



OPEN A sustainable and green HPLC-PDA technique for the simultaneous estimation of Post-COVID-19 syndrome co-administered drugs with greenness and whiteness assessment

Passant M. Medhat¹, Manal Mohamed Fouad^{1,2}, Hany H. Monir³ & Nermine S. Ghoniem³✉

COVID-19 has been a growing global concern in the past four years. Several syndromes associated with this multi-organ viral infection have been observed since the outbreak. According to estimates, 10–15% of people with SARS-CoV- infection may have post-COVID-19 syndrome. Even months after infection, common residual signs and symptoms include myalgia, exhaustion, shortness of breath, rapid heartbeat, stroke, and memory and cognitive impairment which can negatively affect survivors' quality of life and may consequently lead to their death. Therefore, it is necessary to think about potential therapy options for dealing with both short and long-term impacts. Paracetamol (a common analgesic and antipyretic) and Dexketoprofen Trometamol (a non-steroidal anti-inflammatory drug) are used together to relieve post-COVID symptoms like myalgia (muscle pain) and headache. Additionally, to prevent thrombotic events, Rivaroxaban is recommended for 35 days following discharge. Thus an eco-friendly HPLC-DAD technique was developed for simultaneous quantification of Paracetamol, Dexketoprofen Trometamol, and Rivaroxaban which are co-administered for treatment of post-COVID-19 syndrome. The suggested method was found to be linear in the concentration ranges of 3.00–45.00 µg/mL, 0.5–50.00 µg/mL, and 0.15–20.00 µg/mL, and a limit of detection down to 0.531 µg/mL, 0.095 µg/mL and 0.047 µg/mL for Paracetamol, Dexketoprofen Trometamol and Rivaroxaban, respectively. This method was effectively used to quantify the studied drugs in their bulk powder and spiked human plasma with high percentage recoveries (96.55–99.46%). The suggested approach was validated per International Conference on Harmonization (ICH) requirements and found to be within the acceptable ranges. The method was developed using Green Analytical Chemistry (GAC) principles, with the solvents used and run time having a significant effect on the method's greenness. "Non-toxic" ethanol served as the organic modifier in the mobile phase, moreover, the total run time was 12 min making it suitable for the routine analysis of the mentioned drugs in plasma samples. To get a full image of the method's greenness profile; two most recent greenness assessment tools, the Green Analytical Procedure Index (GAPI), and the Analytical GREENness metric (AGREE), were employed, with White Analytical Chemistry (WAC) principles proving its environmental safety.

Keywords Post-COVID syndrome, HPLC-DAD, Paracetamol, Dexketoprofen Trometamol and Rivaroxaban.

COVID-19 pandemic, which was first identified in Wuhan, China, in December 2019, spread quickly to become the largest acute pandemic tragedy in human history^{1–4}.

¹Analytical Chemistry Department, Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA), 11787 6th October City, Egypt. ²Analytical Chemistry Department, Faculty of Pharmacy (Girls), Al-Azhar University, Nasr City, Cairo, Egypt. ³Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, Kasr El-Aini Street, Cairo ET-11562, Egypt. ✉email: nermine.ghoniem@pharma.cu.edu.eg

According to WHO, 75 nations reported COVID-19 cases and 43 countries reported COVID-19 deaths during the 28-day period from January 8, 2024, to February 4, 2024. During this period, there were over 119,000 new hospital admissions and over 1500 new ICU admissions recorded. Recent data reported that during the period from (January 29 to February 4, 2024) about 10% of SARS-CoV-2 PCR showed a positive result, which proves that this virus is still life-threatening to date⁵.

Common symptoms of COVID-19 include fever, myalgia, and exhaustion combined with more respiratory symptoms such as sore throat, cough, and shortness of breath. Since the pandemic started, neurological symptoms involving the central and peripheral nervous systems have also been discovered^{6–8}. Furthermore, COVID-19 impacts all organs and systems.

Although it may take weeks to recover from the coronavirus disease, some patients' symptoms continue even after the first infection. It was found that many patients still suffer from long-term complications such as brain fog (which is a cognitive disturbance), fatigue, shortness of breath, myalgia, headache, palpitation, heart failure, and neurological diseases such as stroke and lung diseases as shown in Fig. 1^{9–11}. This condition is therefore referred to as post-COVID-19 syndrome^{12,13}.

Paracetamol (PAR); N-(4-hydroxyphenyl) acetamide, (Fig.S1(a)) possess analgesic, anti-inflammatory, and antipyretic properties. It blocks cyclooxygenase (especially COX 2), an enzyme involved in the transmission of pain in the brain, acting with extremely precise targeting. It has a different way of action than other painkillers, yet it nevertheless relieves pain throughout the body¹⁴.

Dexketoprofen Trometamol (DEX), chemically known as (2 S)-2-[3-(benzoyl) phenyl] propanoic acid (Fig.S1 (b)). It is the active optical isomer of ketoprofen, used to treat painful musculoskeletal disorders like osteoarthritis and low back pain, post-operative pain, toothache, and dysmenorrhea¹⁴. This combination alleviates pain in conditions such as severe muscle pain, headache, mild migraine, tooth pain, rheumatoid arthritis, and osteoarthritis¹⁵.

Rivaroxaban(RIV),5-chloro-N-((5 S)-2-oxo-3-[4-(3-oxomorpholin-4-yl)phenyl]-1,3-oxazolidin-5-yl)methyl)thiophene-2- carboxamide,((Fig.S1 (c)), is an oxazolidinone based anticoagulant, is a powerful inhibitor of factor X¹⁶. It is approved to treat deep vein thrombosis (DVT), prevent recurrent DVT and pulmonary embolism (PE) after an acute DVT in adults, and prevent thromboembolism in individuals with atrial fibrillation¹⁷.

