ENVIRONMENTAL CHEMICAL CONTAMINANTS

Comparative Greenness Metric Estimates for Content Uniformity Testing of Anti-Cov-2, GS-5734 in Commercial Vials: Validated Micellar Electrokinetic Chromatographic Assay

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Abstract

Background: The antiviral drug GS-5734 remdesivir is a new phosphoramidate prodrug developed initially as a treatment for Ebola virus which then proved to have antiviral properties against other viruses. After clinical trials, it was the first antiviral to be approved by the U.S. Food and Drug Administration in 2020 to treat severe coronavirus (COVID-19) cases. The widespread current pandemic gave an urge to its fast production and marketing. Thus, new analytical methods must be available for its analysis in a fast and easy manner with low cost to be applicable in all laboratories.

Objective: In the current study, a green and economic micellar electrokinetic chromatographic (MEKC) method is proposed for remdesivir analysis.

Methods: A fused-silica capillary (58.5 cm × 50 μm id, 50 cm effective length) with 20 mM borate buffer (pH 9) and 25 mM sodium dodecyl sulfate was used under a positive potential of 30 kV at 25 °C with detection at 245 nm.

Results: Remdesivir analysis was achieved in approximately 5 min. The method proved to be linear in range of 1–50 μg/mL with correlation coefficient, r > 0.999.

Conclusion: The MEKC method proposed was applied to the analysis of remdesivir in its commercial vials. The method was validated per International Conference on Harmonization guidelines.

Highlights: Green chemistry has been the focus of the analytical community in the past few years. This method is considered green due to its low energy and solvent consumption without sacrificing the method’s sensitivity or selectivity. The method’s green profile has been assessed by different greenness assessment scales to ensure the method is eco-friendly and can be used in the pharmaceutical industry.
cardiovascular and/or respiratory conditions are of higher risk to severe complications caused by this virus (3).

Since the beginning there has been no known special treatment for the new COVID-19. Thus, testing the efficacy and safety of pre-existing nucleoside analogues or protease inhibitors that are used for other viruses was fundamental in order to develop a specific therapy rapidly against Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (1, 4).

Remdesivir, GS-5734 (RD, Figure 1) was initially developed by Gilead Sciences as an Ebola virus antiviral drug (5). RD is extensively metabolized forming GS-704277 (an intermediate metabolite) which is then phosphorylated to the nucleoside triphosphate active form, GS-443902, which selectively hinders viral RNA polymerases. RD served as a COVID-19 treatment because of its antiviral efficiency in numerous studies (2, 4, 6). Several clinical trials led the U.S. Food and Drug Administration (FDA) to allow emergency use for RD to treat COVID-19 (7, 8). For treating COVID-19, as proposed by Gilead, a 200 mg dosage of RD was prescribed on day 1 and a 100 mg dose for the following 9 days through intravenous administration (1).

A literature survey reveals few recent analytical methods for RD analysis in dosage forms or biological matrixes (1, 9–15). Therefore, it is crucial to develop a simple, fast, sensitive, and accurate method for RD monitoring during COVID-19 to be used as a routine tool for RD QC analysis.

Meanwhile, the United Nations adopted environmental sustainability goals in 2015 targeting a “Global Green Agenda 2030” where chemistry is required to meet an “Affordable & Clean Energy” goal (16). Thus, green analytical chemistry (GAC), has gained more attention in the past years (17, 18). The main goal of GAC is to reduce the impact of different types of analysis (pharmaceutical, food, or industrial) on the environment (19). Besides, it is much highlighted in less developed countries where using sophisticated methods and high-cost instrumentation is replaced with relatively low-cost and readily available ones (18).

Capillary electrophoresis (CE) is one of the greenest analytical techniques due to low solvent consumption and use of buffer systems where organic solvents, such as acetonitrile, are rarely used. A typical CE column is very narrow and electroosmosis, the main dominating elution force in CE, has a flow rate of sub-μL/min. Thus, the total eluent consumption per run is negligible (20). Consequently, CE compared with different HPLC modes reported for RD is considered much greener (19, 21, 22). Also, CE is capable of separation with high peak capacity and efficiency in a short time using small sample volume and providing flat flow with narrower peaks and better resolution, compared to HPLC-pumped parabolic flow (21, 22).

