with that of a standard untreated solution of the drug. The behavior of RMD under different conditions is illustrated in Table S5 (supplementary file 1).

Exposing RMD to 1 M HCl for 30 min at room temperature caused a degradation of about 15.6% of its potency without the detection of any additional degradation peaks in the densitogram (Fig. 1b).

Concerning alkaline hydrolysis, treating the cited drug with 0.1 M NaOH for 15 min at room temperature led to almost complete degradation. While mixing RMD with a diluted solution of NaOH (0.003 M) for 30 min at room temperature resulted in the most extensive degradation (loss of 18.8% of its peak area) with the appearance of two additional degradation peaks at Rf 0.01 and 0.03 (Fig. 1c). From the chemical structure of RMD, a hydrolytic degradation hypothesis was presented in scheme (1) (Fig. S4, in supplementary file 1) comprising all possible hydrolytic degradation products. This hypothesis is based on the existence of three structural groups in RMD that are liable to hydrolysis which are the carboxylate and phosphamide esters in addition to the nitrile group which could be hydrolyzed to its corresponding amide and carboxylic acid. Using HPTLC-MS technique, the degradants bands at Rf 0.01

![Fig. 3. Kinetic study of the acidic (a) and alkaline (b) hydrolysis of RMD at different strengths of HCl and NaOH, respectively.](image-url)
and 0.03 were analyzed and the resulted MS spectra had m/z 423.1 and 537.1, respectively. We have suggested that the degradant at Rf 0.01 resulted from hydrolysis of carboxylate ester of RMD, followed by cyclization on to the phosphorous displacing the phenoxide group to yield a cyclic anhydride (Fig. 2a). It is noteworthy that the previously suggested degradant matches one of RMD metabolites resulted from the biological hydrolysis of its carboxylate ester mediated by esterase enzymes (Eastman et al., 2020). Concerning the chemical structure of the degradant at Rf 0.03, it is proposed that both nitrile and carboxylate ester groups were subjected to hydrolysis to their corresponding acids (Fig. 2b).

Furthermore, heating RMD with 30% H2O2 at 80 °C for 30 min triggered degradation of about 10.4% of RMD along with the detection of one degradation peak at Rf 0.75 (Fig. 1d). It is suggested that the drug underwent hydrolytic oxidation. Hydrolysis of carboxylate and phosphamide esters as well as hydrolysis of nitrile group to acid amide or carboxylic acid were suggested. Oxidation of amino group to nitro and oxidation of hydroxyl groups to their oxidized forms were proposed (scheme (2) in Fig. S5, supplementary file 1). The analysis of the inspected band at Rf 0.75 was performed via HPTLC-MS showing m/z of 347.4 on the MS chart (Fig. 2c) matching the structure of one of the proposed hydrolytic oxidation degradants (compound I) (scheme (2) in Fig. S5, supplementary file 1).

On the other hand, RMD was found to be relatively stable under neutral and photolytic degradation conditions (Fig. 1e and f) which were resulted in loss of about 3% of the drug potency.

It is worthy to mention that the degradation hypothesis presented in schemes (1 & 2) (Figs. S4 and S5, supplementary file 1) agrees with a previously reported hypothesis included in a published accelerated stability study of RMD using HPLC-DAD technique (Hamdy et al., 2021).

Indeed, these findings represent some preliminary data about the structural interpretation of RMD degradation products utilizing their corresponding ESI-MS spectra alone, and a degradation hypothesis of RMD based on previously reported degradation studies.

3.5. Degradation kinetic studies

Acidic and alkaline hydrolysis were carried out in HCl and NaOH solutions of different strengths at room temperature for 1 h, respectively. The developed method was exploited to study RMD degradation kinetics. This was achieved by calculating the remaining concentration of RMD (Ct) after specified time (t) intervals. It was observed that linear relationships were achieved by plotting the log RMD remaining concentrations versus time using different concentrations of HCl or NaOH (Fig. 3). The degradation process followed first order kinetics. Nevertheless, there were two reactants in the reaction media where one reactant was found in a large amount. Thus, the term of pseudo first-order kinetics was given to these reactions. From the slopes of the straight lines, we could calculate the relevant degradation rate constant and half-life (t1/2) for each degradation condition by applying the following equations (Mourad et al., 2016; Gawad and Belal, 2017; Marzouk et al., 2022):

\[
\log C_t = \log C_0 - \frac{kt}{2.303}
\]

\[
t_{1/2} = 0.693/k
\]

where, C0 and C0 are RMD concentrations estimated at a given time t and at zero time, respectively, k is the apparent first order rate constant and -K/2.303 is the slope of the line.