Thus, Paracetamol and Dexketoprofen Trometamol combination are co-administered by relieved COVID-19 patients for treatment of post-COVID-19 symptoms such as myalgia and headache¹². Moreover, after being

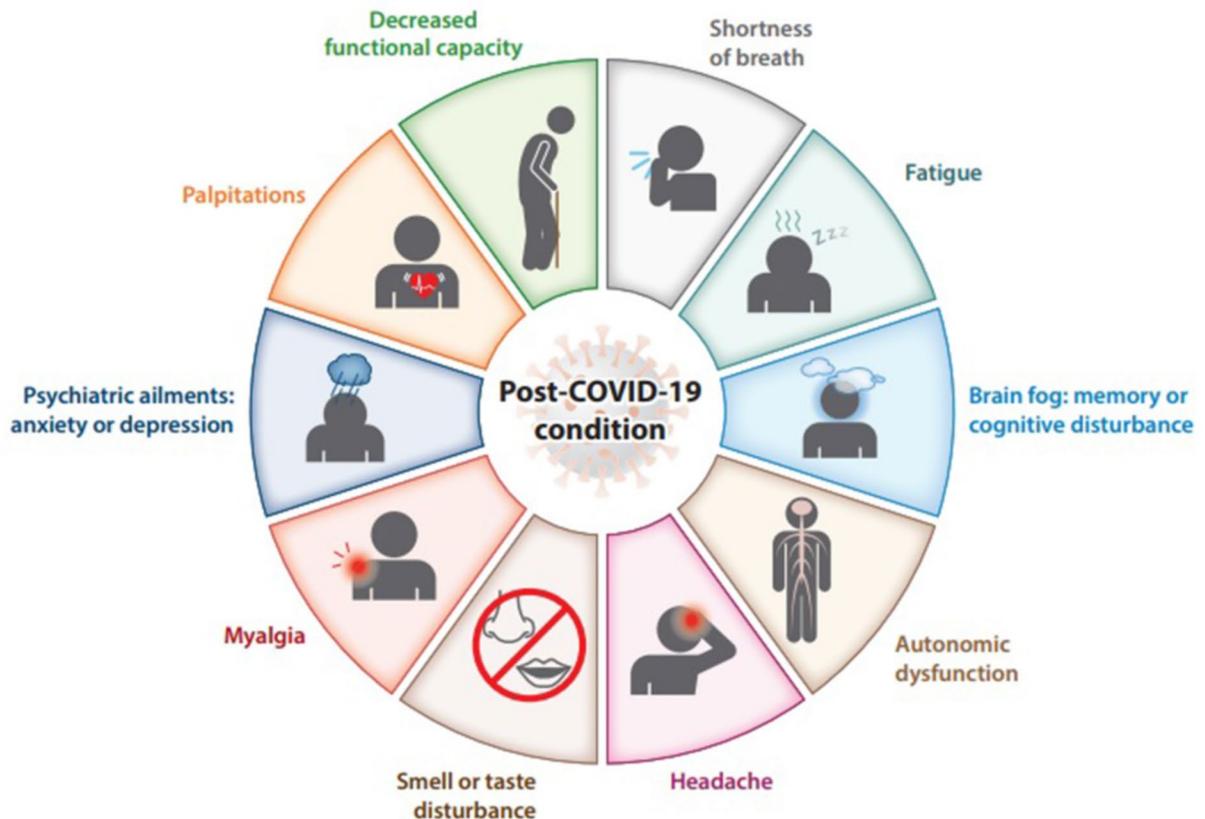


Fig. 1. Common manifestations of the post-COVID-19 condition¹².

discharged from the hospital, patients with COVID-19 are at risk for thrombotic events; however, extended thromboprophylaxis is recommended by the administration of Rivaroxaban for 35 days post-discharge¹⁸.

The literature reveals several chromatographic^{14,19} and spectrophotometric methods²⁰ for the simultaneous determination of PAR and DEX in their combined dosage form. On the other hand, some spectroscopic^{21–23}, chromatographic^{24–31}, and electrochemical methods³² were also reported for the determination of Rivaroxaban either alone or in combination with other drugs. However, there are no published HPLC/UV methods for simultaneously determining PAR, DEX, and RIV in any biological fluid. Consequently, we aimed to develop and validate a green, sensitive, cost-effective chromatographic method for the simultaneous determination of the mentioned co-administered drugs in bulk powder and spiked human plasma. This method successfully detected PAR, DEX, and RIV in biological fluid, making it a more cost-effective alternative to hyphenated GC-MS and LC-MS methods. The greenness of the procedure was evaluated both qualitatively and quantitatively using two commonly employed Green Analytical Chemistry (GAC) evaluation techniques: the Green Analytical Procedure Index (GAPI)³³ and the Analytical GREENness measure (AGREE)³⁴. Moreover, White Analytical Chemistry (WAC) principles³⁵ are used to confirm their environmental impact.

Experimental study

Instrumentation

HPLC separations were carried out with a Waters 2695 Alliance HPLC system equipped with a Waters 996 photodiode array detector and the column was Kromasil Phenyl (150 mm x 4.6 mm, 5 μ m). Sonication of the samples was carried out using Elma Schmidbauer GmbH Sonicator (model S30H, Singen, Germany). Centrifugation of the spiked samples was performed using Sigma Centrifuge (model no. D-37520 Osterode am Harz, Göttingen, Germany).

Samples

Pure samples

Paracetamol certified to be (100.90 \pm 1.310) according to reference method¹⁹ was obtained from (Amriya Pharmaceutical Industries, Amriya, Alexandria, Egypt.), Dexketoprofen Trometamol (100.01 \pm 1.578)¹⁹ was brought from (Utopia Pharmaceutical Industries, 10th of Ramadan, Sharqia, Egypt), and Rivaroxaban (99.66 \pm 1.890)¹⁶ was obtained from (National Organization of Drug Control and Research Centre (NODCAR), Omraniya, Giza, Egypt). Diclofenac sodium (DCL) was used as an internal standard (I.S.) and was kindly supplied from (Egyptian International Pharmaceutical Industries (EIPICO), 10th of Ramadan, Sharqia, Egypt).

Reagents.

- All reagents were of HPLC-grade without any further purification. HPLC grade ethanol, formic acid, acetonitrile, methanol, ethyl acetate, and sodium hydroxide were purchased from Fisher Scientific (USA).
- Water was double distilled by Automatic Water still (Sci Finetech, Seoul, South Korea).
- Fresh human plasma was purchased from Vaccines and Sera Company (VACSERA) (Cairo, Egypt). The study was approved by the Research Ethics Committee of the Faculty of Pharmacy, Cairo University with ethics approval; No: AC(3243).

Standard solutions

Stock solutions of PAR, DEX, and DCL containing (1 mg/mL) were prepared separately by dissolving 0.025 g of each drug in 25 mL of mobile phase (0.1% formic acid in water and ethanol (50:50 v/v), except for RIV stock solution; it was prepared by dissolving the drug in acetonitrile: water (80:20 v/v). The stock solutions were then further diluted with the mobile phase to obtain working solutions of concentration (0.1 mg/mL). RIV was prepared in this solvent, unlike the other two drugs due to its insolubility in ethanol or methanol.

Chromatographic conditions

Gradient chromatographic elution was achieved on Kromasil Phenyl column (150 mm x 4.6 mm i.d, particle size 5 μ m) as a stationary phase and a mobile phase containing 0.1% formic acid in water (A): ethanol (B), operated as follows: (95:5 v/v) for 1.5 min, then scaling up to (30:70 v/v) from (7.5 to 8.0 min) and finally returns back to initial concentration from (9.0 to 12.0 min). The flow rate was adjusted to 1.5 mL/min at 25°C. UV detection was carried out at 254.0 nm using a photodiode array detector and the injection volume was 10.0 μ L.