The aim of this work is to highlight the benefit of CE and MEKC (micellar electrokinetic capillary chromatography), a CE mode having principles of both chromatography and electrophoresis, as a green analytical technique to analyze, for the first time, a recently FDA-approved drug molecule: remdesivir, GS-5734 and to be a routine method for its analysis. To ensure our goal, four different greenness assessment tools have been used to evaluate the method greenness.

**Experimental**

**CE Instrument**

An Agilent 7100 series CE instrument (Waldbronn, Germany) was used in this study together with Agilent ChemStation software and a deactivated fused-silica capillary (58.5 cm × 50 μm id, 50 cm effective length). The RD assay was performed at 245 nm using a diode-array detector (DAD). Injection was hydrodynamic (50 mbar pressure for 20 s) and the applied voltage was 30 kV.

**Materials and Chemicals**

Filtered, distilled water was used throughout the experimental work. RD pure material (purity of 99.38%) was obtained from Selleckchem (Houston, TX, USA). Sodium hydroxide from El-Nasr Chemical Ind. Co. (Giza, Egypt), sodium dodecyl sulphate (SDS) and boric acid from Oxford Lab Chem (Mumbai, India) were used. HPLC grade methanol was obtained from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). Remdesivir-Eva Pharma injection vials (Eva Pharma, Cairo, Egypt) labeled to contain 5 mg/mL RD were bought from the commercial market.
**Results and Discussion**

**Optimization of the Proposed MEKC Method**

Most pharmaceutical compounds are neutral so the basic CZE (capillary zone electrophoresis) is not suited for their separation because it separates analytes based on differences in their electrophoretic mobilities only. MEKC, a mode of CE, separates neutral compounds as it is based on differential partitioning, like traditional chromatography, between a mobile aqueous phase and a micellar pseudo-stationary phase (23).

To achieve best electrophoretic analysis of RD, buffers of different pH and concentrations were tested including acetate buffer pH 4.7, phosphate buffer pH 7.4, and borate buffer pH 9. However, CZE using buffers solely did not achieve the required peak symmetry for RD.

This can be attributed to the fact that pH of the BGE has a crucial role in analyte separation in CZE, especially for analytes that have weak acidic or weak basic properties. In order to achieve an optimum pH for the proposed method, the pKa of the studied drug was considered. RD has an acidic pKa value of 10.23 and a basic pKa value of 0.65 (24) and found to be predominantly in the unionized form (25) in the pH range 3–10 and is only completely ionized at a pH higher than 12 which is not applicable in capillary electrophoresis.

As previously mentioned, only ionized compounds can have a differential migration in CZE; neutral compounds cannot be separated. Thus, simple CZE methods can only be used for the analysis of different charged compounds (26). Neutral compounds can be analyzed by MEKC whose charged micelles make uncharged compounds move in the electric field. Therefore, based on these facts, simple CE was not favorable for the analysis of RD, and that was proved to be true practically by the distorted peak shape and migration with the electroosmotic flow (EOF) in different buffers tried.

Thus, MEKC mode was tried by adding SDS above its critical micelle concentration (CMC) to the buffer to form micelles. The micelles act as a pseudo-stationary phase and interact with the analytes by partitioning mechanisms, similar to traditional chromatography. MEKC achieved better resolution and peak symmetry for RD compared to traditional CE.

The following parameters were optimized for the RD assay using MEKC:

(a) Buffer pH and type.—In comparing three different buffers, each one at its pKa (acetate, 20mM, pH 4.7; phosphate, 20mM, pH 7.4; and borate, 20mM, pH 9) combined with 25mM SDS, it was observed that acetate buffer showed a split RD peak, while borate and phosphate buffers showed optimum peak shape and symmetry (between acceptable limits of 0.8–1.2). Taking into consideration the total run time, borate buffer was the best as the migration time of RD was 5.24 min compared to phosphate buffer (9.45 min). Furthermore, different pH values (8–10) of the borate buffer had no effect on migration time nor peak shape. For CE it is much better to have the pH as close to the pKa of the buffer as possible to provide better buffer capacity and pH stability, so finally pH 9 was chosen as it is the pKa value of borate buffer.

(b) Buffer concentration.—High buffer and SDS concentrations lead to high viscosities and currents so should be avoided. Viscosity variations in the BGE may lead to band broadening and poor peak shape. It also affects sample injection and the migration time of the analyte that, in turn, can affect the reproducibility of the analysis (26). Different concentrations of 10, 20, and 50 mM borate buffers at pH 9 were examined and it was found that increasing the buffer concentration increased the migration time. Thus, a buffer concentration of 20 mM was chosen to reduce analysis time while maintaining good peak shape.