The rate constants and half-lives under different degradation conditions are gathered in Table 2. As expected, under stress conditions, increasing HCl or NaOH strengths resulted in decreasing RMD half-life at room temperature. RMD was found to be highly unstable in alkaline medium as illustrated form its half-life upon exposure to 0.005 M NaOH being very short (around only 1 h). Moreover, it is unstable in acidic medium but in a lesser extent as indicated from rate constants and half-lives together with concentrations used from both HCl and NaOH.

3.6. Greenness appraisal and comparison of the proposed method with published ones

Green analytical chemistry (GAC) aims at encouraging analytical chemists to take into consideration the effect of their analytical procedures on the environment, health and personal safety. GAC necessitates the implementation of dedicated tools to accurately measure the level of greenness of any analytical procedure and hence to compare different analytical procedures to precisely indicate the method that provides the best compromise between being environmentally benign and achieving a good analytical performance.

| Acidic hydrolysis at room temperature | K (h⁻¹) | t1/2 (h) |
|--------------------------------------|---------|----------|
| HCl strength (M)                     |         |          |
| 1.0                                  | 0.3144  | 2.20     |
| 0.5                                  | 0.2306  | 3.01     |
| 0.2                                  | 0.1078  | 6.43     |
| Alkaline hydrolysis at room temperature | K (h⁻¹) | t1/2 (h) |
| NaOH strength (M)                    |         |          |
| 0.005                                | 0.6015  | 1.15     |
| 0.003                                | 0.3602  | 1.99     |
| 0.001                                | 0.2867  | 2.42     |
For preliminary judgement on the greenness of the developed method we employed the simple semi-quantitative Analytical Eco-scale (AES) tool (Gałuszka et al., 2012). While, for the sake of obtaining a more comprehensive comparison between our method and different published analytical procedures in terms of their ecological effect, it is highly recommended to combine more than one GAC metric, where synergistic results allow for rational ranking of these procedures (El-Kafrawy et al., 2022a; El-Kafrawy et al., 2022b). This is usually achieved when specially combining the inclusive Green Analytical Procedure Index (GAPI) metric (Plotka-Wasylka, 2018) and the more informative analytical greenness (AGREE) tool (Pena-Pereira et al., 2020).

Concerning the AES tool, it classifies the method’s greenness according to a total numerical score. This score is calculated by subtracting penalty points from a total of 100. These penalty points are allocated for each aspect that negatively affects the method’s greenness. These aspects include toxic solvents or reagents, volume of generated waste, energy demanded by the technique and occupational hazards. When the method’s total score is above 50, the procedure is regarded green, if the method’s total score is above 75, it is regarded excellent green, while scores below 50 represent inadequate green procedure (Gałuszka et al., 2012). After calculation of AES score of the developed method, it obtained a score of 90, thus it is regarded as excellent green method (Table S6, supplementary file 1).

For comparison of the proposed method with previously published stability indicating HPLC methods (Ibrahim et al., 2021; Hamdy et al., 2021; Ramakrishna Reddy et al., 2021; Surabhi and Jaina, 2021), we first applied the GAPI tool to attain an inclusive greenness evaluation and rational ranking of the methods. This metric offers a thorough ecological evaluation of the whole method starting from sample preparation step up to the final determination step. It is represented as a colored pictogram composed of 15 sections referring to 15 evaluated parameters including sample collection, preservation, transportation, solvents and reagents used, energy consumed, waste generated and other instrumental parameters. Each section is colored by either green, yellow or red indicating low, medium or high negative impact on the environment. After visual inspection of resulted GAPI pictograms of the methods under study, we found that the proposed HPTLC method together with the recently published HPLC method (Ibrahim et al., 2021) are ranked as the greenest ones since they obtained the highest number of green zones and lowest number of red zones [the developed HPTLC method obtained 6