Procedures

Calibration curves

Different aliquots from PAR, DEX, RIV, and DCL working standard solutions (0.1 mg/ mL), were accurately transferred into a set of 10-mL volumetric flasks and the volume was completed to mark with the mobile phase, to obtain final concentrations of (3.00–45.00 μ g/mL) PAR, (0.50–50.00 μ g/mL) DEX, (0.15–20.00 μ g/mL) RIV, and 25 μ g/mL I.S (DCL). Ten μ L of each solution was injected in triplicates to the HPLC and the chromatograms were obtained using the same procedures as mentioned under “Chromatographic conditions”. The calibration curves were constructed by plotting the relative peak area versus the respective concentration and the regression equations were computed.

Spiked human plasma samples preparation

Human plasma aliquots of 200.0 μ L were individually transferred into a set of 15 mL centrifugation tubes containing serial dilutions of PAR, DEX, RIV, and DCL. The samples were vortexed for 60 s followed by the addition of 800.0 μ L methanol for protein precipitation to obtain final concentrations ranging from (3.0–45.0 μ g/mL), (0.5–45.0 μ g/mL), and (0.5 to 45.0 μ g/mL) of PAR, DEX, and RIV, respectively, in addition to 25 μ g/mL I.S

(DCL). Then the tubes were then centrifuged for 10 min at 6000 rpm. Each tube's supernatant was filtered using syringe filters (0.25 μm). Ten μL of each solution was injected in triplicates to the HPLC and the chromatograms were obtained using the same procedures as mentioned under "Chromatographic conditions".

Results and discussion

The proposed method showed reasonable results, confirming its accuracy, precision, and selectivity. The approach has various advantages, including minimal solvent usage, great sensitivity, very small injection volume, and a short run time. Moreover, it offered superiority over other reported HPLC-DAD^{14,19} and spectrophotometric²⁰ methods for the determination of Paracetamol and Dexketoprofen Trometamol in terms of sensitivity as shown in Table S1. These advantages enable this analytical method to be used in pharmacokinetic research so it can be used as a cheaper alternative method to hyphenated GC-MS and LC-MS methods.

Optimization of the chromatographic conditions

Several trials were conducted to obtain the optimal chromatographic conditions for separating the targeted analytes. Various mobile phase solvents, ratios, and column types were tested. Initially, a Kromasil C₁₈ (150 mm x 4.6 mm, 5 μm) column with a mobile phase consisting of 0.2% Tri ethylamine adjusted to pH 3.0 by orthophosphoric acid and acetonitrile, in gradient elution mode, was tried. This setup led to overlapping between Rivaroxaban and Dexketoprofen peaks. Switching to a longer column as Kromasil C₁₈ (250 mm x 4.6 mm, 5 μm) improved peak sharpness but did not enhance the resolution. Further trials employed different chromatographic conditions and elution modes. For example, Kromasil C₁₈ (150 mm x 4.6 mm, 5 μm) column was tested with a mobile phase of (solution (A): 95% of orthophosphoric acid (pH adjusted to 3.0 by NaOH): 5% acetonitrile) while (solution (B): 70% acetonitrile: 30% orthophosphoric acid (pH adjusted to 3.0 by NaOH)) in gradient elution mode. Additionally, an Inerstil C₁₈ (150 mm x 4.6 mm, 5 μm) column with a mobile phase of 0.2% tetra butyl ammonium hydrogen sulfate adjusted to pH 6.0 by sodium hydroxide and acetonitrile in ratio (60:40 v/v), in isocratic elution mode. Despite these variations, resolution remained inadequate. Three different internal standards were tried; Caffeine, Prednisolone, and Diclofenac sodium. Caffeine and Prednisolone internal standards were excluded in our study as Caffeine interferes with Paracetamol peak, while Prednisolone interferes with the Rivaroxaban peak. Finally, Diclofenac sodium was the chosen internal standard due to its effective resolution to the other studied drugs. According to the previous trials, the best separation between Paracetamol, Rivaroxaban, Dexketoprofen, and Diclofenac sodium internal standard was obtained using a Kromasil Phenyl column (150 mm x 4.6 mm, 5 μm) as a stationary phase, 0.1% formic acid in water: ethanol which was operated in gradient elution mode as the mobile phase, flow rate 1.5 mL/min, injection volume 10.0 μL and UV detection at 254.0 nm. The obtained chromatogram of the studied drugs is shown in Fig. 2 with retention times 3.1, 7.0, 7.8, and 8.7 min for Paracetamol, Rivaroxaban, and Dexketoprofen Trometamol and Diclofenac sodium, respectively.

Method validation

The developed method was validated according to ICH guidelines³⁶.

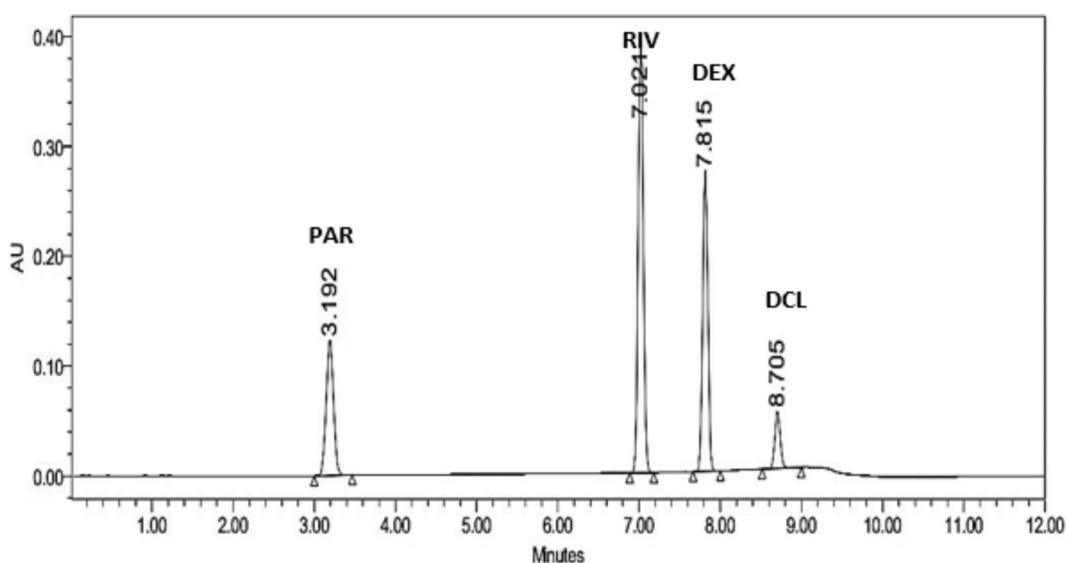


Fig. 2. HPLC chromatogram showing simultaneous separation of PAR (200 $\mu\text{g}/\text{mL}$), RIV (200 $\mu\text{g}/\text{mL}$) and DEX (200 $\mu\text{g}/\text{mL}$) in pure form in presence of Diclofenac sodium (25 $\mu\text{g}/\text{mL}$), as an internal standard at 254.0 nm.