(c) SDS concentration.—The effect of SDS concentration was also studied by inclusion of 15, 25 or 50 mM SDS in the BGE. It was found the SDS concentration effect was similar to that of the buffer. As SDS concentration increased, migration times increased. Therefore, 25 mM SDS was selected as it was the best compromise.

(d) Diluting solvent selection.—For choosing the optimum diluting solvent that gives the best sensitivity and resolution, both BGE and water were tested with water giving better peak shape. This can be attributed to sample high-field stacking due to the lower ionic strength of water which causes ions to migrate faster and stack as a sharp peak (22).

(e) Applied voltage.—Different applied voltages (15–30 kV) were tested using the optimized BGE. It was found that resolution of the RD peak was not affected by changing the potential but migration time increased by decreasing the applied voltage due to a decrease of the EOF. Thus, a voltage of 30 kV was selected for effective RD assay within acceptable time limit.

(f) Sample injection time.—Injection time affects both peak area and height. Thus, to choose the optimum injection time, varying injection times from 3 to 22 sec were tried at 50

**Procedure**

(a) Preparation of running buffer/background electrolyte.—The background electrolyte (BGE) consisted of 20 mM borate buffer (pH 9) containing 25 mM SDS. The borate buffer was prepared by weighing and dissolving 0.124 g boric acid and 0.04 g sodium hydroxide in 100 mL distilled water then adjusted to pH 9. For 25 mM SDS preparation: 0.722 g SDS was dissolved in 100 mL of the prepared buffer, and sonicated for 10 min.

(b) Conditioning of the capillary.—Before the first run of each working day, the capillary was rinsed well for 15 min with 0.5 M NaOH, followed by distilled water for another 15 min. The capillary was then flushed and rinsed for at least 5 min using 0.1 M NaOH to ascertain the activation of the inner wall of the capillary. Finally after rinsing with distilled water for 5 min, the capillary was left to equilibrate for 10 min with the BGE. The capillary was also rinsed using the BGE for 2 min before each new injection.

(c) Standard solutions and calibration graphs.—RD standard stock solution (1000 µg/mL) was prepared in methanol and kept in a refrigerator at 4°C. Accurate volumes (10–500 µL) of RD stock solution were transferred into 10 mL volumetric flasks and then, diluted to volume using distilled water to give a linearity concentration range of 1–50 µg/mL. Each sample was injected in triplicate and the calibration graph of the proposed MEKC method (using the average RD peak area of each sample against its corresponding concentration) was then plotted.

(d) Analysis of dosage form.—Two vials, each containing 100 mg RD were reconstituted with 0.9% (v/v) saline infusion to a volume of 250 mL. Prepared stock solution (800 µg/mL) was then diluted with water to give the required concentrations of working solutions for subsequent MEKC analysis.
mbar. Generally, increasing injection time caused peak height and area to increase. However, further increase of injection time beyond 20 s (the chosen optimum time) led to peak shape deformation (broadening) and loss of symmetry.

Detection wavelength.—The developed method allowed the efficient assay of the analyzed drug at 245 nm (RD $\lambda_{\text{max}}$) in 5.24 min. Figure 2 represents a typical electropherogram of the MEKC assay of the cited drug. The calculated system suitability parameters for RD assay were all within the acceptable limit showing retention factor ($k'$) of 1.434, theoretical plates (N) of 43,317, and USP tailing factor of 1.091.

Regarding the reported methods for analysis of RD in its injection vials, they have been compared to the proposed CE method in online Supplemental Table 1 in terms of conditions and parameters. In terms of greenness, which is the main goal for analytical chemistry nowadays, the proposed CE method is better than the HPLC one as it consumes less solvent (which is non-toxic) and consequently generates a lower amount of waste.

Although the reported spectrofluorimetric technique is also green but for future applications in complex mixtures, CE will be a better option as it does not require further mathematical treatment due to spectral overlaps, nor dependent on the native fluorescence of other components in mixture. Moreover, fluorimetric measurements are not as robust as CE ones because the former are highly sensitive to pH change and the presence of oxygen. Meanwhile, the other reported methods for analysis of RD in biological samples, as serum and plasma, are chromatographic methods mainly based on mass spectroscopic detection which are laborious, expensive and time-consuming, in addition to lacking greenness due to huge solvent and energy consumption in these methods which makes them unsuitable for routine QC analysis of RD in its commercial vials.