| Method                        | Mobile phase                                                                 | Run time | Flow rate (mL/min) | Waste (mL/sample) | GAPI Pictogram            | AGREE Pictogram |
|-------------------------------|------------------------------------------------------------------------------|----------|--------------------|------------------|---------------------------|-----------------|
| Developed HPTLC method        | Ethyl acetate: ethanol (96: 4).                                              | –        | –                  | 0.75 mL/sample    | ![GAPI Pictogram](image1) | 0.77            |
| Published HPLC (Ibrahim et al., 2021) | Mixture of 0.025 M Brij-35, 0.1 M sodium lauryl sulfate (SLS) and 0.02 M disodium hydrogen phosphate, adjusted to pH 6 using phosphoric acid | 9 min    | 1 mL/min           | 9 mL/sample       | ![GAPI Pictogram](image2) | 0.77            |
| Published HPLC (Surabhi and Jaina, 2021) | Acetonitrile: water (50:50)                                                  | 10 min   | 1 mL/min           | 10 mL/sample      | ![GAPI Pictogram](image3) | 0.66            |
| Published HPLC (Hamdy et al., 2021) | Acetonitrile: distilled water (acidified with phosphoric acid, pH 4) (55: 45). | 10 min   | 1 mL/min           | 10 mL/sample      | ![GAPI Pictogram](image4) | 0.63            |
| Published HPLC (Ramakrishna Reddy et al., 2021) | Mobile phase A: Buffer solution, by mixing 1 mL of orthophosphoric acid (85%) in 1 L purified water, pH adjusted to 3 with KOH solution. Mobile phase B: Acetonitrile: methanol: water (70:20:10). Phases A & B are eluted in a gradient mode. | 75 min   | 0.7 mL/min         | 52.5 mL/sample    | ![GAPI Pictogram](image5) | 0.55            |
| Published HPTLC (Noureldeen et al., 2022) | Ethyl acetate: methanol: ammonia (8:2:0.2)                                  | –        | –                  | 1 mL/sample       | ![GAPI Pictogram](image6) | 0.74            |
green zones, 8 yellow zones and 1 red zone while the published HPLC method (Ibrahim et al., 2021) obtained 7 green, 6 yellow and 2 red zones. Then the two reported HPLC methods (Hamdy et al., 2021; Surabhi and Jaina, 2021) acquired the same number of green, yellow and red zones (4 green, 9 yellow and 2 red zones) and ranked as lower in greenness. Finally, the reported HPLC method (Ramakrishna Reddy et al., 2021) is ranked as the poorest in greenness as it acquired the greatest number of red zones (4 green, 7 yellow and 4 red zones) (Table 3).

Upon implementing the recent AGREE tool, a more comprehensive and informative appraisal of the methods’ greenness was obtained. This tool is a downloadable software that evaluates the method’s greenness based on the 12 GAC principles (SIGNIFICANCE) (Galuszka et al., 2013). After input of the 12 variables of each method, a circular pictogram is automatically generated comprising 12 sections colored from deep green to red color with a final numerical score in the middle. The score is a fraction of unity ranging from 0 to 1, as the score is near 1 the method is considered greener. From the illustrated AGREE pictograms (Table 3) the developed HPTLC method and the reported HPLC method (Ibrahim et al., 2021) acquired the highest AGREE score (0.77) and are ranked as the greenest methods among all studied methods. The developed HPTLC method employs the smallest sample size (5 μL), provides the highest analytical throughput (30 analytes/h), all solvents and reagents are biobased (ethanol and ethyl acetate) and generates the lowest waste volume (0.75 mL). Regarding the reported HPLC method (Ibrahim et al., 2021), although it employs a mobile phase free from organic components (Brij, sodium lauryl sulfate and disodium hydrogen phosphate), it possesses a lower analytical throughput (6.7 analytes/h) and generates a greater waste volume (9 mL/sample).

The reported HPLC method (Surabhi and Jaina, 2021) is ranked as the second green method (AGREE score = 0.66) as it employs higher sample volume (10 μL), lower analytical throughput (6 analytes/h), less green solvents and reagents (acetonitrile) and greater waste volume (10 mL/sample). Moreover, the high flammability of acetonitrile represents an operator’s health threat.