Linearity

The proposed chromatographic method was found to be linear for PAR, DEX, and RIV over the range of (3.00–45.00 µg/mL), (0.50–50.00 µg/mL) and (0.15–20.00 µg/mL), respectively. Calibration curves were constructed by plotting the relative peak area versus the respective concentrations and the assay validation parameters are shown in Table 1.

Regression equations were found to be:

$$Y = 0.1268x - 0.0257 \text{ (Correlation coefficient } (r) = 1 \text{) (For PAR).}$$

$$Y = 0.09782x - 0.0014 \text{ (Correlation coefficient } (r) = 1 \text{) (For DEX).}$$

$$Y = 0.1181x + 0.0016 \text{ (Correlation coefficient } (r) = 1 \text{) (For RIV).}$$

Accuracy

The accuracy of the proposed method was evaluated by determination of % recoveries of four different concentrations (10.00, 13.50, 20.00, and 26.00 µg/mL) for PAR, (2.50, 8.00, 13.00, 18.00, and 27.00) for DEX and (0.50, 4.00, 10.00, and 20.00/mL) for RIV using the corresponding regression equation. The method was found to be accurate with good mean % recoveries and standard deviation ranging from (100.21–101.20 ± 0.850–1.374), as shown in Table 1.

Precision

The interday and intraday precision were evaluated by analyzing three concentrations of the drugs measured in triplicate (20.00, 25.00, and 35.00 µg/mL) for PAR, (13.00, 20.00, and 30.00 µg/mL) for DEX and (4.00, 10.00, and 20.00 µg/mL) for RIV within the same day to estimate intra-day precision and on three successive days to estimate inter-day precision. The low values of %RSD obtained in both cases within the range of (0.193–0.988) shown in Table 1 suggest excellent precision.

Selectivity

The selectivity of the proposed method was tested by analyzing blank samples (an extracted supernatant of deproteinized blank human plasma samples) under the same chromatographic conditions. No peaks were detected in the blank sample as shown in Fig. 3(a), proving no interference and hence the selectivity of the proposed method.

LOD and LOQ

The LOD and LOQ were calculated using the SD of intercept and the slope of the calibration curves, showing high sensitivity of the proposed method as displayed in Table 1, where LOD ranged from 0.047 to 0.531 µg/mL and LOQ from 0.143 to 1.608 µg/mL.

Robustness

The suggested method was tested for robustness by examining the impact of small deliberate changes in some chromatographic parameters on the obtained results including flow rate (1.5 ± 0.1 mL/min) and wavelength of detection (± 2 nm). The robustness of the suggested method was checked by calculating % RSD; found to be from 0.061 to 1.570% as shown in Table 1, proving the robustness of the proposed method.

Parameter	PAR	DEX	RIV
Linearity ^a (µg/mL)	3.00–45.00	0.5–50.00	0.15–20.00
Slope	0.1268	0.0978	0.1181
Intercept	-0.0257	-0.0014	0.0016
Correlation coefficient(r)	1	1	1
Accuracy ^a (Mean ± SD)	101.20 ± 0.912	100.45 ± 1.374	100.21 ± 0.850
Intra-day ^{ab} (RSD %)	0.318	0.583	0.193
Inter-day ^{ab} (RSD %)	0.988	0.860	0.979
Robustness (RSD %)*	0.410 ^c 0.325 ^d	0.061 ^c 1.570 ^d	0.514 ^c 0.086 ^d
LOD(µg/mL) ^e	0.531	0.095	0.047
LOQ(µg/mL) ^f	1.608	0.289	0.143

Table 1. Assay validation parameters of the proposed HPLC method for determination of Paracetamol, Dexametopfen Trometamol and Rivaroxiban. ^aMean of three determinations. ^bRelative standard deviations for concentrations (20.00, 25.00 & 35.00 µg/mL) for PAR, (13.00, 20.00 & 30.00 µg/mL) for DEX and (4.00, 10.00 & 20.00 µg/mL) for RIV. ^cSmall deliberate changes in the flow rate (1.5 ± 0.1 mL/min), the tried flow rates were (1.4, 1.5 and 1.6 mL/min). ^dSmall deliberate changes in wavelength of detection (± 2 nm) were made. The tried wavelengths were 252.0, 254.0 and 256.0. ^eLOD = 3.3 × SD of intercept/slope. ^fLOQ = 10 × SD of intercept/slope.