Method Validation

The method’s validation parameters were all validated according to the published guidelines of the International Conference on Harmonization (ICH; 27).

(a) Linearity.—The linearity estimated by least squares treatment of the results was confirmed by the high value of the correlation coefficient as shown in Table 1. Performance characteristics values and statistical data are all presented in Table 1. All parameters including the standard deviations of intercept, slope and residuals, the $F$-value and significance $F$ indicate good linearity of this method. Figure 3 shows the calibration and residuals plot of the regression data of RD.

(b) LOD and LOQ.—According to the ICH, the LOD is the analyte’s concentration giving a S/N of 3:1 and for the LOQ, the ratio is 10:1. The values of LOD and LOQ for RD using the proposed MEKC method were calculated and presented in Table 1 confirming the acceptable sensitivity of the developed procedure.

(c) Accuracy and precision.—Determinations ($n = 3$) at three levels (low, medium, and high concentrations within the calibration range) were analyzed on one day and three

| Parameter                      | Value          |
|-------------------------------|---------------|
| Wavelength, nm               | 245           |
| Concentration range, $\mu$g/mL | 1–50          |
| Intercept (a)                 | -0.768        |
| Sa $^a$                       | 0.528         |
| Slope (b)                     | 2.179         |
| $Sb^b$                       | 0.019         |
| RSD, %, of the slope ($Sb\%$) | 0.863         |
| Correlation coefficient (r)  | 0.9998        |
| $Sy/x^c$                      | 0.851         |
| $F^d$                         | 13418.733     |
| Significance $F$              | $9.092 \times 10^{-10}$ |
| LOD, $\mu$g/mL               | 0.289         |
| LOQ, $\mu$g/mL               | 0.963         |

$^a$ Standard deviation of the intercept.
$^b$ Standard deviation of the slope.
$^c$ Standard deviation of residuals.
$^d$ Variance ratio, equals the mean of squares due to regression divided by the mean of squares about regression (due to residuals).

Figure 2. MEKC electropherogram of a standard solution containing 10 $\mu$g/mL RD at 245 nm.
different days for studying the accuracy and intra- and inter-day precision. Statistical evaluation of the precision data showed low RSDs not exceeding 2% (Table 2). The Er% (percentage relative error) values were also less than 2%, proving the accuracy of method.

Selectivity.—Selectivity of the method was confirmed by applying it to analysis of RD in its injection vials successfully without interference (see Supplemental Table 1). Also, the DAD allows verification of peak purity which confirmed there was no sign of any co-eluted peaks. In addition, the overlap of the spectra measured at different points of the RD peak confirms peak purity and selectivity especially given that the threshold value of the noise indicated by the red colored area was not exceeded in the purity plot in Figure 4.

Robustness.—The robustness of the MEKC method was assessed by measuring changes in peak area and migration time after deliberate changes to the method’s parameters. The study was performed on 10 µg/mL sample of RD (n = 3). The studied changes did not affect the tested drug as proved by the RSD, %, values (Table 3).

Assay of Dosage Forms
The proposed MEKC method was used for the assay of RD in its commercially available vials. The electropherogram obtained from the injection vial of RD is shown in Figure 5. Dilution of RD from vials was made using saline infusion in order to simulate the medium in which RD is injected into real to patients to test any possible interference and ensure the method’s selectivity. No additional peaks were detected from inactive components in the vial. DAD also proved the peak purity as mentioned earlier. A comparison of the method in this study to a reported method (15) using the t-test and F-test is given in online Supplemental Table 2. The calculated results show no significant difference between both methods.

Assessment of Method Greenness
Nowadays, environmentally friendly and green practices are adopted in analytical procedures such as using green sample pretreatment, environmentally friendly solvents and reagents, and shortening analysis times. LC is one of the controversial techniques regarding green analysis due to its wide application
in the analytical field using high amounts of dangerous organic solvents which diffuse directly into the environment, provoking a huge risk of toxicity (28). Thus developing other alternative analytical techniques is of great importance. Since greenness assessment of analytical methods has profound importance, several greenness assessment tools have been developed recently (21, 29, 30).