The published HPLC method (Hamdy et al., 2021) is ranked as the third green method (AGREE score = 0.63) as it utilizes a higher sample volume (20 μL) and the operator is exposed not only to the high flammability of acetonitrile but also to the corrosive effect of phosphoric acid used in the mobile phase preparation.

Finally, the published HPLC method (Ramakrishna Reddy et al., 2021) is regarded as the lowest in greenness (AGREE score = 0.55) as it generates the highest volume of waste (52.2 mL/run), provides the lowest analytical throughput (3.2 analytes/h) and employs more toxic and hazardous mobile phase components (methanol, acetonitrile, o-phosphoric acid and potassium hydroxide).

From the previous findings we can conclude that both GAPI and AGREE metrics showed synergistic results proving the final reasonable ranking which considers both the developed HPTLC method and the recently published HPLC method (Ibrahim et al., 2021) equivalent in greenness and ranked as the greenest methods among other stability indicating procedures of RMD. It is worthy to mention that the HPTLC technique is undoubtedly superior to the HPLC one in being a more economic, green and faster alternative depending on parallel assay of multiple samples instead of sequential HPLC analysis of samples and consequently consumes lower volume of mobile phase with minimum generated waste. Also, it is a simpler technique as it neither requires laborious preconditioning of column nor pretreatment of samples. In addition, HPTLC technique deals with cruder samples during impurity profiling without the risk of blockade of the expensive HPLC column (Sherma, 2010; Shewiyo et al., 2012).

Furthermore, the ecological impact of the only published direct HPTLC analysis (Noureldeen et al., 2022) of RMD was evaluated using GAPI and AGREE metrics (Table 3). This method was found of lower greenness than the developed one as its GAPI pictogram encloses lower number of green zones (4 green zones, 10 yellow zones and one red zone) also its AGREE score (0.74) was found lower than our method’s score (0.77). Besides, this method is deemed lower in specificity as it is not a stability indicating assay of RMD (all AGREE reports of developed and reported methods under study are illustrated in Supplementary file 2).

4. Conclusion and recommendations

The proposed HPTLC method represents the first simple, sensitive, specific, economic, fast and ecologically benign stability indicating procedure for the quantification of RMD. Comprehensive greenness appraisal and comparison of the developed method with previously published stability indicating methods were performed using Analytical Eco-scale, GAPI and AGREE metrics. Although the greenness of developed method was found comparable to that of the recently published stability indicating organic solvent-free HPLC method (Ibrahim et al., 2021), the proposed HPTLC technique is deemed superior from GAC perspectives. The proposed method offers shorter analysis time, higher analytical throughput, less laborious sample pretreatment steps, parallel multi-sample analysis instead of sequential manner, consequently it could be regarded as a green and cost-effective alternative to HPLC technique. The developed method was fully validated and successfully applied for the neat separation of RMD from its stress degradation products as well as from co-formulated excipients without any significant interference. Moreover, structural elucidation of degradation products was performed using HPTLC-Ms. Additionally, study of the acidic and alkaline degradation kinetics of the drug was carried out. To our knowledge, no previous analytical reports have tackled the study of degradation kinetics of RMD, hence the proposed method is deemed as the first to report RMD degradation kinetics and to calculate its degradation rate constants and half-lives. Finally, we recommend the proposed HPTLC method as a more suitable method for routine analysis of RMD in quality control laboratories as well as for testing its purity with minimum negative impact on the environment and operator’s health and safety.

Indeed, the present study provides preliminary data about structural elucidation of RMD degradation products based only on their corresponding ESI-MS spectra and therefore more perceptive further studies are recommended for accurate and detailed structural characterization of these degradation products based on the analysis of their MS/MS spectra and correlation with proposed RMD degradation reactions. Another possible area of future research would be the achievement of chemical separation of these degradation products in pure form to investigate their potential toxicity.
Credit authorship contribution statement

Amira H. Abo-Gharam: Data curation, Validation, Formal analysis, Investigation, Writing – original draft. Dina S. El-Kafrawy: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Supervision.

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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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