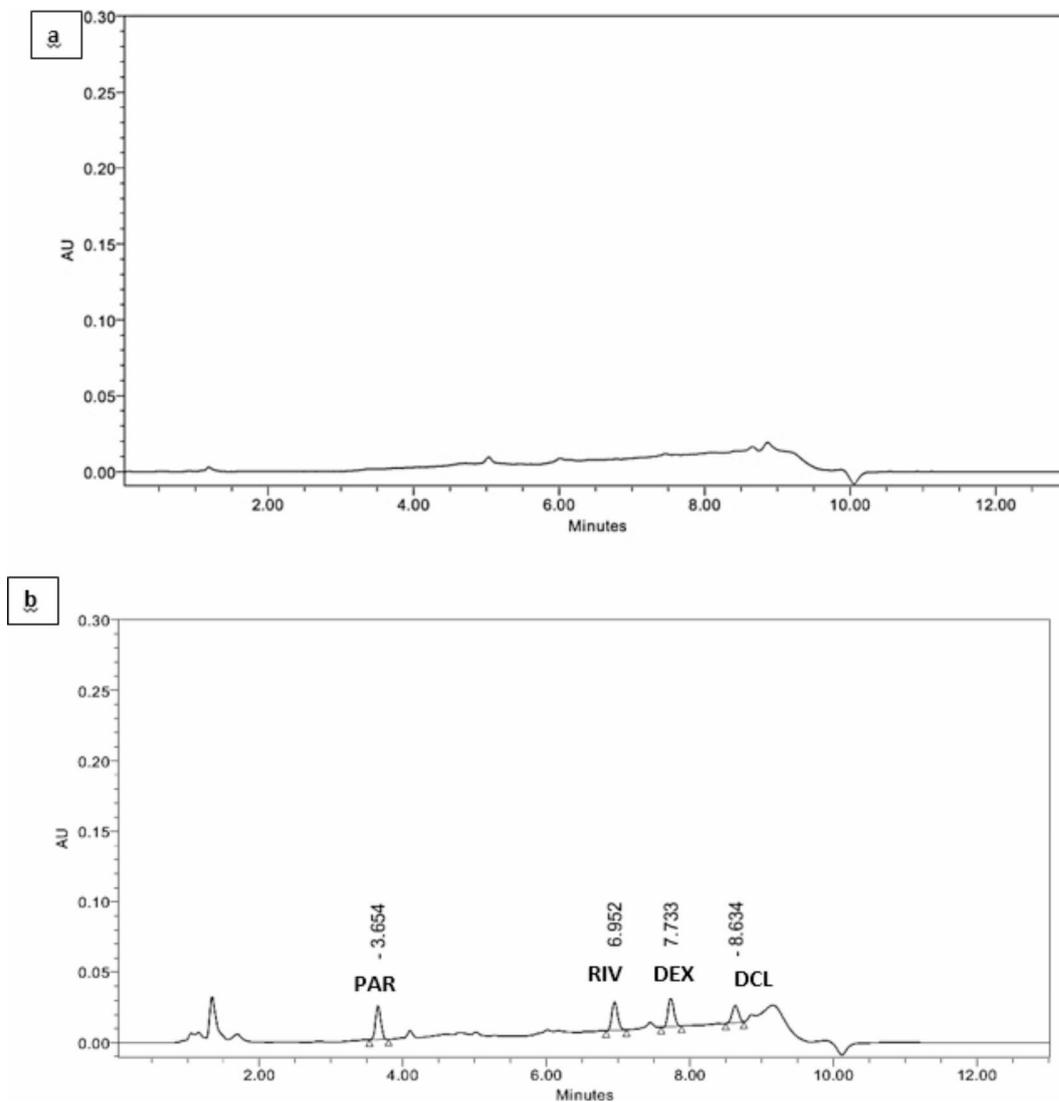


Fig. 3. HPLC chromatogram showing blank human plasma extracted with methanol (a) and spiked mixture of PAR, RIV and DEX (45 µg/mL each) in presence of Diclofenac sodium (25 µg/mL), as an internal standard at 254.0 nm (b).

Parameters	PAR	DEX	RIV	Reference Value ³⁷
Retention time (t_R)	3.19 ± 0.01	7.02 ± 0.01	7.81 ± 0.01	
Capacity factor (K')	1.13	3.68	4.21	1 < K < 10
Resolution (R_s)	2.68		6.66	$R_s \geq 1.50$
Tailing factor (T)	1.01	1.04	1.02	0.80–1.20
Selectivity factor (α)	-	3.26	1.14	$\alpha > 1.00$
Theoretical plates number (N)	5821	53,077	66,656	> 2000

Table 2. System suitability parameters of the proposed chromatographic method.

System suitability

The system suitability parameters should be tested to make sure that the system was correctly functioning during the analysis. Capacity factor (K'), selectivity (α), resolution (R_s), tailing factor (T), and the number of theoretical plates were checked and the obtained results were satisfactory when compared to USP reference values³⁷ as presented in Table 2.

Optimization of the extraction procedure

Optimization of sample preparation is a necessary step for the determination of the investigated analytes in human plasma as it was challenging to recover all analytes from the plasma simultaneously. Thus, two types of sample preparation procedures were tested, such as protein precipitation with methanol, acetonitrile, methanol:acetonitrile (1:1), or liquid-liquid extraction with ethyl acetate in alkaline media. Protein precipitation using either methanol or acetonitrile as an extraction solvent, gave the best recoveries for the three drugs, with the least matrix effect, yet methanol was further chosen rather than acetonitrile as it has a higher green character; Fig.S2 and Table 3.

Application of spiked human plasma

According to their therapeutic levels, the investigated drugs were simultaneously quantified in spiked human plasma. Since the C_{max} of PAR, DEX, and RIV were found to be 13.7, 3.71, and 0.5 $\mu\text{g/mL}$ ^{38–40}, respectively, the sensitivity of the proposed chromatographic method allowed the determination of PAR, DEX, and RIV in spiked plasma as the linearity ranged from (3.0–45.0 $\mu\text{g/mL}$), (0.5–45.0 $\mu\text{g/mL}$), and (0.5–45.0 $\mu\text{g/mL}$) for PAR, DEX, and RIV, respectively, with a good correlation coefficient.

Regression equations were found to be:

$$Y = 0.0881x + 0.1482 \text{ (Correlation coefficient (r) = 0.9995) (For PAR).}$$

$$Y = 0.0737x + 0.3985 \text{ (Correlation coefficient (r) = 0.9998) (For DEX).}$$

$$Y = 0.0917x + 0.0601 \text{ (Correlation coefficient (r) = 0.9993) (For RIV).}$$

Results presented in Table 4, showed good percentage recoveries and %RSD < 15%, proving the absence of any matrix effect and that the sample processing approach is effective in removing any potential matrix interference as shown in Fig. 3(b).

Statistical analysis of results

The statistical comparison of the results obtained by the proposed HPLC-DAD method and reference chromatographic methods for the determination of PAR, DEX¹⁹, and RIV¹⁶, were shown in Table S2. Results revealed no statistically significant difference in accuracy and precision between the suggested and published methods, as the computed t- and F- values were lower than the tabulated ones.

Greenness assessment and whiteness assessment

Green analytical chemistry (GAC) is a concept that demands analytical chemists to consider environmental, health, and safety considerations throughout their work³⁴. The growing recognition of the harmful influence of chemicals on the environment has increased the urgency for the chemistry community to implement greener and more sustainable analytical processes. However, adopting the GAC concept as an alternative to old harmful procedures and generating greener ways of analysis is difficult due to the wide range of analytes and methodologies, sample matrices, and analytical requirements for validation that must be taken into consideration⁴¹.

Some tools have been presented to evaluate the greenness of analytical methodologies. The first and oldest green metric used was NEMI⁴². This qualitative procedure is simple and easy to conduct. The primary drawback was that it does not include and describe all 12 principles of GAC^{33,43}. Another methodology was used for evaluating the greenness of the methods termed Analytical-ecoscale⁴². It is a semi-quantitative technique that incorporates more GAC principles than NEMI and it takes into account the quantity and hazards of each solvent individually, however, it lacks information regarding the structure of the hazard, with no explanation of the causes of the negative environmental effect on the analytical procedure^{33,43}. Thus, two advanced GAC evaluation techniques GAPI³³ and (AGREE)³⁴ were employed to assist the qualitative and quantitative greenness of the established HPLC method to ensure its greenness. White Analytical Chemistry (WAC) principles³⁵ were also tested using the RGB-12 tool to assess their environmental impact.