The Eco-Scale is one of the green assessment tools in use, and it has been found to be a semiquantitative tool (31, 32). It compares the various parameters and steps for the whole analytical process and calculates a final score by subtracting penalty points for each parameter that does not match with green analysis. An Eco-Scale scoring system is as follows (out of 100): excellent greenness score >75, acceptable score >50, and

| Nominal value of RD, µg/mL | Found ± SDa, µg/mL | RSD, %b | Mean recovery, % | E%, %c |
|---------------------------|---------------------|--------|-----------------|--------|
|                           | Within-day          |        |                 |        |
| 5                         | 4.927 ± 0.096       | 1.948  | 98.540          | -1.460 |
| 20                        | 19.691 ± 0.280      | 1.422  | 98.455          | -1.545 |
| 50                        | 50.077 ± 0.955      | 1.907  | 100.154         | 0.154  |
|                           | Between-days        |        |                 |        |
| 5                         | 5.003 ± 0.096       | 1.919  | 100.060         | 0.060  |
| 20                        | 19.753 ± 0.312      | 1.580  | 98.765          | -1.235 |
| 50                        | 50.536 ± 0.701      | 1.387  | 101.072         | 1.072  |

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| 20                        | 19.753 ± 0.312      | 1.580  | 98.765          | -1.235 |
| 50                        | 50.536 ± 0.701      | 1.387  | 101.072         | 1.072  |

*Mean ± SD for three determinations.
% Relative standard deviation.
% Relative error.

Figure 4. (a) Absorption spectrum of 10 µg/mL RD sample (from commercial injection solution) measured at different time intervals across the peak and (b) the purity plot for the RD peak.
inadequate greenness <50. Supplemental Table 3 shows the Eco-Scale assessment for the proposed method with a score of 86 points. The green certificate also classifies methods by colors and letters from A (the greenest) to G. Figure 6a shows the green certificate of the proposed method. The developed method is category B (33).

Another assessment tool called the Green Analytical Procedure Index (GAPI) has been also recently introduced (17). The GAPI pictogram is composed mainly of five major divided pentagrams where each one represents an analytical step in the method. GAPI uses red to indicate bad environmental impact, while yellow and green means intermediate and low impacts, respectively. Figure 6b shows the GAPI assessment of the proposed MEKC method.

A third recent tool is the AGREE (34). AGREE assessment is based on a clock-like graph divided into 12 sections; each represents one of the green analytical chemistry principles. Each section is assessed and represented in a red, yellow, or green color. An overall greenness score (from 0 to 1) is shown inside the clock.

As shown in Figure 6c, the developed MEKC method has an overall AGREE score of 0.92 and a green color meaning low ecological impact. Only one yellow zone was found representing the analysis throughput due to using a small amount of methanol for preparation of RD stock solution.

The National Environmental Method Index (NEMI) is one of the earliest qualitative assessment tools developed even before all methods mentioned above. NEMI judges the greenness of a method using a pictogram of four quadrants where the first quadrant, persistent, bio accumulative, and toxic (PBT), is considered green if the reagents used are not considered PBT by the US Environmental Protection Agency’s Toxic Release Inventory (EPA-TRI; 35). The second quadrant represents “Hazardous” where the chemicals used should not be hazardous, meaning not on the TRI list (35) for the method to be green. The third quadrant “Corrosive” is considered green if the pH of the medium is between 2–12 and finally the last quadrant is concerned with “waste” where it is green if the waste generated is less than 50 g. The NEMI pictogram for the proposed MEKC method is represented in Figure 6d. The reagents and solvents used are not PBT (green). However, methanol (only used in preparation of stock solutions) is on the TRI list (not green). The pH of the method is 9 (green), and the produced waste is less than 50 g.

All our greenness assessment results are in compliance with the latest literature Red-Green-Blue-Model (RGB) which compares CE versus HPLC in terms of potential and usability of each technique. Addition of surfactant in MEKC proved to be an effective way to improve the quality of the analytical results, while maintaining environmental friendliness associated with low waste production. Thus, MEKC proved to better a analytical tool compared to HPLC and conventional CE (36).

Conclusions
A valid, green MEKC method was developed for the assay of RD in bulk and formulation. The method proved to be sensitive, as well
as robust, and its sensitivity and resolution are comparable to other reported chromatographic methods. The method in this study successfully analyzed RD in its injection vials. Assessment of the method greenness was made using four different scales and all proved the method is ecofriendly and green.

Supplemental Information

Supplemental information is available on the J. AOAC Int. website.

Funding

The authors declare that this research is not funded from any source.

Conflict of Interest

All authors declare no conflict of interest.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article. Any further data can be requested from the corresponding author.

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