GAPI tool

GAPI was first developed by Plotka-Wasyłka³³. GAPI provides a qualitative and quantitative review of the entire analytical method, beginning with sample collection till final analysis. The GAPI pictogram can be green, yellow, or red, with green indicating a safe operation and red indicating a non-eco-friendly procedure. The proposed method applies a green approach, where a green mobile phase of ethanol and water mixture was used and a green extraction technique of protein precipitation using methanol was adopted. As shown in Table S3 and Fig. 4(a), yellow color predominates in most of the pentagrams. Zones (2) and (3) are yellow due to using freezing as a physical preservation technique of the sample and the requirement of transport of plasma samples to the lab. Zones (7),(9), (10), and (12) appear to be yellow too due to using green solvent as methanol in the extraction

Drugs	PAR	DEX	RIV
Solvent used	*Recovery %		
Ethyl acetate	32.80	17.24	25.21
Methanol : Acetonitrile (1:1)	77.95	84.75	74.82
Methanol	102.45	99.41	98.24
Acetonitrile	108.79	98.96	100.75

Table 3. The effect of different extraction solvents on the recovery percentage of Paracetamol, Rivaroxiban and Dexametopfen Trometamol in spiked human plasma.

Concentration in spiked human plasma (µg/mL) (PAR: DEX: RIV)	PAR *Recovery %	DEX *Recovery %	RIV *Recovery %
(3:3:3)	91.49	95.33	99.67
(10:10:10)	99.50	101.60	100.40
(30:30:30)	98.67	100.60	98.30
Mean ± SD	96.55 ± 4.404	99.18 ± 3.367	99.46 ± 1.066

Table 4. Determination of Paracetamol, Dexametoprolol and Rivaroxiban in spiked human plasma. * Average of three determinations.

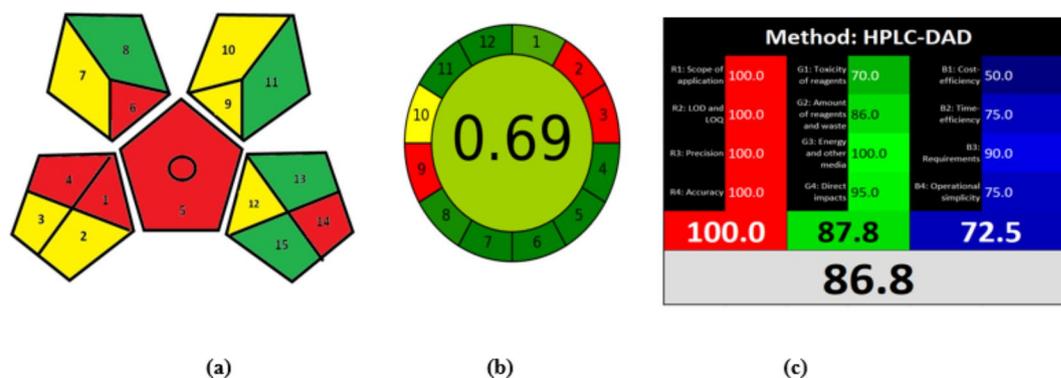


Fig. 4. Greenness and whiteness assessment of the proposed method using GAPI(a), AGREE (b), and RGB-12 tools (c).

technique, using the volume of solvent ranging from (10-100mL) and using ethanol in the mobile phase which is considered according to NFPA moderately toxic (NFPA scale = 2). HPLC uses energy of ≤ 1.5 kWh per sample. Five red regions were found, zones (1) and (4) which are due to using an off-line sample collection technique and storage under special conditions; (freezing). Zones (5), (6), and (14) are red due to the presence of an extraction step and macro extraction technique was applied using methanol and the amount of waste is > 10 mL.

AGREE tool

The recently introduced AGREE tool is a calculator that uses a free software to provide a quick, simple, and complete assessment of the green character³⁴. It is presented as a final score in the center of the pictogram based on fulfilling the 12 green analytical chemistry criteria. The software automatically provides a pictogram with 12 segments. The width of each segment varies according to its significance. Each segment has a distinct color scale that ranges from green to red. In AGREE, the ultimate score that measures the degree of greenness of the approach is a fraction of one. The outcomes vary from zero to one. When it is closer to one, it suggests more green, and when it is close to zero, it indicates insufficient green. As presented in Table S3 and Fig. 4(b), the final score for the developed method is 0.69. This indicates a good greenness profile for the proposed method.

RGB 12 algorithms

Sustainable analytical techniques take into account not only their environmental impact, but also their economic cost, validity, and effectiveness. The White Analytical Chemistry method (WAC) is a useful tool for examining the sustainability of analytical procedures³⁵. As stated by White Analytical Chemistry (WAC), RGB 12 consists of three criteria for assessment (red, green, and blue). Each colour describes a certain aspect. Red; (quality of the method and analytical efficiency), green; (the environmental friendliness and safety aspects), and Blue; (practical and economic aspects). Every criterion in the green, red, and blue principles is evaluated on a scale of 0 to 100. A score closer to 100 indicates a white method, signifying a complete and suitable technique for the application. The suggested chromatographic method demonstrated an outstanding balance between the three characteristics of WAC, scoring an average white score of 86.8 as illustrated in Table S4 and Fig. 4(c). Thus according to WAC evaluation, all principles were equally crucial in maintaining the concept of sustainability.

RGB 12 algorithm Vs. AGREE

During the greenness evaluation of the proposed method, the findings obtained from RGB 12 and AGREE concerning the WAC and GAC principles, respectively, are mainly equivalent. However, one must be alert to the variances in criteria and evaluation procedures. Comparing RGB 12 algorithm with AGREE, RGB 12 algorithm showed several advantages over AGREE as it took into consideration the accuracy, LOD, and cost-effectiveness of the proposed method. (i.e. it published real experimental data). Moreover, it introduces new ideas such as genetically modified organisms (GMOs) or animal use in experiments. RGB 12 algorithm also considers 3 criteria together, red, green, and blue which represent the analytical performance, safety considerations, and financial aspects; respectively. However, this whiteness assessment method has the disadvantage of being subjective as the score is based on the researcher's ethical responsibility. On the other hand, in AGREE more parameters are taken into consideration. AGREE defines the derivatization step separately and the weight for each criterion. Thus, assessing the greenness using GAC and WAC tools enabled us to get a full image of the green and sustainable character of the proposed method^{44,45}. Therefore, it is desirable to employ more than one assessment tool for better evaluation of the green character of the developed analytical procedures⁴⁶⁻⁴⁸.

Conclusion

An HPLC-PDA method was developed and validated for simultaneous determination of co-administered drugs used for the treatment of post-COVID-19 conditions namely PAR, DEX, and RIV in bulk powder, and spiked human plasma. To the best of our knowledge; it is the first method that determines these drugs in a biological sample. The proposed method was validated according to ICH guidelines. This method leverages a green and economical sample preparation approach characterized by high sensitivity and efficient analyte recovery of the

cited drugs from the spiked plasma without peak interference from the biological matrix and with a short run time. Owing to the simplicity and successfulness of the proposed method in determining the proposed drugs in spiked human plasma it is applicable for quality control laboratories, bioequivalence and bioavailability centers. The future research plan is the application of the suggested HPLC-DAD method for quantification of co-administered PAR, DEX, and RIV in healthy Egyptian volunteers who were given Infer-P[®] (325.0 mg of PAR & 25.0 mg of DEX) and Xalerto[®] (20.0 mg of RIV) at the same time. Moreover, calculating the pharmacokinetic parameters as C_{\max} (ng/mL), t_{\max} (h), $t_{1/2}$ (h), AUC_{0-t} , $AUC_{0-\infty}$ (ng.h/mL) and elimination rate constant; K_{el} (h^{-1}).

Data availability

Data will be made available on request. The request for materials should be addressed to P.M (pmedhatn1@gmail.com).

Received: 25 July 2024; Accepted: 3 October 2024

Published online: 31 October 2024

References

1. Organization, W. H. Listings of WHO's response to COVID-19. Available online at: URL (2020). <https://www.who.int/news-room/detail/29-06-2020-covid-timeline> (accessed October 5, 2020) (2021).
2. Turana, Y., Nathaniel, M., Shen, R., Ali, S. & Aparasu, R. R. Citicoline and COVID-19-related cognitive and other neurologic complications. *Brain Sci.* **12**, 59 (2021).
3. Organization W. H.
4. Liu, Y. C., Kuo, R. L. & Shih, S. R. COVID-19: the first documented coronavirus pandemic in history. *Biomed. J.* **43**, 328–333 (2020).
5. WHO, C. O. World health organization. Air Quality Guidelines for Europe. (2020).
6. Baloch, S., Baloch, M. A., Zheng, T. & Pei, X. The coronavirus disease 2019 (COVID-19) pandemic. *Tohoku J. Exp. Med.* **250**, 271–278 (2020).
7. Mao, L. et al. Neurologic manifestations of hospitalized patients with coronavirus disease 2019 in Wuhan, China. *JAMA Neurol.* **77**, 683–690 (2020).
8. Cagnazzo, F. et al. Neurological manifestations of patients infected with the SARS-CoV-2: a systematic review of the literature. *J. Neurol.* **268**, 2656–2665 (2021).
9. Nuzzo, D. et al. Long-term brain disorders in post Covid-19 neurological syndrome (PCNS) patient. *Brain Sci.* **11**, 454 (2021).
10. Rudroff, T., Fietsam, A. C., Deters, J. R., Bryant, A. D. & Kamholz, J. Post-COVID-19 fatigue: potential contributing factors. *Brain Sci.* **10**, 1012 (2020).
11. Salihefendic, N., Zildzic, M. & Huseinagic, H. Ischemic vasculitis as a cause of Brain Disorder's in patients with long covid: Case Report. *Med. Arch.* **75**, 471 (2021).
12. Nalbandian, A., Desai, A. D. & Wan, E. Y. Post-COVID-19 condition. *Annu. Rev. Med.* **74**, 55–64 (2023).
13. Mardani, M. Vol. 15 (Brieflands, (2020).
14. Mulla, T. S., Rao, J. R., Yadav, S. S., Bharekar, V. V. & Rajput, M. P. Development and validation of HPLC method for simultaneous quantitation of Paracetamol and dextropropofol in bulk drug and formulation. *Pharmacie Globale.* **7**, 1–4 (2011).
15. Rao, J. R., Mulla, T. S., Bharekar, V. V., Yadav, S. S. & Rajput, M. P. Simultaneous HPTLC determination of paracetamol and dextropropofol in pharmaceutical dosage form. *Der Pharma Chem.* **3**, 32–38 (2011).
16. Çelebier, M., Reçber, T., Koçak, E., Altınöz, S. & Kir, S. Determination of rivaroxaban in human plasma by solid-phase extraction–high performance liquid chromatography. *J. Chromatogr. Sci.* **54**, 216–220 (2016).
17. Derogis, P. B. M. et al. Determination of rivaroxaban in patient's plasma samples by anti-xa chromogenic test associated to high performance liquid chromatography tandem Mass Spectrometry (HPLC-MS/MS). *PLoS One.* **12**, e0171272 (2017).
18. Ramacciotti, E. et al. Rivaroxaban versus no anticoagulation for post-discharge thromboprophylaxis after hospitalisation for COVID-19 (MICHELLE): an open-label, multicentre, randomised, controlled trial. *Lancet.* **399**, 50–59 (2022).
19. Pokharkar, D. et al. Stability indicating RP-HPLC-PDA method for simultaneous determination of Dextropropofol Trometamol and Paracetamol from tablet dosage form. *Der Pharmacia Letter.* **3**, 49–57 (2011).
20. Kothapalli, L. et al. Simultaneous spectrophotometric estimation of Paracetamol and dextropropofol in pharmaceutical dosage form, Der Pharm. *Der Pharma Chem.* **3**, 365–371 (2011).
21. Seshamamba, B. & Sekaran, C. Spectrophotometric quantification of direct factor xa inhibitor, rivaroxaban, in raw and tablet dosage form. *Glob Drugs th.* **2**, 1–8 (2017).
22. Seshamamba, B. S. V. & Sekaran, C. B. Spectrophotometric Analysis for the quantification of Rivaroxaban in Bulk and Tablet Dosage Form. *Int. J. Med. Pharm. Sci.* **7**, 21–34 (2017).
23. El-Bagary, R. I., Elkady, E. F., Farid, N. A. & Youssef, N. F. A validated Spectrophotometric Method and Thermodynamic studies for the determination of Cilostazol and Rivaroxaban in Pharmaceutical preparations using Fe-Phenanthroline System. *Anal. Chem. Lett.* **7**, 676–688 (2017).
24. Seshamamba, B. S. V., Satyanarayana, P. V. V. & Sekaran, C. B. Application of stability indicating HPLC method with UV detector to the analysis of rivaroxaban in bulk and tablet dosage form. *Chem. Sci. Trans.* **3**, 1546–1554 (2014).
25. Ramisetti, N. R. & Kuntamukkala, R. Development and validation of a stability indicating LC-PDA-MS/MS method for separation, identification and characterization of process related and stress degradation products of rivaroxaban. *RSC Adv.* **4**, 23155–23167 (2014).
26. Walter, M. E. et al. Development and validation of a stability-indicating RP-HPLC method for the determination of rivaroxaban in pharmaceutical formulations. *Lat Am. J. Pharm.* **34**, 1503–1510 (2015).
27. Hadagali, M. D. Determination of rivaroxaban in pure, pharmaceutical formulations and human plasma samples by RP-HPLC. *Int. J. Adv. Pharm. Anal.* **5**, 65–68 (2015).
28. Souri, E., Mottaghi, S., Zargarpoor, M., Ahmadvani, R. & Jalalizadeh, H. Development of a stability-indicating HPLC method and a dissolution test for rivaroxaban dosage forms. *Acta Chromatogr.* **28**, 347–361 (2016).
29. Elesbao Walter, M. et al. Micellar Electrokinetic Capillary Method for the Analysis of Rivaroxaban and its correlation with RP-LC method and Bioassay. *Curr. Anal. Chem.* **13**, 379–385 (2017).
30. Arous, B., Al-Mardini, M. A., Ghazal, H. & Al-Lahham, F. Stability-Indicating Method for the determination of Rivaroxaban and its Degradation products using LC-MS and TLC. *RJPT.* **11**, 212–220 (2018).
31. Alam, P. et al. Ecofriendly densitometric RP-HPTLC method for determination of rivaroxaban in nanoparticle formulations using green solvents. *RSC Adv.* **10**, 2133–2140 (2020).
32. Süslü, İ., Çelebier, M. & Altınöz, S. Electrochemical behaviour investigation and square-wave voltammetric determination of rivaroxaban in pharmaceutical dosage forms. *Anal. Methods.* **6**, 9397–9403 (2014).

33. Plotka-Wasyłka, J. A new tool for the evaluation of the analytical procedure: Green Analytical Procedure Index. *Talanta*. **181**, 204–209 (2018).
34. Pena-Pereira, F., Wojnowski, W. & Tobiszewski, M. AGREE—Analytical GREENness metric approach and software. *Anal. Chem.* **92**, 10076–10082 (2020).
35. Nowak, P. M., Wietecha-Poslusznny, R. & Pawliszyn, J. White Analytical Chemistry: an approach to reconcile the principles of Green Analytical Chemistry and functionality. *TrAC Trends Anal. Chem.* **138**, 116223 (2021).
36. Guideline, I. H. T. Validation of analytical procedures: text and methodology. *Q2 (R1)* 1, 05 (2005).
37. Pharmacopeia, U. in *The United States Pharmacopeia-The National Formulary, The United States Pharmacopeial Convention, Rockville, MD*. 195–197.
38. Van der Westhuizen, J., Kuo, P., Reed, P. & Holder, K. Randomised controlled trial comparing oral and intravenous Paracetamol (acetaminophen) plasma levels when given as preoperative analgesia. *Anaesth. Intensive Care*. **39**, 242–246 (2011).
39. Barbanoj, M. J., Antonijoan, R. M. & Gich, I. Clinical pharmacokinetics of dextetopofen. *Clin. Pharmacokinet.* **40**, 245–262 (2001).
40. Qian, J., Gu, S. & Yan, Y. j. determination of rivaroxaban in rat plasma by ultra-high-performance liquid chromatography–Q-Orbitrap high-resolution mass spectrometry and its application to a pharmacokinetic study. *Biomed. Chromatogr.* **36**, e5491 (2022).
41. Hemdan, A., Magdy, R., Farouk, M. & Fares, N. V. Central composite design as an analytical optimization tool for the development of eco-friendly HPLC-PDA methods for two antihypertensive mixtures containing the angiotensin receptor blocker Valsartan: greenness assessment by four evaluation tools. *Microchem J.* **183**, 108105 (2022).
42. Keith, L. H., Gron, L. U. & Young, J. L. Green analytical methodologies. *Chem. Rev.* **107**, 2695–2708 (2007).
43. Nagieb, H. M., Abdelwahab, N. S., Abdelrahman, M. M., Zaazaa, H. E. & Ghoniem, N. S. Greenness assessment of UPLC/MS/MS method for determination of two antihypertensive agents and their harmful impurities with ADME/TOX profile study. *Sci. Rep.* **13**, 19318 (2023).
44. Habib, N. M., Tony, R. M., AlSalem, H. S., Algethami, F. K. & Gamal, M. Evaluation of the greenness, whiteness, and blueness profiles of stability indicating HPLC method for determination of denaverine hydrochloride and benzyl alcohol in pharmaceuticals. *Microchem J.* **201**, 110733 (2024).
45. Algethami, F. K. & Gamal, M. Development of a simple, eco-friendly HPLC-DAD method for tulathromycin analysis: ensuring stability and assessing greenness. *Microchem J.* **195**, 109511 (2023).
46. Prajapati, P. B., Radadiya, K. & Shah, S. A. Quality risk management based: analytical quality by design approach to eco-friendly and versatile chromatography method for simultaneous estimation of multiple fixed-dose-combination products of anti-diabetic drugs. *J. Pharm. Innov.* **17**, 1–18 (2020).
47. Prajapati, P., Shah, H. & Shah, S. A. Implementation of QRM and DoE-based quality by design approach to VEER chromatography method for simultaneous estimation of multiple combined dosage forms of Paracetamol. *J. Pharm. Innov.* **17**, 1–17 (2020).
48. Gamal, M., Naguib, I. A., Panda, D. S. & Abdallah, F. F. Comparative study of four greenness assessment tools for selection of greenest analytical method for assay of hyoscine N-butyl bromide. *Anal. Methods*. **13**, 369–380 (2021).

Acknowledgements

The authors thank Faculty of Pharmacy, MSA University, 6th October City, Giza, Egypt, for providing the instrument to finish this work.

Author contributions

P.M: Formal analysis, methodology, and writing the original draft, M.F: Supervision, methodology, review & editing, H.M: Supervision, methodology, review & editing and N.G: Supervision, methodology, review & editing. All authors approved the final manuscript.

Funding

Open access funding provided by The Science, Technology & Innovation Funding Authority (STDF) in cooperation with The Egyptian Knowledge Bank (EKB).

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-024-75216-4>.

Correspondence and requests for materials should be addressed to N.S.G.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2024